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MODULATION OF Ca^{2+} EFFLUX AND REBOUNDING Ca^{2+} TRANSPORT IN RAT LIVER MITOCHONDRIA

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The independent pathway for Ca^{2+} efflux of rat liver mitochondria exhibits a sharp temperature and pH dependence. The Arrhenius plot displays a break at 18°C, activation energy being about 117 kJ/mol below 18°C and 59 kJ/mol above 18°C. The pH profile is bell-shaped, with a broad optimum at pH 7.0. These properties of the efflux pathway, together with the membrane potential modulation recently described (Bernardi, P. and Azzone, G.F. (1983) Eur. J. Biochem. 134, 377–383), suggest an explanation for the phenomenon of rebounding Ca^{2+} transport. Addition of a Ca^{2+} pulse to respiring mitochondria causes (i) a phase of rapid Ca^{2+} uptake, leading to a decrease of extramitochondrial free Ca^{2+} to a lower level with respect to that maintained before Ca^{2+} addition, and (ii) a slower phase of net Ca^{2+} efflux, leading to restoration of the steady-state extramitochondrial free Ca^{2+} preceeding Ca^{2+} addition. Evidence is provided that the excess Ca^{2+} uptake is linked to transient inactivation of the efflux pathway due to membrane depolarization. Conversely, the efflux phase is linked to reactivation of the efflux pathway upon repolarization. The efflux component of the rebound cycle and the isolated efflux pathway exhibit similar dependence on temperature, pH and membrane potential.

Introduction

There is now general agreement that mammalian mitochondria possess separate pathways for Ca^{2+} influx and efflux (reviewed in Refs. 1–3). The Ca^{2+} efflux pathway is insensitive to ruthenium red, and is stimulated by Na^+ in mitochondria from heart, skeletal muscle, brain and adipose tissue but not from liver, kidney and lung [4]. There is evidence, however, that heart and brain mitochondria, where Ca^{2+} efflux is usually regarded as Na^+-dependent, also possess a Na^+-independent efflux component [3–6]. Furthermore, it has been recently shown that Sr^{2+} – a Ca^{2+} analogue which is readily transported by the uniporter – inhibits the independent Ca^{2+} efflux pathways both in heart and in liver mitochondria [7]. Despite its importance, little is known about the nature of the Na^+-independent Ca^{2+} efflux pathway, and evidence favouring an electroneutral H^+/Ca^{2+} exchange is circumstantial at best [3,8].

In the course of studies on the regulation of this system, we have described a novel feature of the Na^+-independent Ca^{2+} efflux pathway of liver mitochondria, namely its modulation by membrane potential [9,10]. The rate of ruthenium red-induced Ca^{2+} efflux in fact increased linearly with $\Delta\psi$ as the latter exceeded a value of about 130 mV [10].

Here the parameters affecting the independent

Abbreviations: pCa_0, $-\log[Ca^{2+}]$ outside the mitochondria; $\Delta\psi$, membrane potential; Mops, 4-morpholinepropanesulphonic acid.

Ca^{2+} efflux of liver mitochondria are further analyzed. We show that, unlike the uniporter, the efflux system exhibits a bell-shaped pH profile with a broad optimum at pH 7.0. The system reveals a sharp temperature dependence with a break in the Arrhenius plot at 18°C. This finding, together with the high activation energy below 18°C, indicates that operation of the Ca^{2+} efflux system, like mobile carrier-catalyzed transport processes [11], is much more dependent on the lipid fluidity and/or bilayer structure than the Ca^{2+} uniporter. These properties, together with the $\Delta\psi$-modulation, have been exploited to interpret the process of rebounding Ca^{2+} transport as due to transient inactivation of the efflux pathway following membrane depolarization linked to Ca^{2+} uptake.

