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Dissertação de Mestrado

EFEITO IN VITRO DE ORGANOFOSFORADOS, CARBAMATOS E METAIS PESADOS SOBRE AS ATIVIDADES NUCLEOTÍDÁSICAS E COLINESTERÁSICA EM GLÂNDULA DIGESTIVA DE *Helix aspersa*

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“O rio atinge seus objetivos

porque aprendeu a

contornar obstáculos”

Lao- tse(séc. 5 a.C.), filósofo chinês

“A mente que se abre

a uma nova idéia jamais

voltará ao seu tamanho original”

Albert Einstein (1879-1955), físico alemão

Ao Wagner

pelo carinho, compreensão, paciência,

apoio, companheirismo e amor.

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SUMÁRIO

Resumo

Efeitos adversos devido à contaminação ambiental por pesticidas e metais pesados ocorrem em seres humanos, plantas e animais, onde um dos grupos animais mais atingidos são os moluscos, devido ao seu contato direto com o solo e plantas. O caracol *Helix aspersa* é uma espécie que se destaca pela sua importância econômica, permitindo sua criação e manutenção em cativeiro. Além disso, este animal é bastante utilizado como modelo experimental em estudos de fisiologia e bioquímica. A investigação do papel de purinas em invertebrados tem demonstrado que o sistema purinérgico é bastante diferenciado quando comparado aos vertebrados, uma vez que agonistas e antagonistas clássicos de purinoreceptores são ineficientes. A investigação da relação do sistema purinérgico e colinérgico em moluscos sugere uma ação modulatória do ATP e seus derivados sobre a liberação de acetilcolina. O sistema colinérgico, o qual tem sido utilizado como uma ferramenta para detectar intoxicações por organofosforados, carbamatos e metais pesados também contribui para a diferenciação de parâmetros bioquímicos de vertebrados e invertebrados. A existência de um sistema purinérgico e colinérgico diferenciado em invertebrados, a capacidade de acumulação de poluentes na glândula digestiva e a importância dos moluscos como bioindicadores de contaminação ambiental, este estudo investigou o efeito *in vitro* de pesticidas na forma pura (malation e carbofuran) e comercial (Malatol 500CE® e Furadan 350S®) e de metais pesados (zinco, cobre e cádmio) sobre o metabolismo do ATP, AMP e da acetilcolina na glândula digestiva de *H. aspersa*. Não foram observadas mudanças significativas sobre a hidrólise do AMP na presença do malation e do carbofuran. O malation também não alterou a hidrólise do ATP em nenhuma das concentrações testadas. Na presença do Malatol (5-20 μM), os resultados mostraram uma ativação significativa sobre a hidrólise do ATP, mas não foram observadas mudanças sobre a hidrólise do AMP. Entretanto, o carbofuran promoveu uma inibição da hidrólise do ATP na presença de 1000 μM de pesticida. O Furadan inibiu a hidrólise do ATP em todas as concentrações testadas e foi observada também uma ativação na hidrólise do AMP na presença de 32 nM deste pesticida. Com relação aos experimentos com os metais, o Zinco promoveu uma inibição significativa na hidrólise do AMP nas concentrações de 500 e 1000 μM. Não foram observadas mudanças na hidrólise de ATP. O cobre inibiu significativamente a atividade ATPásica, mas não a atividade AMPásica. Em relação aos efeitos do cádmio, observou-se um efeito inibitório sobre a hidrólise do ATP nas concentrações de 100, 500 e 1000 μM e um similar efeito foi observado sobre a hidrólise do AMP (500 e 1000 μM). Não foram observadas mudanças significativas na atividade da colinesterase na presença dos pesticidas, bem como para os metais pesados testados. Estes achados sugerem que o sistema purinérgico pode ser um alvo da toxicidade induzida por metais pesados e pesticidas e um possível indicador do impacto biológico da exposição a estes agentes tóxicos.

