Thyroxine induces cyclosporin A-insensitive, Ca^{2+}-dependent reversible permeability transition pore in rat liver mitochondria

N.V. Malkevitch, V.I. Dedukhova, R.A. Simonian, V.P. Skulachev, A.A. Starkov*

Department of Bioenergetics, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899 Moscow, Russia

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Abstract The effect of thyroxine on Ca^{2+}-dependent mitochondrial permeability transition has been examined. It is shown that 40 μM thyroxine induces high amplitude swelling and decrease in membrane potential in Ca^{2+}-loaded rat liver mitochondria, both in the presence and absence of cyclosporin A. Thyroxine-induced decrease in membrane potential is partially or completely reversed by addition of EGTA into the incubation medium. Nigericin and ADP are shown to prevent, or significantly delay, the effects of thyroxine on both mitochondrial swelling and membrane potential, whereas nicotinamide potentiates the permeabilisation of mitochondria. It is suggested that thyroxine induced reversible, cyclosporin A-insensitive permeability transition pore (PTP) opening in the inner mitochondrial membrane.

Key words: Permeability transition pore; Cyclosporin A; Thyroxine; Mitochondria

1. Introduction

The Ca^{2+}-dependent permeability transition pore (PTP) in the inner mitochondrial membrane has long been known (see [1,2] for reviews). However, its molecular mechanism still remains obscure. Since the classic studies by Haworth and Hunter [3–5], this is considered to be due to a proteinaceous pore opening in the inner mitochondrial membrane, an event requiring accumulation of Ca^{2+} in the mitochondrial matrix. It is accompanied by high-amplitude swelling and de-energisation of mitochondria.

The pore is affected by a large number of compounds of various chemical nature, which stimulate or suppress the permeability transition; thyroxine (T_4) is one of these compounds. The mechanism by which T_4 stimulates the pore opening is not yet clear, although the involvement of free fatty acids [1] and SH-groups [6] has been suggested. Recently, the enhancement of ADP-ribosylation of adenine nucleotide translocase (ANT) by thyroxine was shown [7]. On the other hand, both ANT and ADP-ribosylation are thought to be involved in PTP regulation (see [2] and refs. therein).

Nowadays, the study of PTP properties and regulation is receiving increased attention due to the proposed key role of PTP in cellular apoptosis [8,9] and in the intracellular defense system against oxygen-derived free radicals [9].

*Corresponding author. Fax: (7) 95-939-3181.
E-mail: stark@energ.genebee.msu.su

Abbreviations: ΔΨ, transmembrane electric potential difference; BSA, bovine serum albumin; DNP, 2,4-dinitrophenol; EGTA, ethylene glycol-*bis*(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, morpholinopropane sulfonate; T_4, L-thyroxine; CS, cyclosporin A; DTE, dithioerythritol; ANT, adenine nucleotide translocase

In a number of recent studies, especially those carried out on intact cells, the sensitivity of the observed effects to a high-affinity pore inhibitor cyclosporin A (CS) is the only criterion to distinguish the involvement of PTP (e.g. see [10]). However, it is known that the sensitivity of PTP to CS depends on the experimental conditions [2]. It was of interest to investigate the characteristic features of pore induction by T_4 which can be considered as a natural modulator of PTP.

Here we present some evidence that T_4 induces the CS-insensitive PTP. The effect of thyroxine is modulated by ADP and nicotinamide. It can be prevented by nigericin and reversed by Ca^{2+} chelation with EGTA.

2. Materials and methods

2.1. Mitochondria

Rat liver mitochondria were isolated by differential centrifugation from 200 g white rats. The animals were decapitated; their livers were excised, cooled down and chopped. The chopped tissue was washed with isolation medium and homogenized in Potter homogenizer. Homogenate was centrifuged for 10 min at 600×*g*, supernatant was filtered through four layers of gauze and centrifuged for 10 min at 12 000×*g*. The resulting pellet was resuspended in isolation medium (10 mM MOPS, (pH 7.4), 250 mM sucrose, 1 mM EDTA, and bovine serum albumin, 1 mg/ml) and centrifuged. The mitochondrial pellet was washed with isolation medium without EDTA and albumin.

