Carbofuran and malathion inhibit nucleotide hydrolysis in zebrafish (*Danio rerio*) brain membranes

Mario Roberto Senger [a,b], Eduardo Pacheco Rico [a,b],
Marcelo de Bem Arizi [a], Denis Broock Rosemberg [a], Renato Dutra Dias [a],
Maurício Reis Bogo [a], Carla Denise Bonan [a,*]

[a] *Laboratório de Pesquisa Bioquímica, Departamento de Ciências Fisiológicas, Faculdade de Biociências,
Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil*
[b] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande
do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil*

Abstract

Carbofuran and malathion are broad spectrum pesticides widely used in agricultural practice throughout the world. Toxicity of these pesticides has been correlated with their inhibitory effects on acetylcholinesterase activity. Nucleotides are extracellular signaling molecules, which trigger multiple biological effects. Studies have demonstrated the co-transmission of acetylcholine and ATP at the nerve endings. The control of neurotransmitter ATP levels is promoted by enzymes named ectonucleotidases, which include nucleoside triphosphate diphosphohydrolase (NTPDase) family and ecto-5′-nucleotidase. Since acetylcholine and ATP are co-released at the synapse and the acetylcholinesterase inhibition is an important target for pesticide action, here we verified the effect of exposure in vitro and in vivo to carbofuran and malathion on ectonucleotidase activities from brain membranes of zebrafish. To verify if carbofuran and malathion have a direct inhibitory effect on NTPDase and 5′-nucleotidase activities in brain membranes of zebrafish, we have tested in vitro concentrations of pesticides varying from 0.25 to 5 mM. Carbofuran, in vitro, inhibited ATP and ADP hydrolysis in an uncompetitive manner, but no effect was observed on AMP hydrolysis. Malathion decreased ATP and ADP hydrolysis in competitive and an uncompetitive manner, respectively, but not altered AMP hydrolysis. After exposure to carbofuran (50 and 500 μg/L) during 7 days, ADP hydrolysis was significantly decreased in both concentrations tested (by 19 and 24.5%, respectively). Malathion, at 500 μg/L, was able to inhibit ADP and AMP hydrolysis (by 28 and 58.5%, respectively). This study has shown that ectonucleotidases from brain membranes of zebrafish can be a potential target for pesticide neurotoxicity.
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* Corresponding author. Tel.: +55 51 3320 3500x4158; fax: +55 51 3320 3612.
  E-mail address: bonan@portoweb.com.br (C.D. Bonan).

1. Introduction

Pesticides are chemicals intentionally introduced to the environment and have become necessary to ensure good harvests in modern agriculture. Overspray and/or runoff of pesticides from agricultural fields may easily contaminate bodies of water, resulting in serious damage to non-target species, including fish. Carbofuran and malathion are broad spectrum carbamate (CB) and organophosphate (OP) pesticides, respectively, that are widely used in agricultural practice throughout the world (Bondarenko et al., 2004). As for other carbamates and organophosphate pesticides, toxicity of carbofuran and malathion has been correlated with its inhibitory effect on acetylcholinesterase (AChE) activity at central cholinergic and at neuromuscular junctions (Ansari and Kumar, 1984; Gupta, 1994; Bretaud et al., 2000). Due to inhibition of AChE, the neurotransmitter acetylcholine (ACh) is less hydrolyzed in synapses, causing abnormal amount of acetylcholine, which leads to overactivation of cholinergic receptors, causing possible toxic effects (Walker, 2001). The inhibition of AChE has been used as an indicator of exposure to CB and OP pesticides in non-target species (Dembele et al., 2000; Roex et al., 2003). However, other effects remain to be considered to completely estimate the neurotoxicity of these contaminants.

