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Stimulation of potassium cycling in mitochondria by long-chain fatty acids

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Abstract

Nonesterified long-chain fatty acids (myristic, palmitic, oleic and arachidonic), added at low amounts (around 20 nmol/mg protein) to rat liver mitochondria, energized by respiratory substrates and suspended in isotonic solutions of KCl, NaCl, RbCl or CsCl, adjusted to pH 8.0, induce a large-scale swelling followed by a spontaneous contraction. Such swelling does not occur in alkaline solutions of choline chloride or potassium gluconate or sucrose. These changes in the matrix volume reflect a net uptake, followed by net extrusion, of KCl (or another alkali metal chloride) and are characterized by the following features: (1) Lowering of medium pH from 8.0 to 7.2 results in a disappearance of the swelling-contraction reaction. (2) The contraction phase disappears when the respiration is blocked by antimycin A. (3) Quinine, an inhibitor of the K^+/H^+ antiporter, does not affect swelling but suppresses the contraction phase. (4) The swelling phase is accompanied by a decrease of the transmembrane potential and an increase of respiration, whereas the contraction is followed by an increase of the membrane potential and a decrease of oxygen uptake. (5) Nigericin, a catalyst of the K^+/H^+ exchange, prevents or partly reverses the swelling and partly restores the depressed membrane potential. These results indicate that long-chain fatty acids activate in liver mitochondria suspended in alkaline saline media the uniporter of monovalent alkali metal cations, the K^+/H^+ antiporter and the inner membrane anion channel. These effects are presumably related to depletion of mitochondrial Mg^{2+}, as reported previously [Arch. Biochem. Biophys. 403 (2002) 16], and are responsible for the energy-dissipating K^+ cycling. The uniporter and the K^+/H^+ antiporter are in different ways activated by membrane stretching and/or unfolding, resulting in swelling followed by contraction.

Keywords: Fatty acid; K^+ uniport; K^+/H^+ antiport; K^+ cycling; Swelling; Transmembrane potential; Energy dissipation; Mitochondrion

1. Introduction

CoA-thioesters of fatty acids are important physiological sources for feeding electrons to the energy-conserving mitochondrial respiratory chain. However, it is known, especially from studies on isolated mitochondria, that nonesterified long-chain fatty acids interact with mitochondrial membranes and interfere with a number of energy-linked membrane functions (for review, see Ref. [1]). Thus, fatty acids are well characterized as weak protonophoric uncouplers in mitochondria. Despite stimulating mitochondrial respiration in the resting state (State 4) by protonophoric activity, fatty acids inhibit mitochondrial respiration under the active state (State 3) and in uncoupled conditions. Several activities of fatty acids seem to be involved in this inhibitory effect, such as interactions with complexes of the respiratory chain, with primary dehydrogenases and with carriers of the substrate transport into the mitochondrion (reviewed in Ref. [1]). Moreover, when mitochondria are exposed to elevated levels of Ca^{2+}, they can undergo permeability transition by opening of a proteinaceous pore, the so-called permeability transition pore, resulting in a nonselective increase in the permeability of the inner membrane to small solutes (see for review, e.g. Ref. [2]). Consequently, mitochondria undergo rapid large-amplitude swelling, paralleled by depolarization of the inner membrane. Fatty acids promote mitochondrial permeability transition effectively at low concentrations [3–9].

We have previously reported [10,11] that long-chain fatty acids can stimulate rapid swelling of rat liver mitochondria in alkaline saline media that is neither due to opening of the

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permeability transition pore nor to the "nonclassical" permeability transition described by Sultan and Sokolove [12,13], but is accompanied by a release of mitochondrial Mg^{2+}. It has long been known that Mg^{2+} depletion increases mitochondrial permeability towards small ions by unmasking the K^+/H^+ exchanger [14–17] (for reviews, see also Refs. [2,18]) and the mitochondrial inner membrane anion channel [19,20]. Moreover, activation of the net electrophoretic K^+ uptake in Mg^{2+}-depleted mitochondria has been also reported [21,22]. In addition, fatty-acid-induced swelling of nonenergized rat liver mitochondria in alkaline KCl media is inhibited in a concentration-dependent manner by quinine [11], a known inhibitor of the K^+/H^+ antiporter [23] and of the inner membrane anion channel [20].

These observations taken together show that long-chain fatty acids can activate ion-conducting pathways of the inner membrane [10,11], which are masked by endogenous Mg^{2+} in freshly prepared mitochondria [14–16,19,20,24,25]. Such unmasking of silent cation transport systems could further suggest that in energized mitochondria, fatty acids are able to initiate an energy-dissipating K^+ cycling across the inner membrane due to the electrophoretic K^+ uptake and K^+ extrusion mediated by the K^+/H^+ antiporter plus the respiratory chain-driven H^+ ejection. The present study shows that such futile, energy-dissipating, K^+-cycling induced by long-chain fatty acids does, in fact, occur in isolated liver mitochondria suspended in saline media at pH 8.0. In addition, we point to a regulatory function of unfolding/refolding of the inner mitochondrial membrane and of stretching forces applied on this membrane in its permeability to ions.