Materials and Methods

The experimental procedures and the preparation of rat liver mitochondria have been described in detail elsewhere [10]. Ruthenium red was purchased from Sigma (St. Louis, MO), and purified according to Luft [12]. The dye solutions were prepared daily, and the concentration of ruthenium red was determined spectrophotometrically on the basis of an ε of 68 per mM per cm at 533 nm [13]. The experiments in which ruthenium red was employed (Figs. 1–3) were carried out in hypotonic media supplemented with cytochrome *c*, in order to enhance the activity of the independent efflux pathway [10]. Qualitatively similar results were, however, obtained in isotonic media. The incubation media are specified in the figure legends. All chemicals were of the highest purity commercially available.

Results and Discussion

Temperature dependence and pH dependence of Ca^{2+} efflux

Fig. 1 analyzes the temperature dependence of ruthenium red-induced Ca^{2+} efflux which reflects the activity of the independent pathway for Ca^{2+} efflux. The Arrhenius plot exhibits a break at about 18°C, and activation energies are about 59 kJ/mol above 18°C and 117 kJ/mol below 18°C. This contrasts with the reported behaviour of the

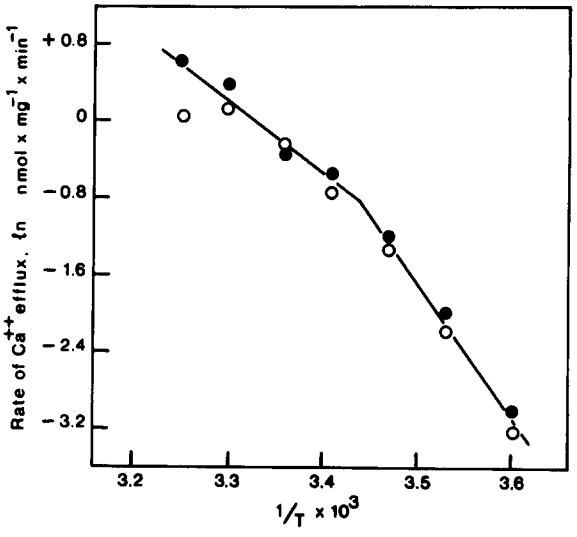

Fig. 1. Temperature-dependence of ruthenium red-induced Ca^{2+} efflux. The incubation medium contained 40 mM choline/10 mM Tris-Mops/5 mM succinate/1.5 mM $MgCl_2$/0.5 mM Pi/1 mg/ml bovine serum albumin/2 μM rotenone/5 μM cytochrome *c*/5 μM Ca^{2+}. The pH was carefully brought to 7.0 at each temperature with either Tris base or HCl. The experiment was started by the addition of 4 mg of mitochondria to a final volume of 4 ml. After attainment of steady-state Ca^{2+} distribution, 0.25 μM ruthenium red was added. Values on the ordinate refer to the rate of Ca^{2+} efflux following addition of ruthenium red. The different symbols refer to different mitochondrial preparations.

uniport Ca^{2+} carrier, which shows no breaks in this temperature range and activation energy of about 42 kJ/mol [11]. Although the mechanism(s) causing the break in the Arrhenius plot are not of easy interpretation, the experiment indicates that the independent Ca^{2+} efflux pathway has a more marked dependence on membrane fluidity than the uniport. The presence of breaks and a high activation energy are typical features of mobile carrier-mediated transport processes [11]. Reported values for H^+ transport via carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone or dinitrophenol and for K^+ transport via valinomycin or nigericin are about 84 kJ/mol below 14°C and 59 kJ/mol above 14°C, respectively [11]. These values are close to those obtained from Fig. 1, and suggest that the independent pathway for Ca^{2+} efflux could be a mobile carrier, possibly a H^+/Ca^{2+} exchanger. The experiment reported in Fig. 2 shows a good correlation between ruthenium