Nucleotides are ubiquitous extracellular signaling molecules, which induce a wide spectrum of biological effects (Burnstock, 1997; Ralevic and Burnstock, 1998; Lazarowski et al., 2003). Extracellular ATP can play a pivotal role in synaptic transmission, acting as a neurotransmitter and/or a neuromodulator (Cunha and Ribeiro, 2000; Burnstock, 2000). Biological responses to extracellular ATP are largely mediated by binding to either G protein-coupled P2Y or ligand-gated P2X receptors (Ralevic and Burnstock, 1998; Communi et al., 2000; Khakh, 2001). There are important mechanisms involved in the control of ligand concentrations, and hence regulate the activation of purinoceptors. Ectonucleotidases constitute a highly refined system for the regulation of nucleotide-mediated signaling, controlling the rate, amount and timing of nucleotide degradation and formation. The hydrolysis of ATP to AMP is catalyzed mainly by a family of ectonucleotidases, named nucleoside triphosphate diphosphohydrolase (NTPDases). The nucleotide AMP is hydrolyzed to adenosine, an important neuromodulator, by the action of an ecto-5'-nucleotidase (Zimmermann, 1992, 1996, 2001). The neuromodulator adenosine exerts its effect through activation of G protein-coupled receptors, named A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 2001).

Burnstock (2004) showed the co-transmission of acetylcholine and ATP at the nerve endings. There is evidence that adenosine can modulate the acetylcholine release (Ribeiro et al., 1996), and the activation of presynaptic nicotinic receptors elicits ATP release from postganglionic sympathetic axons (Kügelgen, 1996). Extracellular ATP is metabolized by a cascade of ectonucleotidases producing adenosine, which it is able to modulate acethylcholine release through inhibitory A_1 or facilitatory A_{2A} receptors (Rebola et al., 2002; Magalhães-Cardoso et al., 2003).

Zebrafish (*Danio rerio*) has many characteristics that make it a useful vertebrate model to study toxicology (Lele and Krone, 1996; Linney et al., 2004). Furthermore, ionotropic P2X and metabotropic P2Y receptors have already been identified in this specie (Kucenas et al., 2003; Schulz and Schöneberg, 2003). Recently, we characterized the presence of an NTPDase and an ecto-5'-nucleotidase activity in brain membranes of zebrafish (Rico et al., 2003; Senger et al., 2004).

Considering that the neurotransmitters acetylcholine and ATP are co-released at the synapse cleft and the inhibition of acetylcholinesterase is the main target for pesticide action, here we verified the effect of exposure in vivo and in vitro to carbofuran and malathion on ectonucleotidase activities from brain membranes of zebrafish.

2. Material and methods

2.1. Animals

Zebrafish were obtained from commercial suppliers and acclimatized for 2 weeks in a 50 L aquarium containing continuously aerated distilled water. The fish of both sexes were kept at $25 \pm 2\,^{\circ}\text{C}$ under a natural light–dark photoperiod. Feeding and maintenance of fish were done according to Westerfield (2000). Procedures for the use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA).

2.2. Chemicals

Carbofuran (95.8%; 2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate, CAS Number 1563-66-2) and malathion (95%; [(dimethoxyphosphinothioyl)thio] butanedioic acid diethyl ester, CAS Number 121755) were kindly donated by FMC Química do Brasil LTDA and Indol do Brasil. Trizma Base, ammonium molybdate, polyvinil alcohol, malachite green, nucleotides, EDTA, EGTA, dimethyl sulphoxide (DMSO), sodium citrate, coomassie blue G, bovine serum albumin, calcium and magnesium chloride were purchased from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.3. Treatments in vitro and in vivo

For the in vitro experiments, carbofuran or malathion were diluted in 70% DMSO solution and used for enzyme assays at the final concentrations of 0.25–5 mM. Selected concentrations of each compound were added to reaction medium before preincubation with the enzyme and maintained throughout the enzyme assay.

For the in vivo treatments, zebrafish were introduced to the test aquarium 10 min after the addition of solutions containing carbofuran or malathion diluted in a 70% DMSO. The final concentration of DMSO in the aquarium was 0.00175%. The animals were maintained in the aquarium test for 7 days in the concentration of 50 or 500 μg/L carbofuran or malathion.

