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Periodate-oxidized ATP stimulates the permeability transition of rat liver mitochondria

Wolfgang Henke *, Thilo Hagen, Klaus Jung, Stefan A. Loening

Research Division, Department of Urology, University Hospital Charité, Humboldt University, Schumannstraße 20 / 21, D-10098 Berlin, Germany

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Abstract

Periodate-oxidized ADP (oADP) and periodate-oxidized ATP (oATP) stimulate the permeability transition in energized rat liver mitochondria measured as the Ca^{2+}-efflux induced by Ca^{2+} and P_i. In the presence of Mg^{2+} and P_i, mitochondria lose intra-mitochondrial adenine nucleotides at a slow rate. oATP induces a strong decrease of the matrix adenine nucleotides which is inhibited by carboxyatractyloside. Under these conditions, Mg^{2+} prevents the opening of the permeability transition pore. EGTA prevents the P_i-induced slow efflux of adenine nucleotides, but is without effect on the oATP-induced strong decrease of adenine nucleotides. This oATP-induced strong adenine nucleotide efflux is inhibited by ADP. oATP reduces the increase of matrix adenine nucleotides occurring when the mitochondria are incubated with Mg^{2+} and ATP. This effect of oATP is also prevented by carboxyatractyloside. oATP is not taken up by the mitochondria. It is suggested that oATP induces a strong efflux of matrix adenine nucleotides by the interaction with the ADP/ATP carrier from the cytosolic side. The induction of the mitochondrial permeability transition by oADP and oATP is attributed to two mechanisms—a strong decrease in the intramitochondrial adenine nucleotide content, especially that of ADP, and a stabilization of the c-conformation of the ADP/ATP carrier. © 1998 Elsevier Science B.V.

Keywords: Mitochondrion; Permeability transition pore; Calcium ion; ADP/ATP carrier; Adenine nucleotide net transport; Periodate-oxidized ATP

1. Introduction

The accumulation of Ca^{2+} and P_i by mitochondria leads to a marked permeability transition of the inner membrane. The discovery that the permeability transition can be inhibited by the immuno-suppressive agent cyclosporin A initiated decisive investigations to discriminate between two hypothesis—an increase of the inner membrane permeability by unspecific changes or by specific pore opening [1]. Increased permeability is now attributed to the opening of a so-called permeability transition pore (PTP). The molecular identity and function of the pore are at present a matter of discussion. Effectors of PTP opening and closure are among others Ca^{2+}, Mg^{2+}, P_i, ADP, reactive oxygen species and polyamines.

Abbreviations: oAMP, periodate-oxidized AMP; oADP, periodate-oxidized ADP; oATP, periodate-oxidized ATP; PTP, permeability transition pore

* Corresponding author. Fax: +49-30-28021402; E-mail: henke@rz.charite.hu-berlin.de

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There is growing evidence that PTP opening is involved in apoptosis [2,3] and ischemic injury [4,5].

The 2′,3′-dialdehyde derivative of ATP (oATP), prepared by periodate oxidation of ATP [6], was used in various studies in order to investigate the active site of ATP-dependent enzymes such as pyruvate carboxylase [6], H^+-ATPase [7] and Ca^{2+}-ATPase [8]. oATP is particularly appropriate for covalent-linkage studies because the reactive moiety is the modified ribose which can only link to the binding site [8]. Surprisingly, preliminary studies with isolated liver mitochondria have demonstrated that oADP and oATP stimulate, in contrast to ADP and ATP [9], the opening of the PTP. Both ADP and ATP inhibit the permeability transition, but the view prevailed that ADP is the only relevant nucleotide. It is generally assumed that ADP affects the permeability transition from the matrix side [10].

The purpose of this study is to investigate whether the periodate-oxidized derivatives of ADP and ATP directly interact with the pore-forming protein and, therefore, might be used for affinity labeling studies. The permeability transition was measured as the Ca^{2+} efflux induced by Ca^{2+} and P_i. This is a well-established approach to investigate the pore opening [10–12].

