The mechanism of palmitoyl-CoA inhibition of Ca^{2+} uptake in liver and heart mitochondria

Michael C. BEATRICE and Douglas R. PFEIFFER

The Hormel Institute, University of Minnesota, Austin, MN 55912, U.S.A.

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The mechanism by which palmitoyl-CoA inhibits Ca^{2+} uptake in liver and heart mitochondria was examined. At a given concentration of palmitoyl-CoA, the extent of inhibition is inversely related to the concentration of the respiratory substrate succinate. Palmitoyl-CoA inhibition of uncoupler-stimulated respiration and respiration stimulated by ionophore-A23187-induced Ca^{2+} cycling is also relieved by high succinate concentrations. These effects of palmitoyl-CoA and succinate concentration are distinct from the increase in inner-membrane permeability, which can be produced by palmitoyl-CoA and Ca^{2+} [Beatrice, Palmer & Pfeiffer (1980) *J. Biol. Chem.* **255**, 8663–8671]. The apparent $K_{0.5}$ of the mitochondrial Ca^{2+} pump is not altered by palmitoyl-CoA. No or negligible effects of palmitoyl-CoA on the Ca^{2+} -uptake rate are observed when ascorbate replaces succinate as an energy source. These findings, together with the known activity of palmitoyl-CoA as a competitive inhibitor of the dicarboxylate carrier [Morel, Lauquin, Lunardi, Duszynski & Vignais (1974) *FEBS Lett.* **39**, 133–138], indicate that palmitoyl-CoA inhibits energy-linked Ca^{2+} transport by limiting the rate of electron transport through limitation of succinate entry into the mitochondria rather than by directly inhibiting the Ca^{2+} carrier.

The ability of mitochondria to transport and retain Ca^{2+} has been intensively investigated (Bygrave, 1977; Hehninger *et al.*, 1978a; Carafoli, 1979; Harris, 1979). Studies have shown that palmitoyl-CoA inhibits Ca^{2+} uptake in both liver and heart mitochondria (Asimakis & Sordahl, 1977; Harris, 1977; Wolkowicz & Wood, 1979, 1980). Although the mechanism has not been established, it has been proposed that this inhibition results from a specific interaction between palmitoyl-CoA and the carrier- Ca^{2+} complex at the outer side of the mitochondrial inner membrane (Wolkowicz & Wood, 1980).

Long-chain acyl-CoA esters are also known to be inhibitors of the adenine nucleotide, dicarboxylate, tricarboxylate and phosphate carriers (Halperin *et al.*, 1972; Harris *et al.*, 1972; Vaartjes *et al.*, 1972; Morel *et al.*, 1974; Shrago *et al.*, 1974). Substrate-transport experiments using de-energized mitochondria have demonstrated that palmitoyl-CoA competitively inhibits the dicarboxylate and phosphate carriers at very low concentrations, with apparent K_i values of 7.1–9.5 μM and 25 μM respectively (Morel *et al.*, 1974). In addition, it is known that phosphate can stimulate the rate of Ca^{2+} uptake by mitochondria (see Bygrave, 1977, for review) and that the maximal rate of Ca^{2+} transport is limited by the maximal rate of electron transport

(Hutson *et al.*, 1976; Heaton & Nicholls, 1976). Together, these findings suggest an alternative mechanism for the inhibitory effect of palmitoyl-CoA on Ca^{2+} uptake, namely that it results from diminished respiratory-substrate and/or phosphate transport. To investigate this possibility, we have examined the relationships between the extent of inhibition of Ca^{2+} and of electron transport and the effect of succinate and phosphate concentrations on these parameters. The results indicate that this is indeed the mechanism of the inhibition of Ca^{2+} uptake produced by palmitoyl-CoA.

