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CHANGES IN MEMBRANE POTENTIAL DURING CALCIUM ION INFLUX AND EFFLUX ACROSS THE MITOCHONDRIAL MEMBRANE

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Summary

1. A depolarisation of the membrane of rat liver mitochondria, as measured with the safranine method, is seen during Ca^{2+} uptake. The depolarisation is followed by a slow repolarisation, the rate of which can be increased by the addition of EGTA or phosphate.

2. Plots relating the initial rate of calcium ion (Ca^{2+}) uptake and the decrease in membrane potential ($\Delta\psi$) to the Ca^{2+} concentration show a half-maximal change at less than 10 μM Ca^{2+} and a saturation above 20 μM Ca^{2+}.

3. Plots relating the initial rate of Ca^{2+} uptake to $\Delta\psi$ are linear.

4. Addition of Ca^{2+} chelators, nitriloacetate or EGTA, to deenergized mitochondria equilibrated with Ca^{2+} causes a polarisation of the mitochondrial membrane due to a diffusion potential created by electrogenic Ca^{2+} efflux.

5. If the extent of the response induced by different nitriloacetate concentrations is plotted against the expected membrane potential a linear plot is obtained up to 70 mV with a slope corresponding to two-times the extent of the response induced by valinomycin in the presence of different potassium ion gradients. This suggests that the Ca^{2+} ion is transferred across the membrane with one net positive charge in present conditions.

Introduction

The driving force of mitochondrial Ca^{2+} transport seems to be a membrane potential with negative intramitochondrial polarity [1—4], which drives the cation electrophoretically into the mitochondrial inner aqueous compartment. Evidence in favour of such a mechanism of transport is that the process can

Abbreviations: MalNEt, *N*-ethylmaleimide; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HOQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide.

also be driven by an artificial K^+ diffusion potential across the membrane created by the ionophore valinomycin [5]. This conclusion is also supported by the fact that Ca^{2+} is passively transferred as a charged species across the mitochondrial membrane in deenergized conditions [2,6]. Determinations of the intra- and extramitochondrial Ca^{2+} and K^+ contents in the presence of valinomycin during respiration suggests that Ca^{2+} is transferred across the mitochondrial membrane with two net positive charges [3]. However, the intramitochondrial Ca^{2+} seems to be bound mainly to phosphate [7] or some other Ca^{2+}-binding substances [8], which casts some doubt on such Ca^{2+} distribution measurements. In some conditions 97% of the intramitochondrial Ca^{2+} appears to be bound [9]. Different Ca^{2+} and K^+ gradients are also obtained in Ca^{2+} distribution experiments with different concentrations of the cations [10]. Furthermore, if Ca^{2+} is translocated with two net positive charges very high gradients of the cation would be created across the mitochondrial membrane as pointed out by Crompton and Carafoli [11] unless an electroneutral Ca^{2+} release mechanism discharges the cation when the intramitochondrial Ca^{2+} concentration rises [9]. Indeed, in certain conditions exchange [12] and cycling [13] of Ca^{2+} occurs across the mitochondrial membrane. Recently a partially electrogenic Ca^{2+} transport model has been suggested [14,15], according to which Ca^{2+} is translocated singly-charged [14] by a MalNEt (N-ethylmaleimide)-insensitive Ca^{2+}/phosphate symporter [15]. Also, an active Ca^{2+} extrusion mechanism in series with the electrogenic uptake mechanism has been proposed [9]. As yet the evidence in favour of an electrogenic Ca^{2+} uptake mechanism has been obtained indirectly [1—4]. Recently has been described a method which enables direct measurement of changes in mitochondrial membrane potential [16]. Thus, the aim of present work was to determine changes in the membrane potential of rat liver mitochondria during Ca^{2+} fluxes across the membrane in order to obtain more information about the driving force and molecular mechanism of Ca^{2+} transport.

Methods and Materials

Rat liver mitochondria were prepared from young male Sprague Dawley rats by a conventional method [17]. Ca^{2+} uptake was measured by the Murexide [18] method using the wavelength pair 540—507 nm or the Arzenoazo method [19] using the wavelength pair 665—685 nm.

The membrane potential was measured with the safranine method [16]. The spectrum of safranine changes in a way typical of stacking upon induction of potentials across the membranes of liposomes [20], bacterial vesicles [21] or mitochondria [16,22]. The response of the probe correlates linearly with the magnitude of potential up to about 200 mV [16]. The spectral shift was calibrated by aid of K^+ diffusion potentials in the presence of valinomycin as described earlier [16]. 524—554 nm was used as wavelength pair. In the Ca^{2+} efflux experiments Arzenoazo III [19] was used to measure the free Ca^{2+} concentration in the presence of different nitriloacetate concentrations in the same conditions as the efflux experiment. The values for free Ca^{2+} agreed fairly well with values obtained by calculation of the free Ca^{2+} concentration using the same stability constants as Reed and Bygrave [23].

