

# Effects of the general anaesthetic Propofol on the $\text{Ca}^{2+}$ -induced permeabilization of rat liver mitochondria

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The molecular mechanism of the  $\text{Ca}^{2+}$ -induced permeabilization of rat liver mitochondria was evaluated by studying a new effect of the commonly used general anaesthetic Propofol (2,6-diisopropylphenol). The compound was found to induce an apparent uptake of  $\text{Ca}^{2+}$  at steady-state in the  $\text{Ca}^{2+}$ -distribution between the medium and the mitochondria, and to inhibit swelling and release of accumulated  $\text{Ca}^{2+}$  induced by inorganic phosphate, *t*-butyl hydroperoxide, diamide or FCCP plus Ruthenium red. The compound did not stimulate the activity of the  $\text{Ca}^{2+}$ -uniporter and it is concluded that the effects seen are due to the inhibition of the  $\text{Ca}^{2+}$ -dependent, unspecific permeability increase. The results suggest two mechanisms whereby Propofol stabilizes the mitochondrial membrane in the presence of  $\text{Ca}^{2+}$ : (i) by interaction with the putative pore, thus causing its closure; and (ii) by scavenging of free radicals thus inhibiting its opening during oxidative stress.

Antioxidant; Calcium; Mitochondria; Nonspecific permeability; Propofol; Swelling

## 1. INTRODUCTION

Mitochondria are able to accumulate  $\text{Ca}^{2+}$  from the surrounding medium in an energy-dependent manner [1]. The uptake of  $\text{Ca}^{2+}$  by the mitochondria will, if a certain threshold of internal  $\text{Ca}^{2+}$  is exceeded, lead to a dramatic increase in the general permeability of the inner mitochondrial membrane with release of  $\text{Ca}^{2+}$ , a fall in  $\Delta\Psi$ , and swelling of the mitochondria [1]. A number of physiological and pharmacological agents of great chemical diversity have been shown to modulate this threshold value. Adenine nucleotides and  $\text{Mg}^{2+}$  raise the threshold and could thus be said to stabilize the mitochondrial membrane whereas so-called  $\text{Ca}^{2+}$ -releasing agents, such as  $\text{P}_1$ , fatty acids, SH-agents and peroxides make the mitochondria more sensitive to  $\text{Ca}^{2+}$ .

Various mechanisms have been proposed to explain the  $\text{Ca}^{2+}$ -induced permeabilization. According to one hypothesis [1], the permeabilization is due to a shift in the equilibrium between hydrolysis and reacylation of membrane phospholipids at the *sn*-2 position towards hydrolysis leading to accumulation of lysophospho-

lipids and fatty acids. It has recently been proposed that the effects of  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -releasing agents may converge to a common ultimate step, the production of oxygen radicals and peroxidized lipids [2] which would cause an increased general permeability of the inner mitochondrial membrane, swelling and possibly the accumulation of lysophospholipids [1]. The permeabilization has also been interpreted as being due to the reversible opening of a pore protein [3]. These mechanisms may, however, be operating simultaneously [4].

This study on the effects of the general anaesthetic Propofol on the  $\text{Ca}^{2+}$ -induced permeabilization was performed in order to obtain more information on the mechanism of the permeabilization. The results indicate that Propofol causes inhibition of the permeabilization by interacting with the pore-forming component, and by scavenging radicals, thereby indirectly inhibiting its opening during oxidative stress. Similar effects have been described the structurally related compound BHT [2].

## 2. MATERIALS AND METHODS

Liver mitochondria were prepared from male rats as described in [5]. The measurements were performed in a medium containing 250 mM sucrose, 10 mM Hepes-Tris, pH 7.40, at a mitochondrial concentration of 1 mg protein/ml and at room temperature. All media were run through a Chelex (Bio-Rad Laboratories, Richmond, USA) cation exchange column equilibrated with NaCl. The medium was purged with oxygen for 30 min prior to use when indicated. Propofol was purchased from Lancaster (Strasbourg, France), distilled twice at a pressure of 15 mm Hg and recrystallized in methanol/ $\text{H}_2\text{O}$  (3:1). Crystals were dried and dissolved in ethanol at a concentration of 100

