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Fatty Acids Induce Chloride Permeation in Rat Liver Mitochondria by Activation of the Inner Membrane Anion Channel (IMAC)

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The inner membrane of freshly isolated mammalian mitochondria is poorly permeable to Cl^-. Low, nonlytic concentrations ($\leq$30 μM) of long-chain fatty acids or their branched-chain derivatives increase permeation of Cl^- as indicated from rapid large-scale swelling of mitochondria suspended in slightly alkaline KCl medium (supplemented with valinomycin). Myristic, palmitic, or 5-doxylstearic acid are powerful inducers of Cl^- permeation, whereas lauric, phytanic, stearic, or 16-doxylstearic acid stimulate Cl^- permeation in a lesser extent. Fatty acid-induced Cl^- permeation across the inner membrane correlates well with the property of nonesterified fatty acids to release endogenous Mg^{2+} from mitochondria. Myristic acid stimulates anion permeation in a selective manner, similar as was described for A23187, an activator of the inner membrane anion channel (IMAC). Myristic acid-induced Cl^- permeation is blocked by low concentrations of tributyltin chloride (IC_{50} $\approx$1.5 nmol/mg protein). Moreover, myristic acid activates a transmembrane ion current in patch-clamped mitoplasts (mitochondria with the outer membrane removed) exposed to alkaline KCl medium. This current is best ascribed to the opening of an ion channel with a single-channel conductance of 108 pS. We propose that long-chain fatty acids can activate IMAC by withdrawal of Mg^{2+} from intrinsic binding sites.

KEY WORDS: Mitochondria; fatty acid; patch clamping; inner membrane anion channel.

INTRODUCTION

K^+ and Cl^- are the most abundant ions in the cell, both in the cytosol and within the mitochondrial matrix compartment. Since mitochondria generate a high, inside negative membrane potential, an uncompensated electrophoretic uptake of K^+ in the matrix due to K^+ leaks across the inner membrane, followed by the passive transmembrane permeation of Cl^-, would destroy the morphological and functional integrity of mitochondria. Therefore, Mitchell postulated in the early 1960s that the joined operation of a putative K^+/H^+ antiporter and of the respiratory chain-driven H^+-ejection is the essential device to compensate electrophoretic K^+ uptake (Mitchell, 1961). Later, the properties of the K^+/H^+ antiporter were extensively characterized, and the transporter was ascribed to an 82-kDa protein (for a review see, Bernardi, 1999; Brierley *et al.*, 1994). In addition to the K^+/H^+ antiporter, the existence of K^+ uniporters or of K^+ channels in the inner membrane has been demonstrated by swelling assay using isolated mitochondria and by patch-clamp measurements with mitoplasts (mitochondria with outer membrane removed) from various tissues (Antonenko *et al.*, 1994; Bernardi *et al.*, 1989; Inoue *et al.*, 1991; Siemen *et al.*, 1999; Xu *et al.*, 2002). Furthermore, it is generally believed that Cl^- permeates

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Key to abbreviations: IMAC, inner membrane anion channel; A23, A23187; TBT, tributyltin chloride; FFAs, free fatty acids; Lau, lauric acid; Myr, myristic acid; Pal, palmitic acid; Phyt, phytanic acid; Stea, stearic acid; 5-Doxyl, 5-doxylstearic acid; 16-Doxyl, 16-doxylstearic acid; Val, valinomycin; Nig, nigericin; BSA, bovine serum albumin; Succ, succinate; RC, respiratory chain; Tris, tris(hydroxymethyl)-aminomethane; HEPES, *N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid].

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the inner membrane by means of the inner membrane anion channel (IMAC) (for a review see, Beavis, 1992).

The inner membrane of freshly isolated mammalian mitochondria is poorly permeable to Cl^-. In a recent study, we reported that nonesterified myristic acid (C14:0) initiates a rapid large-scale swelling when applied in micromolar concentrations ($\geq$5 μM, corresponding to 5 nmol/mg protein) to isolated nonenergized rat liver mitochondria suspended in slightly alkaline KCl medium (Schönfeld *et al.*, 2001). This swelling was accompanied by the release of endogenous Mg^{2+} to the surrounding medium (Schönfeld *et al.*, 2001, 2002). In these experiments, the medium was supplemented with the potassium ionophore valinomycin to ensure rapid equilibration of K^+ across the inner membrane. Therefore, swelling indicates that myristic acid stimulates Cl^- permeation in mitochondria. However, myristic acid failed to initiate Cl^- permeation when mitochondria were in neutral KCl medium. In addition, the induced Cl^- permeation was not due to protonophoric activity of myristic acid, since FCCP, a potent protonophore was not able to initiate mitochondrial swelling at alkaline KCl medium (Schönfeld *et al.*, 2000).