2.4. Membrane preparation

Brain membranes were prepared according Barnes et al., (1993). Whole zebrafish brains were homogenized in 60 volumes (v/w) of chilled Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a glass-Teflon homogenizer. The homogenate was centrifuged at $1000 \times g$ for 10 min and the pellet was discarded. After removing the nuclear and cell debris, the supernatant was centrifuged for 25 min at $40,000 \times g$. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris–citrate buffer, and recentrifuged for 20 min at $40,000 \times g$. The final pellet was resuspended and used in the enzyme assays. The material was maintained at 2–4 °C throughout preparation.

2.5. NTPDase and 5′-nucleotidase assays

NTPDase and 5′-nucleotidase assays were performed according Rico et al., 2003 and Senger et al., 2004, respectively. Brain membranes of zebrafish (3–5 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the ecto-5′-nucleotidase activity) in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C. The reaction was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM and stopped by the addition of 200 μL 10% trichloroacetic acid. The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (P_i) (Chan et al., 1986). Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity is expressed as nanomole of P_i released per minute per milligram of protein. All enzyme assays were run at least 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 ± S.D. and analyzed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P < 0.05$ as significant.

3. Results

To evaluate a possible direct effect of carbofuran and malathion on NTPDase and 5′-nucleotidase activities we have tested in vitro concentrations varying from 0.25 to 5 mM of each pesticide. In previous experiments, we have tested five different concentrations of DMSO, which were present in the reaction medium for each concentration of the pesticide tested:

0.7% (0.25 mM); 1.4% (0.5 mM); 2.8% (1 mM); 8.4 % (3 mM) and 14.0% (5 mM). There are no significant difference on ATP, ADP and AMP hydrolysis in the presence of DMSO at the different concentrations tested (data not shown). Therefore, controls adding DMSO in a final concentration of 14% (used to achieve the most concentrated solution of pesticides tested in vitro) were used for the subsequent experiments and did not alter significantly the ectonucleotidase activities.

Carbofuran, presented a significant effect on ectonucleotidases in a milimolar range, inhibiting in vitro ATP hydrolysis in the concentration ranged from 1 to 5 mM (28–59%), but ADP hydrolysis was only inhibited at 3 and 5 mM carbofuran (35 and 45%, respectively (Fig. 1A)). There were no significant changes on AMP hydrolysis in the presence of carbofuran in all concentrations tested (Fig. 1B). However, malathion was able to alter ectonucleotidase activities from 0.25 to 5 mM range, inhibiting in vitro both ATP (24–48%, respectively) and ADP hydrolysis (31–59%, respectively) (Fig. 2A). There were no significant changes on AMP hydrolysis in all concentrations tested (Fig. 2B).
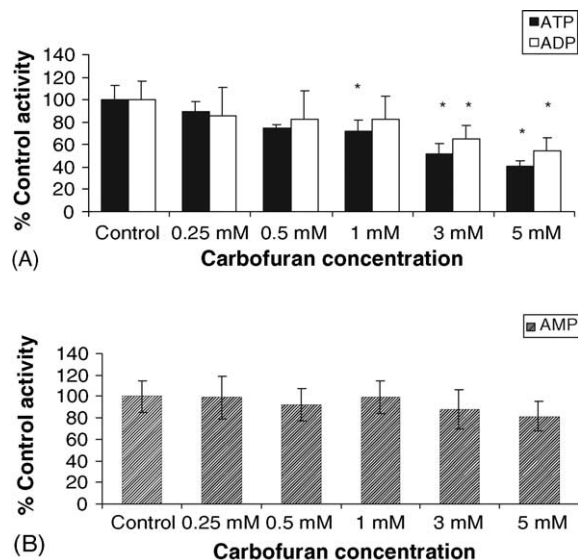