All spectrophotometric measurements were made in an Aminco DW_2 spectrophotometer.

Reagents: FCCP was kindly donated by Dr. P.G. Heytler. EGTA, oligomycin and valinomycin were obtained from Sigma Chemicals Co., St. Louis, Mo.; Safranine from E. Merck A.G., Darmstadt. Ruthenium red was obtained from BDH Chemicals Ltd., Poole, U.K. and was recrystallized according to Luft before use [24]. All the other reagents were commercial products of highest pure quality.

Results and Discussion

Changes in membrane potential in energized conditions during Ca^{2+} influx

An addition of Ca^{2+} to respiring mitochondria causes a decrease in the mitochondrial membrane potential ($\Delta\psi$). The decrease in membrane potential (depolarisation) coincides well with the uptake of Ca^{2+} (Fig. 1). After the Ca^{2+}-induced depolarisation a prolonged repolarisation phase is seen (Fig. 2). During this repolarisation no net change in extramitochondrial Ca^{2+} can be measured (not shown) suggesting that a cycling of Ca^{2+} (electrogenic influx versus electroneutral efflux) prolongs the depolarisation. Additions of phos-

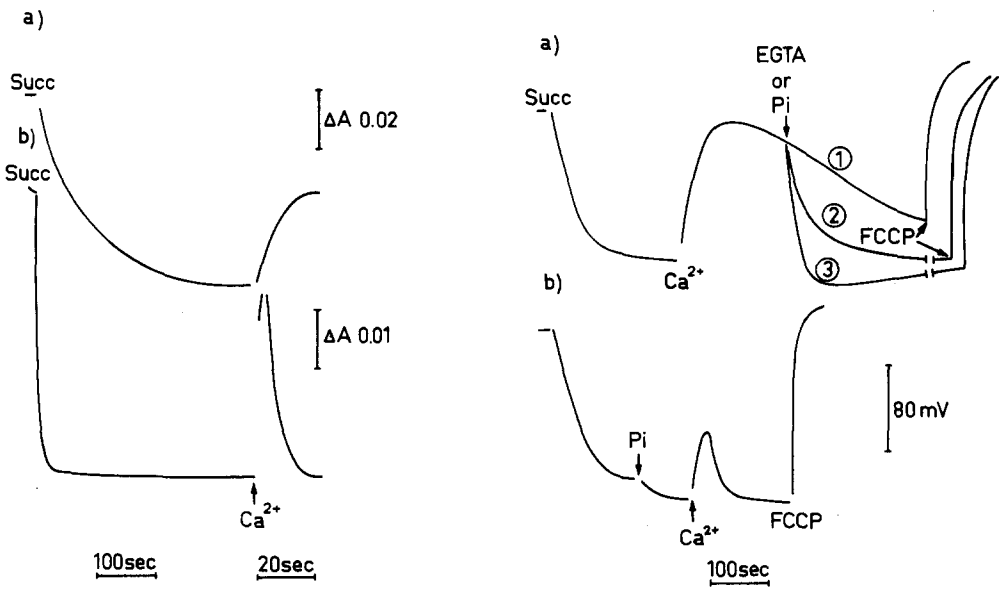

Fig. 1. Depolarisation of the mitochondrial membrane during Ca^{2+} uptake. Mitochondria (1 mg/ml) were incubated in a medium containing 0.25 M sucrose, 20 mM Tris · HCl, 10 mM HEPES, pH 7.4 (sucrose/Tris/HEPES), 10 μM rotenone, 4 μg/ml oligomycin and 10 μM safranine (a) or 40 μM Arzenoazo (b). Additions: 8 mM succinate (succ) and 10 μM $CaCl_2$ (Ca^{2+}) as indicated. Downward deflection polarisation (a) or decrease in medium Ca^{2+} (b).

Fig. 2. Effect of phosphate and EGTA on the Ca^{2+} induced depolarisation of the mitochondrial membrane. Conditions as in Fig. 1 (a). In (a) additions: 8 mM succinate (succ), 20 μM $CaCl_2$ (1,2,3, Ca^{2+}), 4 mM KH_2PO_4 (3, P_i) or 0.5 mM EGTA (2) and 7 μM FCCP as indicated. In (b) 4 mM KH_2PO_4 added before Ca^{2+}.