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Abbreviations: BHT, butylated hydroxytoluene; tBHP, *t*-butylhydroperoxide; DMNTA, 2,2-dimethylnitriilotriacetate; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone;  $\text{PLA}_2$ , phosphatidyl 2-acylhydrolase (EC 3.1.1.4);  $\text{P}_1$ , inorganic phosphate; Propofol, 2,6-di-isopropylphenol; RLM, rat liver mitochondria;  $\Delta\Psi$ , mitochondrial membrane potential

mM. Cyclosporin A was kindly provided as a gift by Dr P. Juutilainen (Sandoz Oy, Helsinki).

Mitochondrial swelling was measured by following the decrease in light scattering at 540 nm with a Shimadzu UV 3000 spectrophotometer (Shimadzu Corp., Japan). The oxygen consumption was measured polarographically using a Clark-type electrode. The distribution of  $Ca^{2+}$  between the solution and the mitochondria was measured with a  $Ca^{2+}$ -selective electrode [6] or with the arsenazo III method [7]. The activity of the uniporter was measured according to the method described in [8]. DNMTA was kindly provided by Dr Hans Kröner (University of Düsseldorf). The activity of the mitochondrial phospholipase  $A_2$  was measured by HPLC [9].

### 3. RESULTS AND DISCUSSION

#### 3.1. $Ca^{2+}$ -distribution and swelling during steady-state conditions

RLM that had been suspended in sucrose medium containing 1 mM  $P_i$  were able to take up and retain 60 nmol  $Ca^{2+}$ /mg protein (Fig. 1, solid line). The steady-state external  $pCa^{2+}$  stayed at about 5.7 until anoxia when the intramitochondrial  $Ca^{2+}$  was released. A slow continuous swelling could be observed during the steady-state phase (Fig. 1, solid line), probably indicating an ongoing process where some of the RLM became permeabilized, released their  $Ca^{2+}$  and accumulated sucrose [3]. When Propofol (80 nmol/mg protein) was added, there was an apparent uptake of  $Ca^{2+}$  (Fig. 1, broken line) and the steady-state concentration decreased in the medium. This could be due to either the activation of  $Ca^{2+}$  influx of the intact mitochondria or to the inhibition of  $Ca^{2+}$  efflux. The question was addressed by measuring the activity of the

$Ca^{2+}$  uniporter in the presence of propofol using the method of Kröner [8]. Propofol failed to exhibit any effect on the uniporter (not shown) indicating that the compound caused a decrease in the steady-state  $Ca^{2+}$  concentration by inhibiting the  $Ca^{2+}$  efflux. This interpretation was further supported by the observation that the slow continuous swelling observed during the steady-state phase was partly inhibited by Propofol (Fig. 1, broken line). This probably indicates that Propofol causes the inhibition of  $Ca^{2+}$  release by inhibiting the  $Ca^{2+}$ -induced increase in the general permeability of the inner mitochondrial membrane. This interpretation was further tested by comparing the effects of Propofol with those of cyclosporin A which is known to specifically inhibit the  $Ca^{2+}$ -induced permeabilization [1]; the effect of cyclosporin A (0.5 nmol/mg protein) on  $Ca^{2+}$  distribution and swelling (Fig. 1, dotted lines) were clearly similar to the effects of propofol.