Fig. 1. In vitro effect of varying concentrations of carbofuran on NTPDase (A) and ecto-5′-nucleotidase (B) in zebrafish brain membranes. Bars represent the means ± S.D. of at least four different experiments. The control ATPase, ADPase and AMPase activities (no carbofuran added) were 668.3 ± 84.7, 141 ± 23.2 and 29 ± 4.6 nmol P_i min^{-1} mg^{-1} of protein, respectively. Asterisk (*) indicates significantly different from control group ($P \leq 0.05$).
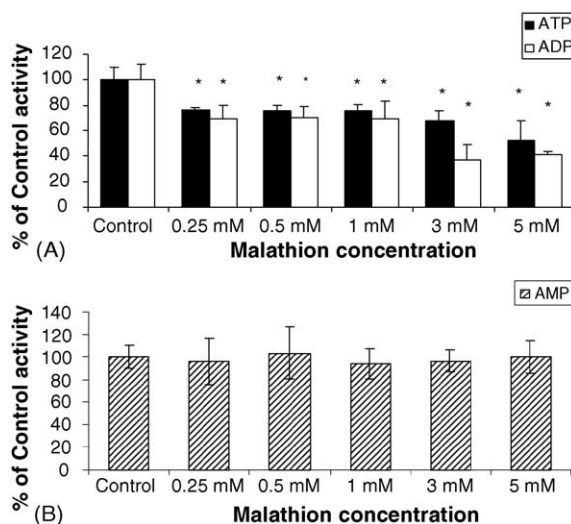

Fig. 2. In vitro effect of varying concentrations of malathion on NTPDase (A) and ecto-5′-nucleotidase (B) in zebrafish brain membranes. Bars represent the means ± S.D. of at least four different experiments. The control ATPase, ADPase and AMPase activities (no malathion added) were 622.23 ± 58.8, 143.3 ± 17.8 and 22 ± 2.24 nmol P_i min^{-1} mg^{-1} of protein, respectively. Asterisk (*) indicates significantly different from control group ($P \leq 0.05$).

The kinetics of the interaction of carbofuran and malathion with NTPDase activity in zebrafish brain membranes were also determined (Figs. 3 and 4). Lineaweaver–Burk double reciprocal plots were analyzed over the range of ATP and ADP concentrations as substrates (0.1–0.250 mM) in the absence and in the presence of carbofuran (Fig. 3) and malathion (Fig. 4). The data indicated that the inhibition of ATP (Fig. 3A) and ADP (Fig. 3B) hydrolysis by carbofuran was uncompetitive because the K_M and V_{max} values decreased with increasing carbofuran concentrations. The inhibition promoted by malathion on ATP hydrolysis seems to be competitive because the K_M value increased and the V_{max} did not change significantly (Fig. 4A). Malathion inhibited ADP hydrolysis in an uncompetitive manner (Fig. 4B).

