***In vitro* and *in vivo* activation of mitochondrial membrane permeability transition pore using triiodothyronine**

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Short title:

Triiodothyronine activation of mitochondrial permeability transition pore

Summary

Using a novel method for evaluating mitochondrial swelling (Drahota et al. 2012a) we studied the effect of calcium (Ca^{2+}), phosphate (P_i), and triiodothyronine (T_3) on the opening of mitochondrial membrane permeability transition pore and how they interact in the activation of swelling process. We found that 0.1mM P_i, 50µM Ca^{2+} and 25µM T_3 when added separately increase the swelling rate to about 10% of maximal values when all three factors are applied simultaneously. Our findings document that under experimental conditions in which Ca^{2+} and P_i are used as activating factors, the addition of T_3 doubled the rate of swelling. T_3 has also an activating effect on mitochondrial membrane potential. The T_3 activating effect was also found after *in vivo* application of T_3. Our data thus demonstrate that T_3 has an important role in opening the mitochondrial membrane permeability pore and activates the function of the two key physiological swelling inducers, calcium and phosphate ions.

Key words

Rat liver mitochondria, membrane permeability transition pore, thyroid hormones

Introduction

Thyroid hormones play an important role as modulators of cell energy expenditure and thermogenesis. Their effect was explained as the uncoupling of mitochondrial oxidative phosphorylation (Harper and Seifert 2008). The mechanism of thyroid hormone action on cell bioenergetics is very complex (Harper and Seifert 2008, Davis and Davis 1996, Guerrieri et al, 1998, Zhang and Lazar 2000, Lani et al. 2001, Mráček et al. 2005, Cheng et al. 2010). Their direct action on mitochondria can be seen through energy dissipation and also through mitochondrial swelling induced by activation of water transport to mitochondria (Raaflaub 1953, Tapley 1956, Lehninger 1960). When the membrane permeability transition pore (MPTP) was discovered to be an important mechanism in activating cell apoptotic processes, it was also demonstrated that this pore is involved in the pathogenesis of diseases such as cardiomyopathies, neuropathies, liver diseases and diabetes (Rasola and Bernardi 2011 Halestrap and Richardson 2015, Bernardi and Di Lisa 2015, Karch and Molketin 2014). Attention has also been paid to characterizing the many factors that modulate MPTP function in various organs (Crompton et al. 1988, Halestrap and Richardson 2015), factors including thyroid hormones (Kalderon et al. 1995, Castilho et al. 1998, Venditti et al. 2003, Yehuda-Shnaidman et al. 2010). However, in spite of the great effort made by researchers, the molecular mechanism of MPTP function as well as the mechanism through which various factors, including thyroid hormones, can regulate its gating have remained obscure (Yehuda-Shnaidman et al. 2014).

There are several reasons why the mechanism of triiodothyronine (T_3) action on MPTP function is so difficult to unravel, the first of which is the fact that MPTP is defined only through its function, i.e. as Ca-activated cyclosporine-inhibited mitochondrial swelling. However, the structural arrangement of MPTP has yet to be precisely defined. It is known that the pore is formed by many protein subunits; however, their number is continuously increasing (Vianello et al. 2012, Karch and Molketin 2014) and some of them have also been dismissed as adenine nucleotide translocator (Kokoszka et al. 2004) or phosphate translocator (Gutiérrez-Aguilar et al. 2014), when genetically modified mice without particular protein were accessible. Some authors have even proposed a role for ATP synthasome component subunit c in the pore structure and function (Alavian et al. 2014).

It is therefore very difficult to localize the target of T_3 action and analyze interactions between factors participating in the regulation of the pore opening. Another problem in these studies is that the main method used for evaluating MPTP function is now more than 60 years old and presents information about the swelling process only in graphical form. A useful method estimating the calcium retention capacity using fluorescent probe calcium green was introduced (Fontaine et al. 1998). This method gives important additional information about the swelling process but fails to provide in the digital form values of maximum swelling rate required for comparative studies of the various factors of action and interaction.

In our previous paper we tried to improve the classical method for measuring mitochondrial swelling (Drahota et al. 2012a). Merely by taking the simple derivative of the classical swelling curve we obtained two additional parameters in digital form which characterized the swelling process: the maximum swelling rate (dA520/10s) and the time (s) required to reach the maximum rate. The extent of the swelling (dA520/9min) can be calculated from classical swelling curves.

Using this novel method enabled better characterization of the differences in the swelling process of mitochondria isolated from various organs (Drahota et al. 2012b) or age-dependent changes (Milerová et al. 2010).

In this paper we concentrated our attention on evaluating the effect of T_3 on MPTP regulation. On isolated rat liver mitochondria we tested interactions between calcium and phosphate ions and T_3 in the regulation of pore opening using three parameters characterizing the swelling process.

Methods

Chemicals

All chemicals used were of the highest commercially available purity from Sigma (Sigma Aldrich Co. Germany).

