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Na^+/H^+ exchange in mitochondria as monitored by BCECF fluorescence

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The recently developed method of loading isolated heart mitochondria with the fluorescent pH indicator, BCECF, was applied to monitor the Na^+_o/H^+_i exchange process from the matrix side of the membrane. The Na^+-induced changes in the pH of the matrix (pH_m) showed that: (i) the Na^+_o/H^+_i exchange followed Michaelis-Menten kinetics with respect to external Na^+ with a K_m of approx. 20 mM; (ii) in contrast to this, the dependence of the exchange rate on the matrix $[H^+]$ did not obey the Michaelian model. No Na^+-induced alkalinization occurred above a pH_m of 7.45 ± 0.09 ($n=4$). Below this value the reciprocal of the transport rate and that of the matrix $[H^+]$ deviated upwardly from the straight line. The results suggest that internal H^+ might exert allosteric control on the mitochondrial Na^+/H^+ exchange process.

Na^+/H^+ exchange; Matrix pH; Biscarboxyethylcarboxyfluorescein; (Mitochondria)

1. INTRODUCTION

The mitochondrial inner membrane contains a Na^+/H^+ exchange route [1-3]. Investigation of this transport pathway revealed that both external Na^+ [3,4] and Li^+ [4] was exchanged for internal H^+ according to simple Michaelis-Menten kinetics. External H^+ inhibited the exchanger acting at a single binding site [4]. Certain derivatives of amiloride were shown to be potent inhibitors of the antiporter [4,5]. Further characterization of the exchange process has been hindered by the lack of appropriate method for studying the interaction between matrix protons with the exchanger. The fact that internal H^+, beside being a substrate, acts as a regulator of the Na^+/H^+ exchanger of several bacterial and animal cell membranes [6-9] prompted us to investigate this problem also in the case of mitochondria.

Our method was based on recent findings obtained in this and other laboratories that isolated mitochondria could be loaded with the lipid-soluble forms of certain Ca^{2+} and H^+-sensitive fluorescent dyes [10-15]. After hydrolytic cleavage of the ester bonds within the matrix, the indicator molecules (quin2, fura-2, indo-1 for Ca^{2+} and BCECF for H^+) became entrapped and reflected the intramitochondrial activities of the corresponding ions under various metabolic conditions.

We report here, that fluorescence measurements performed on BCECF-loaded mitochondria proved to be suitable for the time resolved monitoring of the rapid Na^+_o/H^+_i antiport and the determination of its dependence on pH_m. The results suggest that the mitochondrial Na^+/H^+ exchanger might be modulated by the intramitochondrial pH.

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Abbreviations: BCECF/AM, the acetoxymethylester form of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein; CCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; pH_m, pH of the intramitochondrial matrix; $\Delta\psi$, mitochondrial membrane potential; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediaminedehydrochloride; TPP^+, tetraphenylphosphonium

2. MATERIALS AND METHODS

Rat heart mitochondria were prepared as in [10].

Mitochondria (approx. 50 mg protein/ml) were incubated in the presence of 10 μM BCECF/AM for 20 min at 20°C. Dye-

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loaded mitochondria were then diluted 20-fold with the ice-cold final preparation medium, (75 mM sucrose, 225 mM mannitol, 0.4 mM Tris/EGTA, 0.4 mM K^+-EGTA, 5 mM Tris/Cl, 0.2 mg albumin/ml, pH 7.4) recentrifuged and resuspended to obtain the stock suspension (40–60 mg/ml). Mitochondria (0.8–1 mg) were suspended in 2 ml of basic medium containing 100 mM KCl, 80 mM sucrose, 10 mM K^+-Mops, 2.5 μg/ml oligomycin, 1 μM rotenone, pH 7.0. Fluorescence was monitored with a Jobin-Yvon JY3 spectrofluorimeter at room temperature. The excitation and emission wavelengths were 500 nm (4 nm slit) and 530 nm (4 nm slit), respectively. Calibration of fluorescence in terms of matrix pH (pH_m) was carried out similarly as described in [15] by dissipation of the transmembrane pH gradient with the simultaneous addition of the K^+/H^+ exchanger, nigericin (0.5 μg/ml) and the protonophore, CCCP (1 μM). The pH and the fluorescence of the suspension were determined in parallel after sequential additions of several pulses of 2 N KOH or HCl. This 'in situ' calibration was chosen because the pK of BCECF was found to be different in the mitochondrial matrix and in an external buffer [15]. The signal was corrected for the fluorescence present in the supernatant after sedimentation of mitochondria (approx. 15% of the fluorescence of the Triton-treated sample). This externally located dye escaped from the mitochondria probably during the resuspension procedure, because it was present from the very beginning of the experiments. Further leakage of the dye from mitochondria kept on ice was negligible up to 3 h. The relation between fluorescence and pH_m was strictly linear in the pH range of 6.6–7.8 ($r > 0.99$ in all experiments).