2. Materials and methods

2.1. Materials

Periodate-oxidized derivatives of AMP, ADP and ATP, purine compounds, used as standards for HPLC analysis, and arsenazo III were purchased from Sigma (Deisenhofen, Germany) and carboxyatractyloside from Boehringer Mannheim (Mannheim, Germany). All other chemicals were of highest purity commercially available.

2.2. Isolation of mitochondria

Fed adult male Wistar rats weighing 200–250 g were used. The isolation of liver mitochondria was performed by differential centrifugation [13], applying a solution of 210 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl (pH 7.4), and 0.5 mM EDTA. The mitochondrial pellet was washed twice. For the second washing step, EDTA was omitted. Finally, the mitochondria were resuspended in the isolation medium without EDTA. The protein content was determined by the Biuret method with bovine serum albumin as the standard [13].

2.3. Adenine nucleotide efflux and net uptake

The mitochondria were incubated at 30°C in the resuspension medium containing 2 mM KH_2PO_4, 5 mM $MgCl_2$ and 5 mM glutamate and malate. This solution was used as the standard incubation medium. After indicated time periods, the mitochondria were centrifuged at 4°C and washed once. The final pellet was resuspended in the isolation medium at a protein content of about 4 mg/ml. Adenine nucleotides were extracted with perchloric acid and determined enzymatically as described [14].

2.4. Ca^{2+}-efflux measurements

The mitochondria were preincubated in the resuspension medium, containing Ca^{2+}, oADP or oATP as well as 5 mM glutamate and malate, at a protein content of about 1.5 mg/ml at 37°C for 1 min. The Ca^{2+}-efflux was induced with 2 mM KH_2PO_4 and traced with 50 μM arsenazo III at 650 nm and 690 nm using the microplate reader HT III and the EIA/KIN-Star software, version 7.0 (Anthos Labtec Instruments, Salzburg, Austria).

2.5. Dissociation constants of the Ca^{2+}-complexes of arsenazo III and oATP

The dissociation constant of the Ca^{2+}–arsenazo III complex was determined by titrating arsenazo III (0 to 0.1 mM) with several Ca^{2+} concentrations (0 to 0.5 mM) and that of the Ca^{2+}–ATP complex by titrating 0.05 mM arsenazo III plus Ca^{2+} (0.01 to 0.1 mM) with oATP (0.05 to 2 mM). The calculation of the dissociation constants is based on the conservation equations of the total concentrations of arsenazo III, Ca^{2+} and oATP and accounts for the fact that arsenazo III and the Ca^{2+}–arsenazo III complex contribute to the absorption with different extinction coefficients [15]. Applying a nonlinear regression procedure [16], extended by an iterative algorithm to compute the concentration of the different species

involved in the complex formation [17], the dissociation constants of the Ca^{2+}–arsenazo III complex and Ca^{2+}–oATP complex are estimated to be 10.5 μM and 128 μM, respectively.

2.6. Uptake studies with periodate-oxidized ATP

The uptake of oATP was investigated by an HPLC method [18]. A 1090M HPLC system with diode array detector (Hewlett Packard, Vienna, Austria) and a narrow-bore column, 100 mm × 2 mm I.D., with an integrated guard column, 7 mm × 2 mm I.D., (Knauer, Berlin, Germany) filled with ODS Hypersil (Shandon, UK) were applied. The mobile phase A consisted of 10 mM $NH_4H_2PO_4$ (pH 5) and 2 mM tetrabutylammonium bromide and the mobile phase B contained 10 mM $NH_4H_2PO_4$ (pH 6.5), 0.5 mM tetrabutylammonium bromide and 25% (v/v) acetonitrile. The gradient elution started with 10% of mobile phase B, increased linearly to 80% during 35 min and continued for 10 min.