2. Materials and methods

2.1. Preparation of mitochondria

Liver mitochondria were prepared from adult female Wistar rats (average weight 150–180 g) by differential centrifugation according to our standard protocol [10]. The final pellet was resuspended in 0.25 M sucrose at a protein concentration of 25–35 mg/ml. Protein content in the mitochondrial stock suspension was determined by a modified biuret method.

2.2. Incubations

The standard incubation medium (also defined as the KCl medium) contained 120 mM KCl, 5 μM cytochrome *c*,

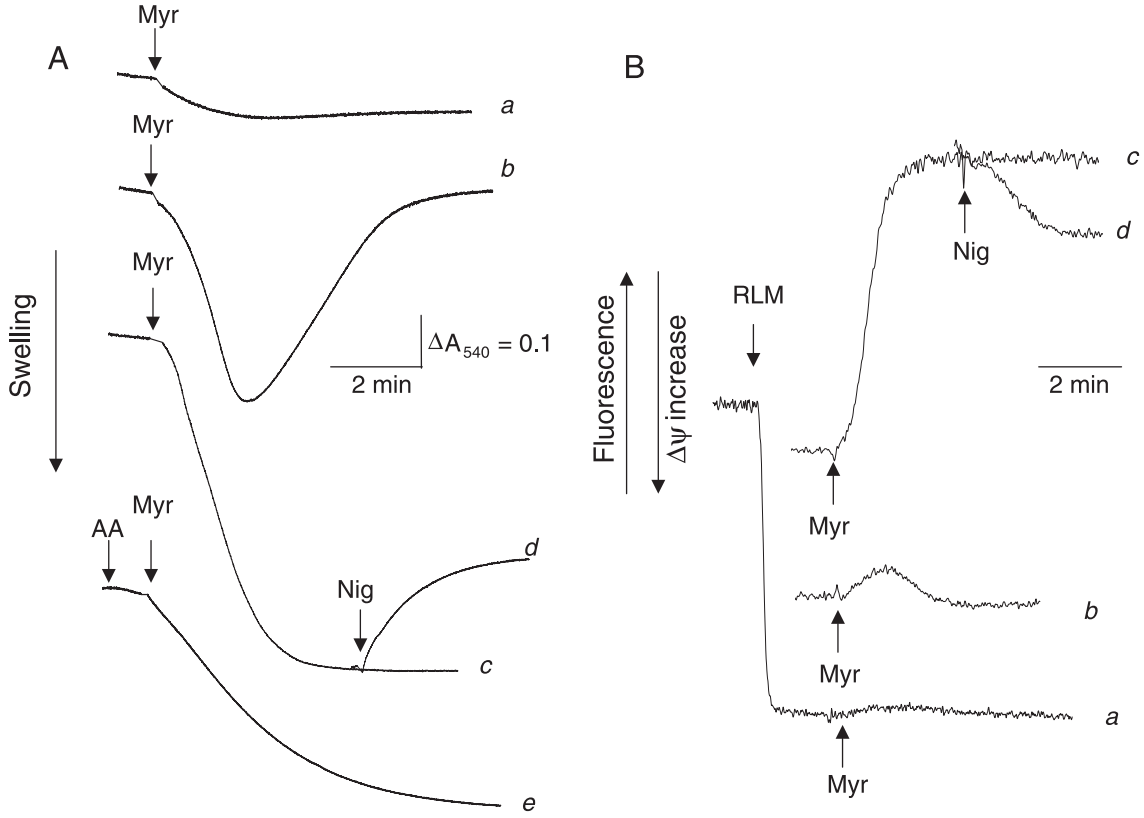

Fig. 1. Myristate-induced changes in mitochondrial matrix volume (A) and membrane potential (B). Mitochondria (RLM, 1.0 mg protein/ml) were suspended in the KCl medium adjusted to pH 8.0 and supplemented with glutamate and malate as described in Materials and methods. Myristate (Myr) was added at the following amounts (in nmol/mg mitochondrial protein): traces a and e, 10; trace b, 22.5; traces c and d, 40 (corresponding to final concentrations of 10, 22.5 and 40 μM, respectively). Other additions: 2 μM antimycin A (AA) and 1 μM nigericin (Nig).

5 mM glutamate, 5 mM malate, 10 mM Tris, 0.5 mM EDTA and 1 μM cyclosporin A; pH was adjusted to the required value with HCl. Other applied saline media contained alkali metal salts, with chloride or nitrate as anions, at a concentration of 120 mM, and other additions as in the KCl medium. The media were supplemented with cyclosporin A to prevent a possible opening of the permeability transition pore in the presence of added fatty acids. Cytochrome *c* was added to compensate for a possible partial loss occurring during swelling in saline media. The loss of cytochrome *c* from mitochondria is known to decrease the activity of the respiratory chain [21,26]. Unless indicated otherwise, mitochondria were suspended in these media at the concentration of 1 mg protein/ml and incubated at 25 °C.

2.3. Mitochondrial swelling

Swelling of mitochondria was measured as changes in the absorbance at 540 nm in a Varian Cary 3E spectrophotometer equipped with a thermostated and magnetically stirred sampling unit. Swelling was induced after 1 min of equilibration of mitochondria in saline media or 240 mM sucrose medium (supplemented with the additions as in the KCl medium) and was quantified by the initial rate of the decrease of light absorbance using the photometer software.