Abstract

Adverse effects due to environmental contamination by pesticides and heavy metals occur in humans, plants and animals, which mollusks are one the most affected groups, since these animals are in direct contact with the soil and plants. The garden snail (*Helix aspersa*) is a specie with economical importance, occurring its breeding and maintenance on controlled conditions. Furthermore, this snail has been used as an experimental model for biochemical and physiological studies. Investigations about the role of purines in invertebrates have showed that the purinergic system is differentiated when compared to vertebrates, since classical agonists and antagonists of purinoceptors are inefficient. The investigation about the relation between purinergic and cholinergic systems in mollusks suggests a modulatory action of ATP and its derivatives on acetylcholine release. The cholinergic system, which has been used as a tool to detect intoxications by organophosphorous, carbamates and heavy metals also contribute for the differentiation of biochemical parameters between vertebrates and invertebrates. Considering the existence of differentiated purinergic and cholinergic systems in invertebrates, the accumulation capability of pollutants in digestive gland and the importance of mollusks as bioindicators of environmental contamination, this study investigated the effect *in vitro* of the pesticides in the commercial (Malatol 500CE® and Furadan 350S®) and pure (malathion and carbofuran) forms and heavy metals (zinc, copper and cadmium) on the ATP, AMP and acetylcholine metabolism in the digestive gland of *H.aspersa*. There were no significant changes in AMP hydrolysis in the presence of malathion and carbofuran. Futhermore, malathion did not alter ATP hydrolysis in any concentrations tested. In the presence of Malatol 500CE® (5-20 μM), the results have shown a significant activation on ATP hydrolysis, but not in AMP hydrolysis. Carbofuran, at 1000μM, promoted an inhibitory effect on ATP hydrolysis. In contrast, Furadan 350S® inhibited ATP hydrolysis at all concentrations tested and it also was found an activation of AMP hydrolysis in the presence of 32 nM. In relation to experiments with heavy metals, the zinc (500 and 1000 μM) promoted a significant decrease on 5´-nucleotidase activity. However, it was unable to promote changes on ATP hydrolysis. Cupper (25 and 50 μM) inhibited significantly the ATPase activity, but did not alter 5´-nucleotidase. In relation to effects of cadmium, it was possible to observe an inhibitory effect on ATP hydrolysis in the concentrations of 100, 500 and 1000μM and a similar effect on AMP hydrolysis was observed at 500 and 1000μM. There were no significant changes on cholinesterase activity in the presence of pesticides, as well for heavy metals tested. These findings suggest that purinergic system can be a target of the toxicity induced by heavy metals and pesticides and a possible indicator of biological impact to exposure to these toxic agents.

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LISTA DE ABREVIATURAS

AMP- adenosina 5'- monofosfato

ADP- adenosina 5'- monofosfato

ATP- adenosina 5'- trifosfato

GABA- acido γ - aminobutírico

NTPDase- nucleosídeo trifosfato difosfoidrolase

OP- organofosforado

CB- carbamato

Zn- Zinco

Cu- Cobre

Cd- Cádmio

ChE- colinesterase

AChE- aceticolinesterase

1. INTRODUÇÃO

1.1. O Filo Mollusca e a espécie *Helix aspersa*

O Filo Mollusca compreende um grupo animal bem diferenciado que se caracteriza pela presença de um corpo mole, não segmentado, com celoma reduzido, simetria mais ou menos bilateral e geralmente envolvido por uma concha calcária. Predominam amplamente em ambiente marinho, sendo abundantes nas águas doces e estão bem representados no ambiente terrestre. Apesar de terem uma organização fundamental simples e primitiva, apresentam uma extraordinária diversidade de formas (THOMÉ, 1985).

O filo é dividido em 7 classes, sendo a classe Gastropoda a maior delas (cerca de 35.000 espécies vivas e 15.000 formas fósseis). Entre os gastrópodes, a subclasse Pulmonata contém os caracóis terrestres mais diferenciados e bem sucedidos, além de muitas formas de água doce. A característica deste grupo é a transformação da cavidade do manto em um pulmão. A subclasse Pulmonata compreende as ordens Systelommatophora, Basommatophora, e Stylommatophora, as quais apresentam como características fundamentais a ausência de brânquias e opérculos e um sistema nervoso concentrado e simétrico, com exceção da ordem Basommatophora na qual existem algumas espécies marinhas que exibem opérculo e brânquias secundárias.

Na ordem Stylommatophora (pulmonados superiores) distinguem-se animais terrestres com 2 pares de tentáculos, sendo que os olhos encontram-se na ponta do par superior. Os Stylommatophora são encontrados por todo o planeta, incluindo até mesmo as regiões desérticas, e desenvolveram uma ampla

variedade de adaptações fisiológicas e comportamentais para enfrentar os problemas ambientais adversos (RUPPERT & BARNES, 1994; BARNES & HARRISON, 1994).

