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Involvement of palmitate/Ca^{2+}(Sr^{2+})-induced pore in the cycling of ions across the mitochondrial membrane

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ABSTRACT

The palmitate/Ca^{2+}-induced (Pal/Ca^{2+}) pore, which is formed due to the unique feature of long-chain saturated fatty acids to bind Ca^{2+} with high affinity, has been shown to play an important role in the physiology of mitochondria. The present study demonstrates that the efflux of Ca^{2+} from rat liver mitochondria induced by ruthenium red, an inhibitor of the energy-dependent Ca^{2+} influx, seems to be partly due to the opening of Pal/Ca^{2+} pores. Exogenous Pal stimulates the efflux. Measurements of pH showed that the Ca^{2+}-induced alkalization of the mitochondrial matrix increased in the presence of Pal. The influx of Ca^{2+} (Sr^{2+}) also induced an outflow of K^+ followed by the reuptake of the ion by mitochondria. The outflow was not affected by a K^+/H^+ exchange blocker, and the reuptake was prevented by an ATP-dependent K^+ channel inhibitor. It was also shown that the addition of Sr^{2+} to mitochondria under hypotonic conditions was accompanied by reversible cyclic changes in the membrane potential, the concentrations of Sr^{2+} and K^+ and the respiratory rate. The cyclic changes were effectively suppressed by the inhibitors of Ca^{2+}-dependent phospholipase A_2, and a new Sr^{2+} cycle could only be initiated after the previous cycle was finished, indicating a refractory period in the mitochondrial sensitivity to Sr^{2+}. All of the Ca^{2+}- and Sr^{2+}-induced effects were observed in the presence of cyclosporin A. This paper discusses a possible role of Pal/Ca^{2+} pores in the maintenance of cell ion homeostasis.

1. Introduction

Mitochondria play an important role in supporting Ca^{2+} homeostasis and signaling within the cell [1–3]. The system of mitochondrial Ca^{2+} transport, which has been studied since the middle of the past century [4], is well characterized from the functional point of view, and substantial progress has been achieved in revealing the identity of its components in the last few years. The identification of the mitochondrial Na^+/Ca^{2+} exchanger in 2010 [5] and the Ca^{2+} uniporter (MCU) in 2011 [6,7], both of which have been studied for a long time [8–12], filled in the major functional gaps in the mitochondrial Ca^{2+} transport. Additionally, other proteins were discovered that participate in the mitochondrial Ca^{2+} exchange: ryanodine receptor [13], leucine zipper EF hand-containing transmembrane protein 1 (Letm1) [14] and uncoupling proteins 2 and 3 (UCP2/3) [15]. Several reviews have been published recently to describe Ca^{2+} exchange in mitochondria and to connect the scheme of exchange to the functional data obtained on both isolated and intact organelles [3,11,12,16–18].

It is now clear that mitochondria serve as Ca^{2+} "dampers", quenching endoplasmic Ca^{2+} spikes in the vicinity of the reticulum by accumulating the ion in their matrix. The mitochondrial Ca^{2+} exchange system is therefore balanced for rapid Ca^{2+} uptake and slow Ca^{2+} release; as emphasized in [19], the maximal rate of Ca^{2+} influx via MCU is approximately 70-fold higher than the combined rate of all of the efflux pathways. Thus, mitochondria are under a constant potential threat of Ca^{2+} overload.

The overload of mitochondria with Ca^{2+} will eventually lead to the permeability transition (PT) in the inner mitochondrial membrane and, as suggested by Bernardi and his colleagues, PT can be a Ca^{2+} release mechanism, working in emergency situations [19,20]. However, PT is not selective; PT will lead to a rapid equilibration of all of the gradients of low-molecular solutes. Therefore, to function as an emergency Ca^{2+} release mechanism, while not leading to the loss of functional activity of the organelles, PT should be transient. In this respect, Cheng and his colleagues monitored mitochondrial PT by registering the superoxide flashes resulting from the burst acceleration of electron transport

