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The Na^+-independent Ca^{2+} efflux system in mitochondria is a $Ca^{2+}/2H^+$ exchange system

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The mechanism of the Na^+-independent Ca^{2+} efflux system in mitochondria has not been elucidated as yet. With the aid of cyclosporin A, an inhibitor of the Ca^{2+}-induced 'pore', and using a variety of inhibitors, uncouplers and ionophores, it is possible to demonstrate, unequivocally, that this process is driven by ΔpH. The efflux is not affected by $\Delta\psi$, thus suggesting an electroneutral $Ca^{2+}/2H^+$ exchange mechanism. Parallel measurements of the rate of Ca^{2+} efflux and ΔpH, as modulated by valinomycin and nigericin, indicate that the rate of efflux is a function of the magnitude of ΔpH.

Mitochondria; Ca^{2+}; $Ca^{2+}/2H^+$ exchange; ΔpH; Cyclosporin A

1. INTRODUCTION

Mitochondria contain several distinct Ca^{2+} transport systems (for recent reviews see 1,2). The most active system is the Ca^{2+} uniporter which catalyzes electrogenic Ca^{2+} uptake. An electroneutral, Ca^{2+}/Na^+ efflux system, which is activated by external Na^+ is found in mitochondria from most tissues, and is particularly active in brain and muscle mitochondria. In addition, another Ca^{2+} efflux system which is Na^+-independent, appears to exist in mitochondria from all tissues. However, the mechanism of this system, which was originally suggested to be an electroneutral $Ca^{2+}/2H^+$ exchange carrier, has not been elucidated as yet. Despite initial suggestions that Ca^{2+}-efflux is driven by H^+-uptake [3-5], no unequivocal link between Ca^{2+}-efflux and H^+-uptake was established and the predicted dependence of the efflux on ΔpH could not be demonstrated [1,2]. A complicating factor, no doubt, is the existence of an additional, nonspecific, pathway for Ca^{2+} efflux which can be induced in mitochondria under a variety of conditions. High concentrations of Ca^{2+} in the mitochondrial matrix induce an increase in the inner membrane permeability to small solutes which collapses $\Delta\mu_H$ and causes Ca^{2+} efflux. This process which is activated by many ions, substrates and chemicals [1] contributes to the Na^+ independent Ca^{2+} efflux and could not be separated from the putative $Ca^{2+}/2H^+$ exchange. It is not clear as yet whether this process represents the activation of a protein 'pore' [2] or a lipid-mediated leak [1]. Nevertheless, it has been argued that under most Ca^{2+} transport assay conditions, a process of continuous, nonsynchronous, cycle of 'pore' opening, release of matrix-Ca^{2+} content, and a resealing with reuptake of Ca^{2+}, can fully account for the observed Na^+-independent efflux [2]. Thus, according to this view, the putative, specific, Na^+-independent Ca^{2+}-efflux system does not exist at all.

Recently, it was found that the immunosuppressive drug, Cyclosporin A, is a potent inhibitor of the Ca^{2+}-induced 'pore' [6,7]. This finding made it possible to re-investigate the existence and the mechanism of the Na^+-independent Ca^{2+}-efflux system. As shown below, we found that once the Ca^+-induced 'pore' is inhibited by Cyclosporin A, the existence of a specific $Ca^{2+}/2H^+$ exchange system can be demonstrated clearly and unequivocally.

2. MATERIALS AND METHODS

Rat liver mitochondria were prepared by the conventional differential centrifugation method as described [8], except that EGTA (0.1 mM) was included during the washing steps. Rat brain mitochondria were prepared by the conventional ficol gradient method [8], except for the addition of EGTA to the washing steps. Rates of Ca^{2+}-efflux were measured from the absorbance changes (685-675 nm) of the Ca^{2+} indicator Arsenazo III with an Aminco DW2A double-beam spectrometer, as described previously [10]. The basic medium was composed of 0.22 M mannitol, 80 mM sucrose, 1 mM $MgCl_2$, 0.5 mM KCl, 2 mM Tris-P_i and 10 mM Tris-Cl pH 7.4. Rat liver mitochondria (1 mg/3 ml) were incubated with 50 μM Arsenazo III and 2 μM rotenone. The experiment was started by the addition of 5 mM Tris-succinate and 30 nmoles Ca^{2+}. When most of the Ca^{2+} was accumulated from the medium, the efflux was initiated by the addition of 3 nmoles of Ruthenium Red (RR). ΔpH was calculated from the