Animals

Male Wistar rats (BioTest Konárovice, Czech Republic) weighing 220-250g were used for the experiments. The rats were housed at 23±1°C, 55±10% humidity, and air exchange 12-14 times/h and 12h light-dark cycle period. The animals had free access to a standard laboratory diet (ST-1, Velaz, Czech Republic) and tap water. In *in vivo* experiments triiodothyronine dissolved in saline was administered intraperitoneally in a single dose or in three doses at 24-hour intervals at a concentration of 200 µg/kg body weight. Control rats received an equivalent amount of saline solution. All rats were sacrificed 24 hours after the final administration of triiodothyronine or saline. All animals received care according to the guidelines set by the Animal-Welfare Body of the Charles University, Prague, Czech Republic, and the International Guiding Principles for Biomedical Research Involving Animals. The animals were sacrificed in a light ether narcosis by exsanguination from aortic bifurcation. The livers were removed, washed in a cold isolation medium, and cut into small pieces.

Isolation of mitochondria

Mitochondria were isolated as described previously (Drahota et al. 2014). The cut and washed tissue was homogenized at 0^{o}C by a teflon-glass homogenizer in an isolation medium containing 220mM D-mannitol, 70mM sucrose, 2mM HEPES, 0.2mM EGTA, and 0.5mg of fatty acid free bovine serum albumin (BSA) per ml, with a pH of 7.2. The 5% homogenate was centrifuged for 10 min at 600xg and the resulting supernatant for 10 min at 6800xg. The mitochondrial sediment was washed twice in the isolation medium without BSA and EGTA and suspended in the same medium. Isolated mitochondria were stored at 0°C. Mitochondrial swelling and other determinations were measured immediately after isolation; we did not use the mitochondria longer than two hours after isolation.

Determination of mitochondrial swelling

Mitochondrial swelling, as previously mentioned (Drahota et al. 2012a), was estimated as a decrease in the absorbance at 520nm at 30^{o}C in a Shimazu UV 160 spectrophotometer. The basic swelling medium contained 125mM sucrose, 65mM KCl, 10mM HEPES, and 5mM succinate, with a pH of 7.2. K-phosphate, T_3 and Ca^{2+} were added as indicated in the figures. One minute after mitochondria reached an absorbance of about 1 (amount 0.4mg/ml) swelling was induced by addition of $CaCl_2$ solution. The decrease in absorbance was detected in 10s intervals for a further 10min. We obtained three parameters of the swelling process in digital form: (a) the extent of the swelling (dA520/9min); (b) the maximum swelling rate (dA520/10s); (c) the time(s) required to reach the maximum rate.

Each figure is representative of at least 3 different experiments using different mitochondrial preparations; all experiments gave identical results.

Determination of mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\psi$) was detected according to Wasilewski et al. (2004) using the cationic dye safranin O. Safranin O uptake by mitochondria (Akerman and Wikstrom 1976) was determined from fluorescence quenching monitored at wavelengths of 495 nm (excitation) and 586 nm (emission) with an AMINCO-Bowman Series 2 Luminescence Spectrometer. The measurement was performed in 1 ml of a basic swelling medium containing 125mM sucrose, 65mM KCl, 10mM HEPES, with a pH of 7.2. First, safranin O (10μM), glutamate (10mM), malate (2.5mM) and mitochondria (0.1 mg prot./1 ml) were added to the medium. T_3 and Ca^{2+} were added as indicated in the figures.

Determination of proteins

Protein content was determined by the Lowry et al. (1951) method using bovine serum albumin as a standard.

Results

For evaluating the T_3 activation effect we selected concentrations of the main swelling inducer - calcium ions that do not induce maximum values of the swelling rate and allow for the study of T_3 activation. Fig. 1 and Table 1 demonstrate that in the presence of 0.1mM K-phosphate, 50μM Ca^{2+} yields a swelling rate of about 50% of the maximum value obtained at 200μM Ca^{2+}. The extent of swelling nearly reaches maximum values with a slower decrease (see Tab.1). We therefore used 50μM Ca^{2+} in further experiments and compared the extent to which the combination of T_3 with calcium and phosphate ions activates the rate of mitochondrial swelling.

Table 2 demonstrates the values of the swelling rate, the extent of swelling and the time required for the maximum swelling rate, which was extracted from classical swelling curves and curves obtained after their derivation (Fig. 2). It is evident that calcium alone increases spontaneous swelling about five-fold. Triiodotyronine activates spontaneous swelling about seven-fold and 0.1mM phosphate has practically no effect. Calcium induced swelling is activated by phosphate five-fold, while tiiodothyronine provided only two-fold activation. However, calcium and phosphate induced swelling can be more than two-fold increased by triiodothyronine (see Table 2). These data thus indicate that T_3 is directly involved in the regulation of MPTP.

We also tested the concentration dependence of the T_3 activating effect on mitochondrial swelling in the absence of phosphate in the medium. As demonstrated in Fig. 3 and summarized in Table 3, there was a very low calcium-induced swelling (in the absence of T_3). Two-fold increases could be detected already at 1.25μM T_3, and the highest activation was detected at a concentration range of 15-25μM T_3.

We also confirmed previous literature findings (Kalderon et al. 1995, Yehuda-Schnaidman et al. 2010) that T_3 activation of $CaCl_2$-induced swelling can be detected in isolated mitochondria after *in vivo* application of T_3 (Fig. 4 and Table 4). After one dose of T_3 the swelling rate reached 169% of the control values and after three applications 198%. The extent of swelling was increased by one dose of T_3 (143%); after three doses of T_3 we did not see any additional increase in the extent of swelling (Table 4).