Na^+-induced acidification of the extramitochondrial compartment was followed in a Mops-free basic medium by a Radiometer pH-electrode, as detailed in [4].

The membrane potential was measured by a TPP^+-sensitive electrode as in [16].

3. RESULTS AND DISCUSSION

3.1. *The use of BCECF for measuring intramitochondrial pH (pH_m)*

The generation of the fluorescent free BCECF from its non-fluorescent acetoxymethylester form can be followed by the spectra of Triton-solubilized mitochondria run after different incubation times (fig.1A). When dye-loading was carried out at 20°C, after 20 min (the incubation period applied in most experiments) the BCECF content of mitochondria was 90.9 ± 21.7 pmol/mg protein ($n = 7$), corresponding to approx. 50% conversion of the total BCECF/AM added. The process reached saturation over 60 min (fig.1B). After washing and resuspension, no hydrolyzable BCECF/AM remained in the samples as the fluorescence of mitochondria was stable over 10 min even at room temperature (not shown).

The initial pH_m of non-respiring mitochondria incubated in a medium of pH 7.0 was 6.86 ± 0.11

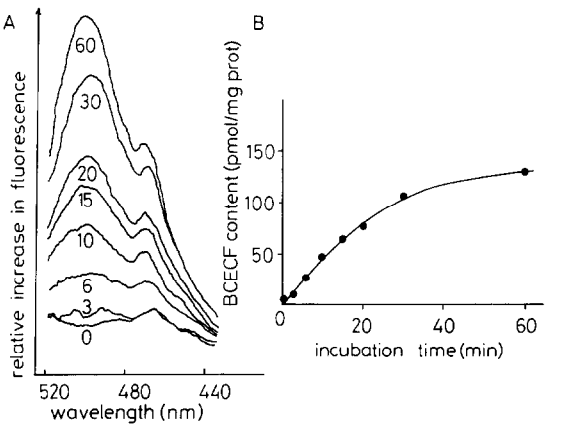

Fig.1. Generation of BCECF from its acetoxymethylester form by rat heart mitochondria. Heart mitochondria were incubated with 10 μM BCECF/AM for different times indicated under the spectral lines. Aliquots of 1 mg mitochondrial protein were then transferred to 2 ml of the basic medium supplemented with 0.063% Triton X-100. (A) Excitation spectra were recorded at an emission wavelength of 530 nm. (B) Time dependence of the formation of BCECF. Absolute amount of the dye in the samples was determined by comparing the fluorescence of incubated and lysed mitochondria with known amount of free BCECF in the presence of lysed unloaded mitochondria.

($n = 12$). On addition of 5 mM succinate the pH_m increased and reached a steady-state after 6–7 min at 7.71 ± 0.08 ($n = 10$). Antimycin A (1 μg/ml) prevented the effect of succinate. Ascorbate (5 mM) plus TMPD (0.125 mM) induced a matrix alkalinization of similar magnitude but the response was more rapid, reaching its maximum after 2 min. Inorganic phosphate (5 mM), due to its symport with H^+, reduced the succinate-caused alkalinization to 7.18 ± 0.05 ($n = 3$). The inhibition of P_i transport by mersalyl reestablished the alkalinization. The fact that these values are both qualitatively and quantitatively in good agreement with previous measurements [13,17] points to the validity of our calibration procedure.