Experimental

Liver mitochondria were prepared from male Sprague-Dawley rats weighing approx. 250 g. The procedure of Johnson & Lardy (1967) was employed, with modified media. The homogenization medium contained 0.23 M-mannitol, 0.07 M-sucrose, 3 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Na^+ salt], pH 7.4, 0.2 mM-EGTA (Na^+ salt) and fatty acid-free bovine serum albumin (4 mg/ml; product no. A6003; Sigma Chemical Co., St. Louis, MO, U.S.A.). EGTA and bovine serum albumin were omitted from the washing and experimental media. Interfibrillar rat heart

mitochondria were prepared by the procedure of Palmer *et al.* (1977), except that 3 mM-Hepes (K^+ salt) was substituted for 5 mM-Mops (4-morpholinepropanesulphonic acid) in the isolation medium. The experimental medium at pH 7.4 contained 0.23 M-mannitol, 0.07 M-sucrose, 3 mM-Hepes (K^+ salt) and 1.7 mM-potassium phosphate.

All experiments were conducted at 25°C with rotenone present at 0.5 nmol/mg of protein. The mitochondrial protein concentration was 1.0 mg/ml. Protein concentrations were determined by the biuret reaction in the presence of 1% deoxycholate, by using bovine serum albumin standards. Additions to the basic medium are described in the Figure legends. Ca^{2+} uptake was followed in the dual-wavelength spectrophotometer (Aminco-Chance DW 2A) by using the cationic indicating dye antipyrilazo III [bis-(4-antipyrilazo)-4,5-dihydroxynaphthalene-2,7-disulphonic acid] with the wavelength pair 720–790 nm (Scarpa *et al.*, 1978). The spectrophotometric tracings were corrected for the non-linear response of the indicator dye by use of a calibration curve. Mitochondrial oxygen consumption was followed with a Gilson oxygraph (Gilson Medical Electronics, Middleton, WI, U.S.A.) equipped with a Clark-type platinum oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). Antipyrilazo III, purchased from K & K Rare Chemicals Co. (Plainview, NY, U.S.A.), was purified as described by Scarpa *et al.* (1978). Palmitoyl-CoA was from P-L Biochemicals (Milwaukee, WI, U.S.A.). The calcium ionophore A23187 was a generous gift from Dr. Robert Hamill, Eli Lilly and Co., Indianapolis, IN, U.S.A.

Results and discussion

Effects of palmitoyl-CoA in liver mitochondria

Fig. 1 demonstrates that the inhibitory effect of palmitoyl-CoA on Ca^{2+} uptake by rat liver mitochondria is seen when succinate, but not ascorbate/*NNN'*-tetramethyl-*p*-phenylenediamine, is the respiratory substrate. With a succinate concentration of 2 mM (Fig. 1a), palmitoyl-CoA at 2 and 6 nmol/mg of protein drastically decreases the rate of Ca^{2+} uptake as compared with the control. With ascorbate/*NNN'*-tetramethyl-*p*-phenylenediamine as substrate (Fig. 1b), palmitoyl-CoA at 2 nmol/mg of protein has no effect on the rate of Ca^{2+} uptake and at 6 nmol/mg of protein only slightly retards it. These findings suggest that the inhibition of Ca^{2+} uptake produced by this agent results from an inhibition of succinate utilization.

If the inhibition of Ca^{2+} uptake results from a competitive inhibition of succinate transport or succinate dehydrogenase, the extent of inhibition should be inversely related to the succinate con-

Fig. 1. Effect of palmitoyl-CoA on the rate of respiration-supported Ca^{2+} uptake in rat liver mitochondria (a) Liver mitochondria (1.0 mg of protein/ml) were preincubated for 1 min in 0.23 M-mannitol, 0.07 M-sucrose, 2 mM-Tris/succinate, 0.5 mM-sodium phosphate, 0.5 μ M-rotenone, 0.1 mM-antipyrilazo III, 3 mM-Hepes (Na^+ salt), pH 7.4. Palmitoyl-CoA was added at 1 min at 0, 2 or 6 nmol/mg of protein as indicated by the numbers beside curves in the Figure. Ca^{2+} uptake was initiated at 2 min by addition of 67 μ M- Ca^{2+} . (b) The conditions were the same as described for (a), except that 3 mM-Tris/ascorbate/0.25 mM-tetramethylphenylenediamine replaced Tris/succinate.

centration. In Fig. 2(a) the rate of Ca^{2+} uptake as a function of succinate concentration is compared in the presence and absence of 2 nmol of palmitoyl-CoA/mg of protein. That palmitoyl-CoA inhibition is most effective at low succinate concentrations supports the involvement of competitive inhibition. The possibility that these effects of palmitoyl-CoA and succinate occur through direct actions at the level of succinate dehydrogenase is considered unlikely, since the inhibitor does not readily enter the matrix space under these conditions (absence of carnitine), and since previous studies have failed to reveal inhibition of succinate dehydrogenase by this compound (Rydstrom, 1972). The possibility that palmitoyl-CoA is inhibiting succinate dehydrogenase indirectly by promoting an increase in the mitochondrial oxaloacetate content is also unlikely, as further discussed below.

