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PALMITOYL CARNITINE: AN ENDOGENOUS PROMOTOR OF CALCIUM EFFLUX FROM RAT HEART MITOCHONDRIA

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Abstract—The effects of the fatty acid ester palmitoyl carnitine (PC) on mitochondrial Ca^{2+} handling and ATP synthesis are described. At low concentrations (5–40 μM) PC was found to produce changes in mitochondrial Ca^{2+} handling, the most significant effect ($P < 0.05$) being the promotion of Ca^{2+} efflux ($EC_{25} = 1.19 \pm 0.11$ μM). Studies on mitochondrial substrate oxidation in the presence of either glutamate plus malate, or succinate, confirmed the ability of PC (10–100 μM) to cause loss of respiratory control as shown by reductions in the Respiratory Control Index for each substrate. It was concluded that the effect of PC on Ca^{2+} transport was due to a direct action on the Na^+–Ca^{2+} antiporter system, whilst the effect on respiration was due to an uncoupling action.

One of the major biochemical changes associated with myocardial ischaemia is the inhibition of mitochondrial β-oxidation of fatty acids [1]. Such inhibition results in the accumulation of long chain acyl CoA in the mitochondrion, and long chain acyl carnitine in the cytosol [2–4]. This increase in fatty acid intermediates is associated with irreversible ischaemic damage resulting from electrophysiological changes [4–7].

In addition to their implication as mediators of electrophysiological changes within the ischaemic or hypoxic myocardium, long chain acyl carnitines, such as palmitoyl carnitine (PC), have also been associated with the appearance of amorphous densities within the mitochondrion and with structural changes of the inner mitochondrial membrane [8, 9].

Destruction of mitochondrial structure, resulting in impaired cardiac metabolism [3] and inhibition of respiration [9], together with inhibition of either the Na^+–K^+ ATPase or the Na^+–Ca^{2+} antiporter system associated with sarcolemmal vesicles [10, 11], and the enhanced release of Ca^{2+} from sarcoplasmic reticular vesicles [12] have all been linked to the "detergent-like" properties of these long chain acyl carnitines [13].

Studies carried out by Piper *et al.* [14] have shown that PC is unable to induce the characteristic changes in ultrastructure observed in mitochondria isolated from ischaemic tissue. These authors conclude that the non-specific detergent-like effects of PC were not sufficient to account for the mitochondrial disruption seen after ischaemia.

Isolated tissue techniques have shown that PC exerts a positive inotropic effect, increasing the Ca^{2+} current in chick ventricular muscle [15]. Similar effects were observed when the level of extracellular Ca^{2+} was increased, thus indicating a direct effect on the membrane surface charge resulting in modification of the Na^+–Ca^{2+} channel system. More recent *in vitro* studies have shown PC to interact selectively with different Ca^{2+} antagonists to displace their [^{3}H] ligands from specific binding sites, thus indicating its potential as an endogenous activator of Voltage Operated Channels (VOCs) [16].

The ability of PC to accumulate within the cell and thus activate Ca^{2+} channels associated with the ischaemic myocardium may account for the deleterious Ca^{2+} overload which occurs in ischaemia following reperfusion [17–21]. We now report the ability of PC to enhance Ca^{2+} efflux from rat heart mitochondria.

MATERIALS AND METHODS

Materials. Palmitoyl carnitine was obtained from Sigma Chemical Co. (London, U.K.) and was dissolved in water. All other laboratory chemicals and solvents were obtained from British Drug Houses Co. (Poole, Dorset, U.K.).

