Ca^{2+} EFFLUX FROM LIVER MITOCHONDRIA INDUCED BY A DECREASE IN EXTRAMITOCHONDRIAL pH

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1. Introduction

The uniport system catalyzing uptake of Ca^{2+} down the mitochondrial electrochemical potential gradient has been characterized in mitochondria isolated from a variety of sources [1–3]. In the last four years much evidence has been amassed to support the existence of a second Ca^{2+} carrier that catalyzes an efflux of Ca^{2+} from the mitochondrial matrix against the electrochemical potential gradient [4–5]. Concurrent operation of these two unidirectional pathways is thought to result in continuous slow cycling of Ca^{2+} across the inner membrane. Such a cycle could provide a sensitive mechanism for controlling intra- and extramitochondrial Ca^{2+} activities if the rate of either or both pathways were subject to modulation [6]. In turn, activities of various Ca^{2+}-regulated enzymes in mitochondria and cytosol could be coordinately modified, with significant impact on cellular metabolism [7].

Two different efflux pathways have been shown to accomplish efflux of Ca^{2+} against the electrochemical potential gradient. (The uniport pathway itself catalyzes efflux in de-energized mitochondria, and an efflux route of potential physiological significance must operate against the potential gradient under energized conditions [6]). A Na^+-dependent efflux from heart, skeletal muscle, and brain mitochondria was discovered by Carafoli and coworkers [8–10] and a similar Na^+-dependent mechanism has been demonstrated in brown adipose tissue [11]. A Na^+-independent Ca^{2+} efflux process has been observed in liver mitochondria [7,12,13]. To date, rather little is known about the mechanism and physiological mode of regulation of the Na^+-independent Ca^{2+} efflux pathway. However, the experiments reported here suggest that change in cytosolic pH may be a physiologically important effector of the rate of Ca^{2+} efflux from liver mitochondria.*

Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; MOPS, 4-morpholinepropane-sulfuric acid; P_i, phosphate

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2. Materials and methods

Mitochondria were isolated from livers of fed 250–300 g rats (Flow Laboratories, Dublin, VA) by conventional differential centrifugation [14]. The isolation medium contained 0.250 M sucrose, 5 mM K MOPS buffer, 1 mg/ml BSA and 1 mM K^+ EDTA, at pH 7.2. BSA and EDTA were omitted from the medium employed for washing the mitochondrial pellets and final resuspension of the organelles. Oxygen uptake was measured using a Yellow Springs oxygen monitor with Clark-type electrode and a strip chart recorder. Temperature was maintained at 26°C by a Haake constant temperature circulator. Calcium uptake and efflux were followed using a Ca^{2+}-sensitive electrode (Radiometer F2112 Calcium Selectrode), a Sigma Trizma combination pH electrode as reference and a Corning 110 pH meter linked to a strip chart recorder. Since the millivolt output of the ion-specific electrode is proportional to the logarithm of Ca^{2+} activity, recordings were graphically transformed to obtain linear Ca^{2+} activity scales. Incubation media contained 100 mM sucrose, 80 mM KCl, 22 mM K MOPS, 1 mM K^+P_i and 1 μM rotenone. Succinate was

employed as respiratory substrate. All experiments were repeated several times with different mitochondrial preparations with similar results.

3. Results and discussion

Rat liver mitochondria that have taken up Ca^{2+} in amounts well below those that uncouple respiratory function will initiate net Ca^{2+} efflux upon the addition of a small amount of H^+ to the suspending medium (fig.1). HCl was added in the experiment shown in an amount sufficient to reduce the pH of the suspending medium by 0.1 unit. Equivalent amounts of HCl, H_2SO_4 or HNO_3 produce identical effects (data not shown). The rate of oxygen uptake did not markedly increase upon induction of efflux. Furthermore, subsequent addition of ADP was followed by a normal respiratory spurt and spontaneous return to the controlled state (fig.1, inset) indicating that the electrochemical potential gradient remained intact. The H^+-induced efflux of Ca^{2+} was not inhibited by ruthenium red, specific inhibitor of the Ca^{2+} uniporter, further ensuring that efflux did not occur by reversal of the uptake porter.

The rate of H^+-induced Ca^{2+} efflux is a function of the magnitude of the Ca^{2+} load accumulated by the mitochondria (fig.2). The maximal rate of efflux observed was approximately 60 ngatoms Ca^{2+}/min/mg protein; this value is comparable to the efflux rate