2.2. Measurements

In the majority of the experiments, the incubation mixture contained 250 mM sucrose, 10 mM Mops–KOH (pH 7.4), 10 mM KCl, 5 mM succinate, 4 mM H_3PO_4, oligomycin (2 μg·ml^{-1}), 2 μM rotenone, 2 μM TPP-Cl. Experiments were carried out at 25°C.

Oxygen consumption was measured at 25°C with a Clark-type oxygen electrode and an LP-7E polarograph.

The ΔΨ changes in mitochondria were estimated using safranine O. An Aminco DW-2000 spectrophotometer was used (the wavelength pair, 523–555 nm). The concentration of mitochondrial protein (0.4–0.8 mg·ml^{-1}) and that of safranine O (8–16 μM) were adjusted to keep the dye/protein ratio 20:1 (nmol dye·ml^{-1}·mg^{-1} protein).

Alternatively, transmembrane potential was monitored by TPP^+-selective electrode as changes in TPP^+ distribution between mitochondrial matrix and incubation medium.

The swelling of mitochondria was measured by absorbance changes at 660 nm using an Aminco DW-2000 spectrophotometer.

Protein concentration was measured by biuret reaction using bovine serum albumin as a standard.

2.3. Chemicals

Rotenone and oligomycin were dissolved in twice-distilled ethanol. Oligomycin, MOPS, and fatty acid-free BSA were from Sigma; EDTA, EGTA, rotenone, and DNP were from Serva; TPP^+ was from Merck, cyclosporin A was from Sandoz.

3. Results

3.1. The thyroxine-induced, CS-insensitive pore opening

We have chosen a 'classical' experimental system to study

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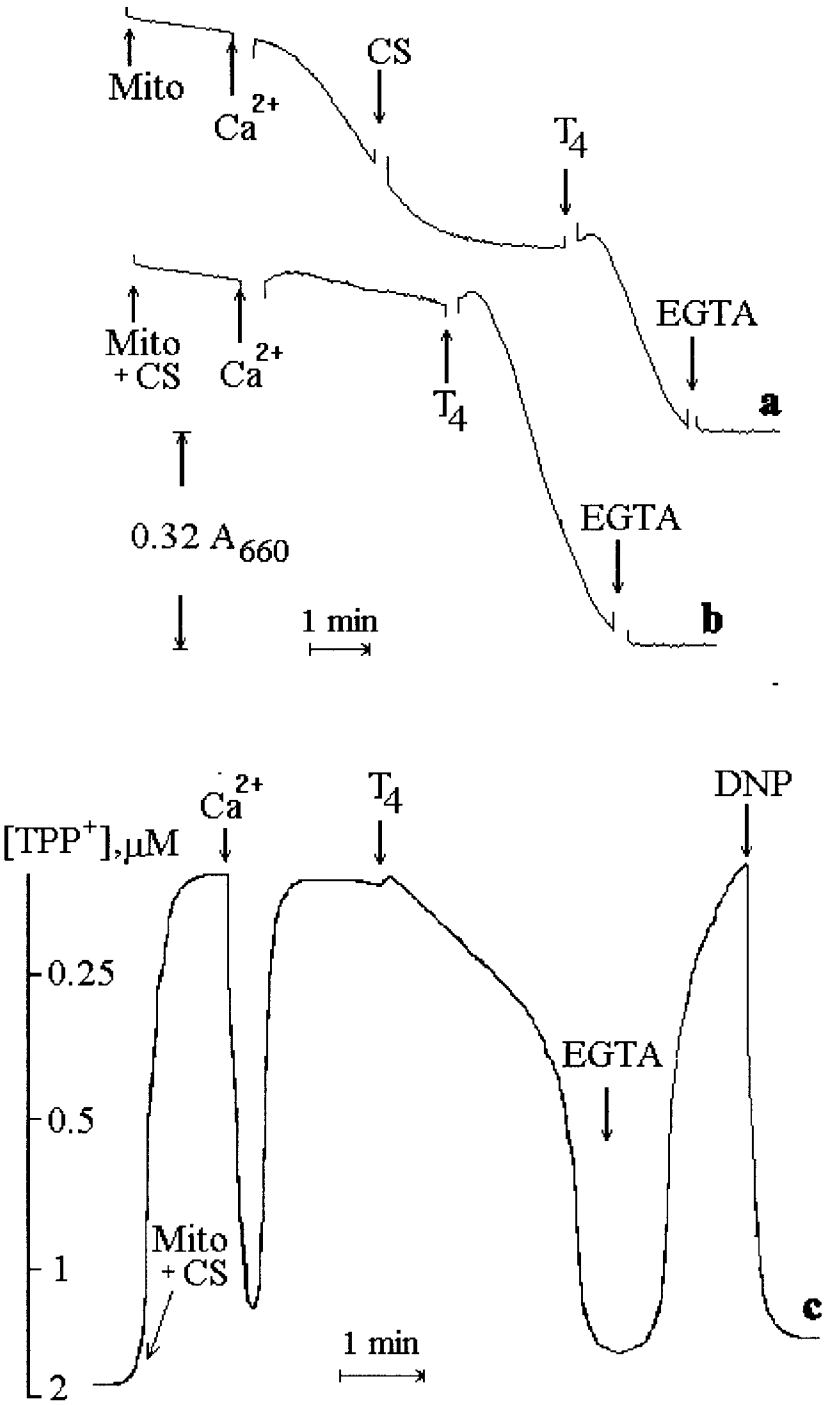

