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THE ROLE OF MITOCHONDRIAL POTASSIUM FLUXES IN CONTROLLING THE PROTONMOTIVE FORCE IN ENERGIZED MITOCHONDRIA

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The two components of the protonmotive force, the pH gradient (ΔpH) and the transmembrane electric potential ($\Delta\psi$), were measured in rat liver mitochondria as a function of K^+ concentration in the suspending medium. It was found that both the rate of formation and the final level of ΔpH upon energization of mitochondria with succinate increased with increasing $[K^+]$. Concomitantly, $\Delta\psi$ decreased so that the level of the protonmotive force remained practically unchanged. Potassium channel opener RP66471 further potentiated both the formation rate and the level of ΔpH. These results are interpreted as showing that the electrophoretic K^+ influx enables the formation of ΔpH by partly compensating charge transfer due to the proton pumping.

Respiring mitochondria eject H^+, thus forming both the transmembrane electric potential ($\Delta\psi$, negative inside) and the pH gradient (ΔpH, alkaline inside). These two gradients together constitute the electrochemical proton gradient (protonmotive force, Δp) which, in line with the chemiosmotic theory (1), provides energy for mitochondrial ATP synthesis. It can be calculated from the electrical capacitance of the inner mitochondrial membrane and the buffering capacity of the inner compartment that the net transfer of 1 nmol H^+/mg mitochondrial protein establishes a $\Delta\psi$ of about 200 mV, whereas it increases intramitochondrial pH by as little as 0.05 pH unit (2). Yet, fully energized mitochondria from most mammalian tissues exhibit $\Delta\psi$ of about 170 mV and ΔpH of about 0.5 unit (2). This implies that redistribution of ions other than H^+ must also take place during mitochondrial energization. Since anions can penetrate the inner mitochondrial membrane essentially in form of the electroneutral anion/anion exchange, this role must be fulfilled by membrane-permeant cations. Although Ca^{2+} can easily cross

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the inner mitochondrial membrane (3), the concentration of free Ca^{2+} in the cytoplasm of the order of 10^{-7} M is too low to account for an appreciable neutralization of $\Delta\psi$ and the concomitant increase of ΔpH. Hence, the most likely candidate for such a function is K^+ whose cytoplasmic concentration is 0.1 M.

It is well established that K^+ can penetrate the inner mitochondrial membrane both as the electroneutral K^+/H^+ exchange and the electrophoretic K^+ influx (4-6). The present study shows that, in fact, electrophoretic K^+ fluxes are likely partly to compensate electric charge transfer produced by the respiratory chain-driven proton pumps, thus promoting the formation of ΔpH.

MATERIALS AND METHODS

Liver mitochondria were isolated from Wistar male rats by the conventional procedure (7) in 75 mM sucrose, 225 mM mannitol, 3 mM Tris-HCl (pH 7.4) and 1 mM EGTA.

The incubation medium contained a mixture of various proportions of 200 mM sucrose and 100 mM KCl containing 0.5 mM EGTA and buffered with 10 mM Tris-HCl (pH 7.4).

Intramitochondrial pH was monitored with the fluorescent pH indicator 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (8). Mitochondria (25-30 mg protein/ml) were loaded with the probe by preincubation in the isolation medium containing 10 μM BCECF acetoxymethyl ester (BCECF/AM) and subsequent washing as described previously (9) except that the preincubation temperature and time were 25°C and 30 min, respectively. Fluorescence was measured in Shimadzu (Kyoto, Japan) model RF-5000 spectrofluorimeter using two excitation wavelengths of 450 and 500 nm and the emission wavelength of 530 nm. Emission ratio at these two excitation wavelengths was a measure of intramitochondrial pH. The system was calibrated by clamping internal pH with the external one by adding nigericin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) in the medium containing 100 mM KCl and titrating medium pH by stepwise additions of small amounts of KOH as exemplified in Fig. 1. External pH was measured with a glass electrode.