Triiodothyronine activation of mitochondrial swelling measured under conditions giving the maximal values of the swelling rate was completely inhibited by cyclosporine A (Fig. 5). These data also confirm a direct effect of T_3 on the functional activity of mitochondrial membrane permeability transition pore.

As an additional support for a direct T_3 action on the mitochondrial membrane permeability transition pore, we tested its effect on the dissipation of mitochondrial membrane potential induced by 50μM $CaCl_2$. Changes in mitochondrial membrane potential were measured by a safranin O fluorescent probe. Our data (Fig. 6) showed that the decrease in the membrane potential induced by calcium can be further potentiated by T_3 in a manner similar to the swelling experiments. T_3 can activate the dissipation of the membrane potential even in the absence of calcium ions.

We may thus conclude that our data indicate that in the absence of calcium and phosphate ions T_3 alone may activate mitochondrial swelling and the rate of this process is further increased by calcium and phosphate. We also confirmed findings of previous experiments (Červinková et al. 1998) showing that the activating effect of T_3 may be detected in isolated mitochondria after the *in vivo* application of triiodothyronine. Using a safranin O fluorescent probe we confirmed the data obtained in the swelling experiments by demonstrating that 25μM T_3 accelerates the discharge of the membrane potential induced by calcium ions.

Discussion

In our experiments we tried to obtain additional data that could help to characterize better the role of T_3 in activating the MPTP opening. Previous data in literature has supported the claim that the T_3 activating effect is indirect due to an increase in the sensitivity of the pore to calcium ions through the mechanism of ROS generation (Castilho et al. 1998). Our data showed that if Ca^{2+}, P_i or T_3 are added separately to mitochondria, both Ca^{2+} and T_3 induce a nearly five-fold increase in the swelling rate, whereas phosphate ions fail to. Phosphate, however, activates both Ca^{2+} as well as T_3 induced rates. The activation of the Ca^{2+} rate was about two-fold higher. The highest swelling rate was observed when all three factors were present (Table 2). This indicates that calcium and phosphate ions cannot reach the maximum swelling rate without T_3. We may conclude that calcium-induced swelling is more activated by phosphate (five-fold) than by T_3 (two fold), but that the Ca^{2+} and P_i induced swelling rate can be further (two-fold) increased by T_3. As evident from Fig. 2, T_3 accelerates the maximum swelling rate, but the maximum extent of swelling attains approximately the same values during the incubation period. This indicates that the membrane’s capacity to swell is not changed by the presence of T_3.

We confirmed the findings of previous literature (Kalderon et al. 1995) which showed that the swelling activation by T_3 can also be observed with isolated mitochondria after the *in* *vivo* application of T_3. This indicates that triiodothyronine can induce changes in MPTP properties that may be observed after isolating mitochondria.

We may thus conclude that applying the derivation of the swelling curves used in this study gives us more information about the swelling process and how various activating factors are involved in the regulation of swelling.

However, for an understanding of the detailed mechanisms through which the opening and closing of MPTP is regulated, more data are still required. There are indications that differences in Ca-induced swelling exist in mitochondrial subpopulations (Saunders et al. 2013). These authors stained mitochondria from rabbit kidneys with molecular probes for cardiolipin content and membrane potential and analyzed using flow cytometry. They found that these subpopulations with different cardiolipin content showed differences in membrane potential, volume, and responses to uncoupling and calcium-induced swelling.

After elucidating the mechanism of pore function and its regulation it will be possible to answer many questions in the various fields of biomedical research since it has been demonstrated that this pore is involved in the pathogenesis of diseases such as cardiomyopathies, neuropathies, liver diseases (Bernardi and Di Lisa 2015, Bonora et al. 2014, Traba et al. 2012) as well as in the process of ageing (Moro et al. 2004). Regulating the function of MPTP could help in the treatment of these diseases.

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Conflict of interest. The authors declare that they have no conflict of interest.

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Figure legends:

Fig. 1: Calcium dependent swelling of rat liver mitochondria. A: Classical swelling curves, B: Swelling curves after derivation. Mitochondria (0.4mg/ml) were incubated in the basic swelling medium with 0.1mM K-phosphate. After one minute of incubation $CaCl_2$ was added**.**