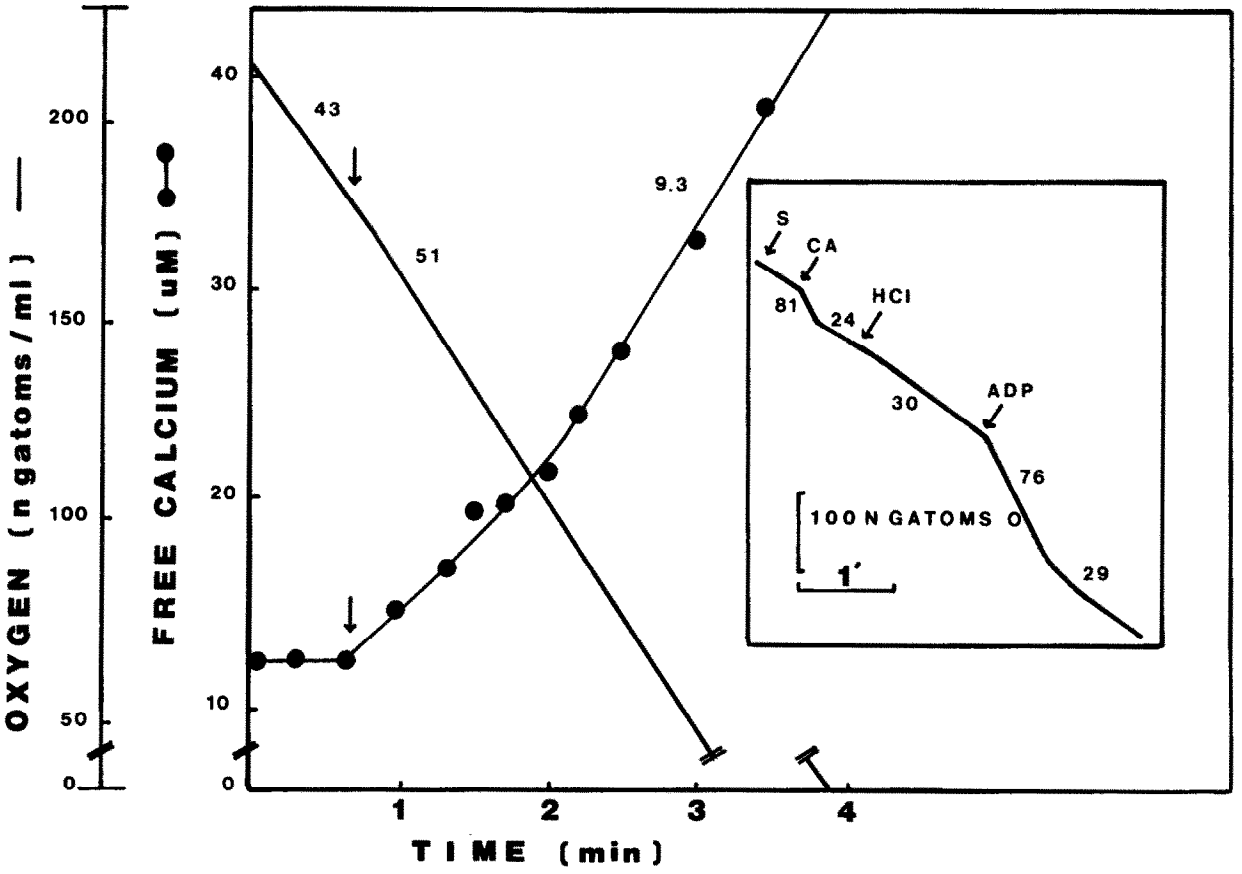

Fig.1. O_2 uptake and Ca^{2+} efflux following addition of H^+ to Ca^{2+}-loaded mitochondria. Data were obtained in parallel experiments. Incubation medium was described in Materials and methods; other conditions as follows: mitochondrial protein, 1 mg/ml; succinate, 11 mM; rotenone, 2 μM; T = 26°C. Mitochondria had accumulated 70 ngatoms Ca^{2+}/mg protein (Ca^{2+} uptake phase not shown) prior to addition of 10 μmol HCl (sufficient to reduce pH from 7.20 to 7.10), where indicated by arrows. Rates of Ca^{2+} efflux and O_2 uptake are indicated by numbers adjacent to traces (units: nagatoms/min/mg protein). (Inset): O_2 uptake observed during Ca^{2+} uptake and H^+ induced efflux, and following ADP addition. Conditions as above except Ca^{2+}, 33 ngatoms/mg protein; ADP, 350 nmol.

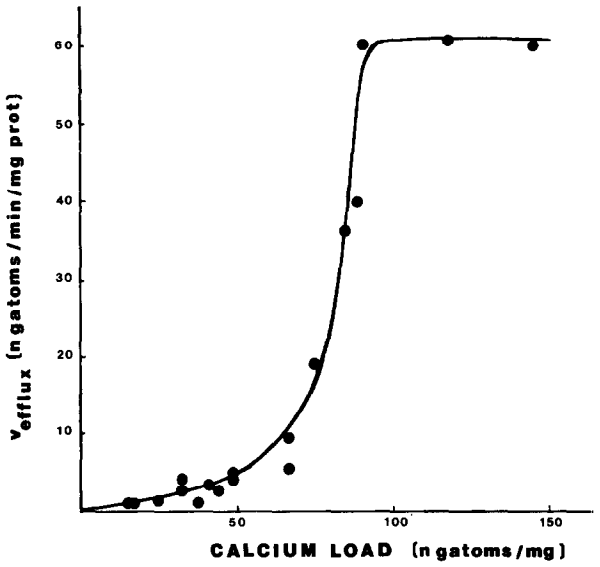

Fig.2. Rates of H^+ induced Ca^{2+} efflux as a function of the previously accumulated Ca^{2+} load. Ca^{2+} uptake was initiated by addition of varying amounts of $CaCl_2$; when uptake was complete, 10 μmol of HCl were added to induce Ca^{2+} efflux. Conditions were otherwise as in fig.1. Points represent data obtained with several different mitochondrial preparations.