3. Results

3.1. Stimulation of the permeability transition by oADP and oATP

ADP inhibits the Ca^{2+}- and P_i-induced Ca^{2+}-efflux. In contrast, oADP leads to a stimulation of the

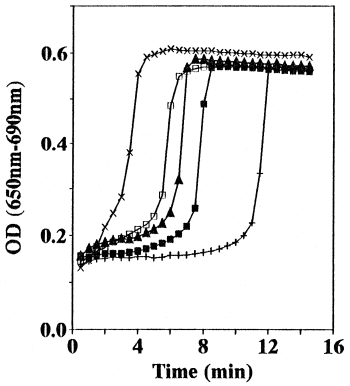

Fig. 1. Stimulation of Ca^{2+}-efflux by periodate-oxidized ADP. Mitochondria were incubated with 60 μM Ca^{2+} as described in Section 2 (▲). The Ca^{2+}-efflux was induced with 2 mM P_i. Further additions: oADP—100 μM (□), 500 μM (×); ADP—1 μM (■), 10 μM (+). Results are representative of three independent experiments including the application of different Ca^{2+} concentrations.

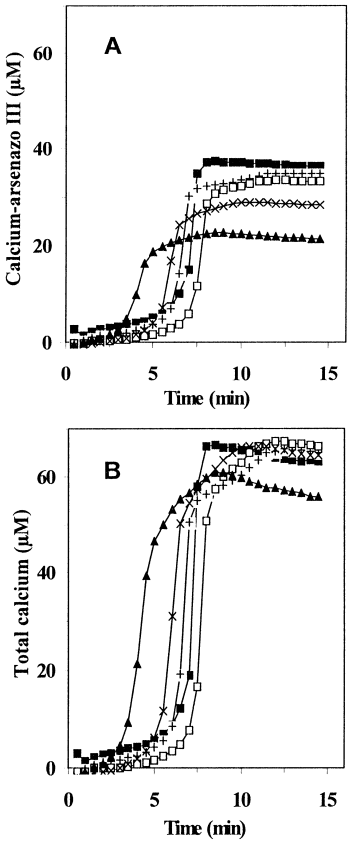

Fig. 2. Stimulation of Ca^{2}-efflux by periodate-oxidized ATP. Mitochondria were incubated with 60 μM Ca^{2+} as in Fig. 1. The following oATP concentrations were applied: 0 mM (■), 0.05 mM (+), 0.25 mM (□), 0.5 mM (×), 1 mM (▲). The concentration of the Ca^{2+}–arsenazo III complex (A) and the total Ca^{2+} in the medium (B) was calculated as described in Section 2. Results are representative of three independent experiments including the application of different Ca^{2+} concentrations.

Ca^{2+}-efflux (Fig. 1). oATP, like oADP, stimulates the permeability transition (Fig. 2B). In the presence of oATP, the absorption of the Ca^{2+}–arsenazo III complex reached at maximal Ca^{2+}-efflux is depressed with increasing oATP concentrations (Fig. 2A). This effect is caused by the complexation of extramitochondrial Ca^{2+} by oATP as proven by calculating the total Ca^{2+}-concentrations appearing in the medium (Fig. 2B). oAMP has no significant effect on the Ca^{2+}-efflux induced by Ca^{2+} and P_i (not shown).

3.2. oATP-induced changes in the matrix adenine nucleotide content

The matrix adenine nucleotide content of liver mitochondria incubated in the presence of 2 mM P_i

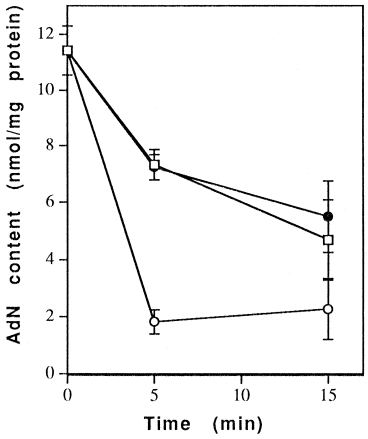

Fig. 3. Stimulation of adenine nucleotide efflux by oATP. Changes in the matrix adenine nucleotide (AdN) content in rat liver mitochondria were measured as described under Section 2 with the following additions: 0 mM oATP (□), 0.5 mM oATP (○) and 0.5 mM oATP and 15 μM carboxyatractyloside (●). Mean ± S.D., $n = 3$.