The direct inhibitory effect of carbofuran and malathion on ectonucleotidase was only observed at extremely high concentrations, which are not expected in the environment. However, several studies have showed that CB and OP compounds may affect second messenger systems, and consequently transduction signal pathways, which could exert a modulatory effect on
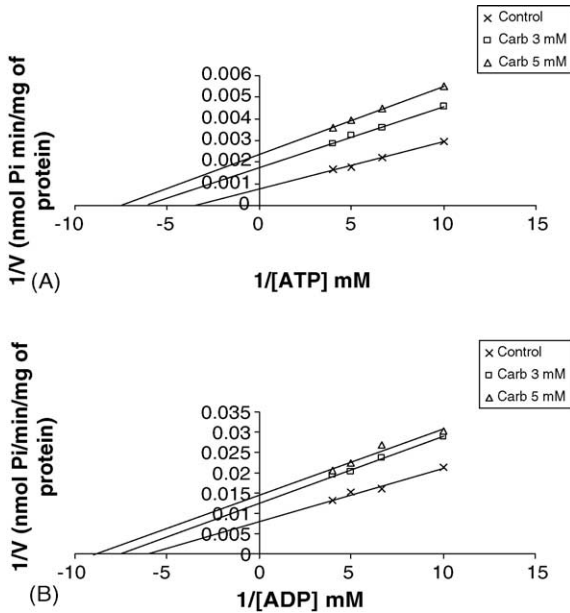
Fig. 3. Kinetic analysis of the inhibition of NTPDase by carbofuran in zebrafish brain membranes. The graphs show double-reciprocal plots of NTPDase activity for ATP (A) and ADP (B) concentrations (0.1–0.25 mM) in the absence (×) and in the presence of 3 mM (□) and 5 mM of carbofuran (△). All experiments were repeated at least 5 times and similar results were obtained. Data represents an individual experiment.
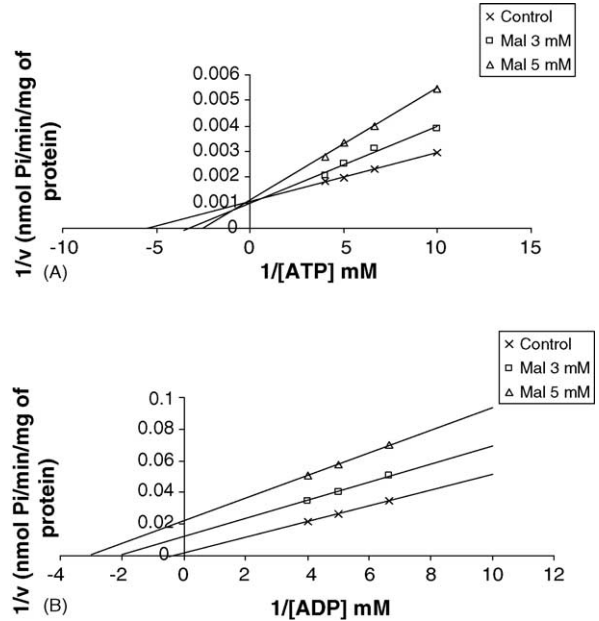

Fig. 4. Kinetic analysis of the inhibition of NTPDase by malathion in zebrafish brain membranes. The graphs show double-reciprocal plots of NTPDase activity for ATP (A) and ADP (B) concentrations (0.1–0.25 mM) in the absence (×) and in the presence of 3 mM (□) and 5 mM of malathion (△). All experiments were repeated at least 5 times and similar results were obtained. Data represents an individual experiment.

ectonucleotidase activities. Therefore, in order to evaluate if ectonucleotidases may be modulated through an indirect effect of pesticides, we have performed in vivo experiments testing lower concentrations, in the concentration range that can be found in the environment (50 μg/L) or can be considered a sublethal concentration for cyprinids (500 μg/L) (Ansari and Kumar, 1984; Bretaud et al., 2002).

In order to evaluate the possible influence of the vehicle DMSO on our results, zebrafish exposed to the DMSO 70% (25 μg/L), used to achieve the most concentrated solution of carbofuran and malathion (500 μg/L), did not present significant changes in ectonucleotidase activities in brain membranes when compared to unexposed control fish (data not shown). Thus, in the following experiments, unexposed fish were used as controls.

After exposure to carbofuran (50 and 500 μg/L) during 7 days, there were no significant changes in ATP and AMP hydrolysis in brain membranes of

carbofuran-treated zebrafish, when compared to control group (Fig. 5A). However, ADP hydrolysis was significantly decreased after exposure to carbofuran at 50 and 500 μg/L (by 19 and 24.5%, respectively) (Fig. 5A). After exposure to malathion (50 μg/L), during 7 days, there were no changes on ATP, ADP and AMP hydrolysis in brain membranes of zebrafish when compared to unexposed group (Fig. 5B). Malathion, at 500 μg/L, was able to inhibit ADP and AMP hydrolysis (28 and 58.5%, respectively) (Fig. 5B).