The extent to which increased succinate can overcome the palmitoyl-CoA inhibition of Ca^{2+} uptake depends on the phosphate concentration. As succinate is increased to 20 mM, the Ca^{2+} -transport rate in the presence of 2 nmol of palmitoyl-CoA/mg of protein and 0.5 mM-phosphate approaches the control value (Fig. 2a), but in the presence of 0.2 mM-phosphate it does not (Fig. 2b). These data suggest that competitive inhibition of phosphate transport by palmitoyl-CoA is also involved in the mechanism of inhibition of Ca^{2+} uptake. This could be a consequence of the ability of phosphate to

Fig. 2. Effect of succinate and phosphate concentrations on palmitoyl-CoA inhibition of Ca^{2+} uptake in rat liver mitochondria

(a) Liver mitochondria (1.0 mg of protein/ml) were preincubated for 2 min in 0.23 M-mannitol, 0.07 M-sucrose, 0.5 mM-sodium phosphate, 0.5 μM -rotenone, 0.1 mM-antipyrilazo III, 3 mM-Hepes (Na^+ salt), pH 7.4. Tris/succinate was in the medium as indicated. Palmitoyl-CoA at 2 nmol/mg of protein was added at 2 min. Ca^{2+} uptake was initiated by additions of 67 μM - Ca^{2+} at 3 min. The rate of Ca^{2+} uptake was not entirely linear with time throughout the experiments. The rates presented were estimated from the earliest recorded portion of the traces, which were nearly linear. O, No palmitoyl-CoA; ●, 2 nmol of palmitoyl-CoA/mg of protein. (b) The conditions were the same as described for (a), except that the sodium phosphate concentration was lowered to 0.2 mM. O, No palmitoyl-CoA; ●, 2 nmol of palmitoyl-CoA/mg of protein.

stimulate Ca^{2+} uptake itself (Lehninger *et al.*, 1967; Reed & Bygrave, 1975), or it could also represent enhanced succinate uptake because of increased intramitochondrial phosphate concentrations (Chappell & Haerhoff, 1967; Chappell, 1968). The slight inhibition of Ca^{2+} uptake produced by 6 nmol of palmitoyl-CoA/mg of protein with ascorbate/tetramethylphenylenediamine as substrate (Fig. 1b) may also result from partial inhibition of the phosphate carrier.

The palmitoyl-CoA-induced decrease in the rate of succinate transport (electron transport) with succinate as the respiratory substrate is shown more directly by its inhibition of uncoupler-stimulated respiration (Fig. 3). A higher concentration of the agent is required to produce a given extent of inhibition when the succinate concentration is increased (Fig. 3a). As was seen for the rate of Ca^{2+} uptake, inhibition of electron transport produced by palmitoyl-CoA at 2.0 nmol/mg of protein is also progressively reversed by increased succinate concentration (Fig. 3b). Competitive inhibition of the dicarboxylate carrier (Morel *et al.*, 1974) can account for the ability of high succinate concentrations to overcome the palmitoyl-CoA inhibition of electron transport, and the similar concentration-dependence for reversal of the two types of inhibition is consistent with a common mechanism.

