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Toxicology in Vitro

Sertraline and clomipramine inhibit nucleotide catabolism in rat brain synaptosomes

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Abstract

The effects of sertraline, a selective serotonin reuptake inhibitor, and clomipramine, a tricyclic antidepressant, were tested on ecto-nucleotidases from synaptosomes of cerebral cortex and hippocampus of rats. Sertraline and clomipramine (100–500 μM) inhibited NTP-Dase, but not ecto-5′-nucleotidase activity in both cerebral cortex and hippocampus. In cortical synaptosomes, sertraline inhibited both ATP and ADP hydrolysis in the concentrations tested. The inhibitory effect varied from 21% to 83% for ATP hydrolysis and 48% to 75% for ADP hydrolysis. The inhibition promoted by sertraline in hippocampal synaptosomes varied from 38% to 89% for ATP hydrolysis and 45% to 77% for ADP hydrolysis. A significant inhibition of cortical NTPDase activity by clomipramine was observed in the all concentrations tested (35–72% and 36–87% for ATP and ADP hydrolysis, respectively). Similar effects were observed in hippocampus (29–91% and 48–83% for ATP and ADP hydrolysis, respectively). There was no inhibitory effect of sertraline and clomipramine on AMP hydrolysis in cerebral cortex and hippocampus. Our results have shown that classical antidepressants inhibit the extracellular catabolism of ATP. Therefore, it is possible to suggest that changes induced by antidepressants on bilayer membrane could affect NTPDase activities and consequently, modulating ATP and adenosine levels in the synaptic cleft.
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1. Introduction

Depressive disorders are chronic conditions that produce both emotional and physical symptoms (Delgado, 2004). Although the pathophysiology of depression is still unknown, there is significant evidence for abnormalities of the norepinephrine (NE) and serotonin (5-HT) neurotransmitter systems in depressive disorders (Fava, 2003). These neurotransmitters can influence the neuroplasticity in the brain and both are involved in mediating the therapeutic effects of most currently available antidepressants (Delgado, 2004).

Depression is one of the most disabling diseases, and causes a significant burden to both the individual and the society. If not treated, it could lead to increased morbidity and mortality (Sobocki et al., 2006). In the last five decades, the psychopharmacology of depression has evolved rapidly, with the development of new antidepressants, showing therapeutic efficacy (Galeotti et al., 2002; Trivedi et al., 2004). The main function of antidepressants is to increase the extracellular neurotransmitter concentrations, inhibiting the metabolism and reuptake. They can also act in synaptic receptors (BezchlibnyK-Butler and Virani, 2004). Because antidepressants have a lag time on their action, it

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is possible that inhibition of neurotransmitter reuptake is not sufficient to explain long-term changes. These antidepressants include monoamine oxidase inhibitors, tricyclic compounds, selective 5-HT and NE reuptake inhibitors, as well as, some atypical drugs (Galeotti et al., 2002)

Clomipramine is a tricyclic antidepressant that acts with a dual role, inhibiting both norepinephrine and serotonin reuptake (Bert et al., 2006). In contrast, sertraline is a selective serotonin reuptake inhibitor (Bailly, 2006). Besides the main action on catecholamines, studies report other effects for these drugs, such as an increase of PKC activity and modulation of the binding properties of cortical NMDA and beta1-adrenergic receptors in an animal model of depression (Harkin et al., 2000; Giambalvo and Price, 2003). Catecholamines can be co-released with ATP, which is considered a neurotransmitter and neuromodulator in central nervous system (CNS) (Burnstock, 2004). Extracellular ATP evokes responses by two subclasses of P2 purinoreceptors, P2X and P2Y (Ralevic and Burnstock, 1998). It has been shown that P2X receptors are coupled to ligand-gated Ca^{2+}-permeable channels, whereas the P2Y receptors have been considered a G-protein-linked (Burnstock, 2004). The signaling actions induced by extracellular ATP are directly correlated to the activity of ecto-nucleotidases since these enzymes trigger enzymatic conversion of ATP to adenosine (Zimmermann, 2001; Robson et al., 2006). Ecto-nucleotidases comprise a group of ecto-enzymes involved in the control of nucleotide and nucleoside levels in the synaptic cleft, which includes NTPDase (nucleoside triphosphate diphosphohydrolase) family and ecto-5′-nucleotidase (Zimmermann, 2001). Four members of the NTPDase family are tightly bound to the plasma membrane via two transmembrane domains, and have a large extracellular region with an active site facing the extracellular side. NTPDase1, 3 and 8 slightly prefer ATP over ADP by a ratio of 1, 3 and 2, respectively. Meanwhile, NTPDase2 prefers triphosphonucleosides (Bigonnesse et al., 2004; Robson et al., 2006).