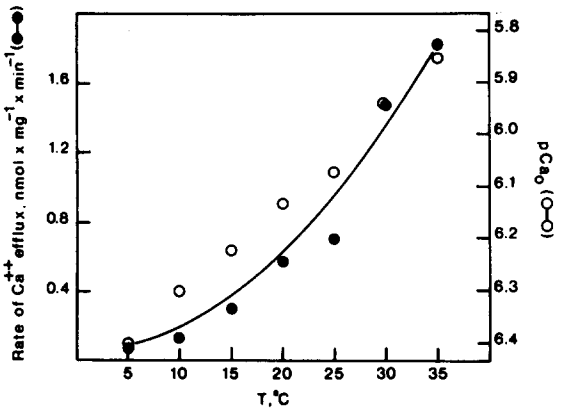

Fig. 2. Relationship between rate of Ca^{2+} efflux and pCa_0 as a function of temperature. Experimental conditions as in Fig. 1. Values on the ordinate refer to the steady-state pCa_0 value (O——O), and to the corresponding rate of Ca^{2+} efflux following the addition of ruthenium red (●——●).

red-induced Ca^{2+} efflux and pCa_0 as a function of temperature. The lower the temperature, and hence the rate of Ca^{2+} efflux, the higher the pCa_0 (i.e., the lower the extramitochondrial free Ca^{2+}). This behaviour confirms that the efflux pathway is more temperature-sensitive than the uniport.

Fig. 3 shows that the ruthenium red-induced Ca^{2+} efflux exhibits a broad pH optimum at pH 7.0. This is an additional indication that ruthenium

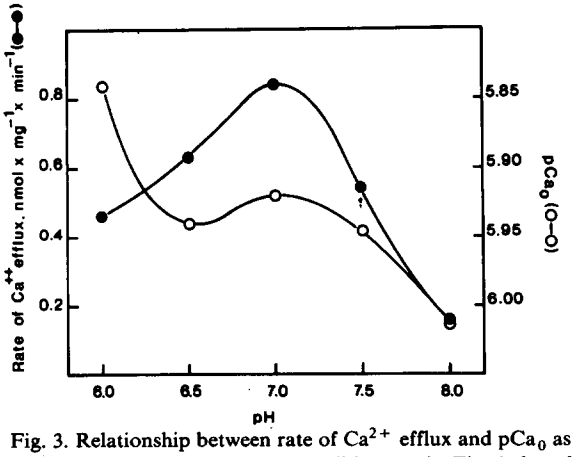

Fig. 3. Relationship between rate of Ca^{2+} efflux and pCa_0 as a function of pH. Experimental conditions as in Fig. 1, but the pH was as indicated and the temperature was 30°C. Values on the ordinate refer to the steady-state pCa_0 value (O——O), and to the corresponding rate of Ca^{2+} efflux following the addition of ruthenium red (●——●).

red-induced Ca^{2+} efflux occurs on an independent pathway, since, in the same pH range, the activity of the uniport increases at increasing pH [14–16]. The pH profile of pCa_0 therefore reflects the relative effect of pH on both the influx and efflux processes. This explains the shoulder at pH 7.0 as due to activation of the efflux pathway (Fig. 3).

Interestingly, the same trend was present in a similar experiment of Nicholls (see Ref. 17, Fig. 10b), but its significance remained undetected, because in that study the efflux rates were not determined [17].

Rebounds in Ca^{2+} transport

The pH dependence and temperature dependence of the isolated Ca^{2+} efflux pathway characterized here, together with the membrane potential modulation recently described [10] will now be utilized to explain the phenomenon of rebounding Ca^{2+} transport.