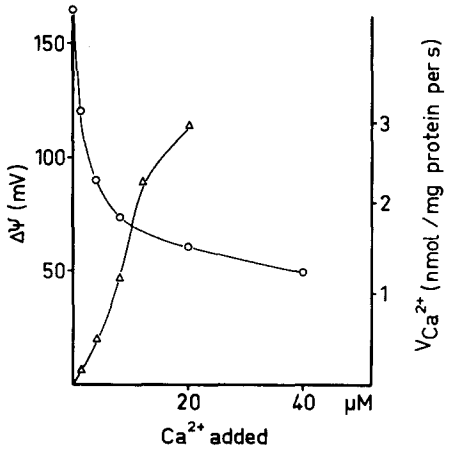

Fig. 3. Membrane potential and initial rate of Ca^{2+} transport as function of the Ca^{2+} concentration. Conditions as in Fig. 1. Membrane potential (○———○) and Ca^{2+} uptake (△———△).

phate or EGTA restore $\Delta\psi$ to its resting level. Phosphate would tend to cause an electroneutral proton influx [25] and thus decreases the proton gradient (ΔpH) created by Ca^{2+} uptake [26] leading to a compensating repolarisation. EGTA immobilizes the external Ca^{2+} and thus would prevent Ca^{2+} cycling. A similar result is obtained by the addition of an effective noncompetitive inhibitor of Ca^{2+} transport [27—29], ruthenium red (not shown). If phosphate is added prior to Ca^{2+} only a transient depolarisation is seen upon the addition of Ca^{2+} (Fig. 2).

Fig. 3 shows the decrease in $\Delta\psi$ and initial rate of Ca^{2+} uptake as a function of the Ca^{2+} concentration. It is seen that a half-maximal change is obtained at Ca^{2+} concentrations below 10 μM, which is near the K_m of transport in present conditions [30—35]. Note also that the plot for the decrease in membrane potential is highly biphasic with no further change after 20 μM Ca^{2+}. This biphasicity of the plot correlates well with the saturation phase of Ca^{2+} uptake (see also refs. 30—35).

Effect of the membrane potential on the kinetics of Ca^{2+} uptake

When the membrane potential is decreased by the addition of increasing concentrations of the respiratory chain inhibitor HOQNO [36], or by subsequent Ca^{2+} additions, and the initial rate of Ca^{2+} uptake is plotted against the membrane potential prior to the Ca^{2+} addition, a linear plot is obtained both at rather high (100 μM) or low (10 μM) Ca^{2+} concentrations (Fig. 4). Note that these plots correspond to current/voltage plots. The linear shape of the plots suggests a simple ohmic relationship in present conditions. When the membrane potential is decreased by HOQNO the plot does not reach the origin. This is probably due to the energy independent membrane potential (Donnan potential) present in deenergized mitochondria (see refs. 16, 37) which could not be able to drive active Ca^{2+} uptake because it is due to an uneven distribution of ions across the mitochondrial membranes [16,37] and does not require energy. The plot reaches origin when subsequent Ca^{2+} additions are made.

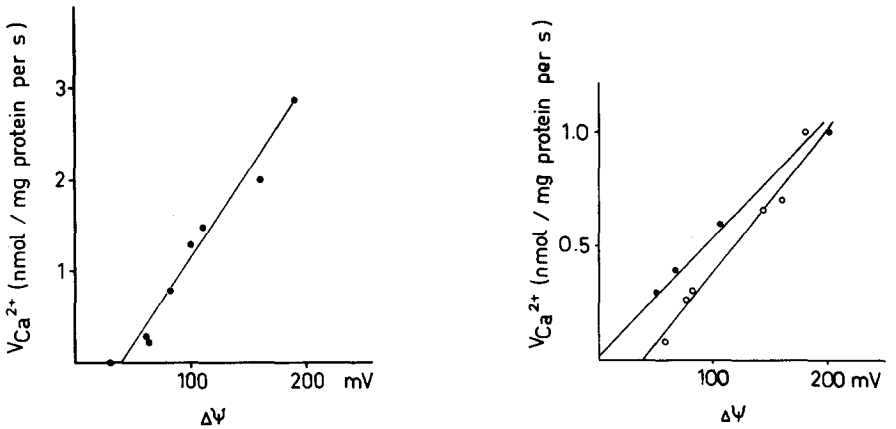

Fig. 4. Initial rate of Ca^{2+} uptake as a function of the membrane potential. Conditions as in Fig. 1. After the response of succinate was completed different concentrations of HOQNO were added, followed by a subsequent addition of Ca^{2+}. Each experimental point comprises one experiment with one HOQNO concentration. In (a) Ca^{2+} concentration 100 μM (●——●), Ca^{2+} uptake measured in the presence of 40 μM Murexide. (b) Ca^{2+} concentration 10 μM (○——○) and Ca^{2+} uptake measured in the presence of 20 μM Arzenoazo III. In b also the inital rate during subsequent addition of Ca^{2+} was measured as a function of the membrane potential (●——●).