Adenosine, a product of ATP catabolism, can evoke its neuromodulatory effects by four subtypes of P_1-purinoreceptors named A_1, A_{2A}, A_{2B} and A_3 (Brundege and Dunwiddie, 1997; Cunha, 2001; Dunwiddie and Masino, 2001). Studies have shown that adenosine administration produces an antidepressant-like effect in the forced swimming test (FST) and in the tail suspension test through an interaction with A_1 and A_{2A} receptors (Kaster et al., 2004). Moreover, it has been shown that hippocampal serotonergic neurotransmission is modulated by hippocampal adenosine receptor subtypes (Okada et al., 1999).

Considering that (i) the adenosine is able to modulate 5-HT release and (ii) ecto-nucleotidases represent one of the most important sources of extracellular adenosine, the aim of this study was to evaluate the effect in vitro of sertraline and clomipramine on the ecto-nucleotidases in synaptosomes from cerebral cortex and hippocampus of rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (age 70–90 days; 220–280 g) from our breeding stock were housed four to a cage, with food and water ad libitum. The animal house temperature was kept between 22 °C and 23 °C with a 12 h light/dark cycle (lights on at 07:00). Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee (CEP 06/03016) of the Pontifícia Universidade Católica do Rio Grande do Sul, Brazil.

2.2. Chemicals

Sertraline, clomipramine, Percoll, Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All reagents used were of analytical grade.

2.3. Synaptosomal preparation

The rats were killed by decapitation, and their cerebral cortex and hippocampus were dissected, homogenized in 10 and 5 volumes, respectively, in an ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.5. The synaptosomes were isolated as described previously (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 mL of the crude mitochondrial fraction was 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 washed twice at 15 000g for 20 min with the same ice-cold medium to remove the contaminating Percoll and 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.

2.4. In vitro treatments

Antidepressants, sertraline and clomipramine, were added to reaction medium before the preincubation with the synaptosomes and maintained throughout the enzyme assays. Antidepressants were tested at final concentrations of 100, 250 and 500 µM.

2.5. Determination of ecto-nucleotidase activities

The reaction medium used to assay ATP and ADP hydrolysis was essentially as described previously (Battastini 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 synaptosomal fraction (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was initiated by the addition of 1 mM ATP or ADP as substrate 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 taken to asses the released inorganic phosphate (Pi) (Chan et al., 1986).

The reaction medium used to assay 5′-nucleotidase activity contained 10 mM MgCl$_2$, 0.1 M Tris–HCl, pH 7.5 and 0.15 M sucrose to final volume of 200 μl (Heymann et al., 1984). The synaptosomal fraction (10–20 μg protein) was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of 1.0 mM AMP as substrate 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 taken to assess the released inorganic phosphate (Pi) (Chan et al., 1986).

In enzyme assays, incubation time and protein concentration were chosen in order to ensure the linearity of the reaction. Controls, with the addition of the enzyme preparation after the addition of trichloroacetic acid, were used to correct non-enzymatic hydrolysis of the substrates. All samples were run in triplicate.

2.6. Protein determination

Protein was measured by the Coomassie Blue method (Bradford, 1976), using bovine serum albumin as a standard.

2.7. Statistical analysis

Data were expressed as means ± SD and analyzed by one-way analysis of variance (ANOVA). A Tukey multiple range test considering $P \leqslant 0.05$ as significant followed the analysis.

3. Results

We evaluated the effect *in vitro* of antidepressant drugs on ATP, ADP and AMP hydrolysis from synaptosomes of cerebral cortex and hippocampus of rats. NTPDase activities were sensitive to antidepressants, clomipramine and sertraline, in cortical and hippocampal brain synaptosomes. In contrast, the ecto-5′-nucleotidase activity demonstrated no sensitivity to the concentrations tested.