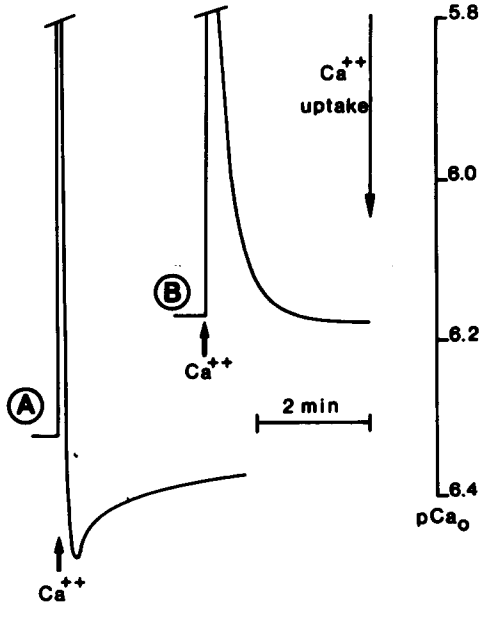

Fig. 4. Rebounding Ca^{2+} transport. Inhibition by Mg^{2+}. The incubation medium contained 0.14 M sucrose/40 mM choline/10 mM Tris-Mops pH (7.0)/5 mM succinate/1 mM Pi/2 μM rotenone/1 μg/ml oligomycin/1 mg/ml bovine serum albumin/5 μM Ca^{2+}. In trace (B), 1.5 mM $MgCl_2$ was added. Final volume 4 ml. The experiment was started by the addition of 8 mg of mitochondria (not shown). When indicated, 40 μM $Cacl_2$ was added.

Fig. 4A shows that addition of a Ca^{2+} pulse to respiring mitochondria induced a biphasic response; (i) a rapid phase of Ca^{2+} uptake, leading to a lowering of extramitochondrial free Ca^{2+} substantially below the level maintained before Ca^{2+} addition and (ii) a slower phase of net Ca^{2+} efflux, tending to restore extramitochondrial free Ca^{2+} to the original level. The rebound in Ca^{2+} transport was abolished by Mg^{2+} (Fig. 4B). Similar rebounds induced by Ca^{2+} transport, measured indirectly on H^+ transport, have been reported in the mid '60s [18,19], but a satisfactory explanation for this behaviour is still lacking. While we will return later on the Mg^{2+} inhibition, all subsequent experiments were carried out in Mg^{2+}-free media in order to characterize this phenomenon.

Energized mitochondria maintain a steady-state Ca^{2+} distribution which, at high membrane potential [17], depends on the relative rates of Ca^{2+} influx on the uniporter and of Ca^{2+} efflux on the independent pathway [1–3]. The rebound cycle essentially consists of an 'excessive' Ca^{2+} uptake following a Ca^{2+} pulse, whereby a lower steady-state extramitochondrial free Ca^{2+} is attained. It may therefore be inferred that during the process of active Ca^{2+} uptake either the uniport is stimulated or the efflux pathway is inhibited, relative to the conditions existing before Ca^{2+} addition. Similarly, the slow Ca^{2+} efflux completing the rebound cycle could be due either to inhibition of the uniport or to reactivation of the efflux pathway.

The electrical capacitance of the inner mitochondrial membrane is very low [20]. Active uptake of substantial amounts of Ca^{2+} therefore requires regeneration of $\Delta\psi$ by H^+ extrusion (see Ref. 3 for a discussion). Since the maximal rate of H^+ pumping by the respiratory chain is slower than the V_{max} of the uniport [21], aerobic Ca^{2+} uptake is accompanied by a transient depolarization of the inner membrane, followed by a repolarization upon completion of Ca^{2+} uptake (Ref. 22; see also Fig. 8). Recent work from our laboratory indicates that the independent Ca^{2+} efflux pathway could be inhibited by lowering the membrane potential below the state-4 value [10]. Taken together, these observations provide a rationale for explaining the rebounds in Ca^{2+} transport: the excess Ca^{2+} uptake could be due to a transient inactivation of the efflux pathway linked to membrane depolarization, whereas the efflux phase reflects its reactivation upon membrane repolarization. The experiments reported below were designed to test this hypothesis.

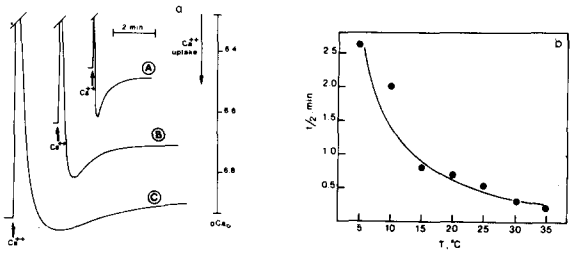

Fig. 5. Temperature-dependence of rebounding Ca^{2+} transport. (a) Experimental conditions as in Fig. 4A, but the temperature was 30°C (trace A), 15°C (trace B) or 5°C (trace C). Where indicated, 10 μM $CaCl_2$ was added. (B) values on the ordinate refer to the half-time of the efflux phase following maximal Ca^{2+} uptake.