and 5 mM Mg^{2+} decreases at a slow rate (Fig. 3). This adenine nucleotide efflux is carboxyatractyloside-insensitive and, therefore, attributed to the $Mg^{2+}ATP/P_i$ carrier [19]. oATP strongly accelerates the decrease in the intramitochondrial adenine nucleotide content (Fig. 3). Five minutes after the addition of 0.5 mM oATP, the shortest time investigated, the adenine nucleotide content decreases to about 15% of the initial value. The oATP-induced adenine nucleotide efflux is inhibited by carboxyatractyloside to the level of the carboxyatractyloside-insensitive efflux in the absence of oATP.

EGTA inhibits the adenine nucleotide efflux occurring in the presence of P_i (Fig. 4). This effect of EGTA is caused by complexation of endogenous Ca^{2+} and illustrates the known fact that Ca^{2+} stimulates the efflux rate of the $Mg^{2+}ATP/P_i$ carrier [19]. In the presence of EGTA, oATP also induces a rapid decrease of matrix adenine nucleotides. ADP inhibits this oATP-induced efflux (Fig. 4).

In mitochondria incubated in the presence of 4 mM ATP, 5 mM Mg^{2+} and 2 mM P_i, the matrix adenine nucleotide content increases over time. This net uptake of adenine nucleotides is carboxyatractyloside-insensitive (Fig. 5) and occurs via the $Mg^{2+}ATP/P_i$ carrier [19]. The increase in the intramitochondrial adenine nucleotide level is reduced by oATP. Carboxyatractyloside restores the increase of matrix adenine nucleotides inhibited by oATP to the rate measured without oATP (Fig. 5).

The observed effect of oATP on the matrix adenine nucleotide content is not caused by the induction of the permeability transition, which has been found

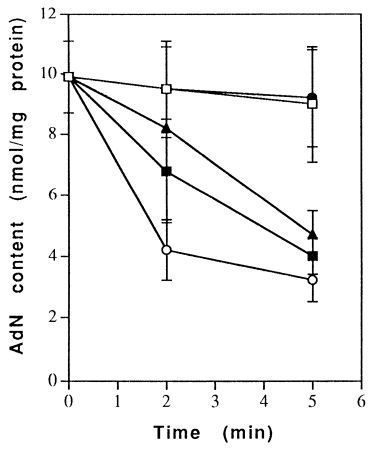

Fig. 4. Inhibition of the oATP-stimulated adenine nucleotide efflux by ADP. Incubations of the mitochondria were performed in the presence of 2 mM EGTA. Additions: 2 mM ADP (□); 0.2 mM oATP and the following ADP concentrations—0 mM (○), 0.2 mM (■), 0.5 mM (▲) or 2 mM (●). Mean ± S.D., $n = 3$.

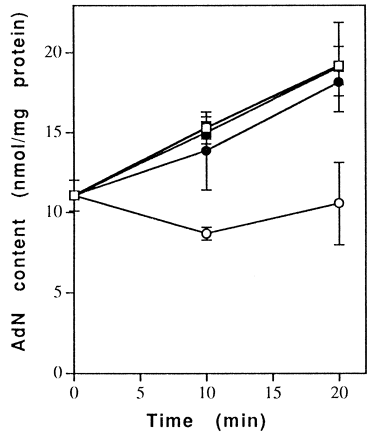

Fig. 5. Effect of oATP on Mg^{2+}ATP net uptake. Mg^{2+}ATP net uptake was measured with 4 mM ATP and 5 mM Mg^{2+} using the incubation medium and no additions (□), 15 μM carboxyatractyloside (■), 4 mM oATP (○), or 4 mM oATP and 15 μM carboxyatractyloside (●). In experiments investigating the effect of oATP, the Mg^{2+} concentration was increased from 5 mM to 9 mM. This change accounts for the formation of the Mg^{2+}–oATP complex and keeps the concentration of Mg^{2+}ATP constant. In comparison with no additions, the effect of 4 mM oATP (○) is statistically significant ($P < 0.01$). Mean ± S.D., $n = 3$.