Methods. Tightly coupled rat heart mitochondria were isolated from female Wistar rats (300–400 g) according to the method of Vercesi *et al.* [22]. Oxygen consumption was measured polarographically at 37° using a Clarke-type oxygen electrode (Rank Bros., Bottisham, U.K.), coupled to a BBC SE 120 pen recorder. Protein was measured by the method of Gornall *et al.* [23]. The reaction medium (3.0 ml) contained 210 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl (pH 7.4) 5 mM potassium dihydrogen orthophosphate and 2 mg mitochondrial protein. After 1 min, State 4 respiration was induced by the addition of either 5 mM sodium glutamate plus 5 mM sodium malate, or 2 mM sodium succinate. One minute later State 3 respiration was induced by the addition of 400 nmol ADP. When present, PC (10–

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100 μM) was added prior to the addition of mitochondria.

Calcium ion movements were followed at 37° using a Corning calcium-specific electrode coupled to a Pentracourt PM 10 pH meter and a BBC SE 120 pen recorder, according to the method of Crompton *et al.* [24]. The reaction medium (10 ml) contained 250 mM sucrose, 5 mM succinate, 2 mM potassium dihydrogen orthophosphate, 5 mM Tris–HCl (pH 7.4) and 12.5 μM calcium chloride. Calcium uptake from the above medium was initiated by the addition of 2 mg mitochondrial protein and changes in extra mitochondrial Ca^{2+} concentration monitored using the Ca^{2+}-specific electrode. The non-specific binding of Ca^{2+} to the mitochondrial membrane was corrected for using samples of mitochondria inactivated by heating to boiling point for 3 min.

All values reported are the means ± SEM of at least five determinations in different preparations. Stimulatory effects are quoted as EC_{25}, and inhibitory effects as IC_{25}; both parameters referring to the concentrations required to produce 25% of the maximal effect.

RESULTS

NAD^+-linked oxidation of glutamate plus malate

Table 1 shows the effects of PC on the NAD^+-linked substrate oxidation of 5 mM glutamate plus 5 mM malate in rat heart mitochondria. PC (10–100 μM) produced a concentration-dependent stimulation of State 4 respiration (substrate and oxygen in excess, ADP absent), the control rate increasing from 41.8 to 86.0 ng atoms O_2 min^{-1} mg $protein^{-1}$. Over the same concentration range (10–100 μM), State 3 respiration (substrate and oxygen in excess, ADP present) was inhibited, the rate decreasing from 295 to 223.6 ng atoms O_2 min^{-1} mg $protein^{-1}$, whilst the Respiratory Control Index (RCI) decreased from 6.3 to 2.5, and the ADP:O ratio decreased from 2.8 to 2.6 (Table 1).

Succinate oxidation

When glutamate plus malate were replaced by 5 mM succinate, the addition of PC produced a similar pattern of effects on mitochondrial respiration. Over the concentration range 10–100 μM, PC produced a concentration-dependent stimulation of State 4 respiration, the rate increasing from 57.8 to 99.1 ng atoms O_2 min^{-1} mg $protein^{-1}$ (Table 1). Similarly, PC produced a concentration-dependent inhibition of State 3 respiration reducing the rate of oxygen consumption from 204.1 to 96.8 ng atoms O_2 min^{-1} mg $protein^{-1}$. Over the same concentration range (10–100 μM) PC reduced the RCI from 3.4 to 1.0, and the ADP:O ratio from 1.85 to 1.04 (Table 1).

Calcium ion movements

Over the concentration range 5–40 μM, PC caused marked effects on the Ca^{2+} handling ability of rat heart mitochondria, producing concentration-dependent decreases in influx rate, total calcium uptake and retention time, and stimulating the efflux rate. Figure 1 shows the effect of PC on the influx rate and total uptake of Ca^{2+} into rat heart mitochondria. Over the concentration range 5–40 μM, PC reduced the rate of Ca^{2+} influx from 265.2 to 160.0 nmol Ca^{2+} min^{-1} mg $protein^{-1}$ (IC_{25} = 26.1 ± 1.5 μM), and the total Ca^{2+} uptake from 251.1 to 62.5 nmol Ca^{2+} mg $protein^{-1}$ (IC_{24} = 17.8 ± 1.7 μM).

