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In Vitro Effects of Thyroid Hormones on Ectonucleotidase Activities in Synaptosomes From Hippocampus of Rats

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SUMMARY

1. Studies have shown that adenosine transport and adenosine A1 receptors in rat brain are subjected to regulation by thyroid hormone levels. Since the ectonucleotidase pathway is an important source of adenosine extracellular, in the present study the in vitro action of T3 and T4 hormones on ectonucleotidase activities in hippocampal synaptosomes was evaluated.

2. T3 (Triiodo-l-thyronine) significantly inhibited, in an uncompetitive manner, the ATP and ADP hydrolysis promoted by ATP diphosphohydrolase activity in hippocampal synaptosomes of adult rats.

3. In contrast, T4 (Thyroxine) only inhibited ATP hydrolysis in an uncompetitive mechanism, at the concentrations tested (100–500 μM), but at the same time did not affect ADP hydrolysis.

4. In the present study, we also investigate the in vitro effect of T3 and T4 on 5′-nucleotidase activity. However, there are no changes in the activity of this enzyme in the presence of T3 and T4 in the hippocampal synaptosomes of rats.

5. These results suggest that thyroid hormones could be involved in the regulation of ectonucleotidase activities, such as ecto-ATP diphosphohydrolase and ecto-ATPase, possibly exerting a modulatory role in extracellular adenosine levels.

KEY WORDS: T3; T4; thyroid hormones; ectonucleotidases; ATP diphosphohydrolase; 5′-nucleotidase.

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INTRODUCTION

Thyroid hormones exert a crucial effect on the growth and development of the central and peripheral nervous systems of mammals that, in general, is retarded in neonatal thyroid deficiency and advanced in hyperthyroidism (Balazs *et al.*, 1975). The presence of nuclear thyroid hormone receptors in adult rat brain has been identified (Schwartz *et al.*, 1994). Furthermore, evidence has demonstrated selective uptake of $[^{125}I]$-T_3 and rapid conversion of Thyroxine (T4) to Triiodo-l-thyronine (T3) in synaptosomal fraction of adult rat brain (Dratman and Gordon, 1996). In addition, alterations in the response of specific brain proteins to thyroid hormones are well documented (Bernal and Nunez, 1995). It has been demonstrated, for example, that in peripheral sympathetic neurons, the catecholamine levels were decreased in the hypothyroid status (Slotkin and Slepetis, 1984).

It is now well established that adenosine is involved in the regulation of a variety of physiological processes in the central nervous system. It acts as an endogenous inhibitory neuromodulator via inhibition of the excitatory neurotransmitters (Dunwiddie and Masino, 2001; Phillips and Wu, 1981). The inhibitory actions of adenosine are mediated by A1 receptors at the presynaptic terminals (Brundege and Dunwiddie, 1997; Dragunow, 1988) and adenosine can be released directly through bidirectional adenosine transporters, or can originate from the extracellular catabolism of adenine nucleotides by the ectonucleotidase pathway (Cunha *et al.*, 1996). In addition, thyroid hormones may also modulate both adenosine transport and adenosine receptors of the A1 subtype, which are widely distributed throughout the central nervous system (Fideu *et al.*, 1994). Adenosine transport and adenosine A1 receptors have been demonstrated to be decreased in synaptosomal preparations from hypothyroid rats (Fideu *et al.*, 1994).

The ectonucleotidase pathway is composed of an ecto-ATPase (NTPDase 2; EC 3.6.1.15), an ecto-ATP diphosphohydrolase (NTPDase 1; ecto-apyrase; EC 3.6.1.5) and an ecto-5′-nucleotidase (lymphocyte surface protein; CD73; EC 3.1.3.5.) (Zimmermann, 1996). Ecto-ATPases are enzymes that hydrolyze preferentially ATP, whereas ecto-apyrase hydrolyzes ATP and ADP to AMP equally well. Both enzymes are expressed in the central nervous system (Kegel *et al.*, 1997). The ecto-5′-nucleotidase, which has also been described in the central nervous system (Bernstein *et al.*, 1978; Schoen and Kreutzberg, 1994), hydrolyzes AMP to adenosine. We have demonstrated that the excitatory neurotransmitter, ATP, is hydrolyzed to adenosine in the central and peripheral nervous system by an enzymatic chain that involves an ATP diphosphohydrolase (NTPDase1; CD39; ecto-apyrase; EC 3.6.1.5.) and a 5′-nucleotidase (Battastini *et al.*, 1991; Sarkis and Salto, 1991).