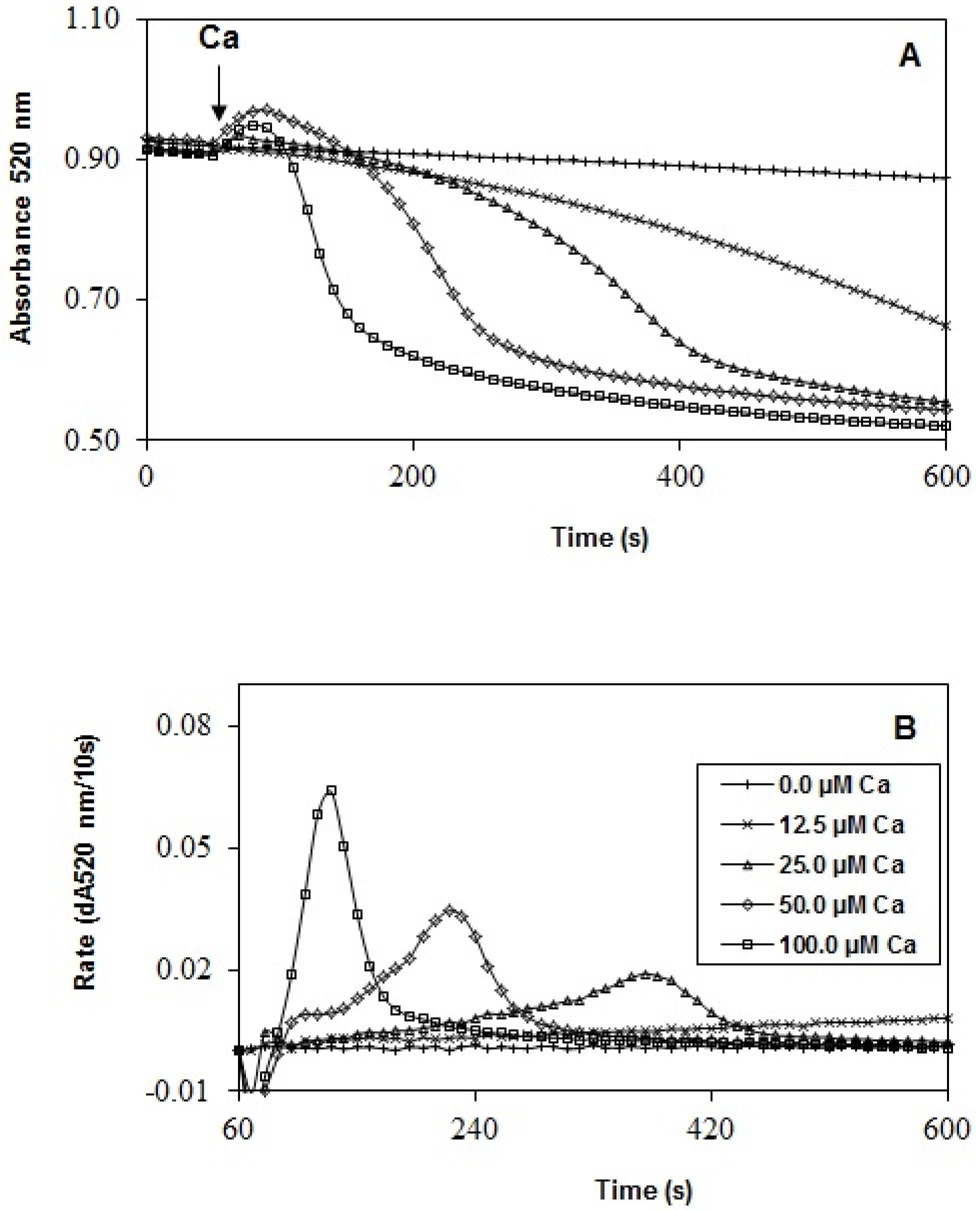


Fig. 2: The effect of calcium, phosphate and triiodothyronine interactions on swelling of rat liver mitochondria. Mitochondria (0.4mg protein/ml) were incubated in basic swelling medium with 0.1mM K-phosphate or 25µM T_3 or with phosphate and T_3 as indicated. T_3 was added immediately after mitochondria; 50µM Ca^{2+} was added after 60s of incubation.

