Long-chain fatty acids promote opening of the reconstituted mitochondrial permeability transition pore

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Abstract Adenine nucleotide translocase–porin–hexokinase complex isolated from rat brain, when reconstituted into phospholipid-cholesterol vesicles, exhibits all properties of the mitochondrial permeability transition pore [Beutner, G., Rück, A., Riede, B., Welte, W. and Brdiczka, D. (1996) FEBS Lett. 396, 189–195]. In the present work, the effect of long-chain fatty acids on such reconstituted pore was examined. Opening of the pore was measured by leakage of either malate or fluorescein sulphonate entrapped inside the vesicles. It was found that myristate and oleate in the presence of 50 or 100 μM Ca^{2+} produced a partial release of the probes in a dose-dependent way. A dicarboxylic fatty acid analogue, that appeared inactive as protonophore in intact mitochondria, exerted no effect on pore opening in the reconstituted system. 100 μM Ca^{2+} alone was without effect. Pore opening by fatty acids in the reconstituted system was partly prevented by cyclosporin A. The pore opening also occurred when the vesicles were incubated in the presence of pancreatic phospholipase A_2. In this case, the opening was decreased by cyclosporin A or serum albumin. These results indicate that long-chain fatty acids elicit opening of the permeability transition pore reconstituted in phospholipid vesicles in a similar way as in intact mitochondria [Więckowski, M.R. and Wojtczak, L. (1998) FEBS Lett. 423, 339–342].

Key words: Mitochondrial permeability transition pore; Reconstitution; Fatty acid; Hexokinase; Phospholipase A_2

1. Introduction

The well-known uncoupling of oxidative phosphorylation by long-chain fatty acids is due to their protonophoric action (for reviews see [1]). Skulachev and coworkers [2,3] have proposed that the adenine nucleotide translocase (ANT) is involved in mediating the transfer of fatty acid, thus providing a rationale for the fatty acid cycling mechanism [4], i.e. a spontaneous movement of the undissociated (protonated) form of the fatty acid from the external leaflet of the membrane to the internal one and a carrier-mediated transfer of the anionic form in the opposite direction. More recently, participation of other mitochondrial carriers, the glutamate/aspartate [5,6], the dicarboxylate [6], the monocarboxylate [7], the tricarboxylate [7] and the phosphate [8] carriers in fatty acid-induced proton permeability of the inner mitochondrial membrane has also been shown (reviewed in [9,10]). Apart from this mechanism, in which long-chain fatty acids supported by mitochondrial carrier proteins function as real protonophores, it has also been proposed [11–14] that fatty acids may promote collapse of the mitochondrial electrochemical proton gradient in an indirect way, namely by opening the permeability transition pore (PTP). Opening of PTP by long-chain fatty acids has already been observed by other authors using the swelling assay [15–17].

PTP is a large non-selective channel that opens under specific conditions as result of Ca^{2+} accumulation in the mitochondrial inner compartment [15] (for reviews see [18,19]). The assembly of PTP is assumed to include ANT [20], porin, cyclophilin D [21] and hexokinase and/or creatine kinase [22,23]. It has been suggested [13,14] that fatty acids may induce PTP opening by interacting with ANT in a similar way as, for example, carboxyatractyloside, another pore-opening ANT ligand [24,25].

The PTP multiprotein assembly has been successfully reconstituted into phospholipid membranes in a functionally competent way [22,23]. The aim of the present work is to examine whether such reconstituted pore can be opened by long-chain fatty acids in a similar fashion as found for the native PTP [14].

2. Materials and methods

2.1. Chemicals

Cyclosporin A was from Sandoz (at present a part of Novartis, Basel, Switzerland); fluorescein-5-(and-6)sulphonate, trisodium salt, from Molecular Probes (Eugene, OR, USA); oleic and myristic acids were from Applied Sciences Laboratories (State College, PA, USA); phospholipase A_2 from bovine pancreas and bovine serum albumin, essentially fatty acid-free, were from Sigma (St. Louis, MO, USA); Sephadex G50 was from Pharmacia (Uppsala, Sweden); and DEAE-52 cellulose was from either Whatman (Maidstone, Kent, UK) or Sigma. 12-(Carboxymethyl-thio)dodecanoic acid, synthesized according to Skrede et al. [26], was a generous gift of Professor Jon Bremer, University of Oslo.