O caracol *Helix aspersa* é uma espécie que pertence à família Helicidae que se destaca pela sua importância econômica. *H. aspersa* é conhecido na França por “escargot” e “Petit-Gris”, nos países de língua inglesa como “the brown garden snail” e no Brasil recebe o nome popular de caracol e até mesmo de caramujo (CHEVALLIER, 1992).

As principais referências na literatura sobre esta espécie tratam da sua importância econômica, destacando assim diversas pesquisas que visam à criação e manutenção deste caracol em cativeiro (RIBAS, 1986; MACHADO, 1993). Além disso, este animal é bastante utilizado como modelo experimental em estudos de fisiologia e bioquímica (GAYTON et al., 1973; VORHABEM et al., 1986; PISU et al., 1999, SNYMAN et al., 2005).

O metabolismo dos gastrópodes terrestres baseia-se na utilização energética de carboidratos e este tipo de substrato assume um papel-chave durante condições ambientais adversas. O hepatopâncreas é um dos vários tecidos de reserva energética em gastrópodes pulmonados. Este órgão, também denominado glândula digestiva, possui a função “hepática”, armazenando substâncias de reserva, como o glicogênio e lipídios, e a função “pancreática” produzindo uma série de enzimas digestivas (VAN WEEL, 1974, GUPPY et al., 1994; BORGES et al., 2004).

Os moluscos em geral, podem servir de alimento para mamíferos, pássaros e outros invertebrados (SYMONDSON & LIDELL, 1993; GRAVELAND et al.,

1994). Com seu alto potencial de acúmulo de poluentes (COUGHTREY & MARTIN, 1976; HOPKIN, 1989; JONES, 1991), lesmas e caracóis podem ser considerados importantes mediadores para a transferência, através da cadeia alimentar, de agentes químicos presentes na vegetação, sendo este considerado um importante aspecto ecotoxicológico (MENTA & PARISI, 2001).

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O glicogênio e o galactogênio são os principais polissacarídeos estocados nos moluscos gastrópodes (SMINIA, 1972; LIVINGSTONE & De ZWAAN, 1983; GERAERTS, 1992). Nesses animais, o glicogênio é encontrado em vários tecidos, enquanto o galactogênio está confinado à glândula de albúme e aos ovos (GOUDSMIT, 1972). O glicogênio, tanto em bivalves, como em gastrópodes, encontra-se armazenado em células especializadas denominadas por Joosse e Geraerts (1983) de células de glicogênio (glycogen cells-GC). Nos gastrópodes, essas células são abundantes na parte anterior do manto e entre os lobos da glândula digestiva (ou hepatopâncreas) e nas gônadas (SMINIA, 1972).

1.2. ATP e seus Metabólitos

O papel do trifosfato de adenosina (ATP) no espaço intracelular como molécula energética, doando grupos fosfato de alta energia e acoplando reações exoergônicas às reações endoergônicas já esta bem estabelecido na literatura. Atualmente, se propõe que o ATP é uma molécula sinalizadora primitiva e ubíqua, que foi mantida como um co-transmissor em quase todos tipos celulares,

desempenhando importantes papéis em estados fisiológicos e patológicos (CHOW et al., 1997; LAMBRECHT, 2000; BURNSTOCK, 2002; BURNSTOCK, 2004).

Como resultado do seu tamanho e carga, o ATP extracelular não se difunde através da membrana plasmática das células (SPERGEL & LAHIRI, 1993). Portanto, uma vez liberado, as respostas celulares para o ATP extracelular e seus produtos são geralmente mediadas por receptores purinérgicos do tipo P2X e P2Y (RALEVIC & BURNSTOCK, 1998). O ATP pode ser armazenado e liberado juntamente com diversos outros neurotransmissores, tais como: acetilcolina, glutamato, norarenalina, serotonina e GABA (BURNSTOCK, 1999; BURNOSTOCK, 2004).

A ação sinalizadora dos nucleotideos é terminada por uma cascata de enzimas localizadas na superfície celular. No caso da degradação extracelular do ATP, o produto final é o neuromodulador adenosina, que age através dos receptores P1, subdivididos em A_1, A_{2A}, A_{2B} e A_3 (DUNWIDDIE & MASINO, 2001). Esta degradação pode inativar a sinalização mediada pelo ATP através dos receptores P2 e aumentar a sinalização mediada pela adenosina através dos receptores P1 (ZIMMERMANN, 2001; KATO et al., 2004).