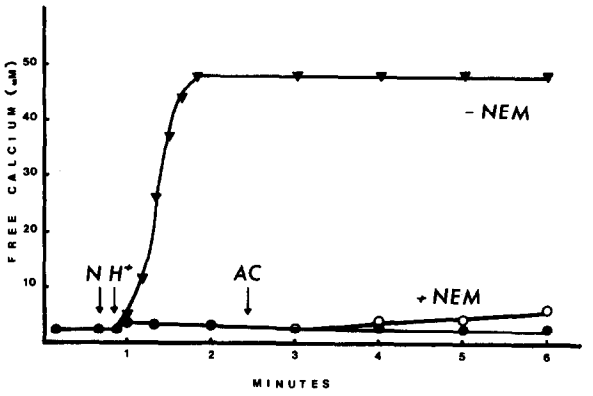

Fig.3. P_i requirement of H^+ induced Ca^{2+} efflux. Mitochondria were allowed to accumulate Ca^{2+} (90 ngatoms/mg protein) and 10 μmol HCl were added to induced efflux as indicated by arrows labeled 'H'. (a) +1 mM P_i, ▼——▼; (b) $-P_i$; or (c) +1 mM P_i +200 μM *N*-ethyl maleimide (added just before HCl at arrow 'N'), ●——●; (d) as in (c) but +5 mM Na^+ acetate added at arrow 'AC', ○——○. Conditions otherwise as described in fig.1.

observed when mitochondria are exposed to an amount of Ca^{2+} just sufficient to cause loss of respiratory control (120–150 ngatoms Ca^{2+}/mg protein). Half-maximal efflux rates were obtained at Ca^{2+} loads of 75–80 ngatoms/mg protein. Replots of the data of fig.2 permit the estimation of a Hill coefficient for the efflux process which is approximately equal to 2.

Induction of efflux by H^+ requires the presence of phosphate. If mitochondria were allowed to accumulate Ca^{2+} in the absence of P_i, addition of HCl, even in larger amounts, failed to evoke significant efflux of Ca^{2+} (fig.3). Similarly, if Ca^{2+} uptake were allowed to occur in the presence of exogenous P_i (0.1–2 mM), and then phosphate movements were blocked by the addition of *N*-ethyl maleimide, inhibitor of the P_i/OH^- antiporter, H^+ addition failed to induce Ca^{2+} efflux (fig.3). If acetate (1–5 mM) was added to the *N*-ethyl maleimide-inhibited mitochondria, to provide an alternate co-anion possibly needed for Ca^{2+} efflux, only a very slow efflux was observed. These findings suggest that phosphate itself, and not just some permeant co-anion, is a requirement for the H^+-induced Ca^{2+} efflux.

Lehninger and coworkers have presented evidence for a Ca^{2+} efflux pathway that they postulate to occur via Ca^{2+}/H^+ antiport and to be regulated by the redox state of the mitochondrial pyridine nucleotides [12, 15]. Pedersen and coworkers have studied Ca^{2+} uptake by inside-put inner membrane vesicles [16]. The uptake into these vesicles is probably synonymous with the normal Ca^{2+} efflux pathway in liver mitochondria. They established a requirement for prior or simultaneous uptake of phosphate into the vesicles before Ca^{2+} uptake could be observed, and acetate was unable to substitute for phosphate [16] as in the work reported here. The H^+-induced Ca^{2+} efflux we have observed probably occurs by the same pathway detected by these workers.

It is of considerable interest that a small (0.1 pH unit) decrease in the pH of the extramitochondrial medium is able to induce efflux of Ca^{2+} even at small intramitochondrial levels. Many physiological phenomena are controlled at least in part by changes in cytosolic (or mitochondrial) Ca^{2+} concentrations [17]. For example, the glycogenolytic response of liver cells to alpha-adrenergic agonists involves a rise in cytosolic Ca^{2+} that is correlated with a decrease in the mitochondrial Ca^{2+} pool [18,19]. The second messenger necessary to convey the stimulus from plasma membrane to mitochondrial inner membrane is unknown, but changes in other ion gradients, including pH gradients, have been proposed to be involved in a number of Ca^{2+}-dependent regulatory phenomena in various

tissues [7,20]. Stimulation of Ca^{2+} efflux from mitochondria by a small decrease in intracellular pH might well be one of the simplest physiological means of regulating that component of the Ca^{2+} cycle.

Experiments designed to characterize the Ca^{2+} efflux induced by H^+ by comparison to: (1) the Ca^{2+} efflux observed when the uniporter is blocked by ruthenium red, and (2) the efflux occurring in conjunction with Ca^{2+} uncoupling of mitochondria will be reported elsewhere.

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