2.2. Isolation of the permeability transition pore complex

PTP was isolated from frozen rat brains as the hexokinase–porin–ANT complex exactly as described previously [22]. Fractions from DEAE-52 cellulose containing the highest hexokinase activity (determined as described previously [22,23]) were pooled and used for reconstitution.

2.3. Reconstitution of the pore complex into liposomes

Pooled fractions containing the highest hexokinase activity were

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Abbreviations: ANT, adenine nucleotide translocase; PTP, mitochondrial permeability transition pore; $\Delta\psi$, mitochondrial transmembrane electric potential

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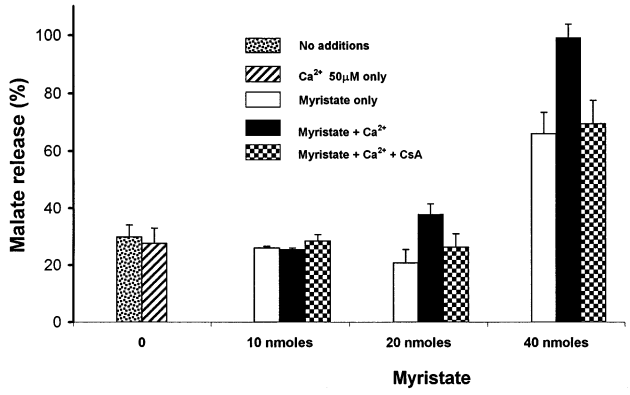

Fig. 1. Effect of myristate and Ca^{2+} on opening of the reconstituted permeability transition pore. Reconstituted vesicles, corresponding to about 2 mg asolectin, were incubated in 275 μl of 125 mM sucrose+10 mM HEPES/NaOH (pH 7.4), as described in Section 2, with or without the indicated amounts of $CaCl_2$ and/or myristate. Cyclosporin A (CsA), when present, was added to the final concentration of 2 μM 10 min before myristate and $CaCl_2$. The incubation was stopped by addition of 0.5 mM EGTA, followed by determination of malate release. Mean values of three experiments ± S.D., except for the effect of 10 nmol myristate where mean values of two experiments ± range, are shown.

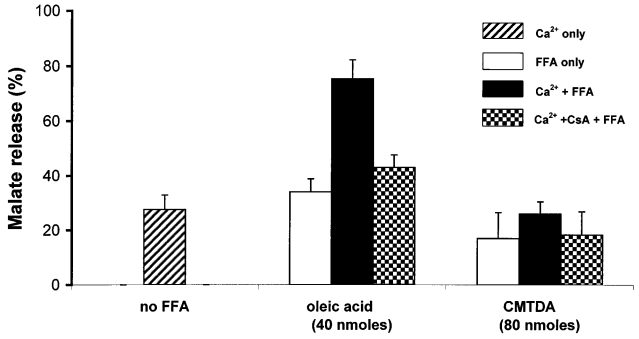

Fig. 2. Effect of 'active' (oleic) and 'inactive' (12-(carboxymethyl-thio)dodecanoic, abbreviated as CMTDA) acids on reconstituted PTP. Experimental conditions were as in Fig. 1. Mean values of three–six experiments ± S.D. are presented.

reconstituted into liposomes prepared from asolectin phospholipids and 2% cholesterol in the presence of 0.06% *n*-octylglucoside essentially as described previously [23]. The vesicles were loaded by sonication with either 5 mM malate or 2 mM fluorescein sulphonate in the reconstitution medium composed of 125 mM sucrose and 10 mM HEPES/NaOH, pH 7.4. After sonication, external malate or fluorescein sulphonate was removed by passing the vesicle suspension through a column (1.5×34 cm) of Sephadex G50.

2.4. Measuring PTP opening by malate release

The vesicles were analyzed for malate release after incubation in 125 mM sucrose+10 mM HEPES, pH 7.4, at room temperature for 15 min in the presence or absence of Ca^{2+}, fatty acids and cyclosporin A. After removal of the vesicles by centrifugation at 400 000×*g* for 45 min at 4°C, malate was determined in the supernatants enzymatically [23].