Diversas enzimas estão envolvidas no controle dos níveis dos nucleosídeos tri- e di-fosfatados. As enzimas da família das NTPDases (nucleosídeo trifosfato difosfoidrolase) hidrolisam purinas e pirimidinas e , até o momento, oito enzimas já foram descritas e caracterizadas dentro desta família, subdivididas em NTPDase 1-8 (ZIMMERMANN, 2001; KUKULSKI et al., 2005). Nesta famíia de enzimas, podemos destacar a ATP difosfoidrolase ou apirase (NTPDase 1, EC 3.6.1.5) e a

ecto-ATPase (NTPDase 2, EC 3.6.1.3). Estas enzimas possuem diversas características em comum, tais como um sítio de hidrólise de nucleotídeos voltado para o espaço extracelular, subunidade catalítica glicosilada, atividade dependente de cátions divalentes (principalmente Mg^{2+} e Ca^{2+}), insensibilidade para os inibidores de diversas ATPases e habilidade para hidrolisar uma ampla variedade de nucleotídeos púricos e pirimídicos di e trifosfatados (ZIMMERMANN, 2001). A distribuição deste grupo de enzimas é muito ampla, sendo descrita em plantas, insetos, em vários tecidos de mamíferos, como por exemplo, na aorta bovina, em células endoteliais, em células do músculo liso, no plasma e eritrócitos de humanos, em pâncreas de porco, na membrana plasmática, em secreções oviductais e seminais, na placenta de ratos e de humanos, no cordão umbilical, na glândula mamária, no útero e pulmão de bovinos e em sinaptossomas do sistema nervoso periférico e central (SARKIS et al., 1995; ZIMMERMANN, 2001). Borges et al. (2004) demonstraram uma atividade ATPásica no gânglio nervoso e na glândula digestiva de *H. aspersa* com características cinéticas diferentes daquelas previamente descritas (PLESNER, 1995; SARKIS et al., 1995; DA SILVA et al., 2002).

Participando da cascata enzimática que hidrolisa ATP a adenosina, podemos destacar a participação da 5'-nucleotidase. A ecto-5'-nucleotidase ocorre em todos os tecidos e é capaz de hidrolisar nucleotídeos monofosfatos (AMP) a nucleosídeos e fosfato inorgânico (SARKIS & SALTÓ, 1991; BATTASTINI et al., 1995; ZIMMERMANN, 1996, ZIMERMMANN et al., 1998; SCHOEN et al., 1999; ZIMERMMANN, 2001). A adenosina é capaz de modular uma série de processos

fundamentais a nível celular em muitos órgãos e tecidos, principalmente no sistema nervoso central (CUNHA et al., 2000).

1.3. Metais Pesados

Atualmente, considerando que nossa economia depende largamente de produtos industrializados e colheitas produtivas, dejetos industriais e agrícolas tornaram-se um grande problema para a saúde humana e do ambiente. A poluição ambiental causada por resíduos de metais pesados e pesticidas é muito relevante pelo seu amplo uso em processos industriais e agrícolas, sendo que muitos efluentes chegam ao meio ambiente sem qualquer tratamento (KUNO et al., 1999; SCHERER et al., 2003).

Os metais ocorrem naturalmente no ambiente, mas desde a Revolução Industrial esta ocorrência tem se ampliado muito com a atividade agrícola e industrial (HOPKIN, 2004).Os metais pesados possuem diversos mecanismos de ação tóxica, sendo o principal a sua ação sobre a estrutura das proteínas, muitas delas com atividade enzimática. Ao alterar as atividades enzimáticas, os metais pesados afetam diversos processos bioquímicos, membranas celulares e organelas. A influência destas substâncias se dá por mecanismos complexos, tais como: interação com metais essenciais por similaridade eletrônica, formação de complexos metal-proteína, inibição enzimática de proteínas com grupos –SH e

injúria de organelas celulares como mitocôndrias, lisossomas e microtúbulos (FERRER, 2003).