Fig.5A shows that the rebound exhibited a marked temperature-dependence, being strongly inhibited at low temperatures. This was mainly due to inhibition of the efflux phase, as indicated by the data of Fig. 5B: as the temperature was lowered from 35°C to 5°C, the half-time of the efflux process increased from 0.2 to 2.5 min. This behaviour reminds the temperature dependence of

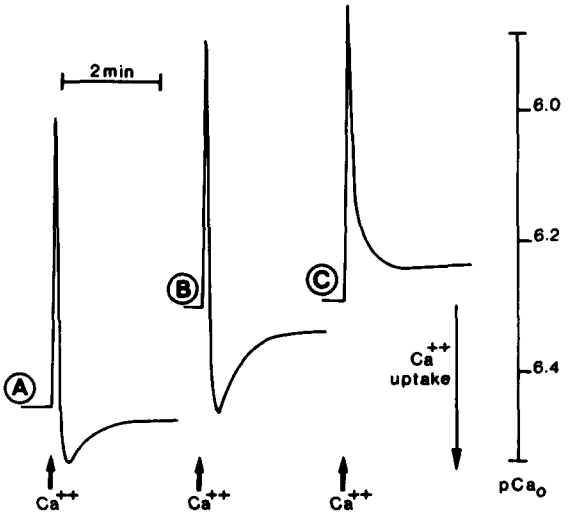

Fig. 6. pH-dependence of rebounding Ca^{2+} transport. Experimental conditions as in Fig. 4A, but the temperature was 30°C and the pH was 8.0 (trace A), 7.0 (trace B) or 6.0 (trace C). When indicated, 10 μM $CaCl_2$ was added.

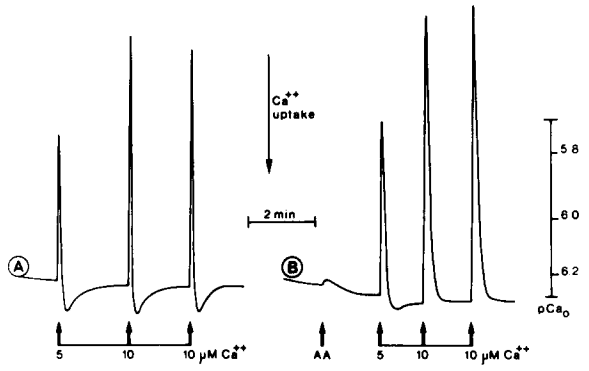

Fig. 7. Effect of antimycin A on rebounding Ca^{2+} transport. Experimental conditions as in Fig. 4A at 30°C. The experiment was started by the addition of 8 mg of mitochondria (not shown). When indicated, $CaCl_2$ and 5 ng of antimycin A per mg protein (AA) were added.

the independent efflux pathway (cf. Fig. 2) and represents an indication of the relationship between the phenomenon of Ca^{2+} rebound and the kinetics of the Ca^{2+} efflux pathway. Fig. 6 shows that the rebound was maximal at neutral pH, while it was clearly inhibited at pH 6.0 and 8.0. Even though a quantitative analysis is difficult, the pH dependence of the rebounds is similar to that exhibited by the isolated Ca^{2+} efflux pathway (cf. Fig. 3).