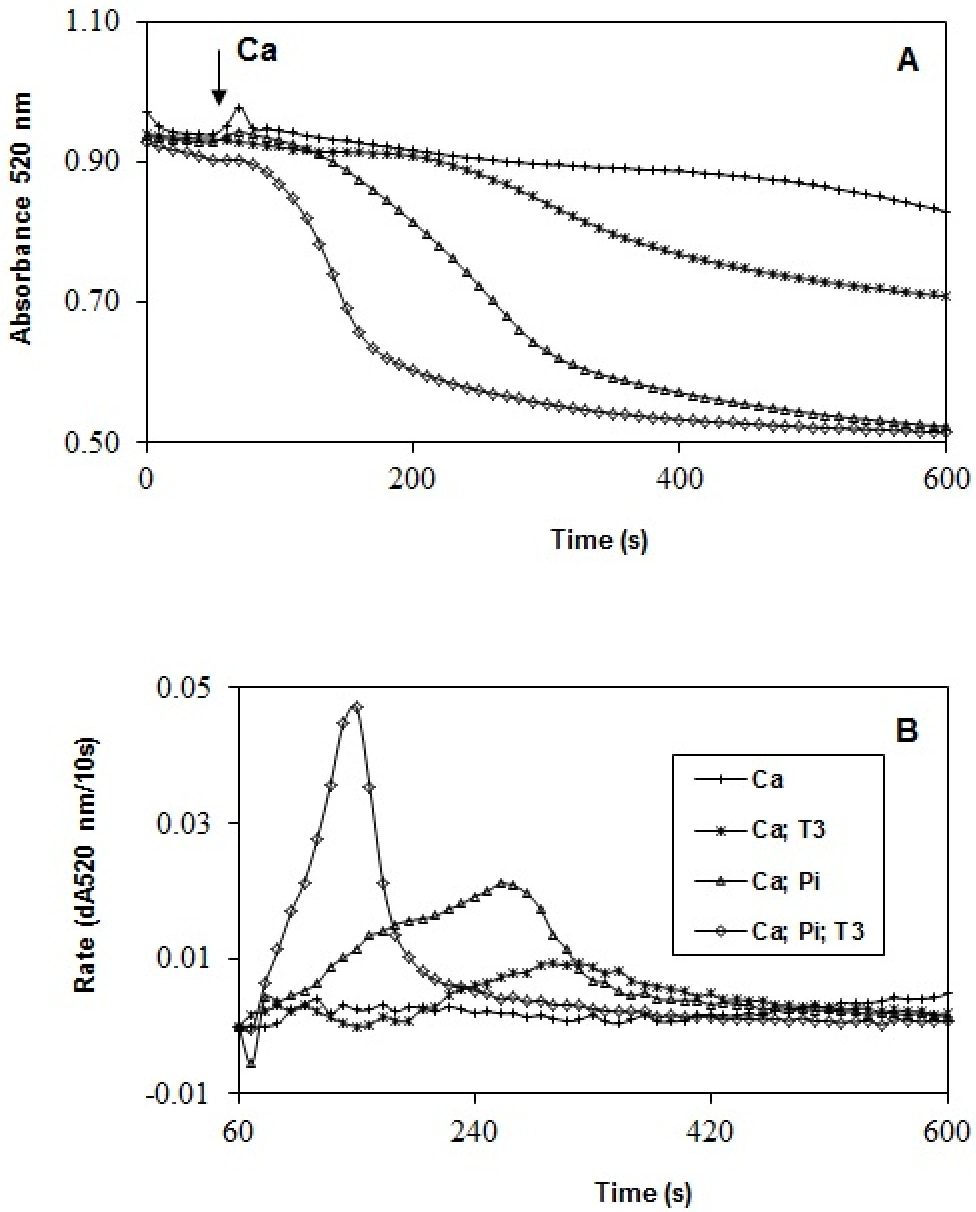


Fig. 3: The effect of T_3 on swelling of rat liver mitochondria. Mitochondria were incubated in the basic swelling medium without phosphate. T_3 was added immediately after mitochondria. Swelling was started by the addition of 50µM Ca^{2+} after 60s of incubation.

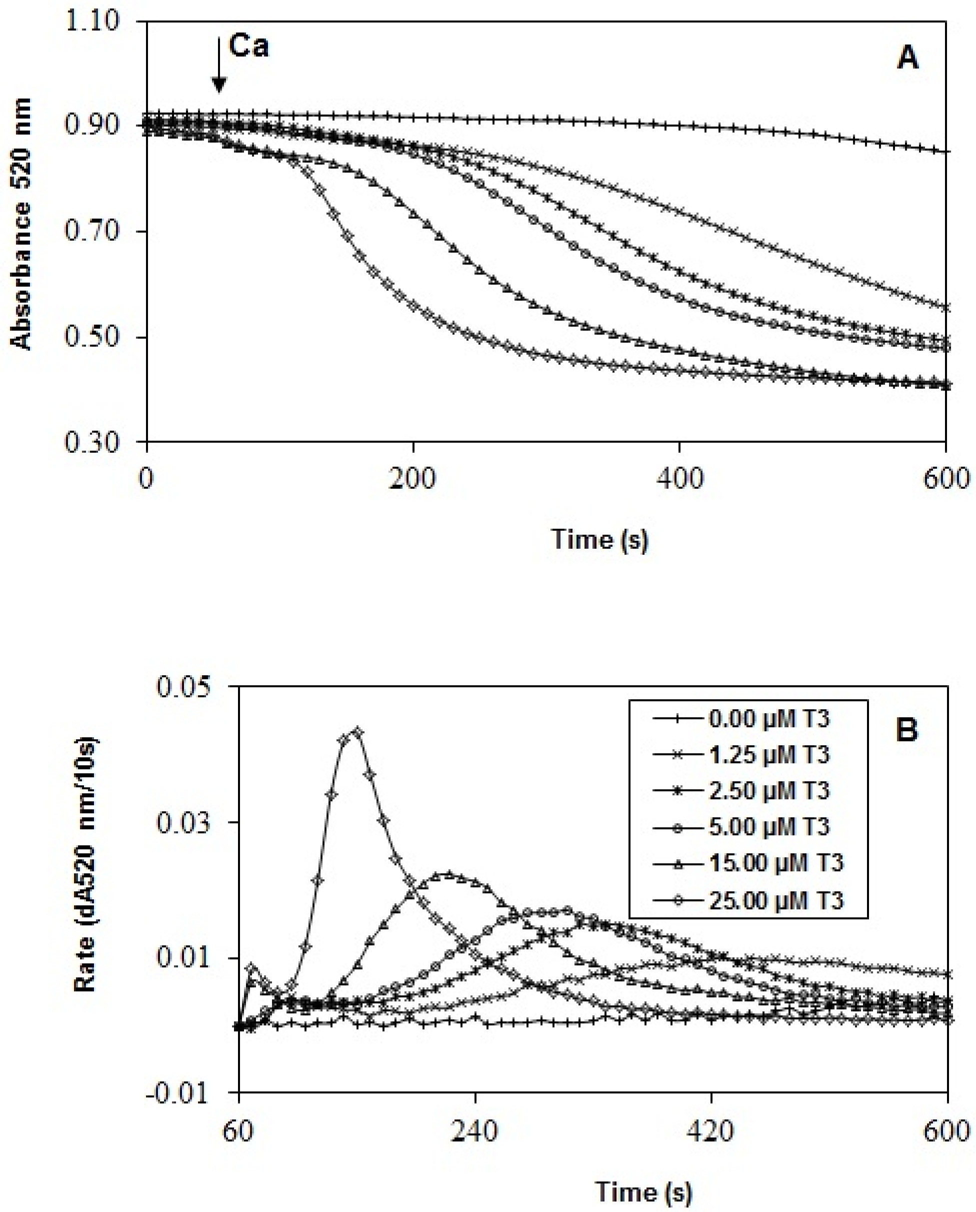


Fig. 4: Swelling of isolated rat liver mitochondria after *in vivo* intraperitoneal application of T_3. T_3 was applied 1x 200 µg/kg body weight or 3x 200 µg/kg body weight. Isolated mitochondria were incubated in the basic swelling medium with 0.1mM K-phosphate. Swelling was started by the addition of 50µM Ca^{2+}.