Fig. 1. The effect of T_4 on swelling and the membrane potential of rat liver mitochondria. For the experimental conditions, see Section 2. b,c: The incubation medium was supplemented with 1 μM Cs. a,b: Swelling of mitochondria; c: changes in mitochondrial ΔΨ measured with a TPP^+-sensitive electrode. Additions: rat liver mitochondria (Mito), 0.8 mg protein·ml^{-1}; 86 μM $CaCl_2$; 1 μM Cs; 40 μM T_4; 1 mM EGTA; 60 μM DNP.

the mitochondrial permeability transition, that is the PTP induction in rat liver mitochondria by Ca^+-loading in the presence of P_i. Fig. 1a shows that the addition of Ca^{2+} to the mitochondrial suspension causes rapid high-amplitude swelling (opening of PTP). The following addition of CS stops the swelling which is 10 induced by 40 μM thyroxine ($C_{1/2}$=20 μM). When CS is added before the mitochondria, Ca^{2+} does not induce swelling until T_4 is added (Fig. 1b). Fig. 1c shows the changes in the mitochondrial transmembrane potential (ΔΨ) measured with a TPP^+-selective electrode. It is seen that the T_4 addition entails a decrease in ΔΨ of the Ca^{2+}-loaded, CS-treated mitochondria. The ΔΨ level can be completely restored by the addition of EGTA. In the absence of CS, T_4 was found to induce PTP much faster and the subsequent EGTA addition restored the ΔΨ only partially (data not shown).

The measurement of the oxygen consumption revealed that T_4 induced an increase in the respiration rate of