In this case $\Delta\psi$ should turn into a proton gradient because of electrogenic Ca^{2+} uptake [1]. However, electrogenic proton translocation is still permitted to occur and hence the membrane potential present is able to drive Ca^{2+} uptake in contrast to the energy independent potential which would be completely collapsed by the initial Ca^{2+} uptake (see below).

Diffusion potential across the mitochondrial membrane induced by Ca^{2+} efflux

When de-energized mitochondria are preincubated in the presence of Ca^{2+}

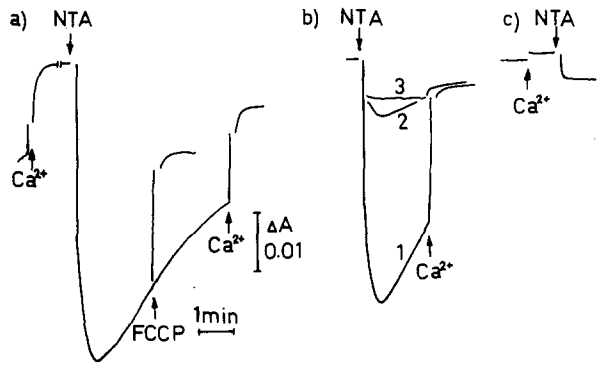

Fig. 5. Polarisation of the mitochondrial membrane during nitriloacetate-induced Ca^{2+} efflux across the mitochondrial membrane. Mitochondria (1 mg protein/ml) were preincubated for 5 min in the sucrose/Tris/HEPES medium containing 10 μM rotenone, 4 μg/ml oligomycin, 5 mM NaCN and 10 μM safranine. In (a) addition of 1 mM $CaCl_2$ (Ca^{2+}) and induction for 2 min. Thereafter 10 mM nitriloacetate was added and 10 mM $CaCl_2$ or 7 μM FCCP subsequently, as indicated. In (b) 1 mM $CaCl_2$ added before the experiment (1,2,3). Ruthenium red 4 μM (3) of La^{3+} 8 μM (2) added prior to 7 mM nitriloacetate. In (c) conditions as in (a) except that 4 μM ruthenium red was added prior to $CaCl_2$.

in order to equilibrate the external concentration with the internal and nitriloacetate is subsequently added, a polarisation of the mitochondrial membrane is seen (Fig. 5). A similar result is obtained with EGTA (not shown). A subsequent addition of FCCP or Ca^{2+} causes a depolarisation. Fig, 5 also shows that Ca^{2+} abolishes the energy independent membrane potential (see Fig. 4 and refs. 16, 37). This potential is completely abolished by the Ca^{2+} concentration used (1 mM). Evidence in favour of these responses being due to the mitochondrial Ca^{2+} translocator is that they can be inhibited by ruthenium red and La^{3+} (Fig. 5), a competitive inhibitor of mitochondrial Ca^{2+} transport [29,38,39]. Rather high concentrations of La^{3+} are needed probably because of chelation of this inhibitor by nitriloacetate. The depolarisation of the energy independent membrane potential is also abolished by ruthenium red (as shown in Fig. 5c) and by La^{3+} (not shown). It is also seen that the response consists partially of a nonspecific absorbance change which can not be abolished by Ca^{2+}. This is probably due to the binding of safranine to the Ca^{2+} chelators. The spectral shifts induced by valinomycin are equal in the presence and absence of nitriloacetate (not shown) indicating that this binding is of no significance for the function of the probe per se. In Fig. 6 a differential spectra of the nitriloacetate induced change is shown.