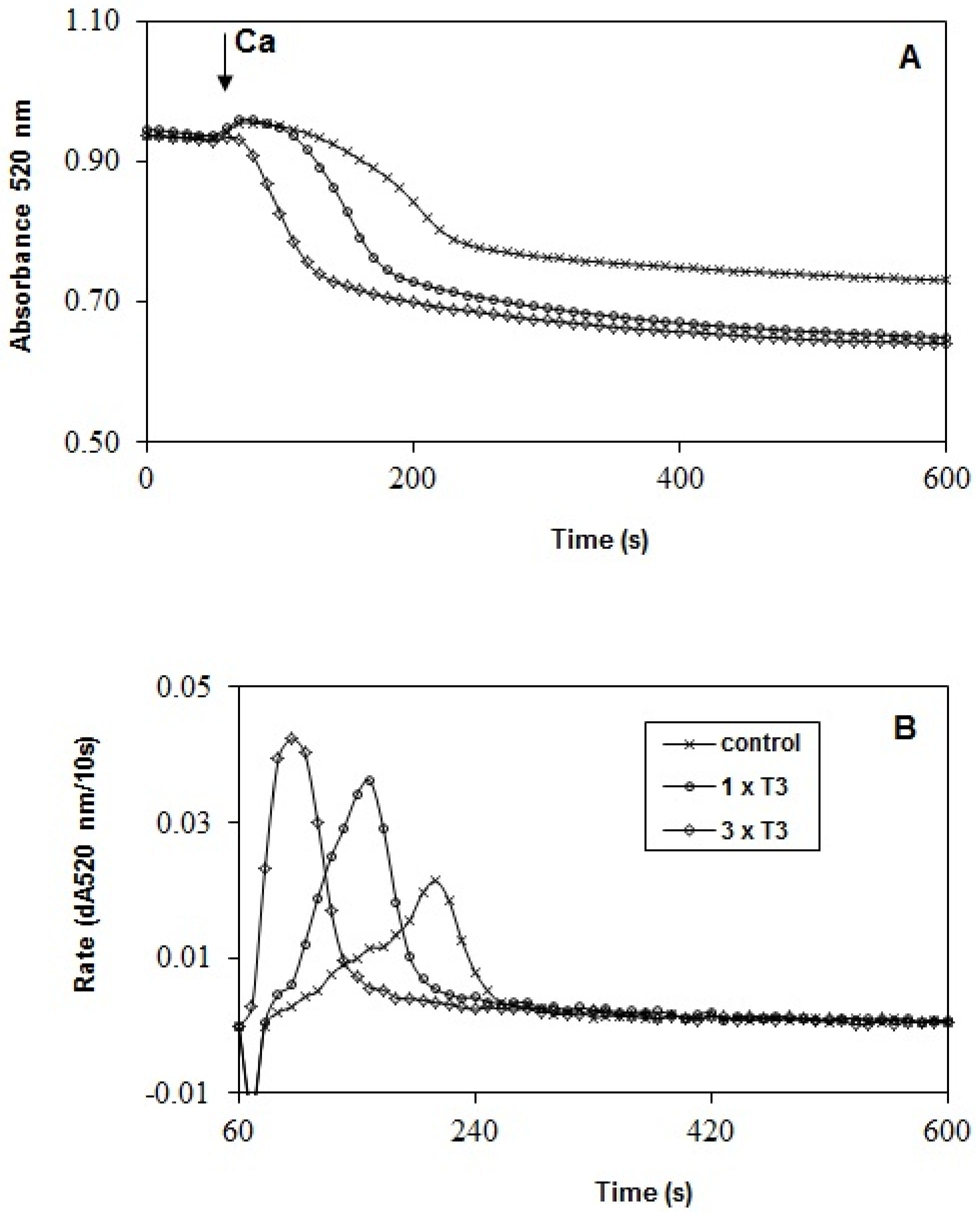
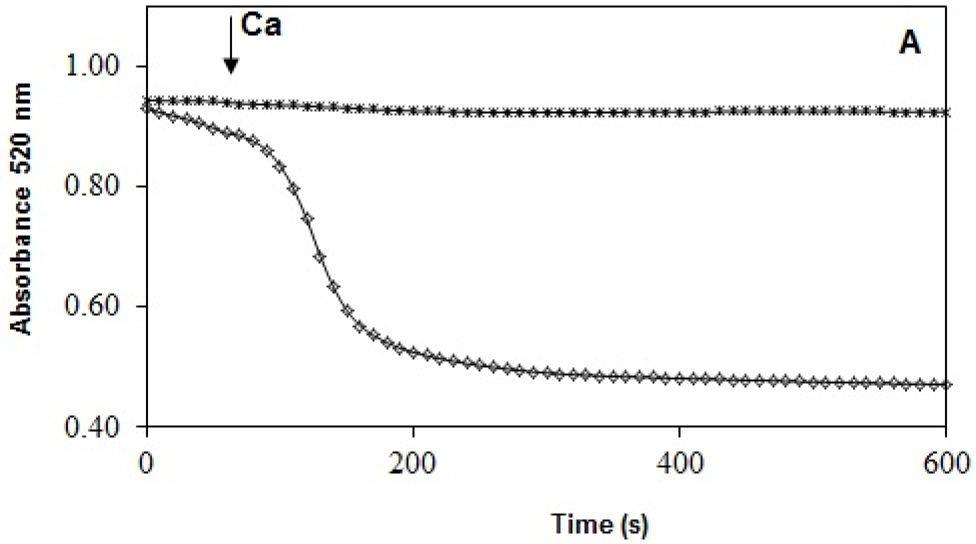