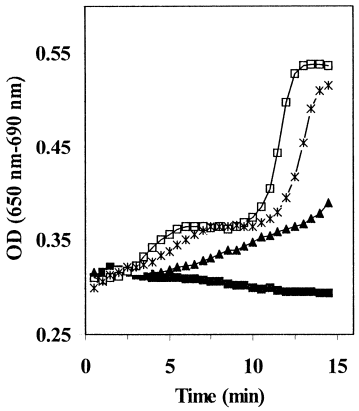

Fig. 6. Prevention of the oATP-stimulated Ca^{2+}-efflux by Mg^{2+}. Mitochondria were incubated with 5 mM Mg^{2+} and 0.5 mM oATP applying the following Ca^{2+} concentrations: 0 μM (■), 40 μM (▲), 60 μM (∗) and 80 μM (□).

to lead to a depletion of adenine nucleotides in mitochondria [20,21]. Without the addition of Ca^{2+} and in the presence of 2 mM P_i, 5 mM Mg^{2+} and 0.5 mM oATP, no permeability increase in energized mitochondria was observed (Fig. 6). The mitochondria incubated under these conditions, however, are able to perform the permeability transition when Ca^{2+} is added.

3.3. Uptake studies with periodate-oxidized ATP

The effect of carboxyatractyloside on both the oATP-induced efflux of matrix adenine nucleotides and the oATP-inhibited increase of matrix adenine nucleotides of mitochondria incubated with Mg^{2+}ATP leads to the assumption that the externally added oATP exerts its effect via the ADP/ATP carrier. The ADP/ATP carrier exchanges extramitochondrial versus intramitochondrial ADP/ATP by a one-for-one mechanism [22]. If the oATP-induced efflux of adenine nucleotides occurs by the same mechanism it should be paralleled by an equimolar accumulation of oATP in the matrix. To measure the oATP taken up by mitochondria we applied an HPLC method. Since oATP is hydrolysed by the F1-ATPase of beef heart mitochondria [23] we also included oAMP and oADP in the standard mixture of purine