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distribution of ^{14}C-DMO and $^{3}H_2O$, using ^{14}C-sucrose as a marker for non-matrix pellet space, as described previously [11]. ^{14}C-DMO or ^{14}C-sucrose and $^{3}H_2O$ were added to a suspension of 3 mg protein/ml. Ca^{2+} content was also increased to give 30 nmoles Ca^{2+}/mg protein. The experiment was carried out exactly as in the efflux assay and the mitochondria separated by centrifugation 2 min after addition of the ionophores.

3. RESULTS

To assess the effect of ΔpH and $\Delta\psi$ on the Na^+-independent Ca^{2+}-efflux we tested the effect of inhibitors, uncouplers and inonophores on the rate of Ca^{2+}-efflux. Figure 1 shows the effects of (a) Antimycin A (AA), an inhibitor of electron transport; (b) FCCP, a protonophore; (c) Valinomycin, an ionophore that catalyzes electrogenic K^+ transport; and (d) Nigericin, an ionophore that catalyzes electroneutral K^+-H^+ exchange, on Na^+-induced Ca^{2+}-efflux. Rat liver mitochondria were allowed to accumulate Ca^{2+}, in the presence of 2 mM P_i. P_i greatly reduces the matrix free Ca^{2+}-concentration, thus reducing the role of the Ca^{2+} concentration gradient as a driving force for Ca^{2+}-efflux (see below). Then the electrogenic uptake by the uniporter was inhibited completely by excess Ruthenium red (RR) and Ca^{2+}-efflux (0.8–1.1 nmol/mg protein/min) was observed. In the absence of Cyclosporin A (Fig. 1A), the addition of Antimycin A (trace a) or FCCP (trace b) strongly stimulated the efflux. Valinomycin (trace c) was without significant effect and Nigericin (trace d) inhibited the efflux. Under these conditions, the effects of FCCP, AA and Valinomycin are not consistent with a role for ΔpH as a driving force in Ca^{2+}-efflux, while the Nigericin effect is. This inconsistency is most probably due to the activating effect of uncouplers and electron transport inhibitors on the Ca^{2+}-induced 'pore'. When Cyclosporin A, which inhibits the opening of the 'pore', is included in the incubation medium (Fig. 1B), the results are quite different. Antimycin A, FCCP, and Nigericin all inhibit the efflux. Since Antimycin A and FCCP collapse both ΔpH and $\Delta\psi$, while Nigericin increases $\Delta\psi$ but collapses ΔpH, it is evident that ΔpH drives the efflux while $\Delta\psi$ is without effect. This is further confirmed by the effect of Valinomycin, which in the presence of Cyclosporin A stimulates the efflux. Since Valinomycin collapses $\Delta\psi$ but increases ΔpH, it is apparent that ΔpH drives the efflux while $\Delta\psi$ is without