Since adenosine is a purinergic messenger that regulates many physiological processes, particularly in the brain, and evidence demonstrates that thyroid hormones can modulate adenosine receptors and transport, investigations regarding the effect of these hormones on the ectonucleotidase pathway, which promotes ATP, ADP, and AMP hydrolysis to adenosine in rat brain are necessary and important.

METHODS

Chemicals

T3 (Triiodo-l-thyronine), T4 (Thyroxine), nucleotides (ATP, ADP, AMP), HEPES, Trizma base, EDTA, and Percoll were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Percoll was routinely filtered through Millipore AP15 prefilters to remove aggregated, incompletely coated particles. All other reagents were of analytical grade.

Subcellular Fractionation

Adult male Wistar rats (aged 60–90 days, weighing 220–260 *g*) from the local breeding colony were used. Animals were kept in a 12-h light/dark cycle (lights on at 7:00 A.M.) at a temperature of $23 \pm 1°C$. Procedures for the care and use of animals were adopted according to the regulations published by the Brazilian Socitety for Neurosciences and Behaviour (SBNeC).

The rats were killed by decapitation and their hippocampus were removed and gently homogenized in 5 volumes of an ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA, and 5.0 mM HEPES; pH 7.5 with a motor-driven Teflon-glass homogenizer. The synaptosomes from hippocampus were isolated as described previously (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 mL of the crude mitochondrial fraction were mixed with 4.0 mL of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were then washed twice at 15, 000 *g* for 20 min with the same ice-cold medium to remove the contaminating Percoll. The synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/mL. The material was prepared fresh daily and maintained at 0–4°C throughout preparation.

Enzyme Assays

The reaction medium used to assay ATP and ADP hydrolysis was essentially as described previously (Battasttini *et al.*, 1991) and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris-HCl buffer, pH 8.0, in a final volume of 200 μL.

The reaction medium used to assay 5′-nucleotidase activity contained 10 mM $MgCl_2$, 0.1 mM Tris-HCl, pH 7.0, and 0.15 M sucrose in a final volume of 200 μL (Heymann *et al.*, 1984).

The concentrations of T3 and T4 used in the assay were in the range of 100–500 μM. The synaptosomal fraction (10–20 μg protein) plus T3 or T4 were added to the reaction mixture and preincubated for 30 min at 37°C. The reaction was initiated by the addition of ATP, ADP, or AMP to a final concentration of 1.0 mM and stopped by the addition of 200 μL 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100 μL samples were used for the assay of released inorganic phosphate (Pi) (Chan *et al.*, 1986). Incubation times and protein concentration

were chosen in order to ensure the linearity of the reaction. Controls with the enzyme preparation added after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in duplicate. Protein was measured by the Coomassie Blue method (Bradford, 1976), using bovine serum albumin as standard.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by the Duncan multiple range test, considering a level of significance of 5%.

RESULTS

The in vitro effect of the T3 and T4 thyroid hormones on ATP, ADP, and AMP hydrolysis was measured in synaptosomes from the hippocampus of adult rats. A significant inhibition of ATP and ADP hydrolysis (34 and 24%, respectively) in relation to control (no T3 added = 100% activity) was observed at a concentration of 100 μM T3. The inhibitory effect upon ADP hydrolysis was concentration-dependent, demonstrating a 45% inhibition in the presence of 500 μM T3. However, the effect of T3 on ATP hydrolysis (43% inhibition at 500 μM) was not concentration-dependent, since there was no difference in inhibition in the range 100–500 μM of T3 (Fig. 1(A)).