Abbreviations: Pal, palmitic acid; Pal/Ca^{2+} pore, palmitate/Ca^{2+}-induced pore; RR, ruthenium red; MCU, mitochondrial Ca^{2+} uniporter; MPT, mitochondrial permeability transition; CsA, cyclosporin A; TPP^+, tetraphenylphosphonium; PLA_2, phospholipase A_2; TFP, trifluoperazine; $AACOCF_3$, arachidonyl trifluoromethyl ketone; P_i, inorganic phosphate; FFA, free fatty acids; $\Delta\psi$, mitochondrial transmembrane potential

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in the respiratory chain [21,22]. The transient character of those flashes confirmed that PT could occur in the form of a "flickering pore".

The flickering mode of mitochondrial PT – when PT occurs transiently and repeatedly – implies the induction of Ca^{2+} cycling across the membrane. In our earlier work, we suggested the activation of such a Ca^{2+} cycle in a specific case of mitochondrial PT — permeabilization of the mitochondrial membrane induced by long-chain saturated fatty acids and Ca^{2+}(or Sr^{2+}) [23]. The ability of palmitic acid (Pal) + Ca^{2+} to trigger a permeability transition in the mitochondrial membrane was shown after the discovery that saturated fatty acids (mainly palmitic and stearic) bind Ca^{2+} with high affinity [24]. It was established that the binding of Ca^{2+} to the fatty acid anions resulted in the formation of non-specific lipid pores; this effect was demonstrated on planar membranes (BLM), liposomes and mitochondria [24–27]. The mechanism of pore formation is supposedly based on the chemotropic phase transition in the lipid bilayer upon the formation of fatty acid/divalent cation complexes in the membrane [25,28]. One of the main features of lipid pores is their ability to close spontaneously with rapid restoration of membrane integrity [25,27], providing for the possibility of Ca^{2+} cycling across the mitochondrial membrane [23].

In this paper, we continue to investigate the phenomenon of Pal/Ca^{2+}- and Pal/Sr^{2+}-induced permeabilization of the mitochondrial membrane and show that the RR-induced efflux of Ca^{2+} from mitochondria described earlier seems to relate to the opening of the Pal/Ca^{2+}-induced pore (Pal/Ca^{2+} pore). The pore opening gives rise to the flux of ions down their concentration gradients: the efflux of Ca^{2+}(Sr^{+}) and K^+ from mitochondria and the influx of H^+ into the organelles.

We have found that under the experimental conditions used, the efflux of K^+ is not inhibited by quinidine, a mitochondrial K^+/H^+ exchange blocker, but the subsequent influx of the ion is blocked by glibenclamide. The addition of Sr^{2+} to mitochondria in a hypotonic medium has been shown to result in the CsA-insensitive cycling of ions and corresponding changes in $\Delta\Psi_m$, with the organelles becoming unresponsive to the subsequent Sr^{2+} additions until the PT cycle is complete. Evidence is obtained showing that PT-associated effects, e.g., cycling of Ca^{2+}(Sr^{2+}) and K^+, oscillations of membrane potential ($\Delta\Psi_m$) and respiration, are related to the activation of phospholipase A_2.

2. Materials and methods

2.1. Materials

$CaCl_2$ and tetraphenylphosphonium chloride were purchased from Merck (Germany); arachidonyl trifluoromethyl ketone ($AACOCF_3$) were purchased from Tocris (Great Britain); trifluoperazine (TFP), quinidine, glibenclamide and all of the common chemicals were purchased from Sigma-Aldrich (USA).

2.2. Isolation of rat liver mitochondria

Mitochondria were isolated from the liver of mature male Wister rats (220–250 g) using a standard differential centrifugation technique. The isolation medium contained 210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES/KOH (pH 7.4). The liver was cooled in the isolation medium, washed to remove blood, pressed through a plate with 1-mm holes and homogenized with a Teflon pestle in a Potter-type glass homogenizer (the tissue to medium w/v ratio was 1:8). The homogenate was centrifuged at 700 *g* (10 min), and mitochondria were sedimented for 15 min at 7000 *g*. The mitochondrial pellet was resuspended in a washing medium containing 210 mM mannitol, 70 mM sucrose, 0.1 mM EGTA and 10 mM HEPES/KOH (pH 7.4) and was centrifuged once more for 15 min at 7000 *g*. The pellet was resuspended in the washing medium (0.1 mL/g of liver) and stored on ice. All of the procedures were carried out at 0–4 °C. The resulting mitochondrial suspension contained 70–80 mg of protein per mL. The concentration of protein was determined by the Lowry method [29].