Figure 2 shows the effects of PC on Ca^{2+} retention time and efflux rate from rat heart mitochondria over the concentration range 5–40 μM. PC was found to produce a concentration-dependent reduction in the retention time reducing it from 16.0 to 3.0 min (IC_{25} = 5.6 ± 0.05 μM), an increase in the efflux rate

Table 1. The effect of palmitoyl carnitine and the NAD-linked substrate oxidation of glutamate plus malate and succinate by rat heart mitochondria

Palmitoyl carnitine (μM)	Mitochondrial respiration ng atoms of O_2 consumed min^{-1} mg $protein^{-1}$				Respiration ratios			
	Glutamate plus malate		Succinate		Glutamate plus malate		Succinate	
	State 4	State 3	State 4	State 3	RCI	P:O	RCI	P:O
Control	41.8 ± 3.8	295.3 ± 6.3	57.7 ± 1.2	204.1 ± 5.5	6.3 ± 0.67	2.8 ± 0.11	3.4 ± 0.12	1.85 ± 0.04
10	46.2 ± 3.5	273.5 ± 11.4	64.5 ± 1.9	203.4 ± 8.9	6.2 ± 0.62	2.9 ± 0.03	3.2 ± 0.08	1.79 ± 0.02
20	53.1 ± 3.2	279.9 ± 13.5	63.3 ± 1.7	195.9 ± 9.9	5.6 ± 0.38	2.95 ± 0.03	3.0 ± 0.14	1.83 ± 0.03
40	57.7 ± 5.3	259.6 ± 1.4	69.8 ± 3.6	174.0 ± 5.8	4.5 ± 0.38	2.85 ± 0.089	2.5 ± 0.10	1.80 ± 0.02
60	65.8 ± 5.5	250.1 ± 2.9	83.8 ± 3.4	165.0 ± 11.1	4.0 ± 0.40	2.8 ± 0.09	2.1 ± 0.17	1.63 ± 0.09
80	75.8 ± 6.1	238.3 ± 6.2	99.1 ± 4.0	126.7 ± 5.6	3.4 ± 0.30	2.7 ± 0.10	1.4 ± 0.13	1.04 ± 0.04
100	86.0 ± 5.7	223.6 ± 7.7	85.8 ± 4.5	96.8 ± 4.5	2.5 ± 0.09	2.6 ± 0.20	1	—

The reaction medium contained 315 μmol mannitol, 105 μmol sucrose, 15 μmol Tris–HCl (pH 7.4) and 5.7 μmol potassium dihydrogen orthophosphate. The experiments were carried out at 37° and each experiment was started by adding 2.0 mg mitochondrial protein to 1.5 ml of the above medium. The mitochondria were allowed to equilibrate for 1 min and State 4 respiration induced by adding 7.5 μmol glutamate plus 7.5 μmol malate or 7.5 μmol succinate. Mitochondria were allowed to respire for 1 min and State 3 respiration induced by adding 400 nmol ADP. When present palmitoyl carnitine (15–150 nmol) was added prior to the addition of mitochondria. The results are the means of five different experiment ± SEM.