The data in Figs. 4 and 5 examine the relationship

between the inhibition of Ca^{2+} uptake and succinate transport (electron transport) in more detail. Ionophore A23187 activates succinate oxidation in mitochondria by establishing, together with the endogenous Ca^{2+} uniporter, a cyclic flux of Ca^{2+} across the inner membrane, dissipating the electrochemical proton gradient (Reed & Lardy, 1972; Pfeiffer *et al.*, 1976). Thus elevated respiration induced by ionophore A23187 is ultimately due to Ca^{2+} transport. Fig. 4 demonstrates that, like uncoupler-stimulated respiration, respiration stimulated by ionophore A23187 is inhibited by palmitoyl-CoA and that this inhibition is progressively relieved by increasing the succinate concentration. These findings support our proposal that the inhibition of Ca^{2+} uptake produced by palmitoyl-CoA results primarily from inhibition of succinate transport. However, it is still possible that the agent also interacts with the Ca^{2+} carrier, as suggested previously (Wolkowicz & Wood, 1980), thereby inhibiting by a second independent mechanism. The experiments in Figs. 1, 2 and 4 might not reveal the second type of inhibition, since the uptake rates were obtained at extramitochondrial Ca^{2+} concentrations well above the 5–10 μM needed to produce a maximal rate of Ca^{2+} uptake (Pfeiffer *et al.*, 1976; Hutson *et al.*, 1976; Heaton & Nicholls, 1976). In Fig. 5, the effect of palmitoyl-CoA on Ca^{2+} uptake (ionophore-A23187-stimulated respiration) is examined at an extramitochondrial Ca^{2+} concentration

Fig. 3. Effect of palmitoyl-CoA and succinate concentrations on dinitrophenol-stimulated respiration in rat liver mitochondria

(a) Liver mitochondria (1.0 mg of protein/ml) were preincubated for 1.5 min in 0.23 M-mannitol, 0.07 M-sucrose, 0.5 μ M-rotenone, 3 mM-Hepes (Na^+ salt), pH 7.4. Tris/succinate was in the medium at: ▲, 10 mM; ●, 2.5 mM; ■, 1 mM. Then 0.1 mM-dinitrophenol was added at 1.5 min and palmitoyl-CoA at 3 min. (b) Tris/succinate was in the medium as indicated; 0.1 mM-dinitrophenol was added at 1.5 min and 2 nmol of palmitoyl-CoA/mg of protein at 3 min. Other conditions were as in panel (a).

which is sufficient to produce only approximately one-half of the maximal uptake rate. These conditions would be most sensitive to any change in the affinity or activity of the endogenous transport system for Ca^{2+} . As shown in Fig. 5, palmitoyl-CoA failed to inhibit Ca^{2+} uptake at concentrations below 4 nmol/mg of protein. At higher concentrations, the subsequent inhibition curve coincided with that for the inhibition of uncoupler-stimulated respiration. These data indicate that palmitoyl-CoA does not decrease the apparent affinity of the carrier for Ca^{2+} . At this low Ca^{2+} concentration, the uninhibited portion of succinate-transport activity is sufficient to support the partially activated rate of respiration until the inhibitor concentration is increased above 4 nmol/mg of protein. In contrast, with uncoupler-stimulated respiration, inhibition begins at a much lower concentration of the inhibitor, since the more rapid rate of respiration requires a more rapid rate of succinate transport. If the inhibitor acted to decrease the affinity of the carrier for Ca^{2+} , inhibition at the $K_{0.5}$ Ca^{2+} concentration would have been apparent at lower concentrations and the two curves would not have coincided above 4 nmol of palmitoyl-CoA/mg of protein.

Effects of palmitoyl-CoA in heart mitochondria

Since the effect of palmitoyl-CoA on energy-linked Ca^{2+} transport has primarily been investigated in heart mitochondria (Asimakakis & Sordahl, 1977; Harris, 1977; Wolkowicz & Wood, 1980), we also examined the mechanism of inhibition in rat heart mitochondria. Only interfibrillar mitochondria were studied, since the apparent K_i of palmitoyl-CoA inhibition of Ca^{2+} uptake was identical for both interfibrillar and subsarcolemmal mitochondria (Wolkowicz & Wood, 1980). Unlike liver mitochondria, the maximal rate of Ca^{2+} uptake by these mitochondria appears to be slower than could be supported by the potential rate of electron transport (J. W. Palmer, unpublished work). However, the inhibitory action of palmitoyl-CoA on Ca^{2+} uptake (Fig. 6a), on respiration stimulated by ionophore-A23187-induced Ca^{2+} cycling (Fig. 6b) and on uncoupler-stimulated respiration (Fig. 6c) is again most effective at low succinate concentrations and is abolished at high substrate concentration. Thus, although a greater percentage inhibition of succinate transport by palmitoyl-CoA would be necessary to diminish Ca^{2+} uptake in these mito-