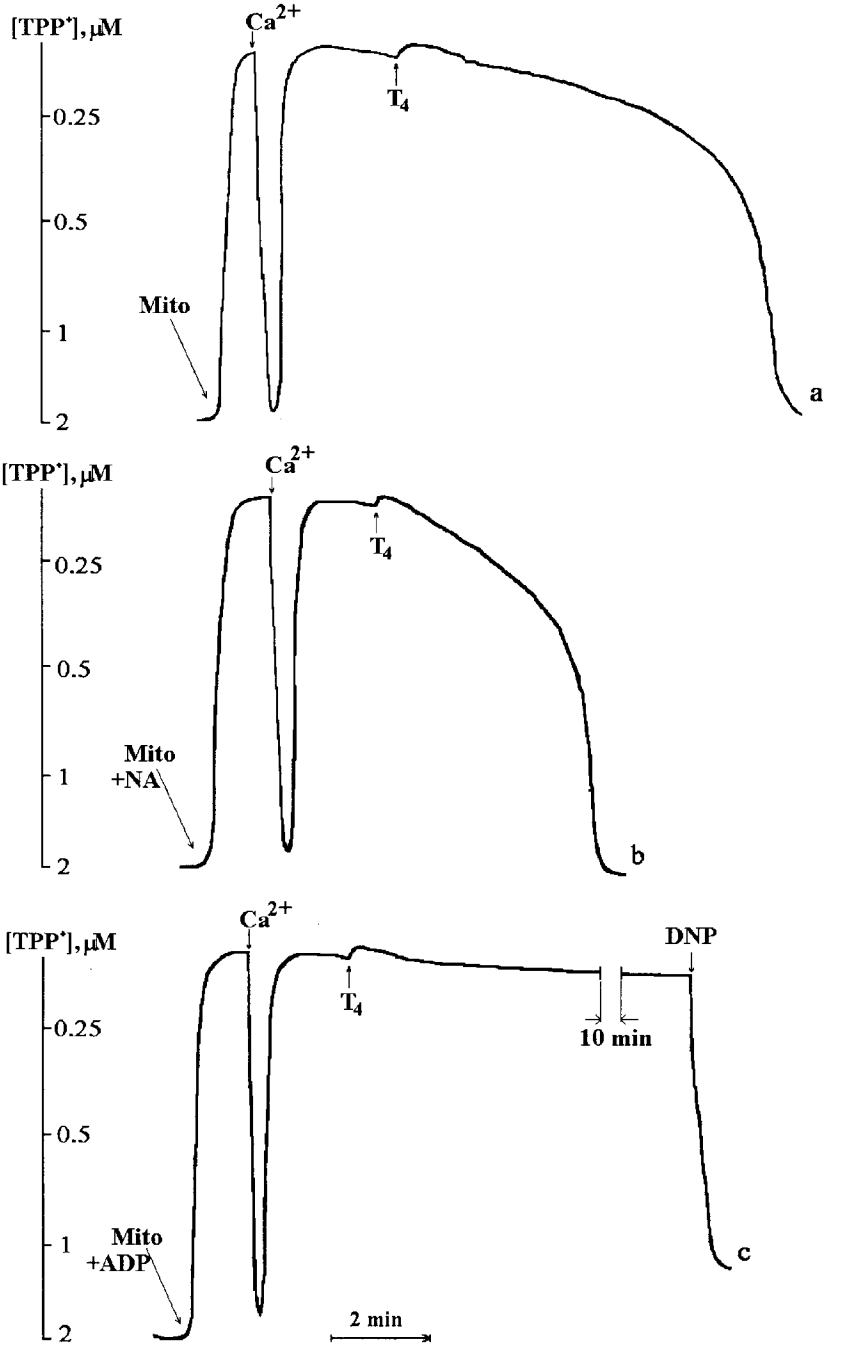

Fig. 2. The effect of ADP and nicotinamide on T_4—induced pore opening. For the experimental conditions, see Section 2. The incubation medium was supplemented with 1 μM CS (a); 1 μM CS and 10 mM nicotinamide (NA) (b), and 1 μM CS and 5 μM ADP (c). Additions: rat liver mitochondria, 0.9 mg protein·ml^{-1}; 86 μM $CaCl_2$; 40 μM T_4.

Ca^{2+}-loaded mitochondria in the presence of CS (data not shown).

3.2. Thyroxine effect is modulated by ADP and nicotinamide

Recently, it was shown that T_4 induced ADP-ribosylation of ANT which was inhibited by nicotinamide [7]. Fig. 2 shows that nicotinamide shortened the lag-period of the T_4-induced $\Delta\Psi$ decrease in Ca^{2+}-loaded mitochondria, whereas 5 μM ADP increased it significantly. The effect of ADP was dependent both on Ca^{2+} loading of mitochondria and P_i concentration in the incubation medium. High Ca^{2+}-loading (>200 nmol Ca^{2+}/mg protein at 4 mM P_i) abolished the effect of ADP (data not shown). It should be noted that under the chosen experimental conditions, the $\Delta\Psi$ level was stable for nearly 1 h of incubation even at high Ca^{2+}-loading of mitochondria (500–700 nmol Ca^{2+}·mg^{-1} protein) in the presence of CS (data not shown).