O impacto da contaminação do ambiente terrestre por metais é de difícil avaliação devido à complexidade do ecossistema do solo, bem como sua alta variabilidade (VAUFLEURY & PIHAN, 2000)

Os metais cádmio (Cd) e zinco (Zn) diferem em termos de importância metabólica para os organismos. O Cd é um metal não essencial e geralmente tóxico para os organismos (DEPLEDGE et al., 1994, ODENDAAL & REINECKE, 1999), enquanto que o zinco, sendo um metal essencial, desempenha um papel importante em vários processos metabólicos (MILLER, 1983; JACKSON, 1989, ODENDAAL & REINECKE, 1999). Entretanto, o zinco pode ser potencialmente tóxico para os organismos se ocorrer em altas concentrações (HARRIS, 1991; BEYER & STORM, 1995). Até o momento, pouco se sabe sobre os efeitos do Cd e do Zn no solo.

Van Straalen e colaboradores (1987) sugeriram que as principais diferenças na ecofisiologia de metais ocorrem devido à sua essencialidade versus sua não-essencialidade para os organismos. Os níveis de metais nutricionais, como Zn e Cu, são regulados, e xenobióticos, como Cd, são acumulados. Entretanto, Zn, Cu e Cd são potencialmente tóxicos aos organismos, se eles ocorrerem em concentrações elevadas (HARRIS, 1991; BEYER AND STORM, 1995). Beeby e Richmond (1989) sugerem que, em moluscos, os metais podem ser acumulados nas conchas e este pode ser um fator de risco para os animais que se alimentam de conchas de caracóis, como os pássaros.

Devido ao amplo efeito exercido pelos metais pesados, não são conhecidos todos os mecanismos de ação tóxica destes compostos (SANDHIR et al., 1994; MYERS et al., 2000). Além disso, muitos fatores afetam os efeitos patofisiológicos dos metais pesados, tais como a sua forma química, via de entrada no organismo, duração da exposição, concentração, idade e espécie do animal (KOSTIAL et al., 1978; MOLLER-MADSEN, 1990).

1.4. Pesticidas e atividade colinesterásica

Pesticidas são substâncias intencionalmente introduzidas no ambiente com o intuito de controlar pragas como insetos, nematódeos e ácaros. Estas substâncias se tornaram indispensáveis na agricultura moderna para garantir boas colheitas. Atualmente, carbamatos e organofosforados são as classes de pesticidas mais comumente utilizadas na agricultura, devido ao grande problema da persistência dos organoclorados no ambiente (BONDARENKO et al., 2004).

O mecanismo tóxico dos carbamatos e organofosforados geralmente é atribuído à capacidade destas substâncias de inibir a atividade da acetilcolinesterase em sinapses colinérgicas do sistema nervoso central e em junções neuromusculares (ANSARI & KUMAR 1984; GUPTA, 1994; SHAO-NON & DE-FANG, 1996). Esta inibição tem como conseqüência o acúmulo de acetilcolina nos terminais nervosos, com possíveis efeitos comportamentais e até letais no animal exposto (LITTLE & FINGER, 1990; STEINBERG et al., 1995; SAGLIO et al., 1996). Entretanto, outros efeitos necessitam ser avaliados para completamente compreender os efeitos neurotóxicos destes contaminantes. A inibição da

atividade colinesterásica tem sido amplamente utilizada como diagnóstico de exposição de invertebrados a contaminantes ambientais (DEMBELE et al., 2000; ROEX et al., 2003).

Poucos estudos relacionando a toxicidade de pesticidas em sistemas de neurotransmissão não-colinérgicos foram realizados até o momento. Da Silva e colaboradores (2003) testaram o efeito *in vitro* de formas puras e comercias de pesticidas carbamatos e organofosforados na atividade da Ca^{2+}-ATPase e da colinesterase em gânglios nervosos do molusco *Phyllocaulis soleiformis* e apenas as formas comerciais inibiram significativamente a atividade das duas enzimas.

2. OBJETIVOS

A ação de enzimas que hidrolisam nucleotídeos em invertebrados é pouco conhecida. Estudos têm demonstrado que o sistema purinérgico de invertebrados apresenta peculiaridades bioquímicas não encontradas em vertebrados (KNIGHT et al., 1992a, 1992b).