Despite the property of fatty acids to promote mitochondrial permeability transition effectively at low concentrations (for review see, Bernardi *et al.*, 2002), there are good reasons to exclude that myristate-induced mitochondrial swelling in alkaline KCl medium was due to opening of the permeability transition pore (PTP). First, it was insensitive to cyclosporin A, a compound known to block PTP (Crompton *et al.*, 1988). Second, it proceeds under conditions that were not typical for PTP opening, i.e., in the absence of Ca^{2+} and phosphate and in the deenergized state. Third, myristic acid was not able to induce large-scale mitochondrial swelling in alkaline sucrose medium. Therefore, we proposed that nonesterified long-chain fatty acids presumably activate an Mg^{2+}-controlled silent anion channel of the inner membrane (Schönfeld *et al.*, 2001). In this paper we have studied the properties of fatty acid-induced Cl^- permeation to identify the underlying biochemical mechanism. We applied swelling measurements to rat liver mitochondria, and in addition, single-channel current measurements to patches excised from mitoplasts. The obtained results reveal that fatty acids trigger the opening of an anion channel with properties similar to those reported for IMAC.

MATERIALS AND METHODS

Materials

Fatty acids, cyclosporin A, antimycin A, rotenone, cytochrome *c*, valinomycin, nigericin, tributyltin chloride, and A23187 were purchased from Sigma (Deisenhofen, Germany). Phytanic acid was purchased from ULTRA Scientific (North Kingstown, USA). Magnesium green-acetoxymethyl ester was purchased from Molecular Probes (Eugene OR). All other reagents were of high purity grade. Fatty acids were applied as 10 mM ethanolic solution.

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 (Schönfeld *et al.*, 2000). The final pellet was resuspended in 0.25 M sucrose at a protein concentration of 25–35 mg/mL. The protein content in the mitochondrial stock suspension was determined by the biuret method.

Swelling Assay

Mitochondrial swelling (1 mg protein/mL) was measured as a change in the absorbance at 540 nm by a Varian Cary 3E spectrophotometer equipped with a thermostated and magnetically stirred sampling unit. Mitochondria were suspended in KCl medium containing 120 mM KCl, 10 mM Tris, 0.5 mM EDTA, and 1 μM cyclosporin A (defined as standard incubation medium). Swelling was measured at 25°C, and was quantified by the initial rate of the decrease of light absorbance using the photometer software.

Determination of Matrix Mg^{2+}

Free matrix Mg^{2+} was monitored fluorimetrically using Magnesium green (MgG). Mitochondria (10 mg protein/mL) were loaded for 5 min at room temperature with MgG-acetoxymethylester (2 μM) in 0.25 M sucrose medium (without additions) at pH 7.4 and washed once in standard incubation medium (pH 7.4). Changes in free [Mg^{2+}] within mitochondria were recorded using a Perkin Elmer LS 50B luminescence spectrophotometer at 510 nm excitation and 535 nm emission wavelengths at room temperatute in MgG-loaded mitochondria.

Patch-Clamp Measurements

Preparation of mitoplasts from rat liver mitochondria and single-channel current measurements with mitoplast were done essentially as described by Loupatatzis *et al.* (2002). In short, aliquots of the mitochondrial stock-suspension were first treated hypotonically (1 min) with a solution composed of 5 mM K-HEPES and 1 mM $CaCl_2$ (pH 7.2). Thereafter, isotonicity was restored by