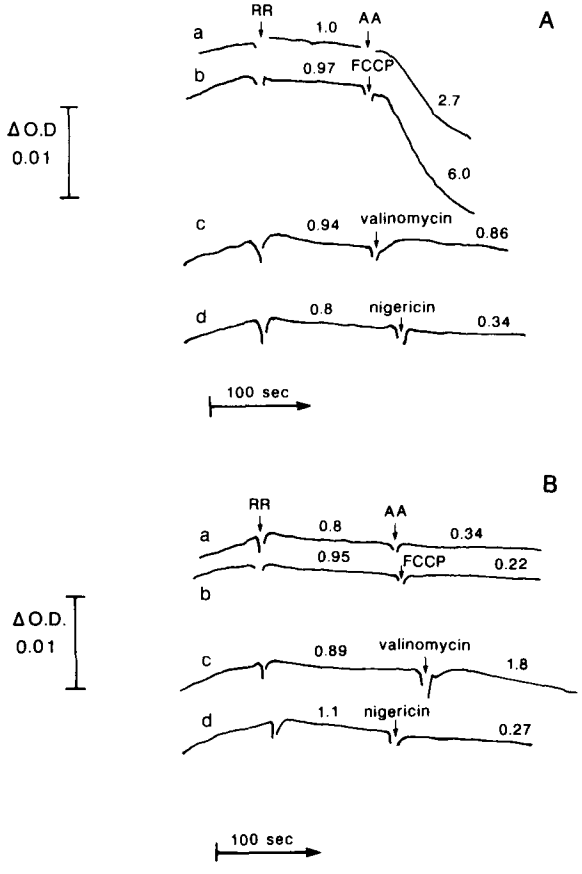

Fig. 1. The effects of ΔpH and $\Delta\psi$ on Na^+-independent Ca^{2+}-efflux. Ca^{2+}-efflux rates were measured as described in Materials and Methods. Part A is without Cyclosporin A and part B is with the addition of 3 μg Cyclosporin A. Trace a, Antimycin A (AA) 30 ng/mg; Trace b FCCP 0.33 μM. Tract c Valinomycin 0.1 μM; and trace d, Nigericin 0.1 μM. Efflux rates (nmol/mg protein/min) are shown above the traces. Rates were calculated from the slopes of the curve 60 s after the addition of reagents. All the results of Fig. 1 are from the same mitochondrial batch.

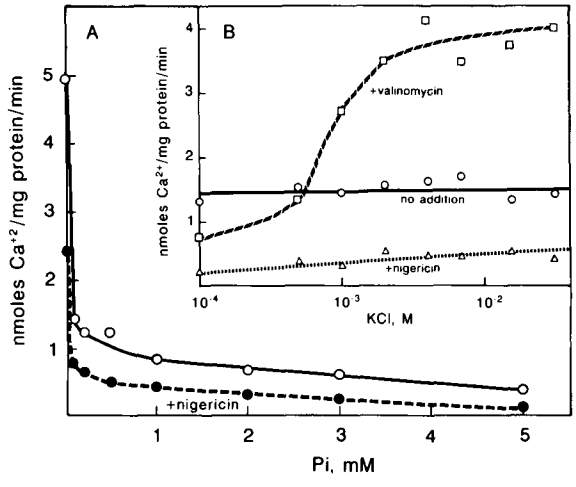

Fig. 2. The effects of P_i (A) and KCl (B) on Na^+-independent Ca^{2+}-efflux. In A, medium and other assay conditions were the same as in Fig. 1 except for the addition of 100 μM ADP and 1 μg/mg Oligomycin. P_i concentration was varied from 0 to 5 mM as indicated. ○, with Cyclosporin A (3 μg/mg); •, with Cyclosporin A and Nigericin (0.1 μM). In B (inset), medium and assay conditions are the same as in Fig. 2A, except for KCl concentrations that were varied from 0 to 32 mM as indicated, and P_i which was 0.2 mM. ○, with Cyclosporin A (3 μg/mg); Δ, with Cyclosporin A and Nigericin (0.1 μM); □ with Cyclosporin A and Valinomycin (0.1 μM). All data points are the averages of 4 rate determinations. The standard errors in these rate determinations were approximately 15%. The experiments of A and B were each performed on the same mitochondrial batch. Similar experiments with other mitochondrial preparations produced similar results.

effect. These results are compatible with the suggestion that the efflux is mediated by a $Ca^{2+}/2H^+$ exchange carrier. Similar results were obtained with brain mitochondria (not shown).