Fig. 5: Inhibition of mitochondrial swelling induced by Ca^{2+} and activated by 25µM T_3 by CsA. Mitochondria were incubated in the basic swelling medium as in Fig. 1. 25µM T_3 and 1µM CsA were added after mitochondria. 50µM Ca^{2+} was added after 60s of incubation.



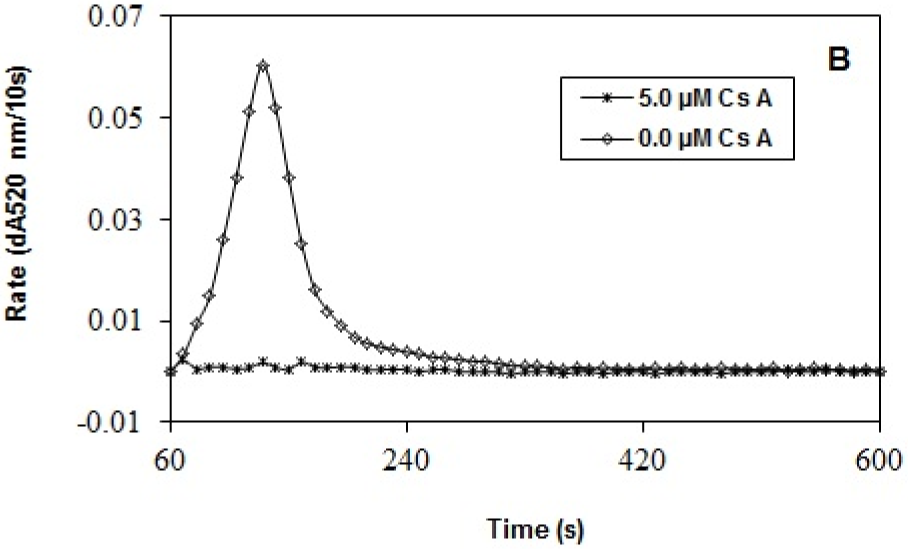
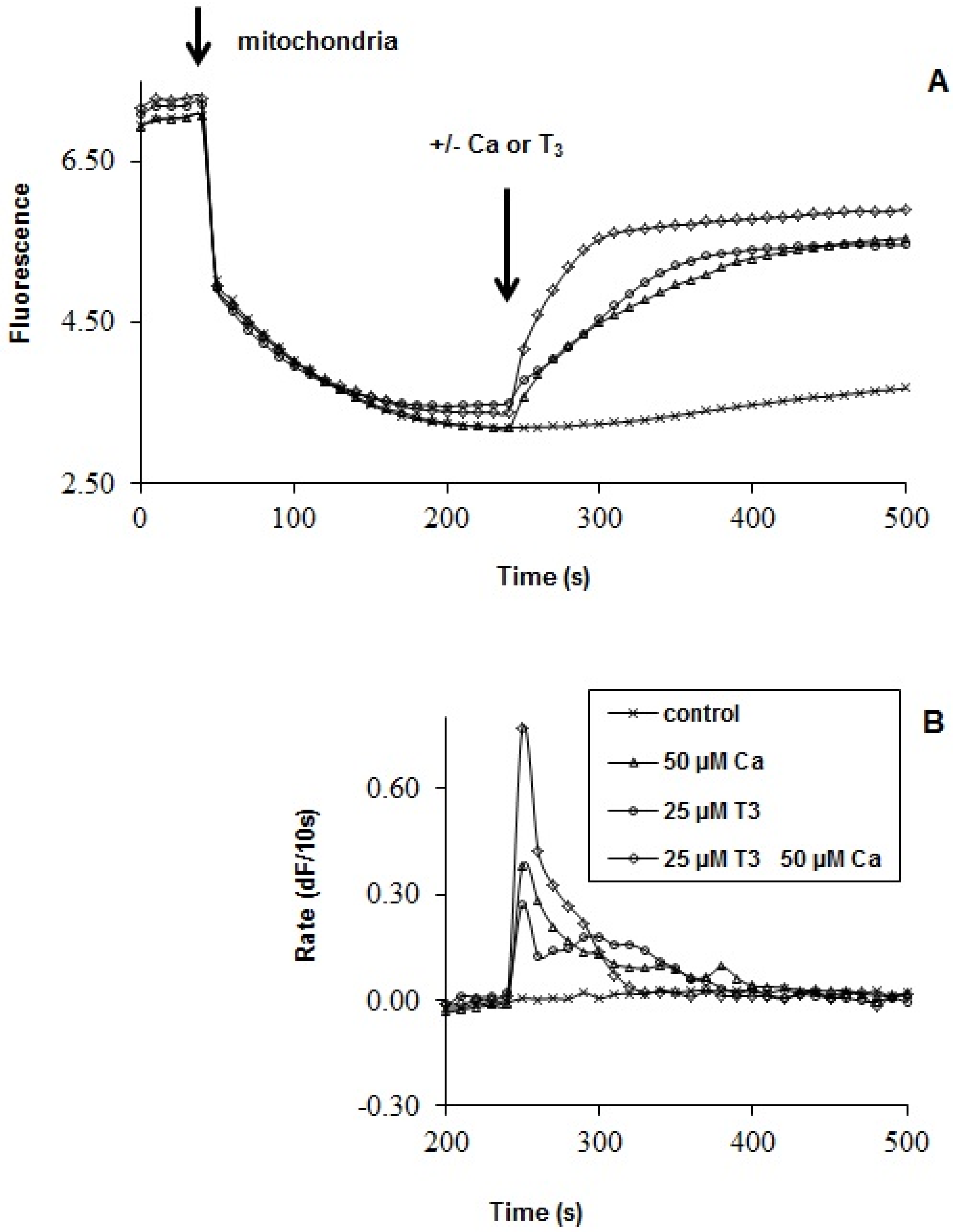