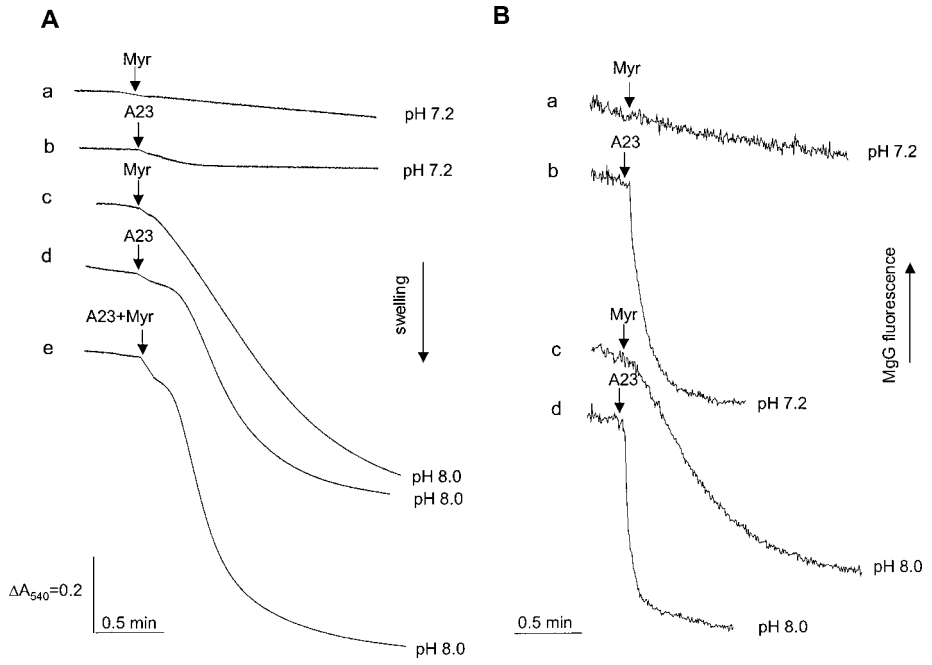

Fig. 1. Effect of myristic acid and of A23187 on swelling of mitochondria in KCl medium and Mg^{2+} depletion. Mitochondria (1 mg protein/mL) were suspended in KCl medium (plus 1 μM antimycin A and 0.5 μM valinomycin) adjusted to pH 7.2 or 8.0. Additions were myristic acid (30 μM, corresponding to 30 nmol/mg mitochondrial protein) or A23187 (1 μM). These experiments are representative of five replicates each. Shown are changes in light absorbance (A) and MgG fluorescence (B).

adding a solution composed of 750 mM KCl, 80 mM K-HEPES, and 1 mM $CaCl_2$ (pH 7.2). The filling solution for the borosilicate glass patch pipettes (resistance of about 17 MΩ; Clark, Pangbourne, UK) contained 150 mM KCl, 20 mM K-HEPES, and 0.1 mM $CaCl_2$ (pH 7.2). Free-floating mitoplasts were approached by means of an electrically driven micromanipulator and attached to the tip of the pipette by gentle suction. Patch-currents were recorded by an EPC-7 amplifier (HEKA electronics, Lambrecht, Germany). Experiments were done in the mitoplast-attached mode. Test solutions were added through the glass capillaries of a peristaltic-pump driven flow system. Currents were low-pass filtered by a 4-pole Bessel filter at a corner frequency of 1 kHz. Data were recorded at a sample frequency of 2.5 kHz by means of the pClamp6 software (Axon instruments, Foster City, CA) which was also used for processing of the data.

RESULTS

Induction of Cl^- Permeation by Alkalinization and Fatty Acids

Nonenergized mitochondria (i.e., in the absence of respiratory substrates and in the presence of respiratory inhibitor antimycin A) suspended in neutral or slightly alkaline (pH $\leq$ 8.0) KCl medium swell only slowly, even when valinomycin enables a rapid influx of K^+ (Fig. 1(A)). But, they start to swell rapidly when myristic acid (30 nmol/mg protein) or the Ca^{2+}/Mg^{2+} ionophore A23187 (1 nmol/mg protein) was added to KCl medium adjusted to pH 8.0. A simultaneous addition of myristic acid plus A23187 does not significantly change the initial swelling. A23187 is known to deplete mitochondria from endogenous Mg^{2+}, thereby increasing dramatically the permeability of the inner membrane towards small ions, such as K^+ or Cl^- (for review, see Beavis, 1992; Brierley *et al.*, 1994). Therefore, changes in endogenous Mg^{2+} content were estimated fluorimetrically using MgG as fluorescence probe (Fig. 1(B)). It can be seen that swelling is paralleled by depletion of endogenous Mg^{2+} at alkaline pH. In addition, contrary to A23187, myristic acid does not decrease Mg^{2+} at neutral pH, indicating that it has no ionophoretic activity to Mg^{2+}.

Figure 2 shows that Cl^- permeation into mitochondria increases heavily when the medium pH (corresponds to the matrix pH in nonenergized mitochondria) was shifted from pH 7.2 to more alkaline (pH 8). In the absence of any long-chain fatty acid this effect was almost absent. The ability of saturated long-chain fatty acids or their branched-chain derivatives to increase the permeability of

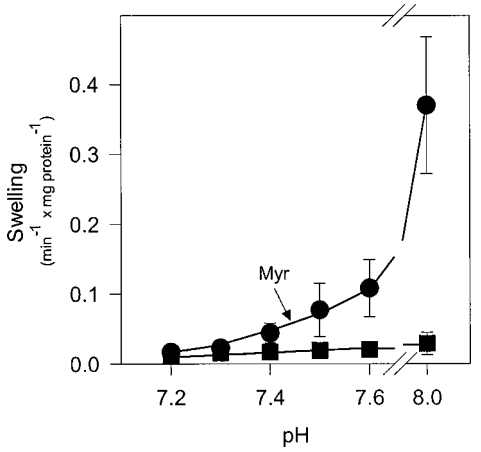

Fig. 2. Alkalinization of the medium pH increased in the presence of myristic acid and the permeability of the inner membrane to chloride dramatically. Mitochondria (1 mg protein/mL) were in KCl media (plus 1 μM antimycin A and 0.5 μM valinomycin) adjusted to pH values as indicated. The final concentration of myristic acid was 30 μM (filled circles). Filled squares were measured without addition of fatty acid. Data are given as means (±SD) obtained from four mitochondrial preparations.