The effect of T4 on ATP and ADP hydrolysis in synaptosomes from hippocampus of adult rats was also evaluated. T4, at a concentration of 100 μM, significantly inhibited ATP hydrolysis (34% inhibition), but ADP hydrolysis was not altered in the presence of this hormone at the concentrations tested (100–500 μM) (Fig. 1(B)).

The kinetics of the interaction of T3 or T4 with ATP and ADP hydrolysis in synaptosomes from hippocampus was determined. The Lineweaver–Burk double reciprocal plot was analyzed over a range of ATP or ADP concentrations (0.1–0.25 mM) in the absence and presence of T3 (100 μM). The data indicate that the inhibition of ATP and ADP hydrolysis by this hormone is uncompetitive in synaptosomes from hippocampus (Fig. 2(A) and (B)).

Considering that T4 produced an inhibitory effect only on ATP hydrolysis, we also evaluated its kinetic interaction in synaptosomes from hippocampus. The Lineweaver–Burk double reciprocal plot was analyzed over a range of ATP concentrations (0.1–0.25 mM) in the absence and presence of T4 (100 μM). Similarly, the data indicate that T4 inhibited ATP hydrolysis in an uncompetitive form in synaptosomes from hippocampus of rats (Fig. 3).

In the present study, we also determined the effect of T3 and T4 on 5′-nucleotidase activity, as it has been proposed that this enzyme participates in the complete hydrolysis of ATP to adenosine in the synaptic cleft. We evaluated the effect of T3 and T4 on the 5′-nucleotidase of synaptosomes from hippocampus in the range of 100–500 μM. Thyroid hormones did not significantly change the 5′-nucleotidase activity at the concentrations tested when compared to the control enzyme activity (no T3 or T4 added) (data not shown).

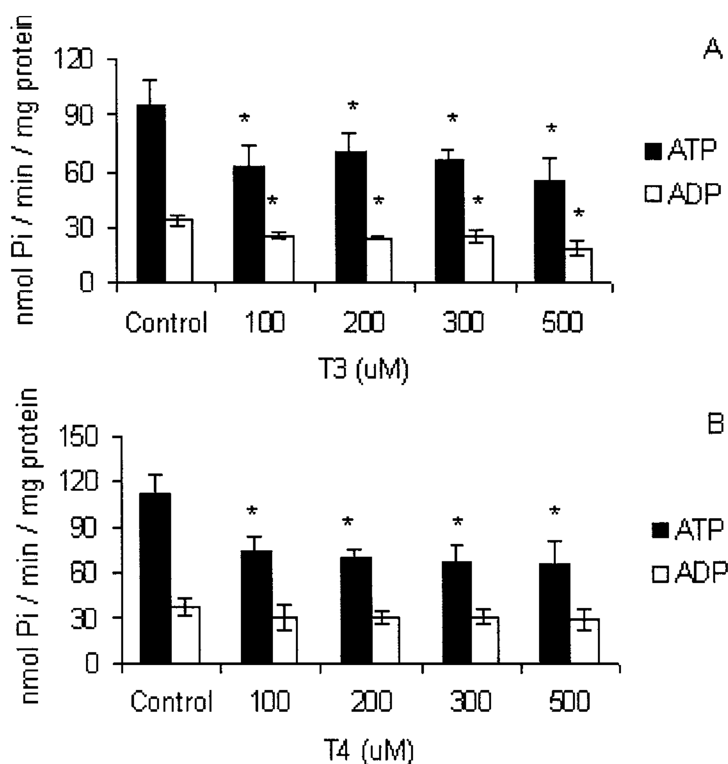

Fig. 1. Effects of T3 (A) and T4 (B) hormones on ATP and ADP hydrolysis promoted by ATP diphosphohydrolase in synaptosomes from hippocampus of rats. Bars represent mean ± SD of three experiments. $^{*}P < 0.05$.