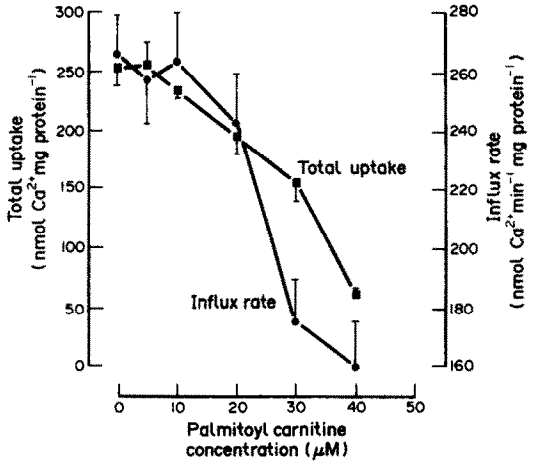

Fig. 1. The effect of palmitoyl carnitine on calcium influx and total uptake in rat heart mitochondria. Calcium fluxes were measured using a calcium-specific electrode. Calcium influx and total uptake was initiated at 37° by the addition of 2.5 mg mitochondrial protein to 10 ml of the reaction medium containing 2500 μmol sucrose, 50 μmol succinate, 20 μmol potassium dihydrogen orthophosphate, 50 μmol Tris–HCl (pH 7.4) and 125 nmol Ca^{2+}. When present palmitoyl carnitine (50–400 nmol) was added to the medium prior to the addition of mitochondria. The results are the means of five different experiments ± SEM.

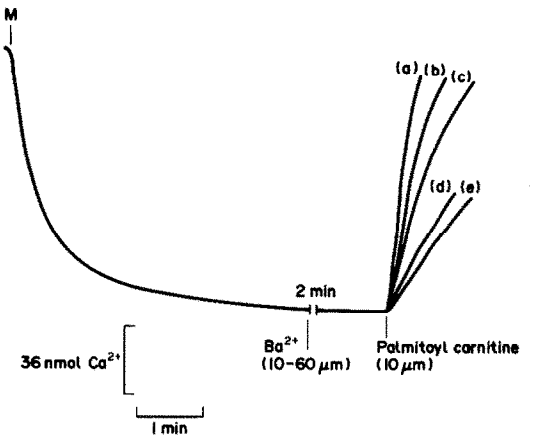

Fig. 3. The effects of palmitoyl carnitine on calcium efflux in rat heart mitochondria. For experimental details refer to legend to Fig. 1. When present palmitoyl carnitine (100 nmol) was added to the medium after the mitochondria had accumulated the Ca^{2+} load (125 nmol) and 3 min after the addition of barium chloride (100–600 nmol): a, control no Ba^{2+}; b, 100 nmol Ca^{2+}; c, 200 nmol Ba^{2+}; d, 400 nmol Ba^{2+}; and e, 600 nmol Ba^{2+}.

from 3.3 to 34.7 nmol Ca^{2+} min^{-1} mg $protein^{-1}$ (EC_{25} = 1.19 ± 0.11 μM).

The addition of PC (1–12 μM) to rat heart mitochondria preloaded with Ca^{2+} (12.5 μM) produced a concentration-dependent stimulation of efflux, the rate increasing from 2.96 to 337.3 nmol Ca^{2+} min^{-1} $protein^{-1}$ (EC_{50} = 0.11 ± 0.007 μM; Fig. 3). In the presence of 12 μM PC, barium chloride (Ba^{2+}; 10–40 μM) a known inhibitor of the Na^+–Ca^{2+} exchange system [25], produced a concentration-dependent reduction in PC-induced Ca^{2+} efflux reducing the rate from 337.3 to 21.0 nmol Ca^{2+} mg protein (Figs 3 and 4).

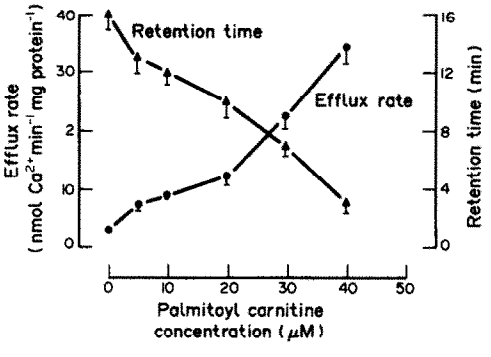

Fig. 2. The effect of palmitoyl carnitine on calcium efflux and retention time in rat heart mitochondria. For experimental details refer to legend to Fig. 1. When present palmitoyl carnitine (10 to 120 nmol) was added to the medium prior to the addition of mitochondria. The results are the means of five different experiments ± SEM.