3.2. *Na^+-induced changes in the matrix pH of BCECF-loaded mitochondria*

The addition of Na^+ caused a rapid alkaline shift in pH_m of non-respiring mitochondria, the extent of which was proportional to the applied Na^+ concentration. For measurement of true initial rate of pH_m changes Na^+ was injected into the cuvette through a thin plastic tube without the interruption of the recording. (see fig.2a). The initial rate of

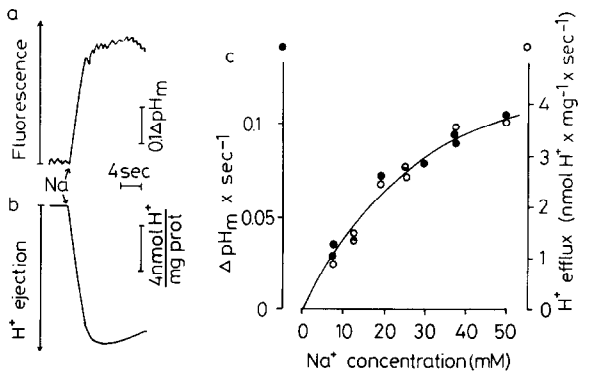

Fig.2. Parallel measurement of Na^+-induced changes in matrix pH and H^+ efflux from BCECF-loaded mitochondria. In a, 1 mg of mitochondrial protein was suspended in 2 ml of the basic medium. In b, 3 mg mitochondrial protein was suspended in 2 ml of Mops-free basic medium and the pH was adjusted to 7.0. Where indicated 20 μl of 5 M NaCl (50 mM) was added and changes in fluorescence (a) or in medium pH (b) were recorded. The initial pH_m was 6.95. In c, the initial rate of the changes in pH_m (●) and that of H^+ release (○) are plotted as the function of the applied Na^+ concentrations.

changes in pH_m and of appearance of H^+ extramitochondrially from BCECF-loaded mitochondria were determined in parallel (fig.2, traces a and b). (The latter was measured as the Na^+-induced acidification of the Mops-free basic medium.) Fig.2 shows that the $[Na^+]$-dependence of these two responses run in parallel, yielding a K_m of about 20 mM for Na^+. This value is in good agreement both with our previous measurements in intact mitochondria [4], and the K_m found in submitochondrial particles [5,18]. Furthermore, the parallel monitoring of changes in pH_m and the amount of ejected H^+ allowed us to estimate the internal buffering power of rat heart mitochondria. This was found to be 38.9 ± 8.4 nmol H^+/pH unit per mg protein (n = 3), which is very close to the value (30–40 nmol/pH U per mg) obtained with less direct methods in liver mitochondria [17]. (Buffering by BCECF itself must have been negligible as its matrix concentration was below 0.1 mM.)

In order to investigate the pH_m dependence of the Na_o^+/H_o^+ transport we had to manipulate pH_m without drastic alteration of the buffering power of the matrix. This was accomplished by addition of different amounts of antimycin A (0–400 ng/mg protein) to mitochondria respiring on succinate. After achieving a steady-state value of pH_m, i.e. when the fluorescence signal was stable over 1 min, 50 mM Na^+ was added and the initial rate of the shift in pH_m was recorded. (The addition of 50 mM Cholin-Cl instead of NaCl did not cause any change in the fluorescence showing that elevation of the osmotic concentration did not interfere with the pH_m measurement.) Fig.3. shows the relationship between pH_m and the initial rate of pH_m changes after the addition of Na^+. The correlation reveals two remarkable characteristics: (i) no Na^+-induced pH_m change occurred above a pH_m value of 7.45 ± 0.09 (n = 4); (ii) the Lineweaver-Burk plot of the data deviated from the straight line indicating that the decrease in the initial rates of pH_m changes with decreasing matrix $[H^+]$ was greater than predicted on the basis of a simple Michaelis-Menten kinetics (see inset).

