Activation of ion-conducting pathways in the inner mitochondrial membrane – an unrecognized activity of fatty acid?

P. Schönfeld[a,*], T. Schlüter[a], R. Schüttig[b], R. Bohnensack[a]

[a] *Institute of Biochemistry, Otto-von-Guericke-University, Leipziger Str. 44, D-39120 Magdeburg, Germany*
[b] *Institute of Clinical Chemistry, Clinical Centre Dresden Friedrichstadt, Friedrichstr. 41, D-01067 Dresden, Germany*

Received 20 December 2000; revised 9 January 2001; accepted 12 January 2001

First published online 29 January 2001

Edited by Vladimir Skulachev

Abstract The effect of non-esterified myristate (C14:0) or dodecyl sulfate was studied on passive swelling of rat liver mitochondria suspended in hypotonic alkaline KCl medium in the absence of the potassium ionophore valinomycin. Both compounds rapidly initiated large-amplitude swelling. However, they failed to initiate swelling when the mitochondria were suspended in hypotonic alkaline sucrose medium. In contrast to myristate or dodecyl sulfate, the non-ionic detergent Triton X-100 initiated swelling of mitochondria in both of the media. The following findings indicate that the inner mitochondrial membrane (IMM) is permeabilized by myristate to K^+ and Cl^- in a specific manner. (i) Swelling initiated by myristate did not respond to cyclosporin A, (ii) the protonophoric uncoupler FCCP was unable to mimic the myristate effect on swelling, and (iii) myristate-induced Cl^--permeation (measured with KCl medium plus valinomycin) was inhibited by *N,N′*-dicyclohexylcarbodiimide, quinine or ATP. Myristate- or dodecyl sulfate-initiated swelling was paralleled by the lowering of endogenous Mg^{2+} content. Both effects, stimulation of swelling and depletion of endogenous Mg^{2+} are correlated with each other. Similar effects have been reported previously for the carboxylic divalent cation ionophore calcimycin (A23187). The A23187-induced swelling has identical inhibiting characteristics on Cl^--permeation with respect to *N,N′*-dicyclohexylcarbodiimide, quinine and ATP as the myristate-stimulated swelling. Therefore, we conclude that non-esterified fatty acids increase the permeability of mitochondria to K^+ and Cl^- at alkaline pH by activating Mg^{2+}-dependent ion-conducting pathways in IMM.

Key words: Fatty acid; Swelling; Mitochondrion; Magnesium; Inner membrane anion channel

1. Introduction

Within cells mitochondria generate a high, inside negative membrane potential ($\Delta\psi$) in a K^+-rich milieu. Under this condition, an uncompensated K^+ uptake in the matrix would initiate swelling and, thereby destroy the morphological and functional integrity of mitochondria. To maintain the mitochondrial physiology, the inner mitochondrial membrane (IMM) contains ion carriers and channels regulating the net ion exchange across IMM (for review see [1–4]). The K^+/H^+ antiporter, a 82-kDa protein enables the release of matrix-K^+ resulting from electrophoretic K^+ uptake. In addition, Cl^- permeates IMM using an anion channel with broad specificity for anions, the inner membrane anion channel (IMAC) [5]. Both, IMAC and the K^+/H^+ antiporter are believed to participate in net salt extrusion from mitochondria [5,6].

Despite of $\Delta\psi$-driven leakage of K^+ into the matrix, an electrophoretic K^+ uptake occurs by a K^+ uniporter [2,7] and an ATP-sensitive K^+ channel [8,9]. In isolated mitochondria, IMAC, K^+/H^+ antiporter and K^+ uniporter are latent, but become activated when mitochondria are suspended in alkaline hypotonic medium and endogenous Mg^{2+} is depleted with the carboxylic ionophore calcimycin (A23187) [5,7,10–12]. It has been proposed that these ion-conducting pathways are regulated by the concentration of free matrix-Mg^{2+}, putatively by disassociation of Mg^{2+} bound to negative regulatory sites [9,12]. Additionally, Mg^{2+} interacts with other membrane constituents, preferably with negatively charged membrane phospholipids [13,14] and, on this manner could change the membrane fluidity [15]. These observations may explain that Mg^{2+} is involved in the regulation of a variety of membrane transport systems (see also [16] and references therein).