We have previously shown that in brain mitochondria, P_i at very low concentrations (<200 μM), greatly inhibits the Na^+-independent Ca^{2+}-efflux while stimulating the rate of the electrogenic uptake of Ca^{2+}, an effect which was attributed to lowering of the matrix free Ca^{2+} concentration. The lowering of matrix free Ca^{2+} by P_i, greatly reduces the magnitude of the Ca^{2+} concentration gradient, and thus, enhances the role of the pH gradient as a driving force for the efflux. At higher concentration of P_i (0.2–5 mM) further inhibition of Ca^{2+}-efflux was observed and the latter effect correlated with the well known P_i-dependent reduction of ΔpH [12]. Fig. 2A shows that Na^+-independent Ca^{2+} efflux in liver mitochondria in the presence of Cyclosporin A is also inhibited by P_i. The inhibition of efflux at high concentrations of P_i (>0.2 mM) is correlated with the reduction of ΔpH [13]. Nigericin further inhibited the efflux and the combination of high P_i and Nigericin inhibited the efflux almost completely. Similar effects of P_i on Ca^{2+} efflux in liver mitochondria were reported previously [14].

The dependence of the Ca^{2+} efflux on the medium potassium concentrations in control, Valinomycin- and Nigericin-treated liver mitochondria is shown in Fig. 2B. There was no dependence on K^+-concentration in the absence of ionophores. The Valinomycin enhancement of efflux was strongly dependent on external K^+. The enhancement increased with K^+-concentration over the mM range in parallel with the effect of K^+ on ΔpH [15]. The inhibition of efflux by Nigericin was stronger at low K^+ and was diminished at high K^+. These differential effects of K^+ on efflux are correlated with its efect on ΔpH. The Nigericin effect on ΔpH is enhanced by increasing the K_{in}/K_{out} ratio while the Valinomycin effect on ΔpH is enhanced by increasing K_{out}. Similar dependence on KCl of the Valinomycin and Nigericin effects on Ca^{2+}-efflux were also observed in brain mitochondria (not shown).

To obtain a quantitative correlation between the magnitude of ΔpH and the rate of Na^+-independent Ca^{2+}-efflux, we measured in parallel the effect of different concentrations of Nigericin and Valinomycin on the rate of Ca^{2+}-efflux and ΔpH. To maximize the effect of Valinomycin and Nigericin on ΔpH, we used 4 mM KCl in the Valinomycin experiments and 0.5 mM KCl in the Nigericin experiments. Fig. 3 shows that increasing the valinomycin concentration increased ΔpH and Ca^{2+}-efflux rate while increasing Nigericin concentrations reduced ΔpH and inhibited the efflux. Fig. 4 shows the relationship between ΔpH and Ca^{2+}-efflux obtained from the results of Fig. 3 and a similar experiment with a different batch of mitochondria. It is clearly observed that the rate of Ca^{2+}-efflux depends on the magnitude of ΔpH over the entire experimental range. The dependence is particularly strong at high ΔpH where 2 ΔpH >> ΔpCa. At lower value of 2 ΔpH, where ΔpH ≃ ΔpCa, the dependence is more moderate. This observation strongly supports the notion that (a) the Na^+-independent Ca^{2+}-efflux system does exist and (b) the process is catalyzed by an electroneutral $Ca^{2+}/2H^+$ exchange carrier.

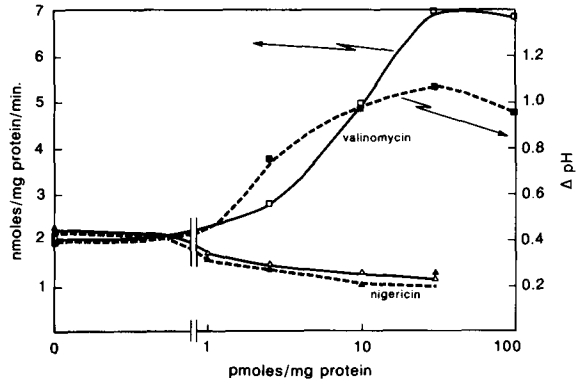

Fig. 3. The concentration dependence of the Valinomycin and Nigericin effects on ΔpH and Ca^{2+}-efflux. Na^+-independent efflux was measured as in Fig. 2B. ΔpH was estimated as described in Materials and Methods. Medium and assay conditions were the same as in Fig. 2B, except that 4 mM KCl was used for the Valinomycin experiments and 0.5 mM KCl was used for the Nigericin experiments. Full triangles (▲) show the effect of Nigericin on ΔpH and empty triangles (△) show the effect of Nigericin on Ca^{2+} efflux. Full squares (■) show the effect of Valinomycin on ΔpH and empty squares (□) show the effect of Valinomycin on Ca^{2+} efflux. Data points of efflux rates are the averages of 4 determinations (standard errors were approximately 15%). Data points of ΔpH determinations are the averages of 3 determinations (standard errors were approximately 10%). All data are from the same mitochondrial batch. Similar experiments with other mitochondrial preparations produced similar results (see Fig. 4).