2.5. Measuring PTP opening by fluorescein sulphonate release

Release of fluorescein sulphonate from the vesicles was followed spectrofluorimetrically in the same medium in rectangular cuvettes (3 ml volume, 1 cm light path) by measuring fluorescence increase at 480 and 510 nm excitation and emission wavelength, respectively [27]. This procedure is based on quenching and de-quenching of some fluorescent dyes on concentration and dilution, respectively, as studied for carboxyfluorescein by Chen and Knutson [28].

3. Results

Phospholipid vesicles reconstituted with the isolated permeability pore complex were loaded with malate. When such vesicles were subsequently incubated in the sucrose medium in the absence of added Ca^{2+}, about 30% of trapped malate leaked out. This leakage was not affected by addition of 50 μM Ca^{2+} alone. However, addition of myristate+50 μM Ca^{2+} greatly potentiated the release of malate, so that at 40 nmol myristate per sample the outflow was complete. Under these conditions, myristate alone liberated only 60% of trapped malate. Preincubation of the vesicles with cyclosporin A, a potent inhibitor of PTP [29], decreased the outflow of malate to the level observed for myristate alone (Fig. 1). It can be, therefore, concluded that the outflow of malate produced by myristate alone was due to the detergent action of the fatty acid, whereas the concerted effects of myristate and Ca^{2+} resulted in PTP opening, as observed previously in intact mitochondria [14].

Similar results were obtained with oleic acid, but not with 12-(carboxymethyl-thio)dodecanoic acid, a dicarboxylic acid that appeared to be inactive as protonophore [30] (Fig. 2).

In order to observe kinetics of the pore opening, the fluo-

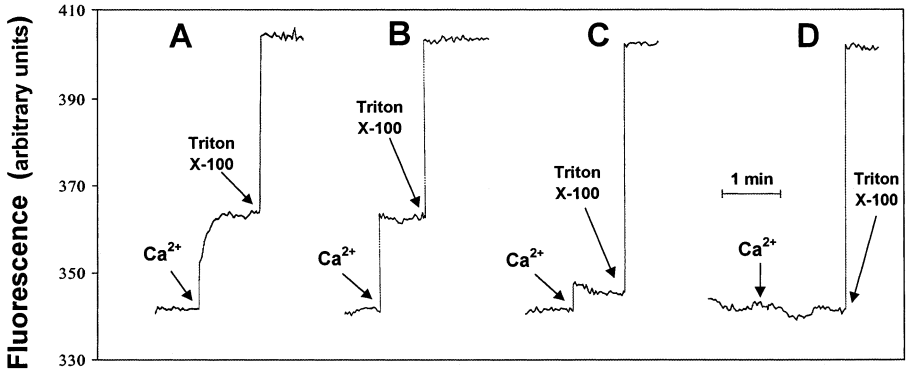

Fig. 3. Effect of fatty acids on opening of the reconstituted PTP measured by the release of fluorescein sulphonate. Vesicles corresponding to about 4 mg asolectin were suspended in 3.0 ml of the same medium as in Fig. 1 and preincubated with the following additions indicated until a plateau (resulting from spontaneous leak and the detergent action of fatty acid) was reached (about 2–3 min, not depicted in the figure): A, 50 nmol myristate; B, 50 nmol oleate; C, 50 nmol oleate+2 μM cyclosporin A; D, 100 nmol 12-(carboxymethyl-thio)dodecanoate. Then, 100 μM $CaCl_2$ was added. Complete release of fluorescein sulphonate was obtained after lysis of the vesicles with 0.05% Triton X-100. 100 μM $CaCl_2$ added in the absence of fatty acid had no effect (not shown). The figure shows one experiment out of two similar ones.