We report in this study that non-esterified long-chain ('free') fatty acids (FFA) applied in the micromolar concentrations can also deplete mitochondria from endogenous Mg^{2+} and initiate large-amplitude swelling of mitochondria. We hypothesize that the previously reported initiation of swelling of mitochondria by FFA [17] is due to the activation of ion-conducting pathways in the IMM.

2. Materials and methods

2.1. Mitochondria

Liver mitochondria were prepared from adult female Wistar rats (average weight 150–180 g) by differential centrifugation according to our standard protocol [17]. Protein content in the mitochondrial stock suspension was determined by a modified biuret method.

2.2. Passive swelling assay

Passive swelling of non-respiring rat liver mitochondria was done as in [17]. In short, an aliquot of mitochondria (1 mg protein) was added to 1 ml of the KCl medium consisting of 120 mM KCl (with and without 0.5 μM valinomycin (Val)), 10 mM Tris–HCl, 0.5 mM EDTA, 1 μM cyclosporin A (CyA), 2 μM rotenone and 1 μM anti-

*Corresponding author. Fax: (49)-391-67 13050.
E-mail: peter.schoenfeld@medizin.uni-magdeburg.de

Abbreviations: FFA, non-esterified fatty acids; Myr, myristate; IMM, inner mitochondrial membrane; PTP, permeability transition pore complex; IMAC, inner membrane anion channel; A23187, calcimycin; MgG, magnesium green; Val, valinomycin; SDS, sodium dodecyl sulfate; CTAMB, cetyl trimethyl ammonium bromide; CyA, cyclosporin A; DCCD, *N,N′*-dicyclohexylcarbodiimide

0014-5793/01/$20.00
PII: S0014-5793(01)02148-2

mycin A. When not otherwise stated, the KCl medium was adjusted to pH 8.0. Mitochondria were preincubated for 2 min in the KCl medium and, afterwards the swelling was initiated by the addition FFA, sodium dodecyl sulfate (SDS), CTMAB, A23187 or Triton X-100 as ethanolic or aqueous solution. Swelling was measured photometrically at 22°C and was quantified by the initial rate of the decrease of light absorbance at 540 nm using the photometer software.

Rupture of the mitochondrial inner membrane by lytic activity of myristate (Myr), SDS or Triton X-100 was assessed by measuring the release of the matrix enzyme malate dehydrogenase (MDH) that was determined photometrically according to [18]. In short, mitochondria were incubated in KCl medium (pH 8.0) with Myr (40 nmol mg protein^{-1}), SDS (40 nmol mg protein^{-1}) or Triton X-100 (0.02%), swelling was terminated after 2 min by addition of bovine serum albumin (1 mg ml^{-1}), the incubation mixture was centrifuged and MDH was measured in the supernatant.

2.3. Determination of endogenous magnesium

2.3.1. Free matrix-magnesium. Mitochondria (10 mg ml^{-1}) were stained for 5 min with 2 μM magnesium green (MgG)-acetoxymethyl ester (Molecular Probes, Eugene, OR, USA) and washed once in KCl medium. Changes in the free matrix-Mg^{2+} concentration were recorded fluorometrically (510 nm excitation/535 nm emission) after adding stock solutions of FFA, SDS or A23187 to a cuvette containing stained mitochondria (1 mg protein ml^{-1}) at room temperature.

2.3.2. Released magnesium. Mitochondria were incubated in KCl medium (1 mg protein/1.3 ml) for 5 min without or with A23187, Myr or SDS at 25°C, followed by rapid sedimentation of mitochondria in a microcentrifuge. Released as well as retained Mg^{2+} was determined in the supernatant and in the mitochondrial pellet by atomic absorption spectroscopy measurements.

2.4. Chemicals

Myr ($C_{14:0}$), palmitate ($C_{16:0}$), stearate ($C_{18:0}$), laurate ($C_{12:0}$), arachidonate ($C_{20:4}$), SDS, cetyl trimethyl ammonium bromide (CTAMB), *N,N′*-dicyclohexylcarbodiimide (DCCD), antimycin A, rotenone and ATP were from Sigma (St. Louis, MO, USA). Quinine, A23187 and CyA were from Calbiochem. β,β′-Methyl-substituted hexadecane α,ω-dioic acid (Medica 16) was kindly provided by Dr. Jacob Bar-Taba, Department of Human Nutrition and Metabolism, Hebrew University, Jerusalem, Israel.