the inner membrane to Cl^- differs considerably. Thus, unbranched fatty acids stimulate Cl^- permeation best when the backbone of their hydrocarbon-chain contained 14 or 16 carbon atoms (see Fig. 3). It might be of interest that the branched-chain phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), a moderate protonophore and promotor of mitochondrial permeability transition like its analogue palmitic acid (Gutknecht, 1987; Schönfeld and Bohnensack, 1997; Wojtczak *et al.*, 1998), stimulates Cl^- permeation less then palmitic acid. As mentioned above, it was found that myristic acid-induced mitochondrial swelling was accompanied by the release of matrix Mg^{2+} (Schönfeld *et al.*, 2001, 2002). Therefore, the release of Mg^{2+} was measured in the presence of fatty acids and their derivatives. When mitochondrial swelling rates were plotted vs. rates of depletion of matrix Mg^{2+}, a good correlation between both parameters was obtained (see Fig. 4). Data obtained with the bivalent-cation ionophore A23187 were included. A23187 stimulates mitochondrial swelling as myristic acid or palmitic acid do, but releases Mg^{2+} much more rapidly. In order to show that the loss of Mg^{2+} is not simply the consequence of swelling, the effect of swelling on the release of Mg^{2+} was also tested. For this purpose, mitochondria were suspended in alkaline isosmolar KSCN medium (SCN^- permeates freely the inner mitochondrial membrane), and the swelling was initiated by addition of valinomycin. In

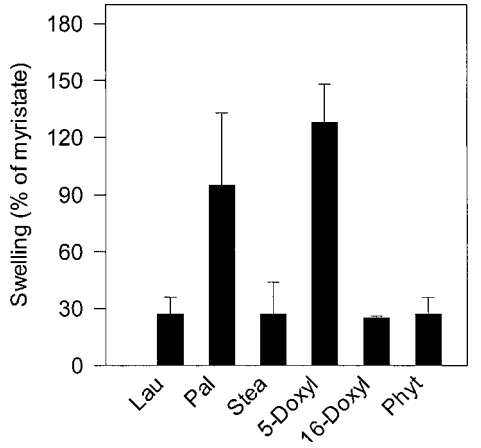

Fig. 3. Effect of various fatty acids on Cl^- permeability in alkaline KCl medium. Mitochondria (1 mg protein/mL) were suspended in alkaline KCl medium (pH 8.0). Additions were lauric acid (Lau), palmitic acid (Pal), stearic acid (Stea), 5-doxylstearic acid (5-Doxyl), 16-doxylstearic acid (16-Doxyl), or phytanic acid (Phyt). The final concentration of fatty acids added was 30 μM. Data are given as means (± SD) obtained from three to four mitochondrial preparations.

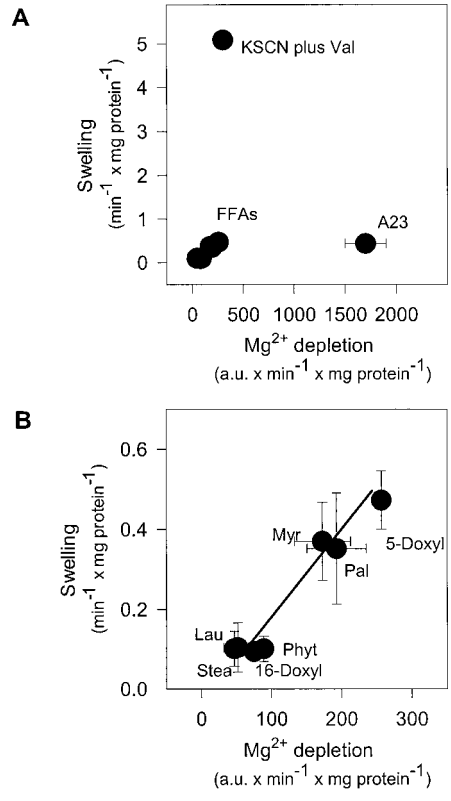

Fig. 4. Relation between mitochondrial swelling and depletion of matrix Mg^{2+} initiated by fatty acids or their derivatives. Rates of mitochondrial swelling were obtained from Fig. 3. Rates of depletion of matrix Mg^{2+} were estimated as described in Materials and Methods. The final concentration of A23187 was 1 μM. The effect of swelling on Mg^{2+} depletion was measured by treating mitochondria with KSCN medium (120 mM, pH 8.0) plus valinomycin. The data points are means (±SD) of three to four mitochondrial preparations.