Fig. 6: T_3-activated changes of mitochondrial membrane potential. Membrane potential measured as described in methods.



Tables:

Additions (μM Ca^{2+})	Swelling Rate (dA_{520}/10s)	Extent of Swelling (dA_{520}/9min)	Time of maximum swelling (sec. after swelling induction)
0	**0.0010 (1%)**	**0.0450 (11%)**	**540**
5	**0.0012 (2%)**	**0.0582 (15%)**	**540**
12.5	**0.0081 (11%)**	**0.2516 (65%)**	**540**
25	**0.0191 (26%)**	**0.3687 (93%)**	**310**
50	**0.0345 (47%)**	**0.3991 (100%)**	**160**
100	**0.0639 (87%)**	**0.4012 (101%)**	**70**
150	**0.0739 (101%)**	**0.3954 (100%)**	**60**
200	**0.0731 (100%)**	**0.3969 (100%)**	**60**

Table 1: Calcium induced swelling of rat liver mitochondria. Data presented were extracted from the swelling curves in Fig. 1. The changes of swelling rate and extent of swelling are expressed in absolute values (dA_{520}/10s) and (dA_{520}/9min). For better clarity, the results were expressed also as a percentage of values obtained at 200μM Ca^{2+} (100%).

Additions	Swelling Rate (dA$_{520}$ /10s)	Extent of Swelling (dA$_{520}$/9min)	Time of maximum swelling (sec. after swelling induction)
No additions	**0.0009**	**0.0173**	**480**
50µM Ca^{2+}	**0.0042**	**0.1229**	**500**
25µM T_3	**0.0065**	**0.2186**	**210**
0.1mM P_i	**0.0014**	**0.0450**	**390**
$P_i + T_3$	**0.0128**	**0.2781**	**150**
$Ca^{2+} + T_3$	**0.0093**	**0.2216**	**240**
$Ca^{2+} + P_i$	**0.0211**	**0.4143**	**200**
$Ca^{2+} + P_i + T_3$	**0.0471**	**0.3846**	**90**

Table 2: Maximum swelling rate: Calcium, Phosphate and Triiodothyronine interactions. Data presented were extracted from swelling curves presented in Fig. 2.

Additions ($\mu M\ T_3$)	Swelling Rate (dA_{520}/10s)	Extent of Swelling (dA_{520}/9min)	Time of maximum swelling (sec. after swelling induction)
0	**0.0042 (10%)**	**0.0730 (16%)**	**530**
1.25	**0.0101 (23%)**	**0.3388 (74%)**	**370**
2.5	**0.0151 (35%)**	**0.4128 (90%)**	**260**
5	**0.0171 (39%)**	**0.4234 (92%)**	**250**
15	**0.0224 (52%)**	**0.4573 (100%)**	**160**
25	**0.0433 (100%)**	**0.4581 (100%)**	**90**

Table 3: The effect of T_3 on swelling of rat liver mitochondria. Data in the table were extracted from Fig. 3A,B. The changes of swelling rate and extent of swelling are expressed in absolute values (dA520/10s) and (dA520/9min). For better clarity, the results were expressed also as a percentage of values obtained at 25μM T_3 (100%).

Additions ($\mu M\ T_3$)	Swelling Rate (dA_{520}/10s)	Extent of Swelling (dA_{520}/9min)	Time of maximum swelling (sec. after swelling induction)
Control	**0.0214 (100%)**	**0.2062**	**150**
1x T_3	**0.0361 (169%)**	**0.2954**	**100**
3x T_3	**0.0424 (198%)**	**0.2953**	**40**

Table 4. The effect of T_3 applied *in vivo* on swelling of isolated mitochondria. Data presented were extracted form swelling curves in Fig. 4.