Fig. 7A shows a series of rebounds induced by

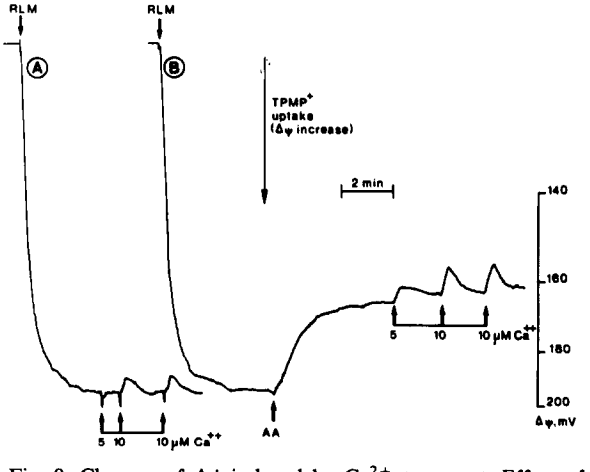

Fig. 8. Changes of $\Delta\psi$ induced by Ca^{2+} transport. Effect of antimycin A. The incubation medium was as in Fig. 4A, plus 6 μM triphenylmethylposphonium ($TPMP^+$). Final volume 5 ml, 30°C. When indicated, 10 mg of mitochondria (RLM), $CaCl_2$ and 5 ng of antimycin A per mg protein (AA).

multiple Ca^{2+} additions to steady-state mitochondria. Addition of 5 ng of antimycin A per mg protein before the Ca^{2+} pulses led to net Ca^{2+} uptake (Fig. 7B), due to inactivation of the efflux pathway [10]. In this case, Ca^{2+} transport did not exhibit the rebounds and Ca^{2+} uptake levelled off, as external free Ca^{2+} approached the value preceeding Ca^{2+} addition (Fig. 7B). Fig. 8 shows a continuous recording of the mitochondrial membrane potential, carried out during Ca^{2+} transport. Ca^{2+} addition caused a transient depolarization, followed by recovery of the same $\Delta\psi$ level preceding the Ca^{2+} pulse (trace A) (cf. Ref. 22). A comparison between Fig. 7A and 8A indicates that the Ca^{2+} uptake phase occurs while the membrane is depolarized, whereas the Ca^{2+} efflux phase coincides with membrane repolarization. Fig. 8B shows that addition of 5 ng of antimycin A per mg protein caused a 40 mV depolarization. As the new steady state $\Delta\psi$ was attained, multiple Ca^{2+} additions led to essentially the same pattern observed in trace 8A; yet no rebounds could be observed during Ca^{2+} transport (Fig. 7B). The simplest explanation is that the independent Ca^{2+} efflux pathway is already inactivated by antimycin-induced depolarization.

This hypothesis permits also a likely explanation for the Mg^{2+} inhibition (Fig. 4). In the absence of Mg^{2+}, at 30°C, the relation between initial rate of Ca^{2+} uptake and external Ca^{2+} concentration is hyperbolical, and the K_m is in the 10^{-6} M region (reviewed in Ref. 1). Under these conditions Ca^{2+} uptake is faster than respiratory chain activity even at Ca^{2+} concentrations near the steady-state value [21]. As a consequence, the membrane potential will remain lower, and hence the Ca^{2+} efflux inhibited, until extramitochondrial free Ca^{2+} is lowered well below the K_m. At this point $\Delta\psi$ will rise to the original level and reactivate Ca^{2+} efflux, with reestablishment of the steady state. In the presence of Mg^{2+} the rate of Ca^{2+} uptake is lowered [23], and the relation between rate of Ca^{2+} uptake and external Ca^{2+} concentration becomes sigmoidal [17,21,24,25], while the apparent K_m is increased by one order of magnitude [21,24]. In this case, the activity of the uniporter falls rapidly as pCa_0 approaches 6.0 (see Fig. 4B). At this point the rate of Ca^{2+} uptake becomes rate-limiting, the membrane repolarizes

and thus the transient inactivation of the efflux pathway is no longer appreciated.

In conclusion, the present paper defines some thermodynamic parameters of the independent pathway for Ca^{2+} efflux in rat liver mitochondria, and shows that significant changes of steady-state extramitochondrial free Ca^{2+} can be obtained by specifically modulating either Ca^{2+} uptake or Ca^{2+} efflux rates. Furthermore, it shows that the depolarization accompanying active Ca^{2+} uptake is effective in inhibiting the Ca^{2+} efflux pathway. This might represent an energy-saving device designed to limit futile Ca^{2+} cycling during the process of Ca^{2+} uptake.

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