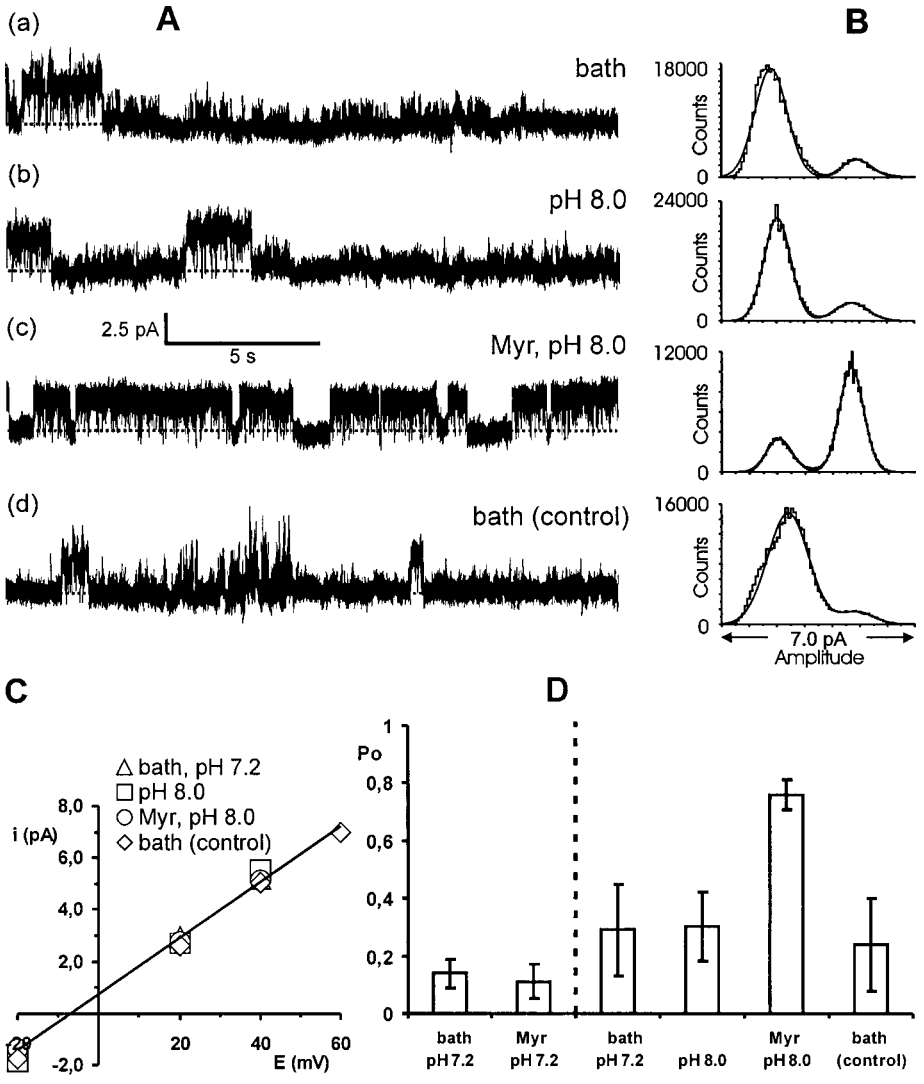

Fig. 5. Myristic acid induces increased activity of an ion channel in mitoplasts at alkaline medium pH. (A) Single-channel recordings: Single-channel current traces of 20 s duration at a holding potential of 20 mV are shown in the absence and in the presence of 200 nM myristate and at pH 7.2 or 8.0. Closed state marked by dotted lines. (B) All-point analysis of the current traces in panel A. Histograms give number of points for different amplitudes. (C) Current–voltage relationships: Relationships were fitted best by a straight line with a slope (single-channel conductance, γ) of 108 pS. (D) Mean open probability ($P_o \pm$ SEM) calculated from histograms of 2–5 comparable 20-s single-channel current segments under each condition. P_o was calculated from three independent experiments.

this case, mitochondria swelled 10 times more rapid as with myristic acid, but the initial rate of Mg^{2+} depletion was about that seen with 5-doxylstearic acid (Fig. 4(A)).