The inhibitory effect of ADP on T_4-induced pore opening depended on ADP concentration in the 1–5 μM range, which indicated involvement of ANT. Earlier, the key role of ANT in controlling the Ca^{2+}-dependent pore state was proposed ([2] and refs. therein). Special experiments showed that the specific inhibitor of ANT, 1 μM carboxyatractylate, completely abolished the action of 5–500 μM ADP. In the absence

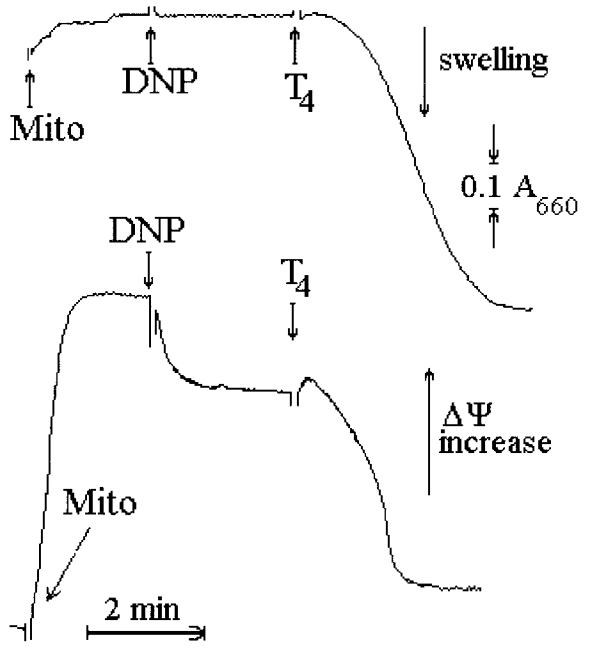

Fig. 3. The effect of 2,4-dinitrophenol on PTP induction in the presence of CS. Incubation medium contained 250 mM sucrose, 10 mM Mops–NaOH (pH 7.4), 10 mM KCl, 5 mM succinate, 3 mM H_3PO_4, oligomycin (2 μg·ml^{-1}), 2 μM rotenone, 1 μM CS, 10 μM safranine O. Additions: rat liver mitochondria, 0.5 protein·ml^{-1}; 40 μM T_4; 15 μM DNP. A: Swelling of mitochondria; B: changes in the ΔΨ.

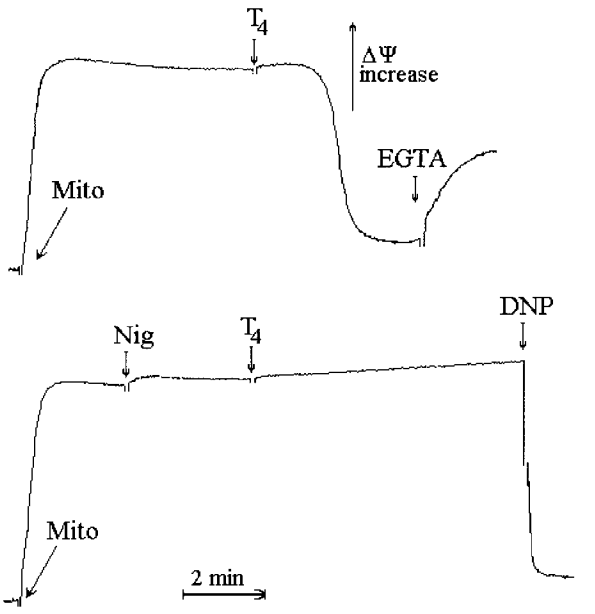

Fig. 4. Protective effect of nigericin on pore induction by T_4 in rat liver mitochondria. Incubation medium contained 250 mM sucrose, 10 mM Mops–NaOH (pH 7.4), 10 mM KCl, 5 mM succinate, 4 mM H_3PO_4, oligomycin (2 μg·ml^{-1}), 2 μM rotenone, 1 μM CS, 16 μM safranine O. Additions: rat liver mitochondria, 0.8 mg protein·ml^{-1}; 200 nM nigericin (Nig); 40 μM T_4; 60 μM DNP; 1.5 mM EGTA.

of T_4, carboxyatractylate did not induce pore opening if CS was included in the incubation medium (data not shown).