3. Results

3.1. Stimulation of passive swelling by Myr

Myr applied at low concentrations (5–10 nmol mg protein^{-1}) to rat liver mitochondria suspended in KCl medium stimulated swelling significantly, whereas lower concentrations of the detergent Triton X-100 were without effect (see Fig. 1A). In order to eliminate possible contribution of the permeability transition, the KCl medium was supplemented with CyA, a potent blocker of the permeability transition pore [4]. In case of lytic action of Myr, it is expected that swelling in mitochondria is initiated irrespective of the medium composition. Indeed, with a higher concentration of Triton X-100 mitochondria swell rapidly in KCl and in sucrose medium (see Fig. 1B). On the contrary, Myr does not initiate swelling of mitochondria in sucrose medium. In addition, at a lower pH of KCl medium (7.4), Myr fails to stimulate swelling in mitochondria [17], whereas Triton X-100 still is effective (not shown). Interestingly, dodecyl sulfate acted similar as Myr, whereas cetyl trimethyl ammonium did not initiate swelling in both media. Finally, MDH activity released from mitochondria during initial Myr-initiated swelling (see Section 2) was practically not higher (8 ± 6% of total activity) than without Myr (3 ± 1% of total activity). In the whole, these observations suggest a specific action of Myr in the swelling of mitochondria in alkaline KCl medium.

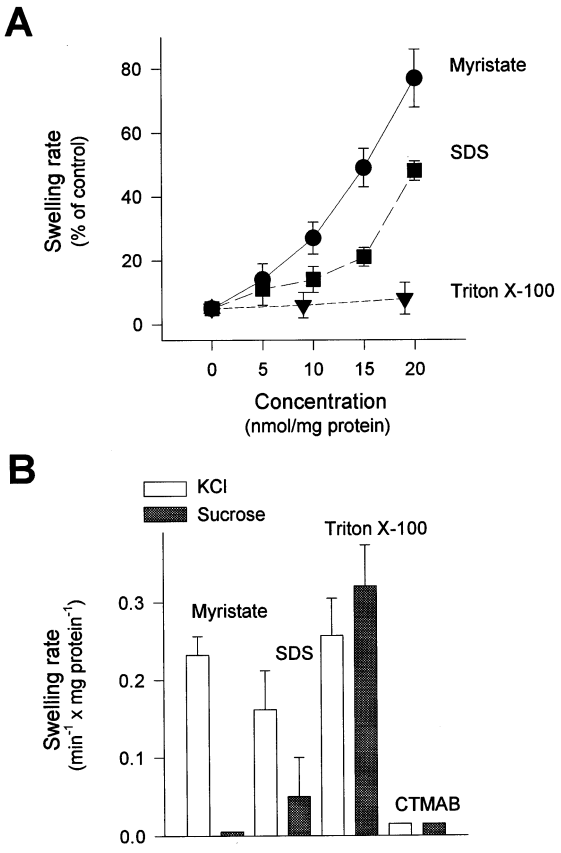

Fig. 1. Passive swelling of mitochondria. A: Concentration dependency of swelling. RLM were suspended in KCl medium and the swelling was initiated by addition of various concentrations of Myr, SDS or Triton X-100. Swelling is expressed in percent of the swelling rate adjusted with 40 nmol Myr mg protein^{-1} (0.383 ± 0.065/min mg protein^{-1} was set 100%). B: Swelling in KCl or sucrose medium initiated by Myr, dodecyl sulfate, Triton X-100 or CTAMB. RLM (1 mg protein) were suspended in 1 ml of KCl medium or of sucrose medium (240 mM sucrose, 10 mM Tris–HCl, 0.5 mM EDTA, 2 μM rotenone, 1 μM antimycin A, 1 μM CyA) adjusted to pH 8. Additions were Myr (40 nmol mg protein^{-1}) or SDS (40 nmol mg protein^{-1}) or Triton X-100 (300 nmol mg protein^{-1}). The data (means ± S.D.) were from three to six mitochondrial preparations.