In cortical synaptosomes, sertraline inhibited both ATP-ase and ADPase activities in all concentrations tested (100–500 μM). The inhibitory effect varied from 21% to 83% in ATP hydrolysis and from 48% to 75% for ADP hydrolysis (Fig. 1a). The inhibition promoted by sertraline in hippocampal synaptosomes varied from 38% to 89% for ATP hydrolysis and from 45% to 77% for ADP hydrolysis (Fig. 2a). No inhibitory effect of sertaline was observed in AMP hydrolysis in both hippocampus and cerebral cortex (Figs. 1b and 2b).
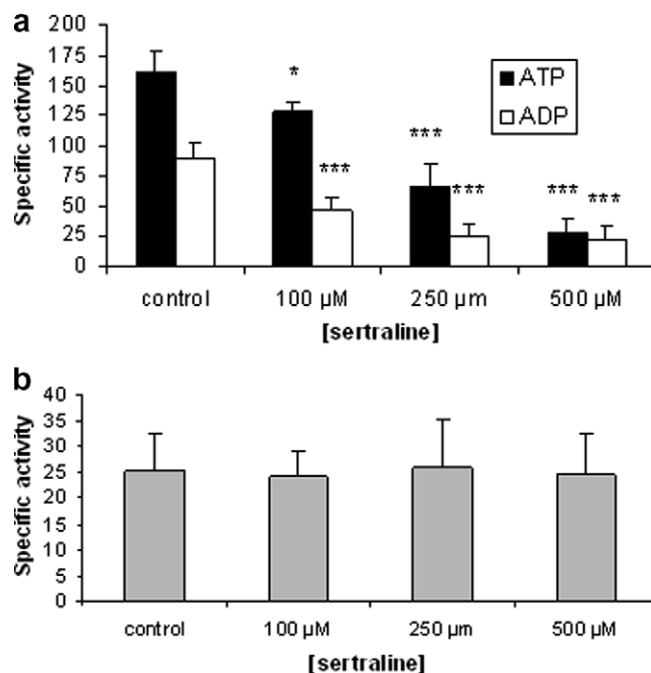

Fig. 1. *In vitro* effect of sertraline on cortical NTPDase (a) and ecto-5′nucleotidase (b) activity in rat brain. Bars represent the mean ± SD of three or more different experiments. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test (*$p \leqslant 0.05$, **$p \leqslant 0.01$, ***$p \leqslant 0.001$ when compared to the control).

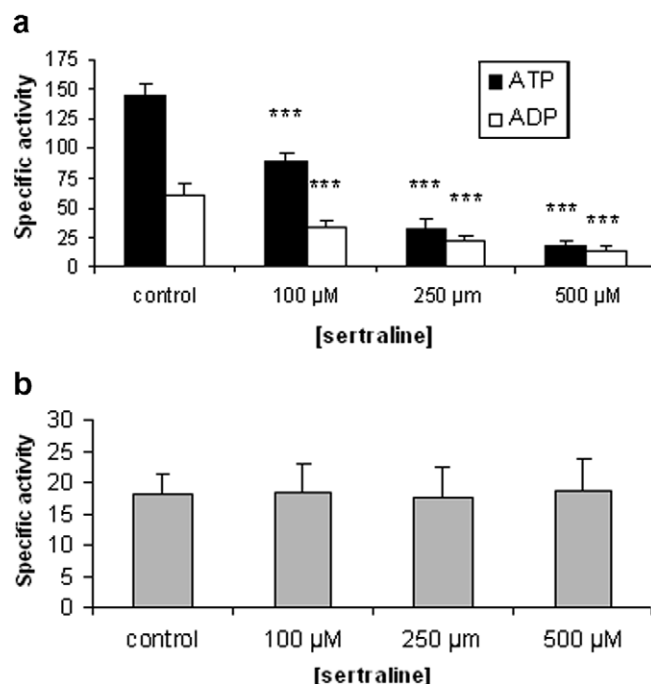Fig. 2. *In vitro* effect of sertraline on hippocampal NTPDase (a) and ecto-5′nucleotidase (b) activity in rat brain. Bars represent the mean ± SD of three or more different experiments. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test (*$p \leqslant 0.05$, **$p \leqslant 0.01$, ***$p \leqslant 0.001$ when compared to the control).