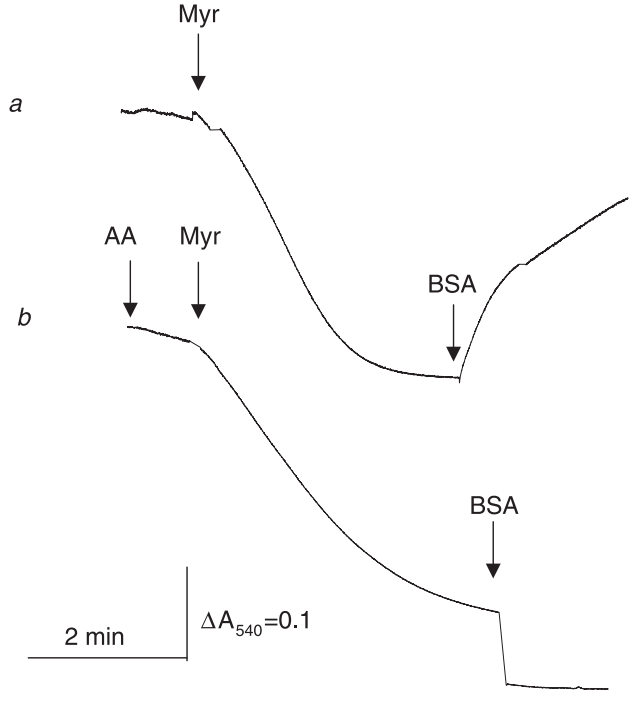

Fig. 2. Contraction of swollen mitochondria produced by serum albumin. Mitochondria (1 mg protein/ml) suspended in the KNO_3 medium adjusted to pH 8.0 and supplemented with other additions as in Fig. 1 were induced to swell by the addition of myristate, 30 nmol/mg protein (Myr). Serum albumin, 1 mg/ml, was added where indicated (BSA). Antimycin A (AA), 2 μM, was present in trace b.

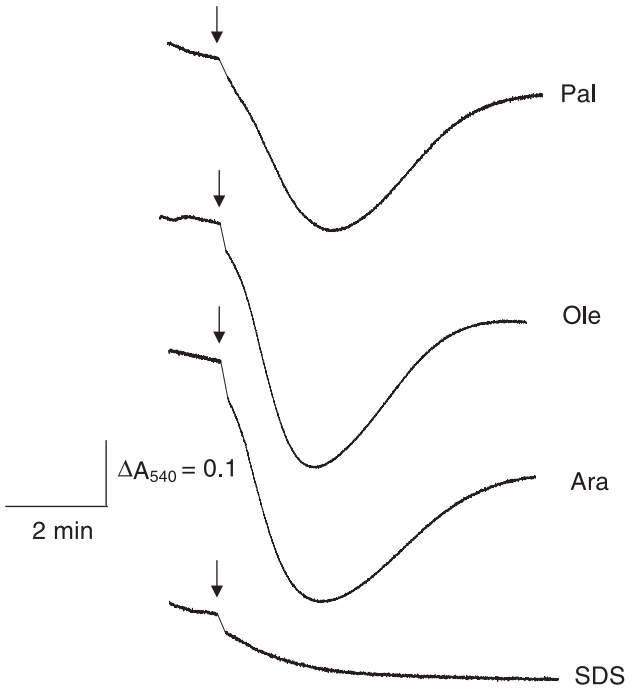

Fig. 3. Capability of various fatty acids to induce swelling–contraction in energized mitochondria. Experimental conditions were as in Fig. 1. Where indicated by the arrow, palmitate (Pal), oleate (Ole), arachidonate (Ara) or sodium dodecylsulfate (SDS) were added to a final concentration of 22.5 μM (corresponding to 22.5 nmol/mg mitochondrial protein).

2.4. Membrane potential

This was evaluated from the uptake of safranine O [27] that was determined from fluorescence quenching [28]

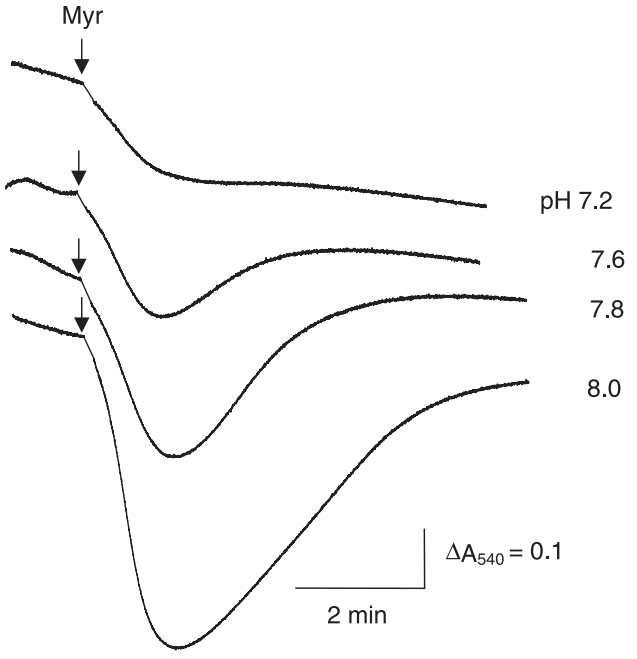

Fig. 4. Effect of pH of the KCl medium on myristate-induced mitochondrial swelling–contraction reaction. Incubations were performed as in Fig. 1A, trace b.