3.3. *Variability of mitochondrial preparations in the sensitivity of the thyroxine effect to Ca^{2+}-loading*

Our experiments revealed that various mitochondrial preparations displaying a similar respiratory control index may differ significantly in the so-called 'Ca^{2+}-retention capacity'. In the context of our experiments, this suggests that some of the mitochondrial preparation did not require Ca^{2+}-loading in order to obtain T_4-induced permeabilisation. Although not new, the phenomenon of variable 'resistance' of mitochondrial to Ca^{2+}-loading has probably never been studied in detail (we failed to find any published data), so the reasons are unclear. According to our experience, such preparations of mitochondria appear more frequently in the periods of November–December and April–May, so this phenomenon is probably somehow related to seasonal changes in metabolism (see [13] for discussion). Despite this, the CS-insensitive PTP induction by thyroxine and be readily demonstrated with this kind of mitochondrial preparations and some obtained data will be shown below.

Table 1
Effect of Ca^{2+}-loading of mitochondria on nigericin protective effect on pore induction by T_4

Nigericin	Ca^{2+} added (μm)	Duration of lag period (s)
−	0	140
+	0	>600
+	8.6	90
+	17.2	81
+	34.4	76
+	68.8	60
−	68.8	48

For experimental conditions, see Fig. 4. Concentration of mitochondria was 0.8 mg protein·ml^{-1}. Nigericin was added before mitochondria. Lag period was estimated as a time interval between addition of T_4 and decrease in the mitochondrial membrane potential by 30%.

3.4. *Thyroxine-dependent PTP induction is not related to the uncoupling action of T_4*

Taking into account the concentration (40 μM) of T_4 employed in our experiments, it was possible that the effect of T_4 was related to the uncoupling action of high amounts of T_4 [11] which had been described in the past. It appeared that under our experimental conditions 40 μM T_4 did not cause an immediate ΔΨ decrease (Figs. 3 and 4) as did, e.g. DNP (Fig. 3). Fig. 3 also shows that 1.5×10^{-5} M DNP fails to induce pore opening although it partially decreases ΔΨ. These data were obtained with a preparation of mitochondria, which did not require the Ca^{2+}-loading to obtain the T_4-induced pore opening. Similar data were obtained with mitochondria which required addition of 20–70 nmol Ca^{2+}·mg^{-1} of protein in order to see the T_4 effect in a minute time scale.

3.5. *BSA and DTE cannot abolish the T_4 effect*

Because involvement of free fatty acids [1] and SH-groups in the PTP induction by T_4 [6] has already been suggested, we tested the influence of dithioerytritol and BSA on the induction of PTP by T_4. DTE is known to protect SH-groups against oxidation, whereas BSA can protect mitochondria from the effects induced by free fatty acids which are produced upon T_4 treatment of mitochondria [12]. These experiments were performed with both kinds of mitochondrial preparations described above. No effect of 1 mM DTE or 0.4 mg/

ml BSA or combination of these agents on PTP induction by T_4 was observed (data not shown).

3.6. Inhibition of the T_4 effect by nigericin

Recently, we described a new effect of T_4 on liver mitochondria which consisted of restoration by T_4 of the membrane potential of mitochondria decreased by azide. The T_4 action was partially mimicked by nigericin [13].

It appeared that negericin abolished the effect of T_4 on the PTP opening (Fig. 4). The protective action of nigericin depends on the Ca^{2+}-loading of mitochondria (Table 1).

4. Discussion

The promoting effect of micromolar concentrations of T_4 on the PTP opening has long been known and is well documented, but it is still poorly understood (see [2] for the refs.). However, the ability of T_4 to induce PTP in a CS-insensitive way has never been reported.

Several features of the T_4 effect on PTP induction have been described in this paper.

(1) Micromolar concentrations of T_4 induce PTP opening which is insensitive to the specific PTP inhibitor CS and is reversible by Ca^{2+}-chelation with EGTA.

(2) Addition of BSA, DTE or both is without effect on the T_4-induced opening.

(3) T_4 efficiency to pore induction was enhanced by nicotinamide and inhibited by low concentrations of ADP.

(4) Nigericin completely or partially (depending on Ca^{2+}-loading of mitochondria) abolished the effect of T_4 on PTP.