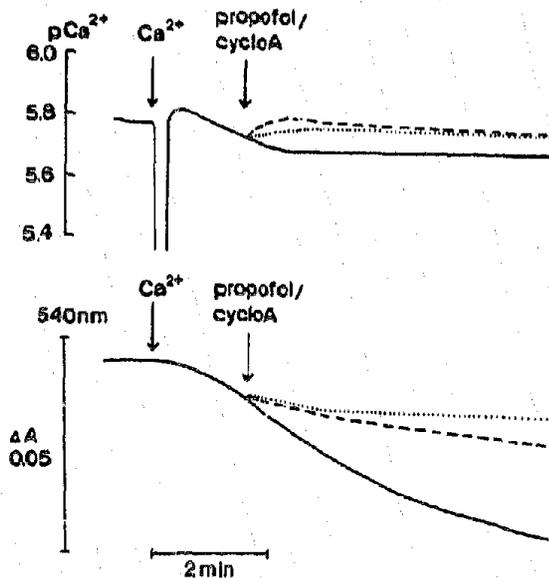


Fig. 1. Parallel measurements of  $Ca^{2+}$ -distribution (top) and mitochondrial swelling (bottom). Control experiment (solid line); Propofol (80 nmol/mg) (broken line) and cyclosporin A (0.5 nmol/mg) (dotted line) were added as indicated.

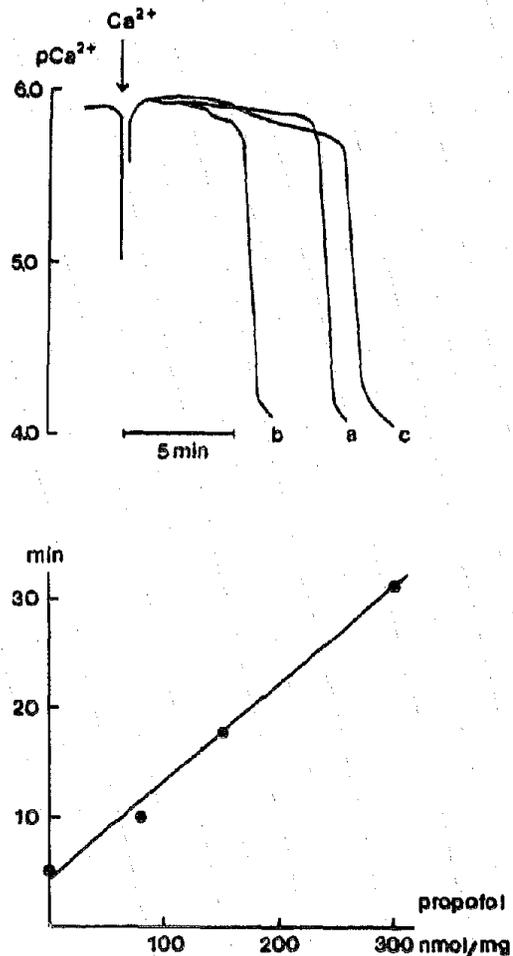


Fig. 2. The efflux of  $Ca^{2+}$  induced by 10 mM  $P_i$ ; (a) under air, (b) under 100%  $O_2$ , (c) under 100%  $O_2$ , 80 nmol Propofol/mg protein added. Bottom: shows  $Ca^{2+}$  retention time under 100%  $O_2$  as a function of the amount of Propofol added.

### 3.2. Inhibition of $Ca^{2+}$ -efflux and swelling in the presence of $P_i$ , tBHP, diamide or FCCP plus Ruthenium red

In order to assess the potency of Propofol as an inhibitor of the  $Ca^{2+}$ -induced permeabilization the effects of the compound were studied under various conditions when the permeabilization is known to be activated, i.e. in the presence of  $P_i$ , tBHP, diamide or FCCP plus Ruthenium red and also under hyperoxic conditions where an enhanced radical production has been shown to take place in cultured cells [10].

This was done by measuring the  $Ca^{2+}$  retention and the swelling of RLM suspended in sucrose medium containing 10 mM  $P_i$  or in the same medium purged with 100%  $O_2$ . In the control experiment ( $P_i$  not added) RLM were able to take up and retain  $Ca^{2+}$  (60 nmol/mg protein) as well under air as under 100%  $O_2$  (not shown); increased  $O_2$  partial pressure did not affect  $Ca^{2+}$  retention. When 10 mM  $P_i$  was included in the medium the accumulated  $Ca^{2+}$  was released after 9 min under air (Fig. 2 top, trace a) and after 5 min under 100%  $O_2$  (Fig. 2 top, trace b). The  $Ca^{2+}$  release was accompanied by swelling (not shown) which was more rapid under 100%  $O_2$ ; it is clear that the RLM were more sensitive to  $Ca^{2+}$  and  $P_i$  under 100%  $O_2$  which

might be attributable to an enhanced production of free radicals during these conditions. When Propofol (80 nmol/mg protein) was added to the medium (purged with 100%  $O_2$ ), the  $Ca^{2+}$  release was inhibited (Fig. 2 top, trace c). The inhibition was concentration-dependent with a maximal effect seen at 300 nmol Propofol/mg protein when the inhibitory effect persisted for more than 30 min (Fig. 2, bottom) - higher concentrations appeared to cause perturbation of the mitochondrial membrane.