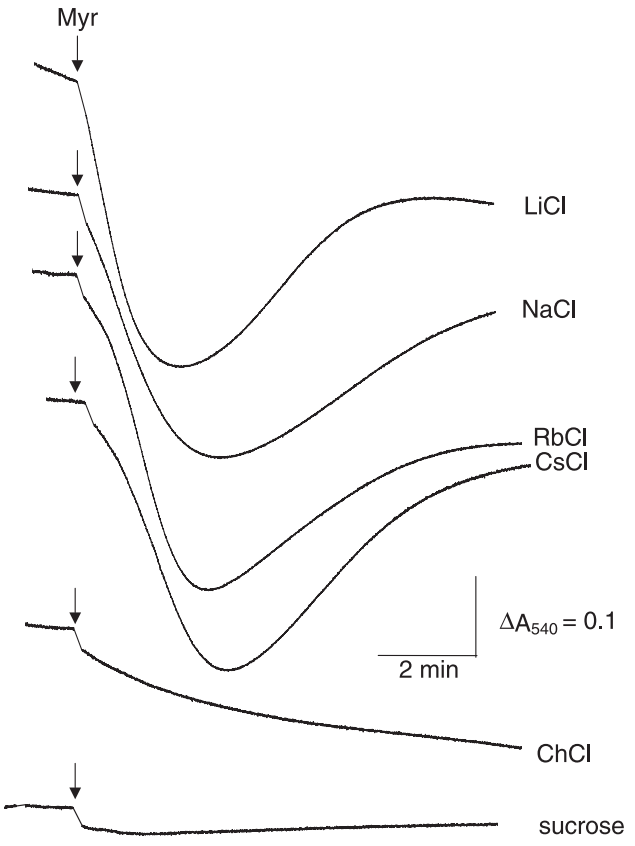

Fig. 5. Effect of the medium composition on the swelling–contraction reaction. Mitochondria were suspended in saline media, in which KCl was replaced by LiCl, NaCl, RbCl, CsCl or choline chloride (ChCl), or the sucrose medium adjusted to pH 8.0. Myristate (Myr) was added to a final concentration of 20 μM (corresponding to 20 nmol/mg mitochondrial protein).

fluorimetrically monitored at the wavelengths of 495 nm (excitation) and 586 nm (emission).

2.5. Oxygen uptake

Oxygen uptake was measured with the Clark-type oxygen electrode using OROBOROS® Oxygraph 2000 (Innsbruck, Austria).

2.6. Chemicals

Myristic, palmitic and oleic acids, sodium dodecylsulfate, bovine serum albumin essentially fatty acid-free, antimycin A, valinomycin and nigericin were purchased from Sigma (St. Louis, MO). Potassium gluconate was from Merck-Schuchardt (Hohenbrunn, Germany). Divalent metal cation ionophore A23187, cyclosporin A, carboxyatractyloside and arachidonic acid were from Calbiochem (Darmstadt, Germany). All other reagents were of high purity grade. Stock solutions of arachidonic acid were made in ethanol (gassed with argon) at 1 or 10 mM concentration and those of other fatty acids in ethanol at 10 mM concentration.

3. Results

Depending on its concentration, myristate added to rat liver mitochondria, suspended in alkaline KCl medium (pH 8.0) and energized with glutamate plus malate as respiratory substrates, induced characteristic changes in the matrix volume, as indicated by changes in light absorbance. Low amounts of this fatty acid, up to 10 nmol per milligram of mitochondrial protein, induced only a minor swelling (Fig. 1A, trace a). When the concentration was in the range of 15–25 nmol/mg mitochondrial protein, mitochondria rapidly swelled immediately after addition of myristate, but contracted shortly thereafter (trace b). This swelling–contraction reaction was very sensitive to the degree of mitochondrial energization. Mitochondria incubated with succinate (plus rotenone) exhibited a similar swelling–contraction as that observed with glutamate plus malate, but they were unable to contract after swelling in the presence of pyruvate plus malate (not shown), a "poor" respiratory substrate couple for rat liver mitochondria. Swelling induced by low concentrations of myristate (10 nmol/mg protein) was strongly enhanced by the respiratory chain inhibitor antimycin A (trace e). In this case, no spontaneous contraction occurred.