2.3. Estimation of the functional parameters of mitochondria

Mitochondrial membrane potential ($\Delta\Psi_m$) was estimated by the distribution of tetraphenylphosphonium (TPP^+) in the mitochondrial suspension and was measured with a TPP^+-sensitive electrode. TPP^+ was added to the incubation medium at the concentration of 1 μM. The concentrations of Ca^{2+}(Sr^{2+}) and K^+ ions in the incubation medium were determined with Ca^{2+}- and K^+-selective electrodes (Nico-Analyt, Russia). Changes in the medium pH were registered by a pH electrode — InLab Micro (Metler Toledo, Switzerland). Changes in the incubation medium pH and the concentrations of TPP^+ and Ca^{2+} were recorded simultaneously in a 1-mL cell with constant stirring at 26 °C (thermostat controlled), using an original multichannel electrometrical system — Record 4 (Russia). The rate of oxygen consumption was measured polarographically with a Clark-type platinum electrode (Oxygraph-2k, Austria) at 26 °C under continuous stirring.

The composition of the incubation medium varied depending on the experimental requirements (see figure legends for details).

To test the effects of the various inhibitors, the inhibitors were added to the mitochondria 2 min before the addition of Ca^{2+}(Sr^{2+}). Stock solutions of the inhibitors were prepared in either ethanol (TFP, $AACOCF_3$) or DMSO (glibenclamide). The final concentration of the solvents in the incubation was <0.1 vol.%.

2.4. Data processing

Each experiment was repeated from 3 to 7 times. The data obtained were processed using Microsoft Excel software and characteristic curves, typical for each of the experiments, were presented in the figures. The curve smoothing (removing the noise from electrodes) was performed by the method of adaptive cubic spline filtering using the Filter package.

3. Results

3.1. Involvement of palmitic acid in the release of Ca^{2+} from mitochondria

Fig. 1 shows the RR-induced Ca^{2+} efflux from mitochondria after the organelles were loaded with Ca^{2+} (70 nmol·mg^{-1} of protein) in a P_i-containing medium, in the absence or presence of palmitate (Pal). As observed, Pal stimulates the Ca^{2+} efflux, and RR enhances this effect.

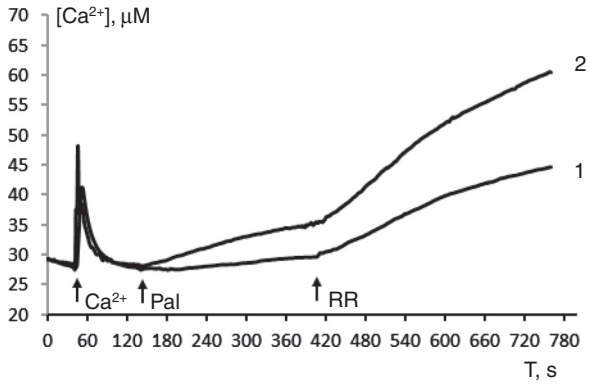

Fig. 1. Ruthenium red (RR)-induced Ca^{2+} release from rat liver mitochondria in the absence (1) or presence (2) of palmitic acid (Pal). Additions: 1) 70 μM $CaCl_2$ and 1 μM RR; 2) 70 μM $CaCl_2$, 30 μM Pal and 1 μM RR. The medium contained 210 mM mannitol, 70 mM sucrose, 1 mM NaH_2PO_4, 5 mM succinic acid, 1 mM rotenone, 10 μM EGTA, 10 mM Hepes-NaOH (pH = 7.4). The concentration of mitochondrial protein was 1 mg/mL.