The effect of Propofol on swelling was also studied in the presence of either tBHP (100 nmol/mg), diamide (100 nmol/mg) or FCCP plus Ruthenium red (5 resp. 3 nmol/mg). RLM suspended in sucrose medium were allowed to accumulate  $Ca^{2+}$  (60 nmol/mg) whereupon the releasing agent was added. All the agents induced rapid swelling (Fig. 3, traces A-C, solid lines); a short lag phase could be observed when tBHP or diamide was present whereas in the presence of FCCP plus Ruthenium red swelling took place without any lag phase. Propofol (80 nmol/mg) caused inhibition of the swelling in all these cases (Fig. 3, traces A-C, broken lines).

These studies with the general anaesthetic Propofol clearly show that the compound is an inhibitor of the  $Ca^{2+}$ -induced permeabilization of RLM. It is well documented that oxidative stress contributes to the induction of increased inner membrane permeability; the effect of Propofol may thus be due to its ability to form a stable radical [11], and consequently to inhibit the propagation of radical reactions. However, Propofol also caused the inhibition of permeabilization under conditions of seemingly low oxidative stress, i.e. in the presence of  $P_i$  (under air) or when the permeabilization was induced by uncoupler plus Ruthenium red. It is thus conceivable that Propofol interacts with the same inner membrane component as cyclosporin A. It might be argued that Propofol inhibits permeabilization by inhibiting the mitochondrial  $PLA_2$  [1]. However, Propofol had no effect on the activity of the enzyme at concentrations where it caused the inhibition of permeabilization, i.e. 80-300 nmol/mg protein (not shown). At even higher concentrations of Propofol a small activation of  $PLA_2$  could be observed; that, however, could be due to perturbation of the membrane.

These studies also indicate that the generation of the  $Ca^{2+}$ -induced increase in the general permeability of the mitochondrial membrane is closely related to the interaction between radicals or oxidized molecules and  $Ca^{2+}$  and that these are among the factors that contribute to pore-formation in the inner mitochondrial membrane.

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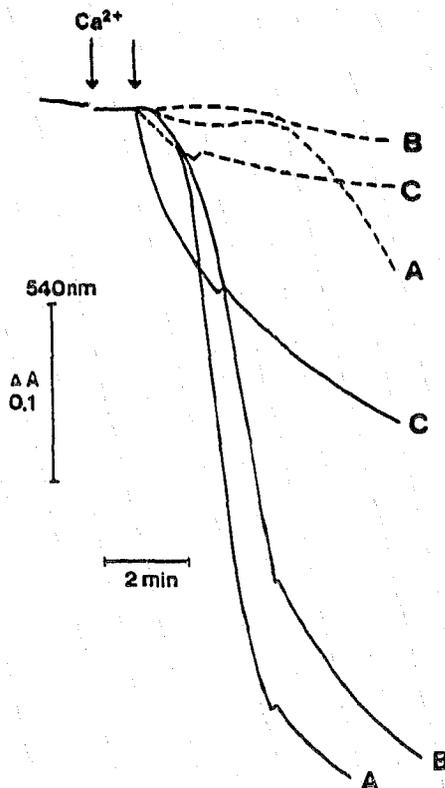


Fig. 3. Swelling induced by  $Ca^{2+}$  in the presence of (A) tBHP, (B) diamide or (C) FCCP plus Ruthenium red. Solid lines, control experiments; broken lines, in the presence of Propofol (80 nmol/mg). The second arrow indicates the addition of the experimental agent.

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