With myristate concentration exceeding 30 nmol/mg protein, the swelling was permanent (trace c). As shown previously [11], this large-amplitude swelling could be

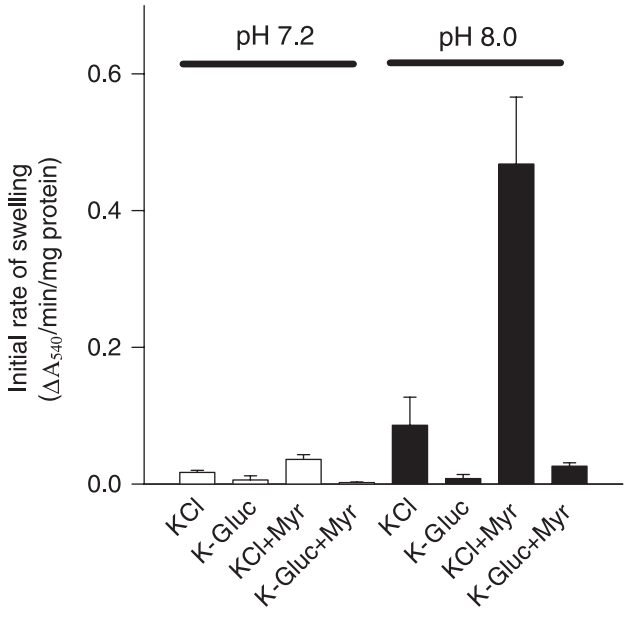

Fig. 6. Myristate-induced permeability of the inner mitochondrial membrane to Cl^-. Mitochondria were suspended in the KCl medium supplemented with 0.5 μM valinomycin and 2 μM antimycin A, adjusted to pH 7.2 or 8.0. Where indicated (K-Gluc), KCl was substituted by potassium gluconate. Myristate (Myr) was added at the amount of 40 nmol/mg protein. Permeability to Cl^- was assessed from the initial rate of swelling. The data are mean values ± S.D. from four experiments with different mitochondrial preparations.

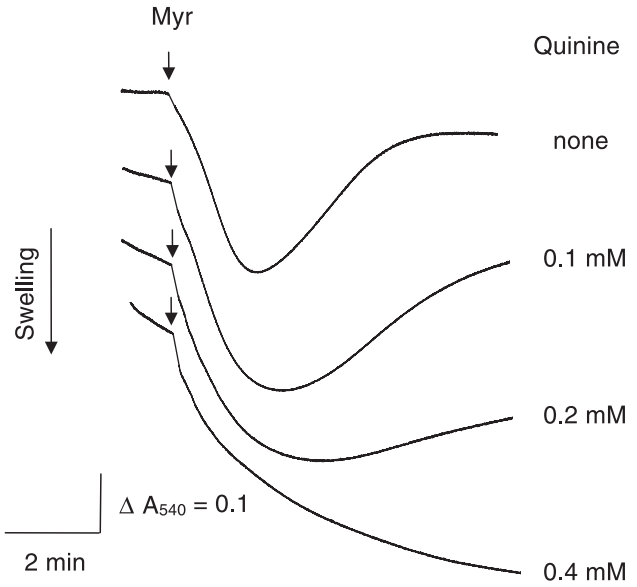

Fig. 7. Effect of quinine on the myristate-induced swelling–contraction of energized mitochondria. Myristate (20 nmol/mg protein) was added to mitochondria (1 mg protein/ml) suspended in the KCl medium at pH 8.0 supplemented with quinine chloride as indicated.

prevented by nigericin, a potent K^+/H^+ exchanger. Here we show that nigericin partly reversed the swelling (trace d). Essentially similar results (not shown) were obtained when the chloride anion in the medium was replaced by nitrate anion, known to easily penetrate the inner mitochondrial membrane, or when the KCl medium was supplemented with 5 mM phosphate.

As shown in Fig. 1B, swelling was accompanied by a decrease in the mitochondrial membrane potential ($\Delta\Psi$), whereas contraction was paralleled by $\Delta\Psi$ increase.

As mentioned above, mitochondria failed to spontaneously contract when the amount of myristate was 30 nmol/mg protein or more. However, they did contract under such conditions upon addition of serum albumin, known to bind nonesterified fatty acids (Fig. 2, trace a). This contraction was again prevented by antimycin A (Fig. 2, trace b).

Palmitate, oleate and arachidonate applied at low concentrations of around 20 nmol/mg protein produced a similar swelling–contraction reaction (Fig. 3) as that obtained with the same amount of myristate (see Fig. 1A, trace b). In contrast, dodecylsulfate did not initiate a swelling–contraction cycle (Fig. 3, bottom trace). When added at higher concentration of 40 nmol/mg protein, it resulted in a slow but steady mitochondrial swelling (not shown). This fatty acid-induced swelling–contraction reaction appeared to be pH-dependent and declined with decreasing alkalinity of the medium (Fig. 4). Replacing the KCl medium at pH 8.0 by a similar isosmotic medium composed of LiCl, NaCl, RbCl or CsCl had no essential effect on the swelling–contraction reaction (Fig. 5). In contrast, mitochondria did not swell after the addition of myristate in alkaline sucrose or choline chloride medium (Fig. 5).