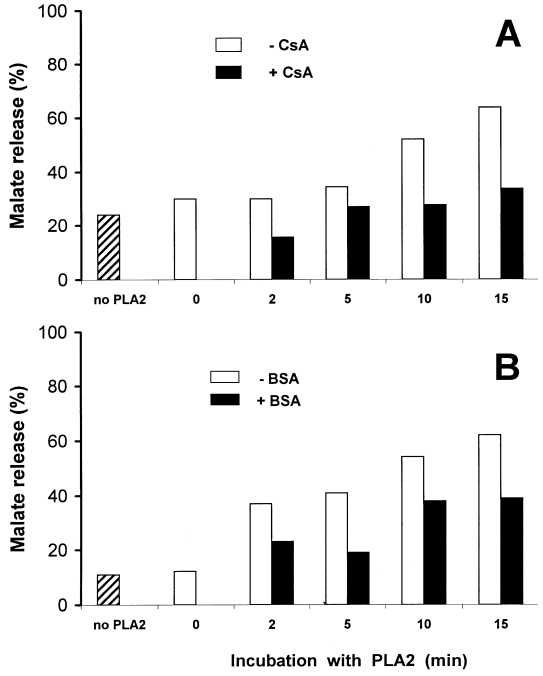

Fig. 4. Effect of phospholipase A_2 on opening of the reconstituted PTP. The reconstituted vesicles were incubated in the presence of 50 μM Ca^{2+} as in Fig. 1, except that pH of the medium was 7.8. The reaction was started by addition of 0.1 unit of phospholipase A_2 (PLA2) per sample and stopped by 0.5 mM EGTA after the time indicated. In panel A, parallel samples (marked in black) were run in the presence of 2 μM cyclosporin A (CsA); in panel B, parallel samples contained 1% bovine serum albumin (BSA). The columns show mean values of two experiments; individual values did not differ by more than 10%.

rescence method was employed. The vesicles were loaded with fluorescein sulphonate instead of malate and outflow of the content was followed fluorimetrically as fluorescence increase. When the vesicles were preincubated with myristate or oleate, addition of 100 μM Ca^{2+} resulted in a very fast release of fluorescein sulphonate (Fig. 3, traces A and B). Hundred μM Ca^{2+} alone had no effect (not shown). The release in the presence of fatty acid and Ca^{2+} was almost completely abolished by cyclosporin A (trace C). No Ca^{2+}-dependent release was observed after preincubation with the 'inactive' dicarboxylic fatty acid analogue (trace D).

The problem was also approached by generating fatty acids in the vesicles by treatment with phospholipase A_2. The vesicles loaded with malate were incubated with pancreatic phospholipase A_2 in the presence of 50 μM Ca^{2+}. This treatment resulted in a time-dependent release of both total and cyclosporin A-sensitive pools of malate (Fig. 4A). The leak was decreased by serum albumin (Fig. 4B). It can be, therefore, inferred that the serum albumin-sensitive malate outflow was due to fatty acid-induced PTP opening, whereas the serum albumin-insensitive portion of the leak might have resulted from the accumulation of lysophospholipids.

4. Discussion

The present results are in line with previous observations [11–17] that long-chain fatty acids may promote opening of PTP in intact mitochondria. As discussed by Schönfeld and Bohnensack [13], this may occur in two different ways: (i) indirectly, by collapsing $\Delta\psi$ due to the protonophoric effect of fatty acid cycling mediated by ANT [4] and other mitochondrial carrier proteins [10], and (ii) directly, by interaction of fatty acids with ANT and stabilization of its 'cytosolic' conformation. The latter possibility was supported by our results [14] showing that synthetic protonophores, such as carbonyl cyanide *m*-chlorophenylhydrazone, used at concentrations that exert protonophoric effect comparable to that of fatty acids, do not open the PTP. On that basis, we have concluded that fatty acids increase the open probability of PTP in a more direct way and not only as protonophores. The present results, showing that fatty acids promote PTP opening in the reconstituted system in which no transmembrane potential exists, provide a further support to this view.

It has been previously suggested [14] that ANT is the primary target of fatty acid action on PTP. Another PTP component, that potentially can be a target for fatty acid effect, is hexokinase. It has been shown previously that non-esterified long-chain fatty acids decrease binding ability of hexokinase to isolated liver mitochondria [31] and to membranes within intact hepatocytes [32]. Another ligand of hexokinase, glucose-6-phosphate, that is also known to desorb hexokinase from mitochondrial membranes and to inhibit its activity (for review see [33]), has been found to open the reconstituted PTP [23]. Thus, non-esterified long-chain fatty acids can be added to the long list of natural, and possibly physiological, regulators of PTP, the list that already includes Ca^{2+}, phosphate [15], $\Delta\psi$, intramitochondrial pH [34], ADP, ATP [16,25] (reviewed in [19]), glucose and glucose-6-phosphate [23].

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