3.2. Effect of fatty acids on mitochondrial Mg^{2+}

Since the above described swelling initiated by Myr is similar as reported for the carboxylic bivalent cation ionophore A23187 [5,7,10–12], the question arises: Does Myr deplete mitochondria from endogenous Mg^{2+}? For examination, the effect of Myr on free matrix-Mg^{2+} was measured applying magnesium green (MgG) as fluorescent probe for Mg^{2+} [19]. RLM loaded with MgG were suspended in alkaline KCl medium (pH 8.0) and the fluorescence of the Mg^{2+}–MgG complex was recorded before and after addition of Myr or A23187. As Fig. 2 shows, Myr decreased the fluorescence of the Mg^{2+}–MgG complex similar as A23187 (trace A and B), suggesting that it lowers free matrix-Mg^{2+}. Interestingly, SDS decreased also the fluorescence, but not as rapid as Myr (trace C). Contrary to A23187, Myr decreased only slowly the fluorescence of the Mg^{2+}–MgG complex when mitochondria were incubated in KCl medium adjusted to pH 7.4 (trace D and E).

We measured at alkaline KCl medium the release of Mg^{2+} in the medium as well as its retention in mitochondria by means of atomic absorption spectrometry. Mitochondria

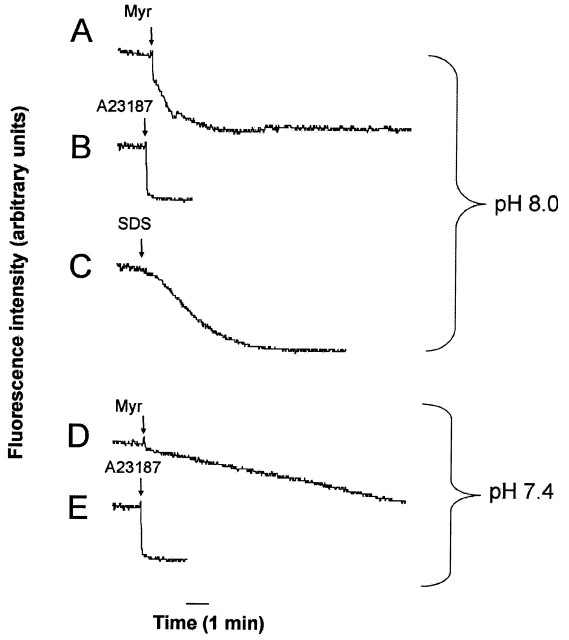

Fig. 2. Decrease of endogenous free matrix-Mg^{2+} by Myr. RLM (1 mg protein ml^{-1}) pretreated with the fluorescent probe McG-acetoxymethyl ester were suspended in KCl medium (pH 7.4 or 8.0). Myr (40 nmol mg $protein^{-1}$), A23187 (1 nmol mg $protein^{-1}$) or SDS (40 nmol mg $protein^{-1}$) were added as indicated.

were incubated for 2 min with Myr, SDS or A23187 and after centrifugation the Mg^{2+} content was measured in samples of the supernatant and pellets. Again, Myr or SDS stimulated the release of endogenous Mg^{2+} from mitochondria, but not in such a quantitative manner as A23187 (see Fig. 3).

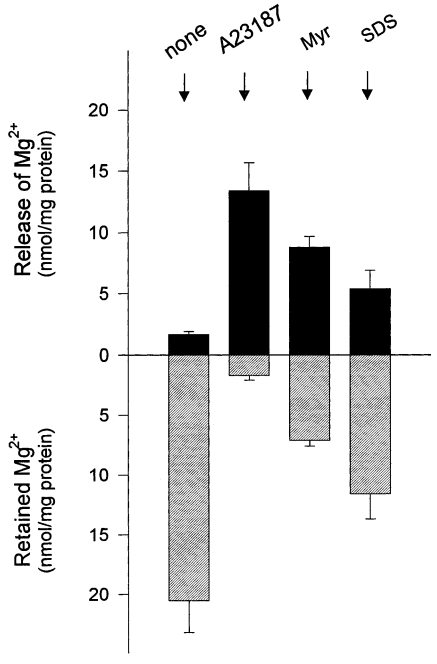

Fig. 3. Effect of Myr on the release of endogenous Mg^{2+} from mitochondria. RLM (1 mg protein/1.3 ml) were incubated in KCl medium (pH 8.0) with Myr (40 nmol mg $protein^{-1}$), A23187 (1 nmol mg $protein^{-1}$) or SDS (40 nmol mg $protein^{-1}$) as described in Section 2. Mg^{2+} released from or retained in RLM was measured by atomic absorption spectrometry. The data (means ± S.D.) were from four mitochondrial preparations.