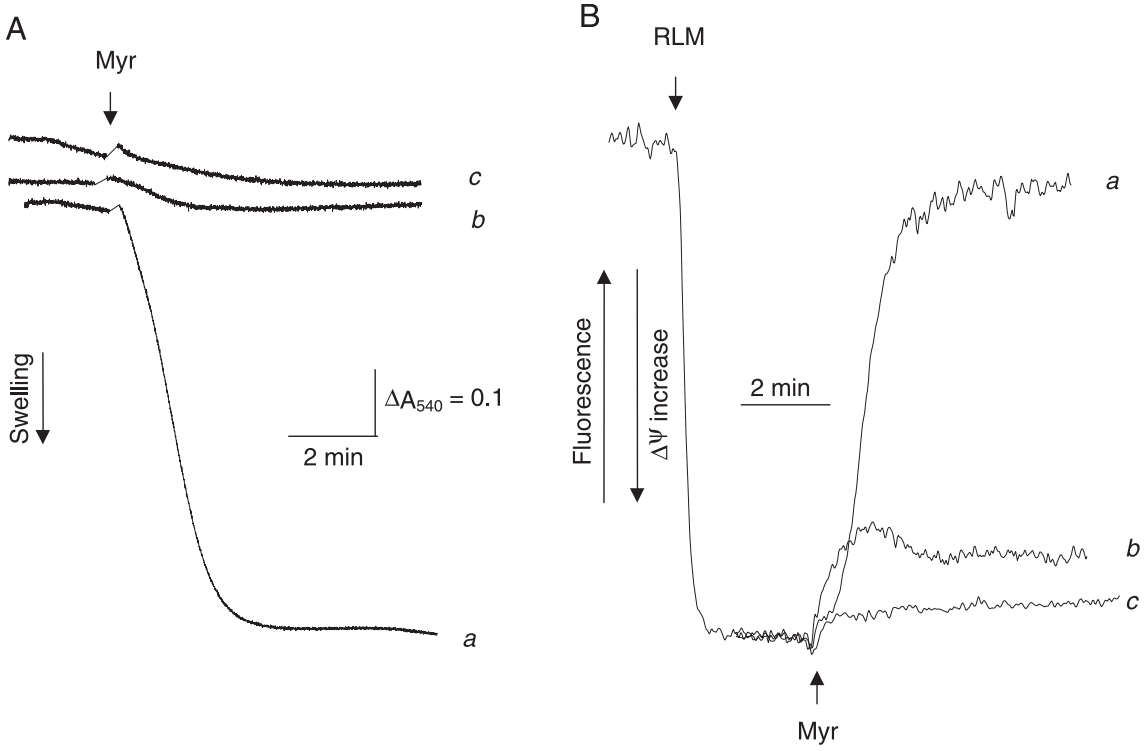

Fig. 8. Effect of myristate on mitochondrial swelling (A) and membrane potential (B). Mitochondria (RLM) were incubated in the KCl medium adjusted to pH 8.0 (traces a and b) or 7.2 (trace c). Myristate was added to a final concentration of 40 nmol/mg protein (40 μM). Incubation sample depicted by trace b contained 1 μM nigericin added prior to myristate (not indicated). The medium for $\Delta\psi$ measurements (panel B) also contained 10 μM safranine O.

Mitochondria suspended in the alkaline KCl medium supplemented with valinomycin and the respiratory inhibitor antimycin A swelled only slowly, but started to swell rapidly when myristate was added (Fig. 6). However, when KCl was replaced with K-gluconate, mitochondria did not respond to myristate. In this experiment, the mitochondrial membrane was freely permeable to K^+ due to the presence of valinomycin. Nevertheless, there was no driving force for K^+ entry because of the low-energy state of mitochondria (due to the presence of antimycin A). Thus, these results clearly indicate that, at alkaline pH, myristate also increases the permeability of the inner mitochondrial membrane to Cl^- presumably by activating the inner membrane anion channel. Gluconate anion is known not to permeate this channel [20]. In this case, Cl^- concentration gradient was apparently the driving force for KCl entry and swelling.

All these results show that myristate and other long-chain fatty acids induce a net influx of monovalent alkali metal cations and chloride anion into rat liver mitochondria, the process that can be followed by respiration-driven extrusion. One of the mechanisms of such extrusion can be the operation of an unselective cation$^+$/H^+ antiporter. To examine this possibility, the effect of quinine on the myristate-dependent swelling–contraction reaction of mitochondria in the alkaline KCl medium was examined. When myristate (20 nmol/mg protein) was added to energized mitochondria pretreated with increasing concentrations of quinine, the swelling phase was not affected but the contraction phase was suppressed (Fig. 7).

The fatty-acid-induced electrophoretic uptake of K^+, combined with the electroneutral K^+/H^+ exchange, should depolarize the inner mitochondrial membrane by dissipating the electrochemical proton gradient built by the respiratory chain. This was found indeed to be the case at high (40 nmol/mg protein) concentration of myristate (Fig. 1B, trace c; Fig. 8B, trace a). At lower concentrations of myristate, 20 nmol/mg protein, the decrease of $\Delta\Psi$ was smaller and transient (Fig. 1B, trace b). However, surprisingly, the depolarization was strongly suppressed when the medium contained the exogenous K^+/H^+ exchanger nigericin (Fig. 8B, trace b; Fig. 1B, trace d), indicating that, in this case, dissipation of the membrane potential was limited by K^+ influx rather than by K^+/H^+ exchange. The degree of myristate-induced depolarization was negligible at pH 7.2 (Fig. 8B, trace c). Comparison of Fig. 8A and B shows that dissipation of the mitochondrial membrane potential ran in parallel to mitochondrial swelling.