If different concentrations of nitriloacetate are added and the response of

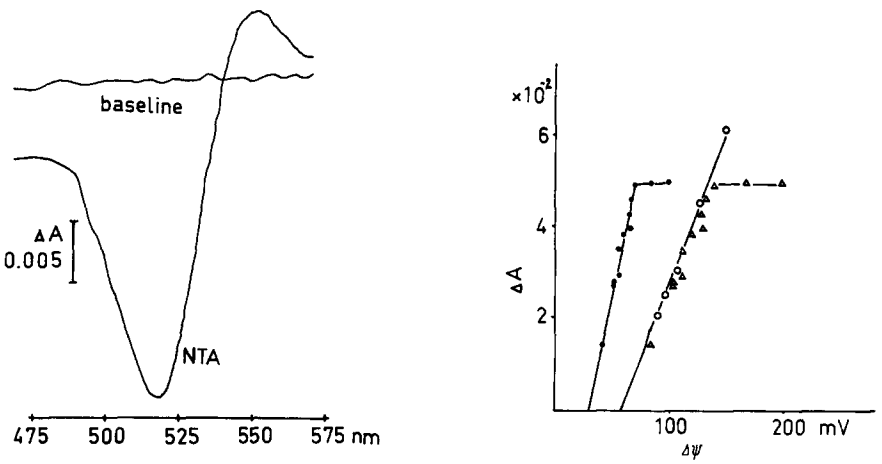

Fig. 6. Differential spectra of the nitriloacetate (NTA)-induced polarisation of the mitochondrial membrane. Conditions as in Fig. 5 (a). The baseline was drawn by scanning with two wavelengths. Thereafter 7 mM nitriloacetate was added to the sample cuvette and the spectra was drawn.

Fig. 7. Response of safranine as a function of expected membrane potential induced by nitriloacetate. Conditions as in Fig. 5. Except that different concentrations of nitriloacetate were added and the expected membrane potential was calculated using Nernst equation, $n = 2$ (●———●) or $n = 1$ (△———△). The extramitochondrial Ca^{2+} concentration was measured in the presence of 50 μM Arzenoazo III in identical conditions. The intramitochondrial Ca^{2+} concentration was assumed to be 1 mM. The valinomycin induced spectral changes were measured in similar conditions except that Ca^{2+} was omitted and different concentrations od KCl were added to the medium. Thereafter 40 ng/ml valinomycin was added and the expected membrane potential was calculated using Nernst equation assuming that the intramitochondrial K^+ is 120 mM (○———○). Abscissa: $\Delta\Psi = (RT/nF)\ln(Cat_i/Cat_o)$.

safranine is plotted against the magnitude of expected membrane potential, assuming that Ca^{2+} is transferred with two charges according to Nernst equation, a linear plot is obtained up to approx 70 mV (Fig. 7). The slope of the plot is almost exactly two-times that of the slope obtained with K^+ diffusion potentials in the presence of valinomycin, suggesting that Ca^{2+} is transferred across the membrane with only one net charge. Thereafter no change in the response of the probe is obtained with an increase in the Ca^{2+} gradient (Fig. 7) up to about 100 mV. If the Ca^{2+} gradient is further increased by large concentrations of EGTA the probe response remains at the same level up to 200 mV (not shown). The reason for this is probably that the Ca^{2+} translocator becomes rate limiting in these conditions.

The results presented suggest that the driving force of mitochondrial Ca^{2+} transport is the mitochondrial membrane potential with negative intramitochondrial polarity as suggested earlier by some workers [1—4]. The following evidence for this has been obtained in the present work:

1. A decrease in the mitochondrial membrane potential during Ca^{2+} uptake.
2. A similar correlation of the initial rate of Ca^{2+} transport and the membrane potential to the Ca^{2+} concentration.
3. A linear relationship between the initial rate of Ca^{2+} uptake and mitochondrial membrane potential.
4. A diffusion potential is created when Ca^{2+} efflux is induced by adding Ca^{2+} chelators (nitriloacetate of EGTA) to a suspension of deenergized mitochondria equilibrated with Ca^{2+}. The results also suggest that Ca^{2+} transferred across the membrane with only one net charge (at least Ca^{2+} efflux). The stoichiometry of Ca^{2+} influx and efflux need not, of course, be the same. However, if the same translocator is used for influx and efflux a similar charge stoichiometry would be expected. The results are in agreement with the proposal of Moyle and Mitchell [14,15], who also have suggested that Ca^{2+} is translocated single charged. The results of Moyle and Mitchell, though, have been questioned by Reynafarje and Lehninger [40], who calculated a net transfer of two charges during Ca^{2+} influx when they measured simultaneously proton ejection, Ca^{2+} uptake and O_2 consumption. A similar result was obtained by Wikström [41], who measured Ca^{2+} uptake and proton ejection in conditions where only the terminal part of the respiratory chain was active and by using potassium ferrocyanide as electron donor. There thus seems to be a clear discrepancy between the calculated charge stoichiometry of Ca^{2+} uptake and the measured charge transfer during Ca^{2+} efflux. However, as much controversy also has been raised recently concerning the stoichiometry between electrogenic proton translocation and electron transfer in the respiratory chain (see, for instance, refs. 42, 43) further studies to clarify this latter point probably will give also much information about the mechanism of Ca^{2+} transport.

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