DISCUSSION

The data reported here demonstrate the in vitro effect of the T3 and T4 thyroid hormones on the hydrolysis of the nucleotides ATP, ADP, and AMP in synaptosomes from hippocampus of adult rats, demonstrating that the thyroid hormone T3 causes a significant inhibition in ATP and ADP hydrolysis. In contrast, the T4 hormone only inhibited ATP hydrolysis. Both hormones inhibited the nucleotide hydrolysis, in the synaptosomes of rat hippocampus, in an uncompetitive manner. The present study also demonstrates that the hormones tested did not significantly change the 5′-nucleotidase activity in the same preparation.

In cultured neural cells, the T3 hormone increased the transport capacity and the number of adenosine transporters (Fideu and Miras-Portugal, 1992). A previous study demonstrated that hypothyroidism causes a decrease in adenosine kinase in fractions obtained from hippocampus, cerebelum, striatum, and hypothalamus (Mazurkiewicz and Saggerson, 1989). These results confirm the involvement of the thyroid hormones in the neuromodulation mediated by adenosine. Thus, the adenosine levels in the central nervous system may be modulated by the thyroid hormones. The inhibitory effects of T3 on ADP and ATP hydrolysis in synaptosomal preparation

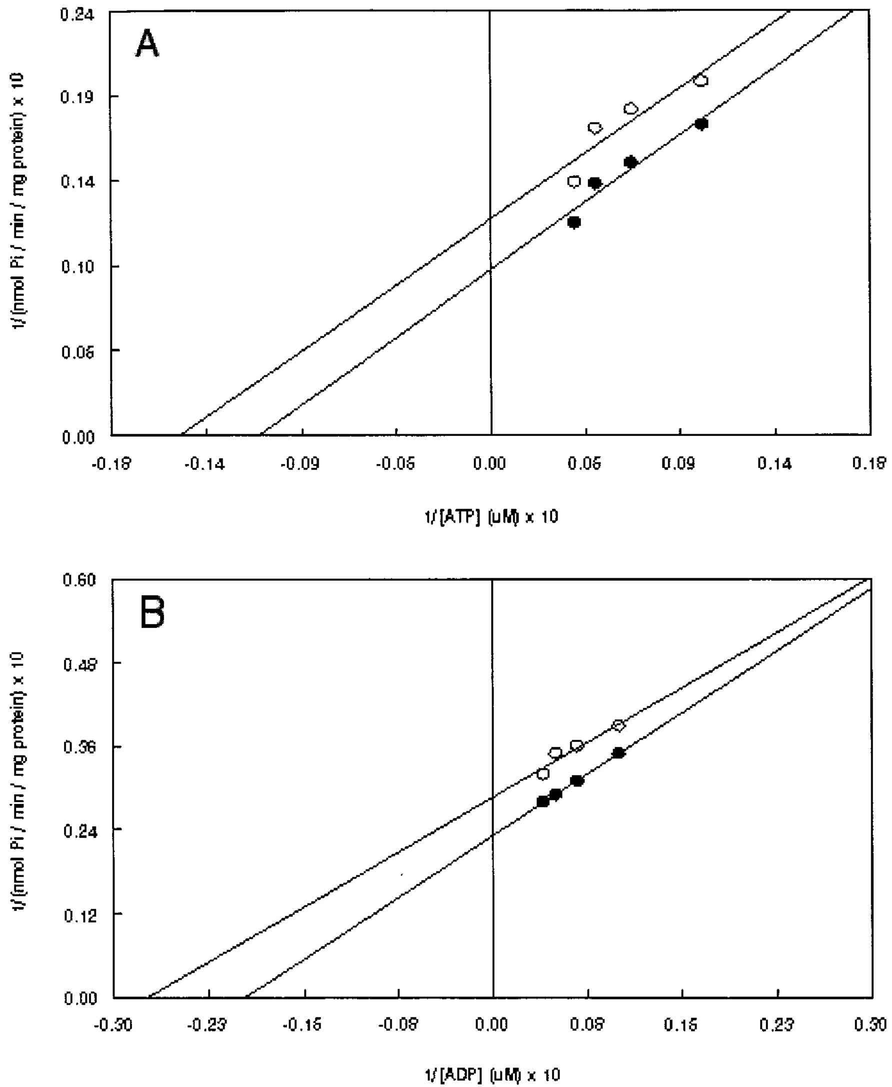

Fig. 2. Kinetic analysis of the inhibition of ATP diphosphohydrolase activity by T3 hormone in hippocampal synaptosomes of rats. The graphs show double-reciprocal plots of apyrase activity for ATP (A) and ADP (B) concentrations (0.1–0.25 mM) in the absence (●) and in the presence of 100 μM T3 (○). All experiments were repeated at least three times and similar results were obtained. Data presented are from individual experiments.