Energy dissipation produced by myristate (and other long-chain fatty acids) in mitochondria suspended in the alkaline KCl medium is also documented by measurements of mitochondrial respiration. In this experiment (Fig. 9), the medium was additionally supplemented with carboxyatractyloside to reduce that portion of oxygen consumption, which was due to the protonophoric activity of long-chain fatty acids [29]. As expected, mitochondrial swelling was accompanied by an increase in O_2 uptake, whereas the

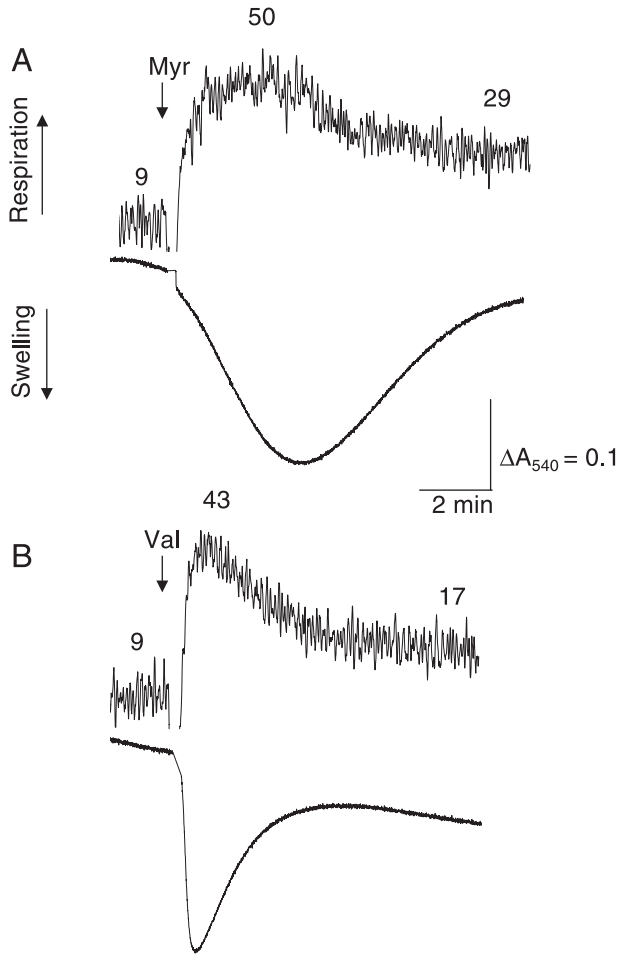

Fig. 9. Comparison of myristate-induced changes in oxygen consumption and swelling of mitochondria. Mitochondria were suspended in the KCl medium (pH 8.0) supplemented with 2 μM carboxyatractyloside. The upper ("noisy") traces in each panel illustrate the rate of oxygen uptake and the lower ("smooth") traces depict the swelling. Numbers at the oxygen traces indicate the rates of respiration expressed in ng atom O/min per mg protein. Myristate (Myr) was added at a concentration of 20 nmol/mg protein (A) and valinomycin (Val) was 0.1 μM (B).

spontaneous contraction was accompanied by a decrease of the respiration rate, which, however, did not return to its resting state level (Fig. 9A). Interestingly, a similar picture was obtained when myristate was replaced by a low concentration (0.1 μM) of valinomycin (Fig. 9B).

4. Discussion

In the present study, we demonstrate that nonesterified long-chain fatty acids (myristate, palmitate, oleate and arachidonate) can produce a characteristic swelling–contraction reaction of energized rat liver mitochondria suspended in moderately alkaline (pH around 8) saline media that has not been reported before. Since the inner mitochondrial membrane at alkaline pH is almost impermeable to KCl in the absence of fatty acids, such swelling–contraction

event reflects a fatty-acid-induced net uptake of KCl (or another monovalent alkali metal chloride) into the matrix compartment and a subsequent extrusion of both the cation and the anion. This observation might be attributed to the ionophoretic activity of fatty acids with respect to monovalent alkali metal cations clearly documented for artificial phospholipid membranes [30,31] and also shown for mitochondrial membranes [32]. However, fatty-acid-mediated ionophoretic K^+ transport alone does not explain why mitochondria do not swell in KNO_3 medium (NO_3^- permeates freely) adjusted to neutral pH (not shown), whereas ionophoretic activity of fatty acids operates in phospholipid membranes over a broad pH range [31].

It has long been known that raising pH of the medium can increase permeability of the inner mitochondrial membrane to ions [33,34]. In addition, more recent studies by Tedeschi's group [35,36], applying patch-clamp techniques to rat liver mitochondria, demonstrated activation of two channels by alkaline pH, one slightly cation-selective (15 pS) and one slightly anion-selective (45 pS). Nevertheless, a massive influx of ions into the matrix compartment of rat liver mitochondria suspended in alkaline KCl medium does not occur unless a fatty acid is added (Fig. 1).