The effect (1) resembles that reported by Novgorodov et al. [14] who showed that a crosslinking SH-reagent phenylarsine oxide can open PTP even in the presence of CS. However, it is not clear whether the pore opening induced by that reagent was reversible. The T_4 effect reported above was reversible upon Ca^{2+} removal with EGTA, thus indicating the absence of irreversible damage to the membrane system.

The lack of DTE effect indicates that the T_4 action is hardly related to oxidation of water-accessible SH groups. This is somewhat in contrast to Harris et al. [15] who reported the dithiothreitol-sensitive stimulatory effect of T_4 on the Ca^{2+} efflux from mitochondria.

An explanation of the T_4-induced pore opening might consist in increased production of free fatty acids [1,16]. Fatty acids are known as pore inductors (see [2] and refs. therein). If so, BSA was expected to prevent, or at least to delay, onset of the permeability transition. However, no effect of BSA was observed in our experiments.

Recently, it was reported by Mowbray and Hardy [7] that 10^{-11} M triiodothyronine addition to hypothyroid rat liver mitochondria increased the rate of operation of ANT, the effect being abolished by nicotinamide. Authors proposed that ANT is regulated in such a way that ADP-ribosylated ANT is stabilised in C-conformation. In these experiments, the nicotinamide treatment of euthyroid mitochondria rendered them indistinguishable from hypothyroid ones [7] stabilising, according to the authors view, the M-conformation of translocase. It is well documented that C-conformation of ANT, as well as ADP-ribosylation of mitochondrial proteins, favors the PTP opening [2]. Due to this, we expected that nicotinamide would diminish the T_4 effect by inhibiting ADP-ribosylation. However, it appeared that nicotinamide enhanced the thyroxine effect on the PTP opening. The main differences between our and Mowbray and Hardy's conditions are that we used (i) much higher T_4 concentrations, (ii) mitochondria isolated from euthyroid animals, and (iii) the CS-supplemented incubation medium. Submicromolar T_4, in our hands, was without measurable influence.

Inhibition by ADP of the T_4-induced pore opening and prevention of the ADP effect by a specific ANT inhibitor, carboxyatractylate, are consistent with the proposed earlier key role of ANT conformation state in PTP regulation [2]. ADP induces the M-conformation of ANT which is unfavorable for pore opening, whereas carboxyatractylate induces the C-conformation. It is noteworthy that our experiments were performed in the presence of CS so the pore was inhibited. Under such conditions, induction of C-conformation of ANT is nog sufficient for pore opening as carboxyatractylate alone did not induced PTP. On the other hand, ADP-induced stabilisation of ANT in M-conformation prevents the pore induction by T_4. Thus, it may be suggested that for PTP induction, interaction of T_4 with the C-conformation of ANT is necessary.

Inhibition by nigericin of T_4-induced PTP opening can be explained by acidification of the mitochondrial matrix under the experimental conditions used (relatively low K^+ concentration in the incubation medium) due to K^+/H^+ exchange. It was shown that matrix acidification decreases the probability of PTP opening and inhibition is competitive to a Ca^{2+}-loading of mitochondria (see [2] for refs.).

It is not clear whether the in vitro effect of thyroxine reported here is related to in vivo hormone action. Some evidence that the thyroid state of an animal affects the mitochondrial PTP properties has been published [2,17]. Liver mitochondria isolated from hyperthyroid animals are more susceptible to permeability transition [17].

The concentration of T_4 used in our experiments is higher than physiological. However, this may result from limitations inherent in the experimental model employed such as isolated mitochondria (absence of cytoplasmic environment and/or possible damage of mitochondria due to the solution procedure). The thyroid state of animals may also be important. It appears interesting to study the PTP induction by T_4 in mitochondria isolated from hypothyroid animals.

It was postulated by one of us [9] that thyroid hormone-mediated uncoupling is the first step in the anti-oxygen cellular defense system. In the framework of this hypothesis, the existence of some direct mechanism underlying the promoting effect of thyroid hormones on mitochondrial permeability transition seems to be logical because the PTP opening in mitochondria is considered as the second step in the chain of events induced by free radicals which results in elimination of radical-producing mitochondria.

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