Considerando a existência de um sistema purinérgico diferenciado em invertebrados, a capacidade de acumulação de poluentes na glândula digestiva e a importância dos moluscos como bioindicadores de contaminação ambiental, este estudo apresentou como objetivo principal a avaliação do efeito *in vitro* de pesticidas na forma pura e comercial e de metais pesados sobre o metabolismo do ATP e da acetilcolina na glândula digestiva de *H. aspersa*. Os objetivos específicos desta dissertação foram os seguintes:

- Avaliar o efeito *in vitro* de carbamatos e organofosforados, puros e de origem comercial, sobre a hidrólise de ATP e AMP em membranas de glândula digestiva de *Helix aspersa*.
- Investigar o efeito *in vitro* de carbamatos e organofosforados na forma pura e comercial sobre a atividade colinesterásica (ChE) em homogeneizado da glândula digestiva de *Helix aspersa*.
- Avaliar o efeito *in vitro* dos metais pesados cobre, cádmio e zinco sobre a hidrólise de ATP e AMP em membranas de glândula digestiva de *Helix aspersa*.

- Investigar o efeito *in vitro* dos metais pesados cobre, cádmio e zinco sobre a atividade colinesterásica (ChE) em homogeneizado da glândula digestiva de *Helix aspersa*.

3. APRESENTAÇÃO DOS ARTIGOS

Os resultados obtidos nesta dissertação originaram três artigos científicos. O primeiro artigo é uma revisão sobre o assunto estudado, o qual foi submetido ao periódico *Environmental Toxicology and Pharmacology*. O segundo artigo verifica o efeito *in vitro* de organofosforados e carbamatos, na forma pura e comercial sobre o metabolismo do ATP, AMP e da acetilcolina na glândula digestiva de *H. aspersa*, submetido para *Toxicology in vitro*. O terceiro artigo refere-se ao estudo realizado sobre o efeito *in vitro* dos metais cobre, cádmio e zinco sobre o metabolismo do ATP, AMP e da acetilcolina na glândula digestiva de *H. aspersa* submetido *a Comparative Biochemistry and Phisiology Part C: Toxicology and Pharmacology.*

Capítulo 1:

Dahm, K. C. S., and Bonan, C. D. ATP and acetylcholine metabolism in mollusks: a review about the influence of organophosphates and carbamates.

(Submetido ao periódico *Environmental Toxicology and Pharmacology*)

ATP and acetylcholine metabolism in mollusks: a review about the influence of organophosphates and carbamates

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Running title: pesticides and neurotransmission in mollusks

Abstract

The garden snail (*Helix aspersa*) is used as bioindicator of pollution. Since these animals are in direct contact with the soil and plants, they may be exposed to several pollutants. Several groups of invertebrates have evolved purinoceptors, which have nucleotides as their main agonists. Nucleotides can be released as signaling substances in the nervous system from both neural and glial cells. Acetylcholine and ATP are co–released in the nervous terminals, where ATP acts as a co-transmitter or modulator of cholinergic synapses. The life span and extent of receptor activation by acetylcholine and ATP are controlled by their hydrolysis performed by acetylcholinesterase (AChE) and nucleoside triphosphate diphosphohydrolases (NTPDases), respectively. Furthermore, other ATP-metabolizing enzymes have been described. Cholinesterase activity in mussel is an useful biomarker of exposure to organophosphates and carbamates. Thus, the analysis of the effects of different pollutants, such as pesticides and heavy metals, on biochemical parameters in mollusks could be an interesting tool to evaluate the level of environmental contamination promoted by these compounds.

Keywords: mollusks, nucleotidases, pesticides, cholinesterases

Introduction

Pesticides are chemicals intentionally introduced to the environment and have become necessary to ensure good harvests in modern agriculture. Overspray and/ or runoff off pesticides from agricultural fields may easily contaminate the environment, resulting in serious damage to species, such as mollusks. Carbamate (CB) and organophosphate (OP) pesticides are widely used in agricultural practice throughout the world (Bondarenko et al., 2004). The toxicity of carbamates and organophosphate pesticides has been correlated with its inhibitory effect on acetylcholinesterase (AChE) activity at central cholinergic and at neuromuscular junctions (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 nontarget species (Shore and Douben, 1994). However, other effects remain to be considered to completely estimate the neurotoxicity of these contaminants. Among these effects, it is important to evaluate the effect of these pesticides on extracellular ATP metabolism, since ATP and acetylcholine are co-released in the synaptic cleft. Therefore, this review aims to emphasize the impact of these contaminants in key species and their possible interaction with purinergic and cholinergic systems in mollusks, which may be considered bioindicators of environmental contamination.