DISCUSSION

The control of Ca^{2+} metabolism is known to be achieved by various mechanisms involving interactions with plasma membranes, intracellular organelles or specific cytosolic binding proteins. Movement of extracellular Ca^{2+} into cells is thought to be regulated by voltage operated channels (VOCs) associated with the cytoplasmic membranes; these channels in turn being controlled by a number of

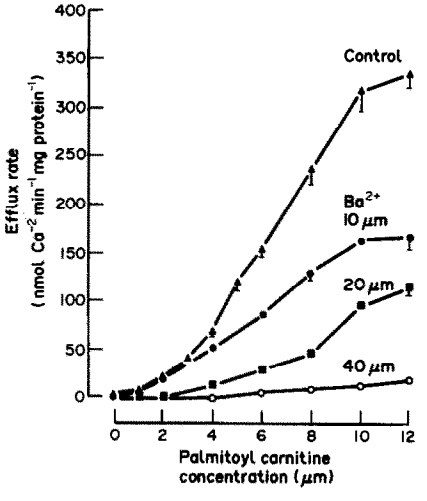

Fig. 4. The effect of barium on palmitoyl carnitine induced calcium efflux in rat heart mitochondria. For experimental details refer to legend to Figs 1 and 3. When present palmitoyl carnitine (10–120 nmol) was added to the medium after the mitochondria had accumulated the calcium load (125 nmol) and 3 min after the addition of barium chloride (100–400 nmol) the results are the means of five different experiments ± SEM.

factors including changes in membrane polarisation [26], changes in surface charge [27, 28], channel phosphorylation [29], interactions with G-proteins [30] or endogenous ligand binding [15, 16].

Initial evidence for endogenous ligand binding was provided by Inoue and Pappano [15] who reported an increase in Ca^{2+} current in avian ventricular muscle following the adminstration of PC. Additional support for this concept has been provided by Spedding and Mir [16] who have suggested that PC acts in a similar manner to the dihydropyridine Bay K8644 as an activator of Ca^{2+}-channels in potassium depolarized smooth muscle, and that it selectively interacts with different types of Ca^{2+} antagonist [31].

These authors have also shown that Bay K8644 and PC bind to similar, but slightly different, dihydropyridine receptor sites within the VOCs. Therefore, the high levels of PC (75–200 μM) known to occur in ischaemic tissue may cause VOC channels to open and produce Ca^{2+} overload.

The mitochondrion is known to play a central role in intracellular Ca^{2+} homeostasis by acting as a "Ca^{2+} sink" [32]. However, the data presented here clearly demonstrate the ability of PC to modify mitochondrial Ca^{2+} transport and energy production. Low concentrations of PC ($< 20\,\mu$M), which were ineffective in preventing mitochondrial ATP synthesis, were found to stimulate Ca^{2+} efflux, whilst at concentrations above 50 μM the amphiphilic nature of PC caused changes in membrane permeability resulting in the uncoupling of substrate oxidation from phosphorylation and consequent loss of respiratory control.

When the two *in vitro* systems are compared in relation to changes in Ca^{2+} movements significant differences appear to exist in both the mode of action of PC and their sensitivity to the acyl carnitine. The effects of PC on mitochondria occur at concentrations well below those necessary to produce effects on smooth muscle, and these data indicate the mechanism of action to be a direct effect on the Ba^{2+}-sensitive Na^{+}–Ca^{2+} antiporter system.

In terms of intracellular Ca^{2+} homeostasis, these results suggest that PC may have a biphasic action on free Ca^{2+} levels within the cell cytoplasm. At low concentrations the stimulation of Ca^{2+} efflux affords organelle protection by preventing Ca^{2+} overload of the mitochondrion, and higher concentrations producing major biochemical changes. It is important to note, however, that the changes in mitochondrial Ca^{2+} handling produced by PC occur at lower concentrations than pharmacological interactions with VOCs in the cell membrane, consequently, the former effect may well by the more important role for PC in the ischaemic cell.

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