The effect of clomipramine on nucleotide hydrolysis was also tested in cortical and hippocampal synaptosomes. A
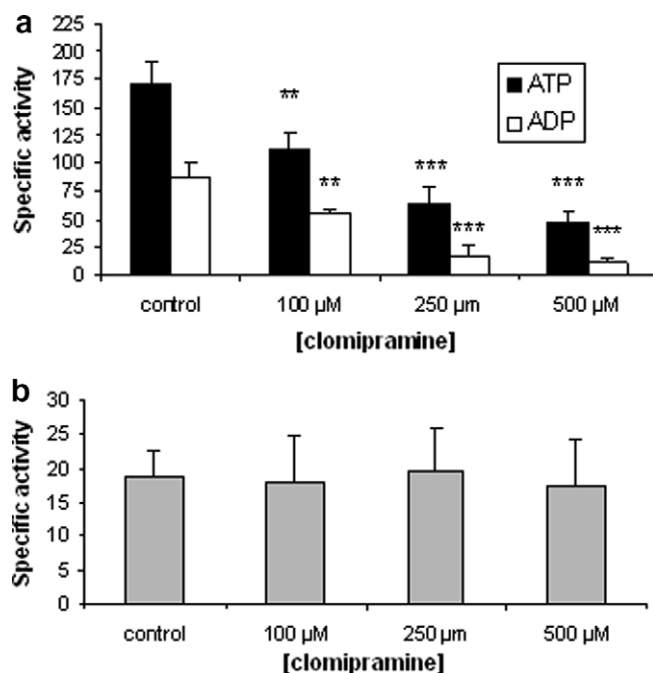
Fig. 3. *In vitro* effect of clomipramine on cortical NTPDase (a) and ecto-5′nucleotidase (b) activity in rat brain. Bars represent the mean ± SD of three or more different experiments. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($^*p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$ when compared to the control).

Fig. 4. *In vitro* effect of clomipramine on hippocampal NTPDase (a) and ecto-5′nucleotidase (b) activity in rat brain. Bars represent the mean ± SD of three or more different experiments. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ($^*p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$ when compared to the control).

significant inhibition of cortical NTPDase activity was observed in the all concentrations tested (35–72% for ATP hydrolysis and 36–87% for ADP hydrolysis) (Fig. 3a). A similar decrease in ATP (29–91%) and ADP hydrolysis (48–83%) was observed in hippocampal synaptosomes (Fig. 4a). Clomipramine failed to inhibit AMP hydrolysis in both brain regions tested (Figs. 3b and 4b).

4. Discussion

The results of the present study demonstrated that NTP-Dase, but not ecto-5′-nucleotidase, activities from cerebral cortex and hippocampus are decreased by the antidepressants sertraline and clomipramine.