Mollusks

The Mollusca comprises one of the largest phyla within the animal kingdom and has adapted successfully, presenting a widespread distribution and being able to survive in both aquatic and terrestrial environments. The gastropod class possesses about 35,000 living species and 15,000 fossil records and is the major class of the mollusks described (Brooks and Storey, 1997).

The successful evolution of mollusks is a consequence of their extraordinary adaptive capacity. Most organisms are exposed to changes, such as temperature, humidity, photoperiodism and energetic substrate availability. This variable environment can induce metabolic and behavioral changes in these organisms. In order to survive, animals can migrate to other places, change their physical characteristics or enter in a hypometabolic condition (Brooks and Storey, 1997).

The brown garden snail *Helix aspersa* was described by O. F. Muller in 1774 from specimens collected in Italy. This plant feeder has disseminated into many parts of the world intentionally as a food delicacy and accidentally by the movement of plants, and by hobbyists who collect snails. The snail was introduced to California in the 1850s as a source of escargot and has been considered to be troublesome as a pest of crops and ornamental plants (Capinera, 2001).

Purinergic system

Nucleotides represent the most ubiquitous class of cell-to-cell signaling substances, eliciting physiological responses in several tissues (Ralevic and Burnstock, 1998). Intracellular ATP is primarily utilized to drive energy-requiring

processes, such as active transport, cell motility and biosyntheses. Since the physiological roles of extracellular ATP and their metabolic products are different, depending on the cell type, these differences also apply to the function, such as platelet aggregation, muscle contraction, vascular tone and cell adhesion (Ralevic and Burnstock, 1998). ATP can be released from stimulated central neurons (Cunha et al., 1996) and even glial cells (Queiroz et al., 1997). ATP can also be released together with other neurotransmitters, such as noradrenaline (Von Kügelgen, 1996), acetylcholine (Vizi et al., 1997), serotonin and GABA (Hugel and Schlichter, 2000).

Over the last decade, substantial evidence has emerged to show that ATP and other nucleoside di and tri-phosphates act as extracellular signaling molecules in virtually all tissues (Cunha, 2001). Extracellular ATP has been established as a signaling molecule, which mediates its actions through two subclasses of P2-purinoceptors, metabotropic P2Y receptors and ionotropic P2X receptor (Cunha, 2001). Seven members of the P2X family have been described: $P2X_1$, $P2X_2$ [$P2X_{2-1}$ (short form)], $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$. Furthermore, seven members of the P2Y family are currently recognized: $P2Y_1$, $P2Y_2$, $P2Y_3$, $P2Y_4$, $P2Y_5$, $P2Y_6$, $P2Y_7$ (Burnstock, 1999).

The G-protein-coupled P2Y receptors typically have seven transmembrane domains, which are well conserved, and an extracellular N-terminus and an intracellular C-terminus that are more variable in structure (Ralevic and Burnstock, 1998). A P2Y receptor has been identified in the frog embryo and shown to be closely associated with the development of the nervous system (Bagdanov et al., 1997).

P2X receptors have two transmembrane domains with short intracellular N- and C-termini and an extensive extracellular loop, which always contains 10 cystines. A special feature of the P2X2 receptor which distinguishes it from the other P2X subtypes, P2Y and P1 receptors, is its great sensitivity to changes in pH, with maximum responses to ATP at pH 6.5 in oocytes (King et al., 1996). It seems likely that there is a nociceptive ATP receptor that is a pore formed by a heteromeric combination of P2X2 and P2X3 units (Lewis et al., 1995). The P2X3 receptor is expressed only in sensory neurons (Chen et al., 1995) in contrast to the P2X4 receptor, which is widely distributed in central and peripheral nervous systems (Buell et al., 1996).

Purine nucleosides and nucleotides effects have been widely studied in several classes of vertebrates (Ralevic and Burnstock, 1998). There is evidence that during the course of their development, several groups of invertebrates have evolved purinoceptors, which do not present a clear physiological relevance (Hoyle and Greenberg, 1988). It has been suggested that ATP is a primitive neurotransmitter since it is well represented in lower vertebrates (Burnstock, 1976). However, few studies have been conducted on adenosine, AMP, ADP and ATP receptor-mediated actions in mollusks (Hoyle and Greenberg, 1988).

The administration of AMP, ADP and ATP to the *H. aspersa* snail produced concentration-dependent contractions, suggesting that purinoceptors are important for evoking the release of acetylcholine from rectum and esophagus (Knight et al., 1992). Furthermore, some evidence seems to indicate a contribution of the neuromodulator adenosine, a product of ATP degradation by ectoenzymes, in metabolic depression in vertebrates and invertebrates (Lazou, 1989).