4. Discussion

In the present investigation, we have shown that carbofuran and malathion can inhibit in vitro and in vivo ectonucleotidase activities in zebrafish brain membranes. Carbofuran, when directly added in the enzyme assays (in vitro studies) produced an inhibition on ATP and ADP hydrolysis in an uncompetitive manner.
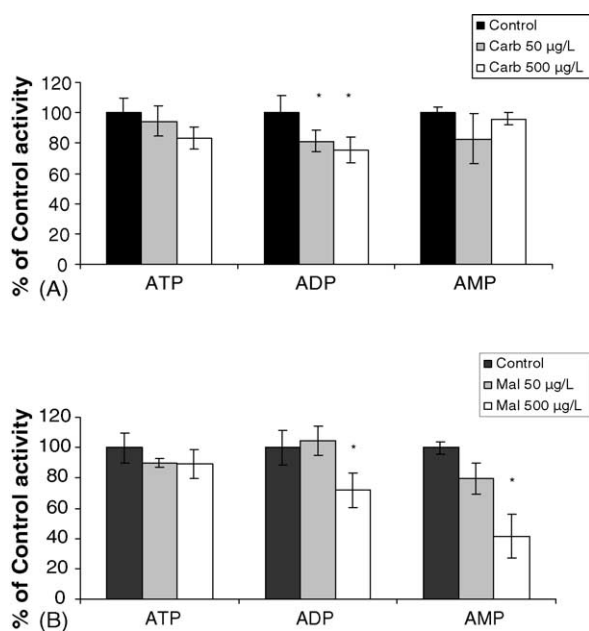
Fig. 5. In vivo effect of carbofuran (A) and malathion (B) on nucleotide hydrolysis in zebrafish brain membranes. Bars represent the means ± S.D. of four different experiments. The control ATPase, ADPase and AMPase activities (no pesticide added) were 689.92 ± 67, 163 ± 18.68 and 24 ± 1.75 nmol P_i min^{-1} mg^{-1} of protein, respectively. Asterisk (*) indicates significantly different from control group ($P \leq 0.05$).

Interestingly, the exposure of zebrafish to carbofuran (50 and 500 μg/L) inhibited only ADP hydrolysis in brain membranes when compared to unexposed group, but ATP and AMP hydrolysis were not modified by this treatment.

Malathion inhibited in vitro ATP and ADP hydrolysis in a competitive and in an uncompetitive manner, respectively. The exposure to malathion inhibited significantly ADP and AMP in the concentration of 500 μg/L after treatment during 7 days. Studies have demonstrated that exposure in vivo to malathion caused a dose-dependent inhibition of acetylcholinesterase in the zebrafish brain (Ansari and Kumar, 1984). The authors tested concentrations ranged from 500 to 1100 μg/L during 7 days of exposure. Inhibition of acetylcholinesterase occurred in all concentration tested varying from 67% in lower doses (500 μg/L; when no zebrafish mortality was observed) to 90% in the higher concentrations (1100 μg/L; when 50% of the fish surviving). The authors suggest that the anti-AChE

activity of malathion may not be the only cause of death because even with 90% of AChE inhibition survival of fish was observed. Our results have shown that, after exposure to malathion at lower doses (50 μg/L), the enzymes involved in the control of nucleotide levels were not significantly altered. However, malathion, at 500 μg/L, produced an inhibition of ADP and AMP hydrolysis (28 and 58.5%, respectively), which lead us to propose that these enzymes, together with acetyl-cholinesterase, could be considered as potential useful indicators of environmental contamination by organic compounds.

Evidence suggests that pesticides can interact directly with cholinergic receptors at or below the concentrations that inhibit AChE, affecting second messenger systems (Ward and Mundy, 1996). Furthermore, OP compounds inhibit calmodulin activity and its active conformation (Pala et al., 1991). Jung et al. (2003) demonstrated that the N-nitroso metabolite of carbofuran, N-nitrosocarbofuran induces apoptosis in mouse brain microvascular endothelial cells, at least in part through the ERK pathway. Considering that (i) in vitro carbofuran and malathion only inhibited ectonucleotidase activities at high concentrations; (ii) at low concentrations, these pesticides are able to alter transduction signal pathways and (iii) c-DNA sequence analysis of NTPDases showed potential casein kinase II, protein kinase C and cAMP and cGMP-dependent protein kinase phosphorylation sites (Kegel et al., 1997; Smith and Kirley, 1998), the results observed for in vivo experiments suggest that pesticides are modulating indirectly ectonucleotidase activities, probably by affecting transduction signal pathways. Furthermore, the divergence between the in vitro and in vivo effects observed on nucleotide hydrolysis reinforces the idea that pesticides did not act directly on ectonucleotidase activities when tested in lower concentrations.