Patch-Clamping Indicates Channel-Opening by Myristate at Alkaline pH

In order to test for the underlying pathway of the Cl^- permeation, we examined whether myristic acid would be able to activate ion channels in the inner membrane. The patch-clamp technique was applied to mitoplasts suspended in neutral or alkaline KCl medium. An alkaline medium pH itself did not induce an ion current across the inner membrane of mitoplasts (Fig. 5, compare trace a with trace b in panel A, and the related histograms in panel B. However, exposing the patch to myristate at alkaline pH clearly induced increased activity of an ion channel (trace c). Replacement of the medium against neutral, myristate-free KCl medium (bath) reduces this activity back to normal (trace d). From the current–voltage

characteristics of the myristic acid-based activity a single-channel conductance of 108 pS can be calculated. Both features, the single-channel conductance and distinct kinetics, suggest that the myristic acid-induced conductance is due to activation of a known anion channel (Borecký *et al.*, 1997).

Anion Selectivity of Myristate-Induced Anion Permeation

Furthermore, we estimated the permeability of the proposed myristic acid-induced anion channel to such anions as sulfate, gluconate, or phosphate. In a second series of experiments, the permeability of the inner membrane to these anions was measured using mitochondria treated with A23187 plus nigericin (a K^+/H^+ exchanger) in order to activate IMAC (Beavis and Powers, 1989). Figure 6 shows that the initial rates of myristate- or A23187-induced mitochondrial swelling vary remarkably in alkaline media (pH 8.0) composed of K-sulfate, K-phosphate, or K-gluconate (supplemented with valinomycin). Most important, myristic acid does only slightly enhance the permeability of the inner membrane to gluconate, in contrast to a strongly elevated Cl^- permeation demonstrated before. A similar discrimination of the permeability of the IM to both of these anions was found with A23187, confirming a previous report by Beavis (1992).

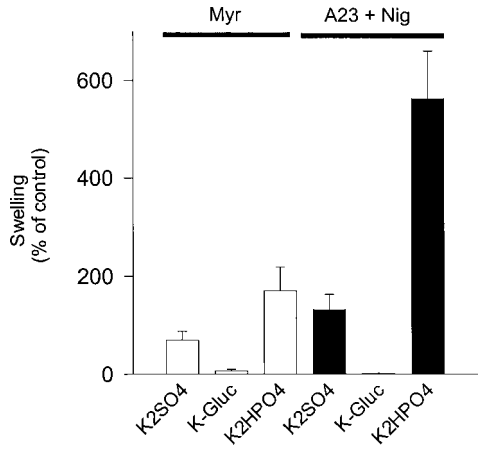

Fig. 6. Permeability of myristic acid- or A23187-treated mitochondria to sulfate, gluconate, or phosphate at alkaline medium pH. Mitochondria (1 mg protein/mL) were suspended in isosmolar alkaline solutions (240 mosmol, pH 8.0) of K-sulfate, K-gluconate, or K-phosphate. Media were supplemented with 2 μM antimycin A plus 0.5 μM valinomycin. Initial rates of swelling were estimated after addition of myristate (30 μM) or after addition of mitochondria pretreated with A23187 (1 μM) plus nigericin (1 μM). For measurement of the phosphate permeation the mitochondria were pretreated with *N*-ethylmaleimide (40 nmol/mg protein) in order to block the electroneutral phosphate/H^+ symporter. Data shown were normalized to swelling rates obtained in KCl medium. The data are means (±SD) obtained from four mitochondrial preparations.

Cl^- Extrusion From Swollen, Nonenergized Mitochondria After Energization

According to our hypothesis, the myristic acid-induced large-scale mitochondrial swelling is secondary to depletion of endogenous Mg^{2+} by nonesterified fatty acids (Schönfeld *et al.*, 2001). Consequently, the proposed anion channel should remain active even after inactivation of the added myristic acid to BSA. In order to test this point, BSA was added for binding of myristic acid, and succinate for energizing of the swollen mitochondria. Figure 7 shows a small contraction of mitochondria after addition of succinate (trace a). Complete extrusion of KCl from mitochondria cannot be expected under these conditions, since succinate-supported polarization of the inner membrane stimulates the valinomycin-mediated electrophoretic K^+ uptake, which decreased the membrane potential again. However, when the K^+/H^+ exchanger nigericin was substituted for valinomycin, the