Different studies have demonstrated the effect of antidepressant drugs in ATPase activities such as Mg^{2+}-ATPase (Nag and Ghosh, 1973), F_0F_1-ATPase (Souza et al., 1994), and Na^+, K^+-ATPase (Zanatta et al., 2001). Sanganahalli et al. (2000) have demonstrated that tricyclic antidepressant (imipramine, desipramine, amitriptyline, and nortriptyline) inhibited Na^+, K^+-ATPase activity in synaptosomal membrane of rat brain. Furthermore, it has been shown that sertraline and clomipramine inhibit the 5-hydroxytryptamine transporter in primary cultures of rat and mouse cortical astrocytes (Bal et al., 1997). Studies have observed that the total uptake and lysosomal trapping of the antidepressants such as imipramine, amitriptyline, fluoxetine and sertraline were higher in the grey matter and neurones than in the white matter and astrocytes, respectively (Daniel et al.,
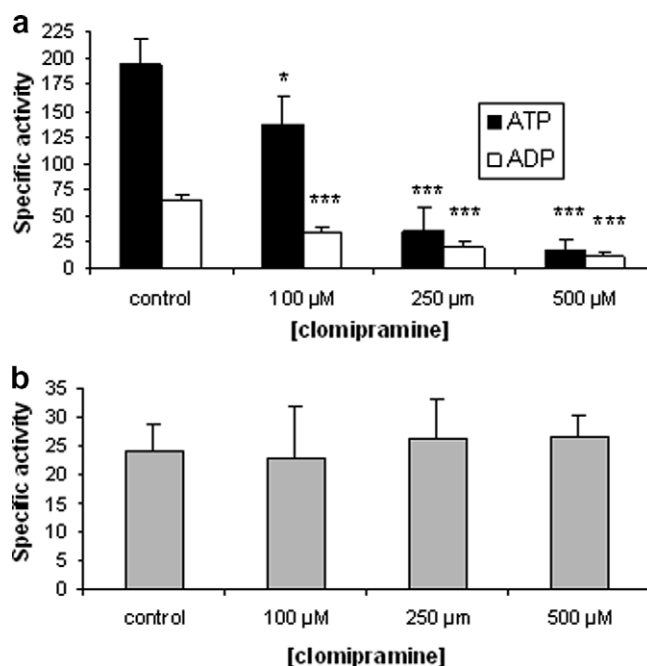
2001). There is evidence that tricyclic drugs alter the structural organization of the lipid membranes. Furthermore, previous studies have shown that fluoxetine and imipramine can alter Na^+, K^+-ATPase activity due to the hydrophobicity of these drugs (Zanatta et al., 2001). Drug interaction with the biomembrane influences the bilayer structure, consequently, modulating processes which range from membrane-bound enzyme activity and receptor binding to membrane permeability and transport (Carfagna and Muhoberac, 1993). Barcellos et al. (1998) have demonstrated that imipramine and desipramine (antidepressants tryciclic) *in vitro* decreased ATP and ADP hydrolysis in synaptosomes from cerebral cortex of rats. The partitioning of the drugs into lipid bilayer affects the membrane fluidity consequently changing the membrane protein function and structure. NTPDases1, 2, 3 and 8 are firmly anchored to the membrane via two transmembrane domains that in the instance of NTPDase1 are important for maintaining catalytic activity and substrate specificity (Grinthal and Guidotti, 2006). Papanikolaou et al. (2005) have shown that removing cholesterol from membranes of NTPDase1-expressing cells reduces ATPase activity to the same extent as solubilization does. However, these authors have suggested that some aspects of cholesterol other than its effect on membrane fluidity is required for native transmembrane helix properties of the enzyme. In addition, several perturbing methods may change common mechanical feature other than fluidity. Lundbaek et al. (2004) have identified bilayer elasticity as a membrane property that is altered by a vari-

ety of structurally unrelated compounds. Changes in elasticity modify the energy required for local membrane deformation associated with a protein conformational change, modifying the total energy barrier between different transmembrane domain conformations. If the balance between stability and mobility is a key feature of the interplay between the transmembrane domains and the active site of NTPDase1, anything that changes bilayer elasticity might change NTPDase activity by altering this balance (Grinthal and Guidotti, 2006). Thus, changes in membrane bilayer environment promoted by the interaction with clomipramine and sertraline may be able to promote the inhibitory effect observed on NTPDase activity. In contrast, ecto-5′-nucleotidase was not altered by clomipramine and sertraline in the doses tested. Ecto-5′-nucleotidase is attached via a GPI (glycosylphosphatidylinositol) anchor to the extracellular membrane (Sträter, 2006). The different effects promoted by antidepressant drugs on NTPDase and ecto-5′-nucleotidase activities can be related to the differences in membrane anchorage of these enzymes.

Adenosine fulfills a double role (Cunha, 2001), acting both as a homeostatic transcellular messenger and as a neuromodulator, controlling neurotransmitter release and neuronal excitability (Fredholm et al., 2005). Studies have shown that the stimulatory effects of A_2 receptor and inhibitory effects of A_3 receptor on hippocampal extracellular 5-HT levels are masked or abolished by the inhibitory effects of A_1 receptor (Okada et al., 1999). The effects of four tricyclic antidepressants, nortriptyline, iprindole, clomipramine and desipramine on adenosine-evoked depressions of the firings of rat cerebral cortical neurones have been studied. Tricyclic antidepressants are potent inhibitors of neuronal uptake of adenosine, which may raise the endogenous adenosine levels (Phillis and Wu, 1982; Phillis, 1984). Moreover, clomipramine and desipramine elicited depressions, which were antagonized by caffeine, an adenosine antagonist (Phillis, 1984). Therefore, it is possible to suggest that changes induced by antidepressants on bilayer membrane could affect NTPDase activities, and consequently, could modulate ATP and adenosine levels in the synaptic cleft. Further studies are required to verify the *in vivo* effect of these drugs on ecto-nucleotidases activities.

In summary, we have shown that clomipramine and sertraline can modulate the ecto-nucleotidase pathway, an important source of extracellular adenosine. These results pointed out for another pharmacological mechanism of these drugs, which can influence their final effects.

Acknowledgements

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