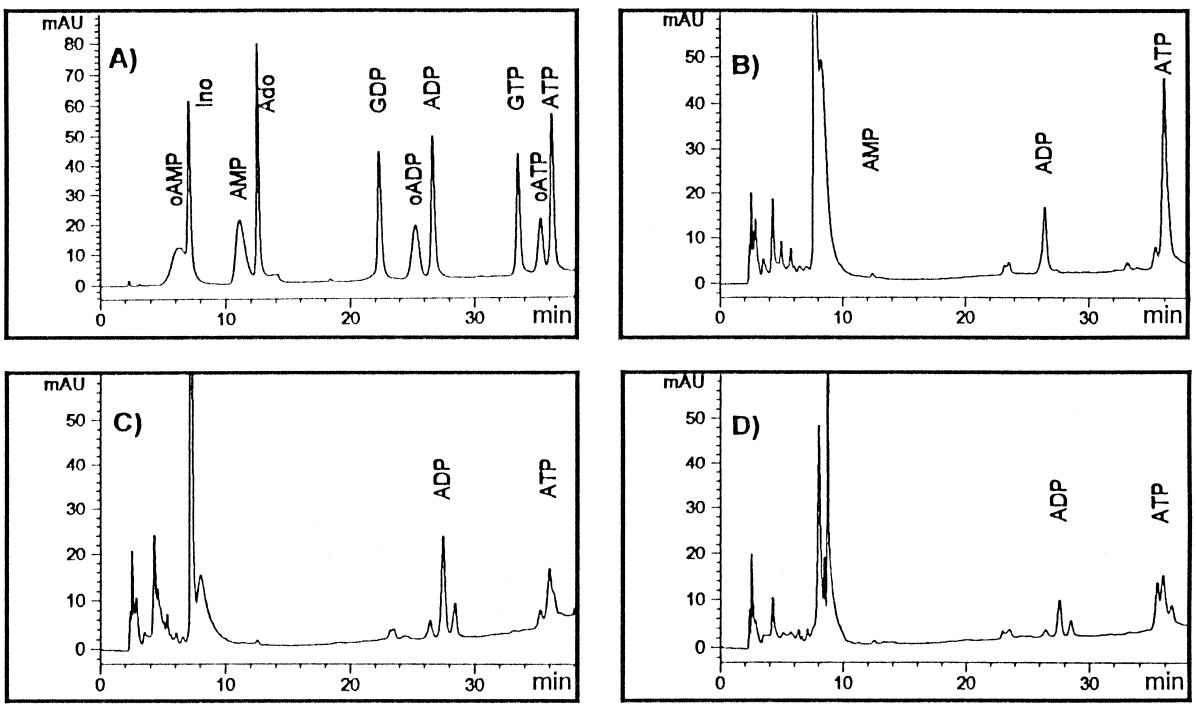

Fig. 7. Uptake studies with periodate-oxidized ATP. Mitochondria were incubated with 0.5 mM oATP in the standard incubation medium. HPLC chromatograms of purine derivatives standards (A) and of neutralized perchloric acids extracts of mitochondria without incubation (B) and after incubation without (C) and with 0.5 mM oATP for 15 min (D). Symbols: Ino—inosine, Ado—adenosine, AMP—adenosine 5′-monophosphate, ADP—adenosine 5′-diphosphate, ATP— adenosine 5′-triphosphate, GDP—guanosine 5′-diphosphate, GTP—guanosine 5′-triphosphate.

derivatives used for the HPLC analysis. Rat liver mitochondria incubated with oATP did not contain oATP, oADP or oAMP. The content of adenine nucleotides was more decreased in mitochondria incubated with oATP than without oATP (Fig. 7).

4. Discussion

Periodate-oxidized ATP induces a rapid decrease in the adenine nucleotide content of isolated liver mitochondria. In principle, a decrease in the matrix adenine nucleotide content may be attributed to the intramitochondrial catabolism of purine nucleotides and the efflux of adenine nucleotides either after the opening of the PTP or via the $Mg^{2+}ATP/P_i$ carrier.

Recently, several reports described that rat liver mitochondria are equipped with enzymes catabolizing intramitochondrial adenine nucleotides [24–27]. The catabolism of matrix adenine nucleotides is favored under deenergized conditions and not influenced by carboxyatractyloside [25]. Since the oATP-induced adenine nucleotide efflux was investigated at energized conditions and an inhibition of the oATP effect by carboxyatractyloside was observed, we conclude that the catabolism of matrix adenine nucleotides is not involved.

Opening of the PTP can be ruled out as the mechanism for the oATP-induced decrease of matrix adenine nucleotides since Mg^{2+} was present in the net transport experiments. Mg^{2+} is a powerful inhibitor of the PTP opening [28–30] and also prevents any permeability increase in the presence of oATP and P_i if no Ca^{2+} is added.

Liver mitochondria [19] translocate $Mg^{2+}ATP$ from the cytosol to the matrix and vice versa. The transport of $Mg^{2+}ATP$ proceeds as an antiport with P_i. Micromolar Ca^{2+}-concentrations stimulate the transport rate. The $Mg^{2+}ATP/P_i$ carrier is involved in the regulation of the matrix adenine nucleotide content [31]. oATP strongly accelerates the adenine nucleotide net loss and diminishes the net accumulation of adenine nucleotides measured at conditions often applied to characterize the $Mg^{2+}ATP/P_i$ carrier [31]. The two effects of oATP on net transport are prevented in the presence of carboxyatractyloside. Since the $Mg^{2+}ATP/P_i$ carrier is insensitive to carboxyatractyloside [19] and carboxyatractyloside affects specifically the ADP/ATP carrier [22], we conclude that the effects of oATP on the adenine nucleotide content are not mediated by the Mg^{2+}ATP/Pi carrier. This conclusion is supported by the finding that ADP inhibits the oATP-induced adenine nucleotide efflux observed at conditions eliminating the activity of the $Mg^{2+}ATP/P_i$ carrier. ADP is a high affinity substrate for the ADP/ATP carrier [22] and a poor substrate for the $Mg^{2+}ATP/P_i$ carrier [31]. Therefore, the observed inhibition suggests a competition of ADP and oATP at the cytosolic adenine nucleotide binding site of the ADP/ATP carrier.