$\Delta\psi$ was determined with tetraphenylphosphonium (TPP^+)-sensitive electrode (10). The potentiometer readings were corrected for TPP^+ binding according to Rottenberg (11) as modified by Żółkiewska et al. (12). To ascertain screening of the surface potential of external leaflets of the membranes also in low K^+ media 2 mM $MgCl_2$ was included in all measurements of $\Delta\psi$.

All measurements were carried out at 25°C.

BCECF/AM was obtained from Molecular Probes (Eugene, Oregon, U.S.A.) and potassium channel opener (1S,2R)-trans-2-benzoyloxy-1-(pyrid-3-yl)cyclohexane-(N-methyl)-carbothioamide (RP66471) from Rhône-Poulenc Rorer (Dagenham, U.K.). RP66471 was used as 100 mM stock solution in dimethylsulfoxide.

RESULTS

When rat liver mitochondria were de-energized by incubation with rotenone, inhibitor of the respiratory chain at the level of NADH dehydrogenase, ΔpH decreased

within a few minutes to the value close to zero. A subsequent addition of succinate (the oxidation of which is not inhibited by rotenone) produced energization of mitochondria which was manifested by an increase of ΔpH as shown by the increase of the BCECF signal (Fig. 1). It appeared that both the initial rate of ΔpH formation and the steady-state level of ΔpH which was attained after a few minutes following energization were increased with increasing [K^+]. The plot of the rate of ΔpH increase *versus* K^+ concentration did not attain saturation up to 100 mM KCl (Fig. 2). It can be deduced from Fig. 2 that K^+ concentration resulting in half-maximal increase of the rate in this [K^+] range (apparent K_{50}) was about 30 mM. The steady-state level of ΔpH was also increased with increasing [K^+] (Fig. 3).