Fig. 4. Effect of palmitoyl-CoA and succinate concentrations on respiration stimulated by ionophore-A23187-induced Ca^{2+} cycling in rat liver mitochondria. Liver mitochondria (1.0 mg of protein/ml) were preincubated for 2 min in 0.23 M-mannitol, 0.07 M-sucrose, 0.5 μM -rotenone, 3 mM-Hepes (Na^+ salt), pH 7.4. Tris/succinate was in the medium as indicated. Then 67 μM - Ca^{2+} was added at 2 min, and 1.0 nmol of ionophore A23187/mg of protein at 3 min. O, No further additions; ●, palmitoyl-CoA at 4.0 nmol/mg of protein was added at 4 min and the subsequent rate of respiration determined.

Fig. 5. Effect of palmitoyl-CoA on respiration stimulated by ionophore A23187 and a limiting Ca^{2+} concentration. O, Liver mitochondria (1.0 mg of protein/ml) were preincubated for 2 min in 0.23 M-mannitol, 0.07 M-sucrose, 0.5 μM -rotenone, 3 mM-Hepes (Na^+ salt), pH 7.4; 10 mM-Tris/succinate and 0.1 mM-EGTA were in the medium. Then 67 μM - Ca^{2+} was added at 2 min, 1.0 nmol of ionophore A23187/mg of protein at 3 min, and palmitoyl-CoA as indicated was added at 4 min. ▲, The data from Fig. 3(a) with 10 mM-succinate are replotted to allow a detailed comparison of the effect of palmitoyl-CoA on ionophore- and uncoupler-stimulated respiration.

chondria, the primary site of action of the inhibitor is again the dicarboxylate carrier.

Relationship between inhibition of Ca^{2+} uptake and the induction of Ca^{2+} release produced by palmitoyl-CoA

Palmitoyl-CoA not only affects Ca^{2+} uptake, but also induces Ca^{2+} release from Ca^{2+} -loaded mitochondria (Asimakis & Sordahl, 1977; Harris, 1977; Wolkowicz & Wood, 1979, 1980). Although kinetic studies have suggested that these two effects are separate and distinct (Wolkowicz & Wood, 1980), this point must be considered in detail, because it is critical to the mechanism proposed here. The release of Ca^{2+} produced by palmitoyl-CoA is accompanied by an oxidation of nicotinamide nucleotides (Wolkowicz & Wood, 1979, 1980), analogous to the oxidation which occurs with other agents that cause Ca^{2+} release.

Some investigators have proposed that the oxidation of nicotinamide nucleotides is the cause of Ca^{2+} release, implying that it could occur before Ca^{2+} release (Lehninger *et al.*, 1978b). If this were the case, one could propose that inhibition of Ca^{2+} uptake produced by palmitoyl-CoA results from an accumulation of oxaloacetate in the mitochondria (produced by malate dehydrogenase), with subsequent inhibition of succinate dehydrogenase, and that succinate overcomes the inhibition by relieving this effect of oxaloacetate. However, we have shown that Ca^{2+} efflux induced by palmitoyl-CoA is due to an increase in the general permeability of the inner membrane brought about by enhanced phospholipase A_2 activity. The oxidation of nicotinamide nucleotides which accompanies this process is not the cause of Ca^{2+} release, but is secondary to the

Fig. 6. Effect of increasing succinate concentration on palmitoyl-CoA inhibition of uncoupler-stimulated respiration, respiration stimulated by ionophore-A23187-induced-Ca²⁺ cycling, and Ca²⁺-uptake rates in interfibrillar heart mitochondria