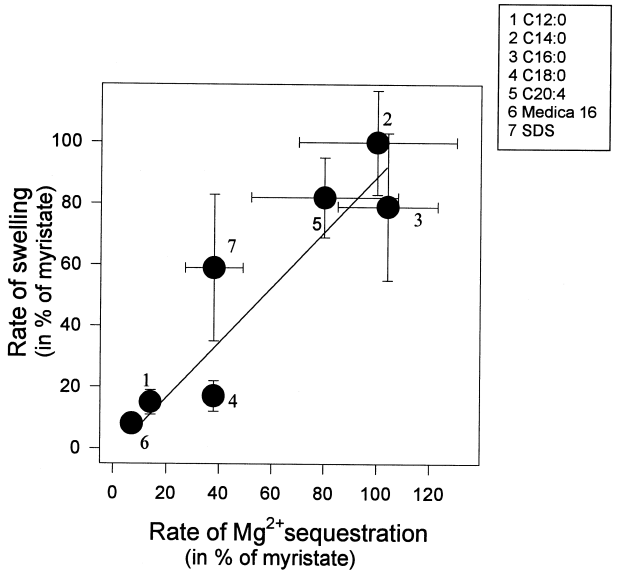

Fig. 4. Dependency of swelling on the decrease of endogenous Mg^{2+} content. Swelling rates were measured with a variety of FFA (40 nmol mg $protein^{-1}$) or SDS (40 nmol mg $protein^{-1}$) as described in Section 2 and normalized to the Myr-stimulated swelling (a swelling rate of 0.232 ± 0.024 min^{-1} mg $protein^{-1}$ was set at 100%). Slopes of FFA-induced quenching of Mg^{2+}–MgG fluorescence were estimated from experiments such as shown in Fig. 1 and normalized to Myr-induced quenching (a fluorescence decrease of 32 ± 9 arbitrary units min^{-1} mg $protein^{-1}$ was set at 100%). The data points are from four to seven mitochondrial preparations.

3.3. FFA-induced swelling and lowering of endogenous Mg^{2+}

Next we examined a potential relationship between the ability of various FFA to decrease free Mg^{2+} and their ability to stimulate swelling of mitochondria. The normalized slopes of the FFA-induced swelling and that of the FFA-induced decrease in the fluorescence of the Mg^{2+}–MgG complex were plotted versus each other (see Fig. 4). Indeed, close proportionality between the FFA-linked lowering of matrix-Mg^{2+} and their ability to stimulate swelling in mitochondria was found.

3.4. Inhibition of passive swelling

An increased permeability of IMM to Cl^- has been attributed to an activation of IMAC, resulting from depletion of endogenous Mg^{2+} at alkaline matrix-pH [5]. Permeation of solutes across IMAC is inhibited by DCCD or quinine [5]. Therefore, we examined the possibility that Myr activates IMAC. For that, mitochondria were suspended in alkaline KCl medium (plus Val; pH 8.0). Under this conditions swelling of mitochondria is only limited by the permeation of Cl^-. Myr-initiated swelling of mitochondria in KCl medium was inhibited by DCCD or quinine, identical to that initiated by A23187 (see Fig. 5). Interestingly, swelling of RLM was also inhibited by ATP in a concentration-dependent manner.

4. Discussion

Recent reports have demonstrated, that FFA stimulate large-amplitude swelling of mitochondria, preloaded with Ca^{2+} and suspended in neutral medium (pH 7.2–7.4), by the opening of the permeability transition pore complex (PTP) [20–28]. In the present paper we studied large-amplitude swell-

ing initiated in mitochondria by Myr in alkaline KCl medium. This swelling does not respond to CyA and is found with mitochondria not preloaded with Ca^{2+}. Thus, Myr-initiated swelling of mitochondria in alkaline KCl medium was obviously not due to an activation of PTP. Therefore, two alternative mechanisms, (1) a lytic action of Myr on mitochondria and (2) an activation of latent ion-conducting pathways in IMM, have to be considered. The results obtained suggest that Myr depletes mitochondria from endogenous Mg^{2+} and thereby activates ion-conducting pathways, allowing the permeation of IMM to normally non-permeable ions (K^+, Cl^-). This conclusion is based on the following findings:

1. Low concentrations of Myr (5–10 nmol mg protein^{-1}) initiate swelling in CyA-treated mitochondria suspended at alkaline KCl medium (Fig. 1A), whereas mitochondria suspended in alkaline sucrose medium do not swell, even in the presence of higher concentration of Myr (40 nmol mg protein^{-1}).
2. The rupture of IMM by lytic action of Myr would stimulate the release of the matrix enzyme MDH. But, we did not found significantly elevated activity of MDH in the supernatant of samples exposed to short-term swelling with Myr (40 nmol mg protein^{-1}).
3. Contrary to Myr, the potent and highly lipid-soluble protonophore FCCP [17] or the cationic surfactant CTAMB does not stimulate swelling of mitochondria in alkaline KCl medium (Fig. 1B), suggesting that Myr interacts specifically with mitochondrial membrane constituents.
4. Myr and dodecyl sulfate decrease free matrix-Mg^{2+} and deplete mitochondria from endogenous Mg^{2+} similar as the ionophore A23187 (Figs. 2 and 3). Depletion of endogenous Mg^{2+} from mitochondria was reported to be related to an increased K^+ or Cl^- permeability and passive swelling in media based on potassium salts [11,29]. The property of FFA to deplete mitochondria from Mg^{2+} is assumed to be caused by their ability to form tight complexes, known as 'fatty acid anion-Mg^{2+} soaps' [30]. Such view is supported by the parallel effects of dodecyl sulfate on swelling and Mg^{2+}-depletion of mitochondria and, in addition, by the missing action of the positively charged cetyl trimethyl ammonium on both.

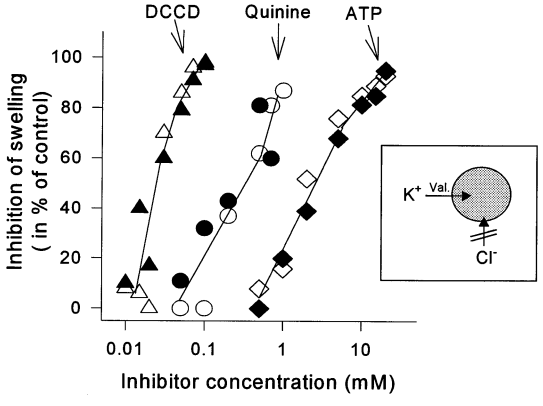

Fig. 5. Effect of DCCD, quinine or ATP on Myr-initiated swelling. RLM (1 mg protein ml^{-1}) suspended in KCl medium (plus 0.5 μM Val) were pretreated with various concentrations of DCCD, A23187 or ATP and, thereafter (2 min) swelling was initiated by addition of Myr (40 nmol mg protein^{-1}) or A23187 (1 nmol mg protein^{-1}). The data points are the mean of three mitochondrial preparations.

5. Since incorporation of FFA in liposomal membranes shifts their p*K*a from 5 to about 8 [31], it is expected that at alkaline medium pH (8.0) the concentration of the Myr anion is higher than at pH 7.4. This could explain why swelling is strongly stimulated when the medium pH was increased from 7.4 to 8.0 [17].
6. The potency of FFA to stimulate swelling of mitochondria in alkaline KCl medium correlates well with their ability to decrease matrix-Mg^{2+} (Fig. 4).
7. Myr-stimulated swelling of mitochondria suspended in KCl-Val medium is inhibited in a dose-dependent manner by DCCD, quinine or ATP, in an identical way as A23187-initiated swelling (Fig. 5).

Taken together, these findings suggest that FFA deplete mitochondria at alkaline pH from Mg^{2+} and activate thereby Mg^{2+}-dependent ion-conducting pathways, e.g. such as IMAC. The exact mechanism of the Myr-induced Mg^{2+} depletion of mitochondria has to be explored in further studies.

Acknowledgements: The authors wish to thank Mrs. H. Goldammer for her skilful help in performing the experiments and express their thanks to Professors Lech Wojtczak (Nencki Institute of Experimental Biology, Warsaw, Poland) and Keith Garlid (Oregon Graduate Institute of Science and Technology, Beaverton, OR, USA) for their valuable comments.