Summarizing the data, it is suggested that oATP decreases the matrix content of adenine nucleotides through an interaction with the ADP/ATP carrier. Since mitochondria incubated with oATP did not contain oATP, oAMP or oADP, the decrease in the matrix adenine nucleotide content cannot be due to a one-for-one exchange of oATP against intramitochondrial ADP or ATP. It is generally assumed that the dimeric ADP/ATP carrier possesses one adenine nucleotide binding site [22]. However, there are several results indicating at least two binding sites. Sequential models of the ADP/ATP carrier with simultaneous occupation by ATP and ADP of both an inner and outer nucleotide binding sites have been proposed [32–36]. Presumably, the reaction of oATP with the adenine nucleotide binding site facing the cytosolic side eliminates the one-for-one exchange mode normally inherent to the ADP/ATP carrier function. The effect of oATP on liver mitochondria is similar to that of P_i in heart mitochondria. Here, P_i induces a carboxyatractyloside-sensitive efflux of matrix adenine nucleotides which occurs via the ADP/ATP carrier [37].

Both oADP and oATP, in contrast to their parent compounds, stimulate the Ca^{2+}-efflux induced by P_i. The induction of the mitochondrial permeability transition by oADP and oATP could be brought about by two mechanisms, through a pronounced decrease in the intramitochondrial adenine nucleotide content and through a direct interaction with the ADP/ATP carrier. The first hypothesis is in accordance with results showing increased rates of swelling in mitochondria depleted of adenine nucleotides by pretreatment with pyrophos-phate [10]. oATP induces a rapid decrease of matrix adenine nucleotides within 5 min. In this

time range, both the adenine nucleotide content is reduced to about 15% of its initial level and the Ca^{2+}-efflux induced by P_i increases abruptly. The overall loss of adenine nucleotides diminishes the matrix content of ADP. Since intramitochondrial ADP is an effective inhibitor of the PTP opening [9,10,38,39] the loss of adenine nucleotides is suggested to stimulate the opening of the PTP. The second hypothesis is supported by the fact that the ADP/ATP carrier has been considered a likely candidate as the pore-forming protein [36,40]. Recently published data lead to the view that the conformational state of the ADP/ATP carrier influences the pore-forming protein [1,29,41]. The binding of carboxyatractyloside to the ADP/ATP carrier, occurring only from the cytosolic side and stabilizing the corresponding carrier orientation, termed c-conformation [36], induces the opening of the PTP [36,40,42]. As shown by HPLC analysis, oATP is not taken up by mitochondria. Therefore, we conclude that the periodate-oxidized analogues mediate their effects from the cytosolic side of the mitochondria. It has been shown that [^{3}H]oADP reacts with heart mitochondria. This reaction is partially inhibited by carboxyatractyloside and, therefore, attributed to the covalent modification of the ADP/ATP carrier [43]. Since oATP induces a carboxyatractyloside-sensitive loss of matrix adenine nucleotides by the interaction with the ADP/ATP carrier, the stabilization of the carrier in the c-conformation caused by the binding of oATP may also promote the pore-opening.

In summary, it is suggested that oADP and oATP stimulate the mitochondrial permeability transition by decreasing the intramitochondrial adenine nucleotide content, especially that of ADP, via the ADP/ATP carrier and by interacting with this carrier from the cytosolic side.

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