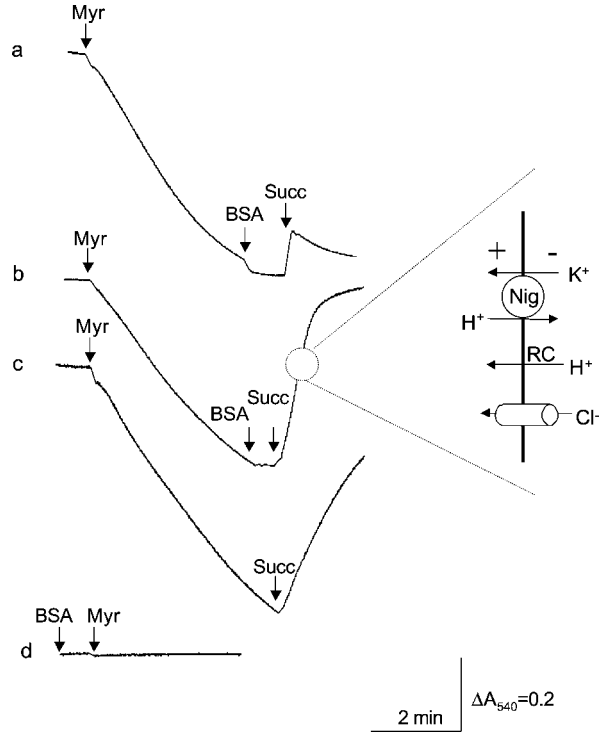

Fig. 7. Energy-driven extrusion of KCl from swollen mitochondria. Swelling of rat liver mitochondria (1 mg protein/mL) in alkaline KCl medium (pH 8.0) was induced by myristic acid (30 μM). The medium was supplemented with 5 μM rotenone, 5 μM cytochrome *c*, and 0.5 μM valinomycin (trace a) or 1 μM nigericin (traces b and c). BSA (1 mg/mL) and succinate (10 mM) were added as indicated.

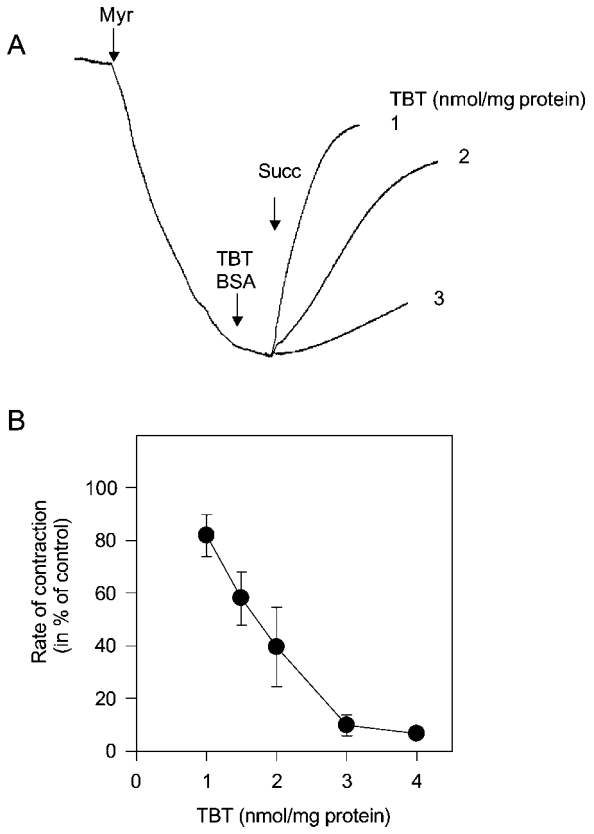

Fig. 8. Inhibition of chloride extrusion by increasing concentrations of tributyltin chloride. (A) Mitochondria (1 mg protein/mL) were treated as in Fig. 7 (trace b), except that tributyltin chloride was added prior to succinate. (B) Initial rates of contraction were referred to those measured without TBT. Data (means ± SD) were obtained from four mitochondrial preparations.

mitochondrial swelling was reversed with and without added BSA (traces b and c). When BSA was added before myristic acid, no swelling was induced, clearly indicating complete inactivation of myristic acid by BSA (trace d).

Triorganotin compounds are not only the most potent inhibitors of IMAC known to date, but they also inhibit its activity selectively (Powers and Beavis, 1991). Cl^- extrusion becomes strongly delayed when low doses of tributyltin chloride (TBT) were added together with BSA and succinate (Fig. 8, panel A). A half-maximal inhibitor concentration of about 1.5 nmol/mg protein was estimated from the dose–response curve for TBT (panel B).

DISCUSSION

Matrix alkalinization in connection with low concentrations of nonesterified myristic acid enhance the permeability of the inner membrane to Cl^-. This study investigated the mechanism that modifies the underlying change in permeability. In view of the similarities between myristic acid-induced anion permeation across the inner membrane, and anion permeation stimulated by A23187, it is likely that long-chain fatty acids activate IMAC. This view is supported by the following observations:

(i) If nonenergized rat liver mitochondria are suspended in alkaline KCl medium (supplemented with valinomycin or nigericin) an exposure to myristic acid plus EDTA initiates large-scale swelling (see Figs. 1(A), 7, and 8), which looks similar to that found with A23187 (see Fig. 1).