(a) Palmitoyl-CoA inhibition of Ca²⁺ uptake. Interfibrillar heart mitochondria (0.67 mg of protein/ml) were preincubated for 2 min in 0.23 M-mannitol, 0.07 M-sucrose, 1.7 mM-potassium phosphate, 0.5 μ M-rotenone, 0.1 mM-antipyrilazo III, 3 mM-Hepes (K⁺ salt), pH 7.4. Then 4 nmol of palmitoyl-CoA/mg of protein was added at 2 min and Tris/succinate at the concentrations indicated at 3 min. Ca²⁺ uptake was initiated at 4 min by addition of 45 μ M-Ca²⁺. O, No palmitoyl-CoA; ●, 4 nmol of palmitoyl-CoA/mg of protein. (b) Palmitoyl-CoA inhibition of respiration stimulated by ionophore-A23187-induced Ca²⁺ cycling. Interfibrillar heart mitochondria (0.67 mg of protein/ml) were preincubated for 2 min in 0.23 M-mannitol, 0.07 M-sucrose, 1.7 mM-potassium phosphate, 0.5 μ M-rotenone, 3 mM-Hepes (K⁺ salt), pH 7.4. Then 45 μ M-Ca²⁺ was added at 2 min, 1 nmol of ionophore A23187/mg of protein at 3 min, and, when indicated, 8 nmol of palmitoyl-CoA/mg of protein at 4 min. O, No palmitoyl-CoA; ●, 8 nmol of palmitoyl-CoA/mg of protein. (c) Palmitoyl-CoA inhibition of dinitrophenol-stimulated respiration. Interfibrillar heart mitochondria (1.0 mg of protein/ml) were preincubated for 2 min in 0.23 M-mannitol, 0.07 M-sucrose, 1.7 mM-potassium phosphate, 0.5 μ M-rotenone, 3 mM-Hepes (K⁺ salt), pH 7.4. Tris/succinate was in the medium as indicated. Then 0.1 mM-dinitrophenol was added at 2 min and, when indicated, 2 nmol of palmitoyl-CoA/mg of protein at 3 min. O, No palmitoyl-CoA; ●, 2 nmol of palmitoyl-CoA/mg of protein.

collapse of the mitochondrial pH gradient (Beatrice *et al.*, 1980). A similar situation is apparent in heart mitochondria (J. W. Palmer & D. R. Pfeiffer, unpublished work). Several factors indicate that this increase in permeability is not involved in the phenomena being studied here.

Important points in this interpretation are that the increase in inner-membrane permeability produced by Ca²⁺ plus palmitoyl-CoA is dependent on the accumulation of exogenous Ca²⁺ (to activate the phospholipase; Beatrice *et al.*, 1980; Pfeiffer *et al.*, 1979) and that the increase in permeability occurs after a lag time which represents the time required for the products of phospholipase reaction to accumulate. The endogenous Ca²⁺ (approx. 5 nmol/mg of protein) is below that required to activate the enzyme significantly, and the concentrations of palmitoyl-CoA employed (2–8 nmol/mg of protein) are too low to increase permeability through a general detergent effect (Beatrice *et al.*, 1980; Wolkowicz & Wood, 1980).

In Figs. 1, 2 and 6(a) of the present paper, the Ca²⁺-uptake rates were obtained from the early portions of the experiment before appreciable Ca²⁺ was accumulated and before the lag period for the permeability increase had passed. Similar experiments (results not shown) demonstrated that the nicotinamide nucleotides remained reduced during

this time, as expected. The data are thus uptake rates for mitochondria which are still intact and which retain a low NAD(P)⁺/NAD(P)H ratio. For Figs. 3, 4, 5, 6(b) and 6(c) the data were obtained either in the absence of added Ca²⁺ or in the presence of excess ionophore A23187, which prevents net Ca²⁺ uptake. Since these conditions also prevent the increase in permeability, these rates also represent activities of intact mitochondria. It is thus clear that the inhibition of Ca²⁺ uptake produced by palmitoyl-CoA and its relief by increased succinate concentration is not dependent on changes in membrane permeability or on the oxidation state of nicotinamide nucleotides. Accordingly the oxaloacetate-succinate dehydrogenase explanation appears unlikely.

A final point considers the mechanism by which the local anaesthetics nupercaine (dibucaine) and butacaine decrease the rate of Ca²⁺ uptake by mitochondria (Harris, 1977). Since these agents can inhibit the dicarboxylate transporter (Barritt, 1979), their inhibition of Ca²⁺ uptake may also be due to their interaction with this carrier.

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