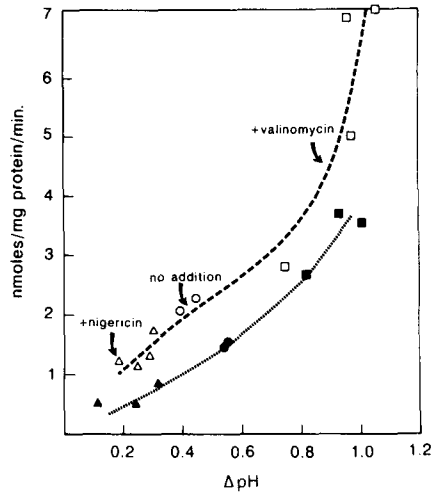

Fig. 4. The relationship between ΔpH and the rate of Ca^{2+}-efflux. Data were taken from Fig. 3 (empty symbols) and from a similar experiment with another batch of mitochondria (closed symbols). Circles (○,●) are controls without ionophores, triangles (▲, △) are with Nigericin and squares (■, □) are with Valinomycin.

4. DISCUSSION

The results of this study clearly demonstrate that the Na^+-independent Ca^{2+}-efflux in mitochondria is an electroneutral process driven by ΔpH. It is necessary to emphasize that this could only be demonstrated when all alternative Ca^{2+} transport systems were blocked. The electrogenic uniporter was blocked by excess Ruthenium red, the $2Na^+/Ca^{2+}$ exchange system was inhibited by eliminating Na^+ from the suspension medium and the Ca^{2+} induced 'pore' was inhibited by excess Cyclosporin A. Mg^{2+} and ADP, both of which also inhibit the 'pore' formation, were also included in most experiments. Although Cyclosporin A does not appear to completely inhibit the phospholipase A_2 mediated permeabilization [1], the latter process is very slow and does not affect the efflux when measured immediately after Ca^{2+} accumulation, as done in our assay. It is not necessary to review here the numerous conflicting studies of Na^+-independent Ca^{2+} efflux of the last decade (reviewed in Refs. 1,2). It is now clear that most of the earlier confusion arises from the inability to separate the $Ca^{2+}/2H^+$ efflux from the Ca^{2+}-induced 'pore'. Cyclosporin A, when used properly, allows complete inhibition of the 'pore' and reveals the existence of the $Ca^{2+}/2H^+$ exchange system. The suggestion that *all* the Na^+-independent Ca^{2+}-efflux is due to a cycle of 'pore' activation and inactivation [2] is shown to be incorrect. However, under a variety of conditions, Cyclosporin A inhibited from 5% to 95% of the Na^+-independent efflux and under some assay conditions the cycle of pore opening and closing appeared to be a significant and even a dominant component of the efflux. In agreement with our conclusion is the demonstration that in the presence of Ruthenium red the equilibrium distribution of Ca^{2+} in liver mitochondria is not altered by the ionophore A23187 (which catalyzes $Ca^{2+}/2H^+$ exchange) [16]. We have previously shown that Nigericin enhances Ca^{2+} retention in liver mitochondria even in the absence of Ruthenium red [17] and this effect too is probably due to inhibition of ΔpH. Because of the inability to demonstrate a flux stoichiometry of $2H^+/Ca^{2+}$ [18,19], alternative mechanisms have been suggested. However, we believe that because of the presence of many, very active, H^+-coupled transport systems in mitochondria, it is not reasonable to expect to be able to measure the slow proton flux of the $Ca^{2+}/2H^+$ exchange system isolated from all other H^+ fluxes as attempted in previous studies. In contrast, the calcium flux of the carrier could be isolated, as demonstrated here, and shown to be driven by the pH gradient.

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