(ii) Mitochondrial swelling induced by myristic acid is paralleled by depletion of mitochondria from endogenous Mg^{2+} at alkaline pH (Fig. 1(B)). Matrix Mg^{2+} is well known to exert negative control on IMAC activity, and A23187, a potent Mg^{2+}/Ca^{2+} ionophore activates IMAC by reducing matrix Mg^{2+} (Beavis, 1992). This probably happens by dissociation of Mg^{2+} bound to negatively regulated binding sites at the integral proteins (Garlid, 1980). Since fatty acids easily permeate the inner membrane, and since they are known to complex free Mg^{2+} ions (Shinohara *et al.*, 1995), we proposed earlier that fatty acids under conditions of alkaline matrix pH activate an intrinsic Mg^{2+}/H^+ exchanger (Schönfeld *et al.*, 2002). Despite different rates of endogenous Mg^{2+} depletion initiated by A23187 or by myristic acid (Fig. 1(B)), the measured rates of induced Cl^- permeation into mitochondria are similar for both effectors (see Figs. 1(A) and 4). This could be explained by a mechanism by which long-chain fatty acids remove Mg^{2+} directly from supposed protein binding-sites at IMAC, whereas A23187, however, primarily depletes the matrix pool of Mg^{2+}, which secondarily initiates the dissociation of Mg^{2+} from its binding-sites. Different activities of fatty acids for stimulation of the transmembrane Cl^- permeation in mitochondria (see Fig. 3) could indicate the specificity of the interaction of fatty acids with the Mg^{2+} binding-sites. A certain structural requirement could explain why shortening or branching of the hydrocarbon-chain reduces the affinity of a fatty acid for an interaction with the Mg^{2+}-dependent regulatory sites of the anion channel.

(iii) Furthermore, myristic acid-induced Cl^- permeation across the inner membrane declines steeply when the medium pH was shifted from the alkaline (pH 8.0) to neutral (see Fig. 2). In the

same pH range, IMAC-mediated Cl^- permeation responded sensitively to a shift in the medium pH (Beavis and Garlid, 1987). Mg^{2+} depletion and alkalinization of matrix pH are known to be main factors in the activation of IMAC (Beavis and Powers, 1989)

(iv) Despite the fact that IMAC transports a wide variety of physiological and nonphysiological anions, some anions, such as gluconate, are transported slowly (Beavis, 1992). Figure 6 shows that liver mitochondria treated with myristic acid or A23187 swell much slower in alkaline K-gluconate medium (pH 8.0) as in alkaline KCl medium. This indicates that nonesterified fatty acids initiate the specificity of the inner membrane to anion permeation, in a similar way as A23187 does.

(v) Previous experiments have revealed that myristate-induced Cl^- permeation in mitochondria becomes inhibited by dicyclohexylcarbodiimide and quinine (Schönfeld *et al.*, 2001). Both chemicals are known to reduce the anion permeation mediated by IMAC (Beavis, 1992). In addition, tributyltin chloride, a potent and selective inhibitor of IMAC-mediated anion permeation (Powers and Beavis, 1991), inhibits the extrusion of Cl^- from swollen mitochondria even if it is applied in a very low concentration (see Fig. 8).

(vi) Finally, patch-clamp measurements at mitoplasts in alkaline KCl medium show that the permeability of the inner membrane to ions becomes strongly increased by myristic acid (see Fig. 5(A)). The myristic acid-induced ion current is best characterized by a conductance of 108 pS. An 108 pS-channel was shown to exhibit identical properties with IMAC (Borecký *et al.*, 1997).

In conclusion, activation of IMAC, and possibly, one or more cation-conducting systems in the inner membrane, such as the K^+ uniport and the K^+/H^+ antiporter (Schönfeld *et al.*, 2003), demonstrates a novel feature of nonesterified fatty acids. Based on the obtained results, fatty acid-induced activation of IMAC seems to be best explained by a direct withdrawal of Mg^{2+} from supposed protein binding-sites at IMAC. A good correlation between initial rates of swelling and those of Mg^{2+} depletion (Fig. 4) is in line with such an activity of fatty acids. This mechanism is further supported by the finding that transmembrane Cl^- permeation operates even after extraction of myristic acid from mitochondria by BSA (Fig. 7). Finally, the findings that long-chain fatty acids can activate different ion-conducting pathways similar to A23187, such as IMAC (this study), K^+/H^+ antiporter and K^+ uniporter (Schönfeld *et al.*, 2003), suggests that Mg^{2+} depletion is the cause of activation.

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