As an explanation for the above observation the following possibilities were considered.

(i) Under the present experimental conditions (pH_o = 7.0, pH_m = 7.5, $[Na]_o$ = 50 mM) Na_o^+/H_i^+ exchange is thermodynamically restricted. To test this possibility the pH of the medium was raised to 7.5. However no alkaline shift in pH_m could be evoked by the addition of Na^+ if pH_m of respiring mitochondria reached or exceeded 7.4. It should be noted that in this case both Na^+ and H^+ gradients favoured Na_o^+/H_i^+ exchange.

(ii) The membrane potential ($\Delta\psi$) formed in respiring mitochondria opposes Na_o^+/H_i^+ exchange. This possibility had to be investigated carefully as in the experiment represented in fig.3 concomitant with the lowering of pH_m also the $\Delta\psi$ was decreased by antimycin A. However, the following finding is against the determinative role of high $\Delta\psi$ in the cessation of Na_o^+/H_i^+ exchange. Mitochondria were allowed to respire on succinate in a medium of pH 7.5 until their pH_m became equal to that of the external space. At this point sufficient antimycin A was added to collapse $\Delta\psi$ (as verified by the TPP^+-electrode) while their pH_m remained unchanged. Subsequent addition of Na^+ resulted only in negligible or no change of pH_m at all. The role of the $\Delta\psi$-driven Ca^{2+} uptake was also excluded, since its inhibition by Ruthenium Red did not alter the observed phenomena.

(iii) High pH_m favours the entry of Na^+ via other transport pathway(s) than the Na^+/H^+ exchanger. Participation of the only well-characterized Na^+ transporter, the Na^+/Ca^{2+} exchanger is highly im-

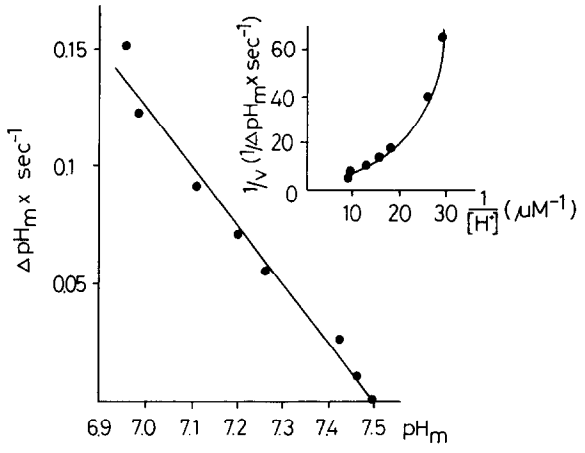

Fig.3. Relationship between pH_m and the initial rate of Na_o^+/H_i^+ exchange. Mitochondria (0.5 mg/ml) were incubated in the basic medium (pH 7) supplemented with 5 mM K^+-succinate. After achieving the maximal fluorescence, different amounts of antimycin A (0–400 ng/mg protein) were added to set pH_m at various values. After obtaining the new steady-state pH_m, 50 mM NaCl was rapidly injected. (Inset) The same data represented as a Lineweaver-Burk plot.

probable as (a), its transport rate is considerably slower [3], and (b), its inhibition by 100 μM $BaCl_2$ [19] had no effect on the pH_m dependence of the Na_o^+/H_i^+ exchange (not shown). Another possibility is the opening of a Na^+ uniport pathway at higher pH_m. However this explanation seems to be unlikely because the addition of 50 mM Na^+ did not increase the rate of succinate-induced O_2-consumption and did not cause any measurable membrane depolarization as followed by the TPP^+-selective electrode.

(iv) In the light of these considerations the above results suggest that the mitochondrial Na^+/H^+ exchanger can be modulated by internal H^+ in a similar way as the plasma membrane antiporter of several cell types: protonation/deprotonation of an H^+-binding site facing to the matrix side might exert allosteric control on the transport pathway. This could provide an explanation for the hindrance of Na_o^+/H_i^+ exchange at high pH_m.

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