A likely explanation of the observed swelling–contraction reaction is that fatty acids activate silent ion-conducting systems in the inner membrane. Such a hypothesis is strongly supported by our finding [10,11] that in alkaline saline media, long-chain fatty acids deplete mitochondrial Mg^{2+}, thus probably activating both the K^+ uniporter and the K^+/H^+ exchanger and, in addition, the inner membrane anion channel. Activation of this latter channel by fatty acids is clearly documented in Fig. 6. Depletion of mitochondrial Mg^{2+} is known to activate K^+ permeability [20,21], K^+/H^+ exchange [14–16,21,37] and anion permeation [19,20]. This has been shown to be more effective under high pH of the medium [21,24]. Therefore, a concerted action of the two factors, alkaline medium and depletion of mitochondrial Mg^{2+} by long-chain fatty acids [10,11], exerts a dramatic effect on mitochondrial volume, membrane potential and respiration as described in the present work. Thus, the swelling–contraction behaviour of mitochondria under such conditions is the resultant of interplay between K^+ influx and K^+ extrusion. It has to be noted, however, that a high rate of K^+ influx depends not only on Mg^{2+} depletion but is also further activate by the actual presence of the fatty acid, as its removal by binding to serum albumin results in a rapid contraction (Fig. 2).

While the swelling of energized mitochondria is caused by the electrophoretic influx of K^+ (accompanied by an inflow of Cl^-), the contraction must result from the electroneutral K^+/H^+ exchange, driven by pH gradient that is built by the respiratory chain, extruding K^+ to the external medium. This is indicated, e.g., by the fact that the contraction phase disappears under the influence of respiratory chain inhibitors (antimycin A) or in the presence of "weak" respiratory substrates and of inhibitors of the K^+/H^+ exchanger (quinine). The latter findings fit well with the observation reported by Bernardi et al. [21] that quinine inhibits spontaneous contraction of swollen mitochondria, which have been treated with the bivalent action ionophore A23187. The question thus arises why the K^+ uniporter is immediately activated whereas the exchanger becomes fully operative after some time only. Such a delay (spontaneous lag) in the activation of the K^+/H^+ exchanger has been also observed in fatty-acid-free incubations [16,21].

Increased permeability for potassium ions of energized mitochondria caused by fatty acid, whatever its mechanism may be, results in a massive influx of K^+ and, consequently, a rapid decrease of $\Delta\Psi$ (Fig. 1B). Subsequent activation of the K^+/H^+ exchanger initiates a futile K^+ cycling that is responsible for a sustained increase of mitochondrial respiration (Fig. 9A). This exchange is driven by pH gradient (maintained by the functioning respiratory chain). And this is, presumably, the reason why $\Delta\Psi$ undergoes only a transient depression (Fig. 1B, trace b). Only in the presence of higher concentrations of fatty acids as in Fig. 1A,B, traces c, and Fig. 8A,B, traces a, the K^+/H^+ exchange is unable to overcome the K^+ influx, the situation that is manifested by a sustained swelling and permanent dissipation of $\Delta\Psi$. This indicates that the K^+ influx is faster than the efficiency of the endogenous K^+/H^+ exchanger. In this situation, the addition of the exogenous K^+/H^+ exchanger, nigericin, helps partly to export some of accumulated potassium ions (followed by the efflux of Cl^-), manifested by a partial contraction (Fig. 1A, trace d) or prevention of swelling (Fig. 8A, trace b). Surprisingly, however, $\Delta\Psi$ becomes also partly restored (Fig. 1B) or its decrease considerably prevented (Fig. 8B, trace b). The latter finding can only be interpreted by assuming that the rate of K^+ uniport is decreased, apparently due to mitochondrial contraction.

These considerations allow us to propose that the K^+ uniporter and the K^+/H^+ exchanger, both of them being activated by Mg^{2+} depletion under alkaline conditions, are sensitive to membrane unfolding/stretching in different ways. The uniporter becomes activated by membrane unfolding, most likely due to a several-fold increase of the surface of the inner membrane that is in a direct contact with the surrounding medium, as first proposed by Azzone et al. [38]. In contrast, the K^+/H^+ exchanger is maximally activated only at a high degree of swelling presumably due to some rearrangement of membrane constituents induced by membrane stretching.

When the rate of the K^+/H^+ exchanger exceeds that of the K^+ influx, a contraction starts. As a consequence, K^+ permeability (equivalent to the K^+ uniporter) decreases because of membrane refolding, whereas the endogenous K^+/H^+ exchanger remains fully activated. Moreover, it seems likely that, in the alkaline medium, membrane stretching or unfolding as such is sufficient to activate the K^+/H^+ exchanger without participation of fatty acids and the resulting Mg^{2+} depletion. Such a conclusion can be drawn from the experiment illustrated in Fig. 9, in which valino-

mycin mimicked the effect of myristate in its action on both mitochondrial volume changes and oxygen uptake. Apparently, mitochondrial swelling resulting from K^+ influx in the presence of valinomycin triggered the K^+/H^+ exchanger and, consequently, K^+ extrusion. A somewhat elevated rate of oxygen uptake that followed the initial rapid rise depicted the futile K^+ cycling. Thus, nonesterified fatty acids under alkaline conditions cause energy dissipation not only by H^+ cycling but also by induction of K^+ cycling. The mechanism underlying fatty-acid-induced swelling–contraction in mitochondria appears to support the view that the permeability of the inner mitochondrial membrane to K^+ and the K^+/H^+ exchange therein are strongly affected by the membrane shape and mechanical forces exerted by membrane stretching [16,21,38]. In this context, it is worthy to recall that activation of ion channels of low specificity by membrane stretching is a common event in bacterial membranes and in eukaryotic plasma membranes [39,40] and therefore most likely represents a general feature of these fundamental biological structures.

Acknowledgements

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