In the literature, studies have described that non-cholinergic neurotransmitter systems can also be affected by toxic effects of pesticides. Gupta et al., (1984) examined the effect of multiple doses of carbofuran on the concentrations of acetylcholine, GABA, norepinephrine, dopamine and serotonin in mice brain. Carbofuran treatment caused significant increase in the levels of these neurotransmitters. The effect of carbofuran on brain catecholamines of goldfish was investigated. The exposure to 50 or 500 μg/L carbofuran during 24 and 48 h caused a significant increase

in catecholamines in distinct brain regions of goldfish (Bretaud et al., 2002). Our results have shown that ADP hydrolysis was significantly decreased after exposure to carbofuran, but there are no significant changes in ATP and AMP hydrolysis. The differential inhibitory effect observed on ATP and ADP lead us to propose that different NTPDases are involved in the control of nucleotide hydrolysis in central nervous system of zebrafish. Four members of the 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 milieu. These enzymes hydrolyze nucleoside triphosphates (e.g. ATP) and diphosphates (e.g. ADP) with different ability. NTPDase1 (CD39) (Wang and Guidotti, 1996) hydrolyzes both ATP and ADP equally well, whereas NTPDase2 (CD39L1) (Kegel et al., 1997) prefers triphosphonucleosides. NTPDase3 (also named CD39L3 and HB6) (Smith and Kirley, 1998) and NTPDase8 (Bigonnesse et al., 2004) slightly prefer ATP over ADP by a ratio of about 3 and 2, respectively. Further studies will be required to determine the members of NTPDase family involved in ATP and ADP hydrolysis in this fraction.

Recently, our laboratory evaluated the in vitro effect of pure and commercial CB and OP pesticides on Ca^{2+}-ATPase and cholinesterase activities in the nervous ganglia of the slug *Phyllocaulis soleiformis*. Surprisingly, only the commercial formulations but not the CB and OP compounds tested inhibited ATPase activity (Da Silva et al., 2003). In regard to this effect of pesticides on ATP hydrolysis, it is known that ATPase activity can be taken as an important index of cellular activity and toxicological tool (Rahman et al., 2000). Moreover, ATPases are target enzymes for organochlorine chemicals that affect conduction of nerve (Jinna et al., 1989). Rahman et al. (2000) demonstrated an inhibition of AChE, Na^+, K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase from rat brain in a dose- and time-dependent manner in the presence of a phosphorothionate. In vitro studies performed in synaptosomes from hen brain showed different effects of some organophosphates on the Ca^{2+}-stimulated ATPase (Barber et al., 2001). Barber et al. (2001) demonstrated that following in vitro exposure OP compounds, Ca^{2+}-stimulated ATPase activity was inhibited by chlorpyrifos, chlorpyrifos-oxon, phenyl saligenin phosphate (PSP) and tri-*o*-tolyl phosphate

(TOTP), but not by parathion, paraoxon or diisopropyl fluorophosphate. In our study, ATP hydrolysis in brain membranes of zebrafish were not altered in vivo for the two pesticides, which leads us to propose that the ATP hydrolysis may be resistant to this chemical aggression. However, in experiments in vitro, high concentrations of the pesticides were tested and the inhibition observed on ATP hydrolysis may be due to direct effect of these pesticides on structure of NTPDases.

Our investigation evaluated the relationship between pesticides, recognized as anticholinesterasic agents, and the enzymes responsible for the hydrolysis of the neurotransmitter ATP to adenosine. This study shows that the purinergic system can be a potential target for neurotoxicity induced by CB and OP pesticides.

Acknowledgements

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