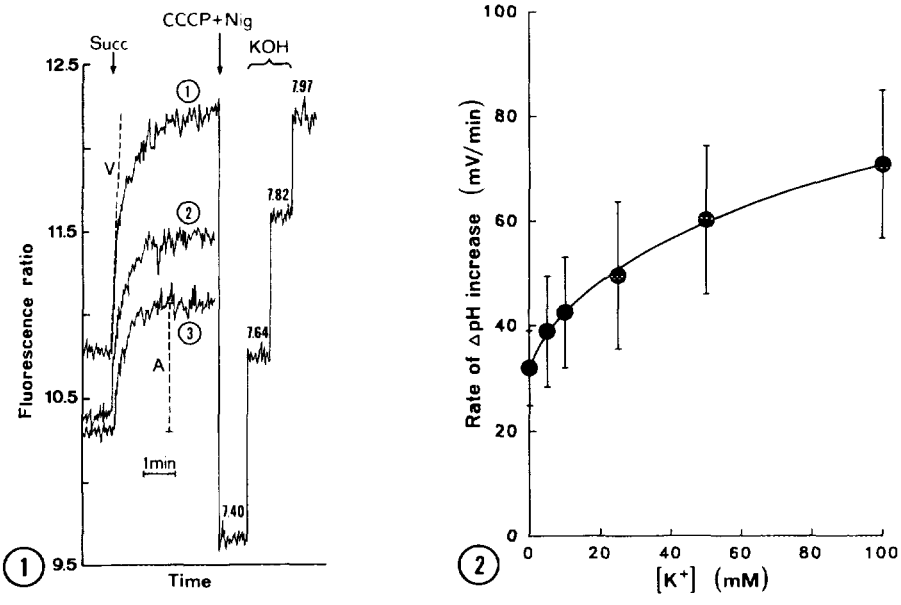

Fig. 1. Formation of ΔpH upon energization of mitochondria. Fluorimeter cuvettes contained 3.0 ml of a mixture of 100 mM KCl and 200 mM sucrose plus 0.5 mM EGTA and 10 mM Tris-HCl (pH 7.4) to obtain K^+ concentrations of 100, 10 and 2.5 mM in traces 1, 2 and 3, respectively, and mitochondria corresponding to 0.6-0.8 mg protein. Rotenone was added to 1.7 μM final concentration and oligomycin to 1.7 μg/ml. After 3-5 min mitochondria were energized by addition of 2.5 mM Tris-succinate (Succ). When the ΔpH increase was completed, CCCP and nigericin (Nig) were added to final concentrations of 0.8 and 0.4 μM, respectively, and the system was calibrated by additions of 5 μl aliquots of 1 M KOH. The numbers at the trace (exemplified for one trace only) indicate pH values measured with glass electrode. Dashed lines: V, initial rate of ΔpH formation; A, final increase of ΔpH.

Fig. 2. Dependence of the rate of ΔpH formation upon K^+ concentration. ΔpH is expressed in mV (one pH unit = 59 mV) and was calculated as illustrated in Fig. 1 (dashed line V and the calibration trace). All conditions are as in Fig. 1. The points and the bars represent mean values ± S.E. for 3 mitochondrial preparations.

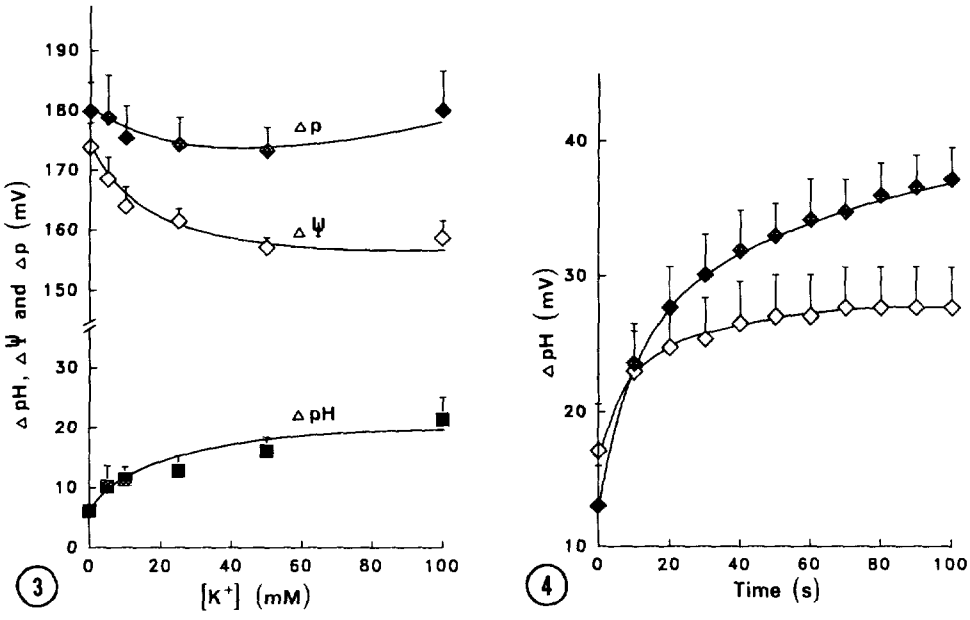

Fig. 3. Effect of K^+ concentration on steady-state values of ΔpH (dashed bar A of Fig. 1), the transmembrane electric potential ($\Delta\psi$) and the total protonmotive force (Δp). Experimental conditions were as described under Materials and Methods and in the legend to Fig. 1. Mean values ± S.E. for 3-4 mitochondrial preparations are presented.

Fig. 4. Effect of potassium channel opener RP66471 on the formation of ΔpH upon energization of mitochondria. Experimental procedure was as described in Fig. 1. The medium contained 5 mM K^+. Energization with succinate is indicated here as the zero time. Open diamonds, control without RP66471; closed diamonds, 100 μM RP66471 was added to the cuvette 1 min prior to energization.