References

[1] Brierley, G.P. and Jung, D.W. (1988) J. Bioenerg. Biomembr. 20, 193–209.
[2] Garlid, K. (1996) Biochim. Biophys. Acta 1275, 123–126.
[3] Brierley, G.P., Baysal, K. and Jung, D.W. (1994) J. Bioenerg. Biomembr. 26, 519–526.
[4] Bernardi, P. (1999) Physiol. Rev. 79, 1127–1155.
[5] Beavis, A.D. (1992) J. Bioenerg. Biomembr. 24, 77–90.
[6] Garlid, K.D. (1988) in: Integration of Mitochondrial Function (Lemaster, J.J., Hackenbrock, C.R., Thurman, R.G. and Westerhoff, H.V), pp. 259–278, Plenum, New York.
[7] Bernardi, P., Angrilli, A., Ambrosin, V. and Azzone, G.F. (1989) J. Biol. Chem. 264, 18902–18906.
[8] Inoue, I., Nagase, H., Kishi, K. and Higuti, T. (1991) Nature 352, 244–247.
[9] Garlid, K.D (2000) Basic Res. Cardiol. 95, 275–279.
[10] Jung, D.W. and Brierley, G.P. (1986) J. Biol. Chem. 261, 6408–6415.
[11] Beavis, A.D. and Garlid, K.D. (1987) J. Biol. Chem. 262, 15085–15093.
[12] Garlid, K.D. (1980) J. Biol. Chem. 255, 11273–11279.
[13] Casal, H.L., Mantsch, H.H. and Hauser, H. (1989) Biochim. Biophys. Acta 982, 228–236.
[14] Merchant, T.E. and Glonek, T. (1992) Lipids 27, 551–559.
[15] Fuyu, Y., Beiqi, G. and Yuguo, H. (1983) Biochim. Biophys. Acta 724, 104–110.
[16] Wachter, C. and Turnheim, K. (1996) J. Membr. Biol. 150, 275–282.
[17] Schönfeld, P., Wieckowski, M.R. and Wojtczak, L. (2000) FEBS Lett. 471, 108–112.
[18] Bergmeyer, H.U. and Bernt, E. (1983) in: Methods of Enzymatic Analysis (Bergmeyer, H.U., Bergmeyer, J. and Grassl, M., Eds.), 3rd edn., Vol. 3., pp. 171–173, Verlag Chemie, Weinheim.
[19] Leyssens, A., Nowicky, A.V., Patterson, L., Crompton, M. and Duchen, M.R. (1996) J. Physiol. 496, 111–128.
[20] Petronilli, V., Carmen, C., Massari, S., Colonna, R. and Bernardi, P. (1993) J. Biol. Chem. 268, 21939–21945.
[21] Brustovetsky, N.N., Egorova, M.V., Gnutov, D., Yu, D., Mokohova, E.N. and Skulachev, V.P. (1993) FEBS Lett. 315, 233–236.
[22] Starkov, A.A., Markova, O.V., Mokhova, E.N., Arrigoni-Martelli, E. and Bobyleva, V.A. (1994) Biochim. Mol. Biol. Int. 32, 1147–1155.

[23] Broekemeier, K.M. and Pfeffer, D.R. (1995) Biochemistry 34, 16640–16649.
[24] Schönfeld, P. and Bohnensack, R. (1997) FEBS Lett. 420, 167–170.
[25] Wieckowski, M.R. and Wojtczak, L. (1998) FEBS Lett. 423, 339–342.
[26] Catisti, R. and Vercesi, A.E. (1999) FEBS Lett. 464, 97–101.
[27] Chavez, E., Zazueta, C. and Garcia, N. (1999) FEBS Lett. 445, 189–191.
[28] Di Paola, M., Cocco, T. and Lorusso, M. (2000) Biochem. Biophys. Res. Commun. 277, 128–133.
[29] Jung, D.W. and Brierley, G.P. (1986) J. Biol. Chem. 261, 6408–6415.
[30] Shinohara, Y., Unami, A., Teshima, M., Nishida, H., van Dam, K. and Terada, H. (1995) Biochim. Biophys. Acta 1228, 229–234.
[31] Sankaram, M.B., Brophy, P.J., Jordi, W. and Marsh, D. (1990) Biochim. Biophys. Acta 1021, 63–69.