may be attributed to an effect of this hormone on the ATP diphosphohydrolase enzyme, since the inhibition occurred in a parallel manner with both nucleotides tested, as well in the kinetic interaction observed. The effect of T4 on ATP hydrolysis may be attributed to a mechanism involving an ecto-ATPase, since no significant effect was observed in ADP hydrolysis.

In addition, the decrease in ATP hydrolysis should be noted, since ATP is currently recognized as an excitatory neurotransmitter in the central nervous system

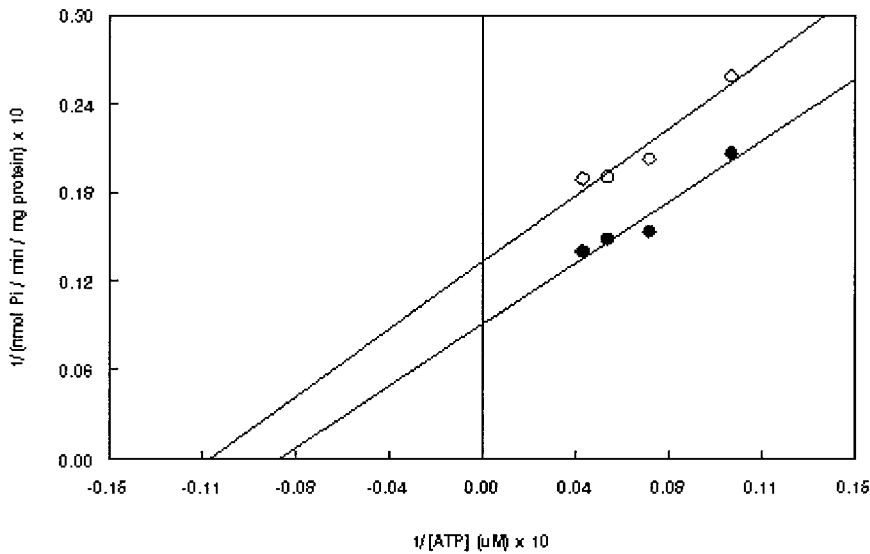

Fig. 3. Kinetic analysis of the inhibition of ATP diphosphohydrolase activity by T4 hormone in hippocampal synaptosomes of rats. The graphs show double-reciprocal plots of apyrase activity for ATP concentrations (0.1–0.25 mM) in the absence (●) and in the presence of 100 μM T3 (○). All experiments were repeated at least three times and similar results were obtained. Data presented are from individual experiments.

(Di Iorio *et al.*, 1998). Therefore, the enzymes that hydrolyze ATP in the brain have a significant role in modulating and controlling excitatory synaptic transmission.

The mechanisms by which these hormones may modulate the ecto-enzymes in the synaptosomal fraction will be studied in future investigations. Most thyroid hormone effects are exerted via a T3-receptor-mediated mechanism of action in the nuclear or mitochondrial compartment and involve modulation of transcription of nuclear or mitochondrial genes. However, a series of rapid thyroid hormone effects has also been demonstrated at the cell membrane level, in part mediated by plasma membrane-bound T3-receptors, ion channels or cytoskeleton-associated mechanisms or by ligand-dependent direct modulation of protein or enzyme function (Davis and Davis, 1996; Leonard and Farwell, 1997). Other membrane enzymes, such as synaptosomal Na^+, K^+-ATPase, have exhibited a dose-dependent inhibition by T3 (Sarkar and Ray, 1993).

Our findings demonstrate the involvement of the thyroid hormones in the purinergic system and further studies respect to the mechanisms of these hormones on the ecto-enzymes will be necessary.

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