The electric component of the protonmotive force ($\Delta\psi$) changed in the opposite way, i.e., it slightly but consistently decreased with increasing K^+ concentration so that the sum of ΔpH and $\Delta\psi$ remained essentially constant (Fig. 3).

A powerful opener of ATP-regulated potassium channel of the plasma membrane, RP66471 (13), increased the rate and the amplitude of ΔpH formation upon energization of mitochondria. This is clearly visible in Fig. 4 where RP66471 was added to mitochondria prior to the energization, but could also be observed as an upward deflection of the fluorescence trace when the opener was added to fully energized mitochondria (not shown). This effect of RP66471 could not be observed in sucrose medium containing no K^+ (not shown).

DISCUSSION

As demonstrated previously (14,15), energization of mitochondria is accompanied by a net uptake of K^+ whereas de-energization in low-potassium media promotes a net efflux. This is compatible with the hypothesis put forward in the Introduction that potassium uptake upon energization partly compensates electric charge transfer produced by the proton pump and thus enables the formation of ΔpH along with $\Delta\psi$. This is further substantiated by the present observation that the rate of ΔpH formation increases with increasing K^+ concentration in the external medium (Fig. 2) and thus with increasing rate of K^+ influx. The final steady-state value of ΔpH also increases whereas that of $\Delta\psi$ decreases at increasing $[K^+]$ so that the resultant protonmotive force remains practically unchanged (Fig. 3).

It was shown previously (14) that the electrophoretic K^+ uptake by mitochondria was partly inhibited by glibenclamide and activated by well known openers of the plasma membrane potassium channel, pinacidil and P1060. On that basis we concluded that this uptake might reflect operation of a potassium channel belonging to the family of ATP-regulated K^+ channels identified in the plasma membrane of cardiac, smooth and skeletal muscles and pancreatic β-cells (16-19). Occurrence of an ATP-regulated K^+ channel in the inner mitochondrial membrane was first described by Inoue et al. (20). This channel was subsequently isolated, partially purified and reconstituted into liposomes by Garlid and his co-workers (21,22) (for review see also Ref. 23). It seems therefore likely that the same channel may function in mediating electrophoretic K^+ fluxes, partly compensating $\Delta\psi$ during mitochondrial energization. Interestingly, the half-saturation value (apparent K_{50}) calculated from the present experiments for increasing the rate of ΔpH formation is about 30 mM K^+ which is almost identical with the K_m of 32 mM of the mitochondrial ATP-regulated potassium channel isolated and reconstituted into liposomes by Paucek et al. (21).

The assumption that K^+ transport accounts for the formation of ΔpH is also supported by the observation (Fig. 4) that both the rate of ΔpH formation and its steady-state level in energized mitochondria are increased by a potent opener of ATP-regulated K^+ channel, RP66471. As shown previously (24), this compound decreased $\Delta\psi$ of energized liver mitochondria by increasing the permeability of the inner mitochondrial membrane to K^+. That both effects are due to activation of a channel rather than to an

ionophoretic action is indicated by the fact that RP66471 does not increase potassium permeability of phospholipid planar membranes (24).

The rate constant for the electrophoretic K^+ influx of 0.11 $nmol \times min^{-1} \times mV^{-1} \times mg\ protein^{-1}$, as calculated previously (15), combined with the uptake of an undissociated penetrant acid, might result in an increase of intramitochondrial osmolarity by about 20 mOsM within one minute in fully energized organelles ($\Delta\psi$ of 190 mV). This is obviously prevented by the electroneutral K^+/H^+ exchange (25). As result, a futile K^+ cycling occurs which may partly account for the resting state respiration of mitochondria (see discussion in Ref. 15).

In conclusion, the present results show that K^+ fluxes in mitochondria are involved in the formation of ΔpH. Their likely sensitivity to ATP may open a way for speculation on how the proportion between the two components of Δp in energized mitochondria, i.e., $\Delta\psi$ and ΔpH, is regulated by intramitochondrial level of ATP or the ATP/ADP ratio.

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