After its release, extracellular ATP can be inactivated by a group of ecto-enzymes named ecto-nucleotidases. The broad substrate specificity of ecto-nucleotidases may thus reflect the broad range of endogenously active ligands rather than a deficit of the enzymes to restrain their catalytic function to defined nucleotides. The final product of the ectonucleotidase chain generally is the respective nucleoside. In the case of ATP, the ecto-nucleotidase chain produces extracellular adenosine, an important neuromodulator. The P1 family corresponds to the adenosine receptors, which are coupled to G-protein and subdivided in four subtypes: A_1, A_3 (inhibitory receptors), A_{2A} and A_{2B} (facilitatory receptors). It should be noted, however, that other nucleosides also elicit physiological responses. These include the nucleosides, inosine (Benowitz et al., 1999) and guanosine (Neary et al., 1996), which can exert trophic effects on nerve cells.

Nucleotidases

Signaling actions of nucleotides require effective mechanisms for inactivation (Zimmermann, 2001). After exerting its actions, ATP can be hydrolyzed to nucleoside adenosine, an important neuromodulator, by a group of enzymes, called ectonucleotidases, located on the cell surface. This group of enzymes includes the nucleoside triphosphate diphosphohydrolase (NTPDase) family, which is represented by ATP diphosphohydrolase (apyrase, NTPDase 1, EC, 3.6.1.5), ecto-ATPase (NTPDase 2, EC 3.6.1.3) and ecto-5'-nucleotidase (EC 3.1.3.5) (Zimmermann, 2001). These enzymes may participate in the control of nucleotide and nucleoside levels in the synaptic cleft and, consequently, in the control of purinergic neurotransmission (Sebastião et al., 1999). For this reason, the

presence of enzymes able to hydrolyze nucleotides may be an important mechanism required controlling the activation and inactivation of these purinoceptors.

The presence and physiological role of enzymes performing ATP and ADP hydrolysis in the mollusks are not fully understood. Our laboratory has characterized a Ca^{2+} -ATPase from the nervous ganglia of *Phyllocaulis soleiformis* (Da Silva et al., 2002). Recently, Borges et al. (2004) demonstrated an ATPase activity in nervous ganglia and digestive gland of H. aspersa with different kinetic characteristics than those previously described (Da Silva et al., 2002). The analysis of these enzyme activities in *H. aspersa* contributed to the understanding of the physiological significance in regulating the extracellular and intracellular nucleotide levels in the snail (Borges et al., 2004). In the digestive gland, the triphosphate nucleotides were hydrolyzed at a higher rate, but the hydrolysis of diphosphate nucleotides is increased in this tissue when compared to nervous ganglia of *H.aspersa* (Borges et al., 2004).

Acetylcholine

Acetylcholine and ATP are co–released in the nervous terminals, where ATP acts as a co-transmitter or modulator of cholinergic synapses. However, it is still unclear whether its modulation is mediated by ATP or by adenosine (Cunha and Ribeiro, 2000). The life span, the duration and extent of receptor activation by acetylcholine and ATP are controlled by their hydrolysis by acetylcholinesterase (AChE; EC 3.1.1.7) and nucleoside triphosphate diphosphohydrolases (NTPDases), respectively (Zimmermann, 2001).

Some authors have shown difficulty in classifying cholinesterases from invertebrates, since these enzymes have apparent affinity for any choline ester, suggesting that they should be classified generally as cholinesterases, and not as acetylcholinesterases or butyrylcholinesterases (Mora et al., 1999).

The inhibition of cholinesterase (ChE) activity has been successfully used to diagnose organophosphorus and carbamate poisoning in invertebrates. However, in invertebrates, the interaction of these compounds with cholinesterases is not homogenous due to differences in sensitivity between different invertebrates, such as mussels and mosquitoes (Mora et al., 1999).

Pesticides

The inhibition of ChE activity is the first toxic action of carbamates and organophosphates, and this effect is one of the most important biomarkers (Shore and Douben, 1994).

Organophosphorus compounds have been used for a wide variety of applications such as therapeutic agents, agricultural chemicals, plasticizers and lubricant fuel additives. However, their most widespread utilization is as insecticides for the control of pests on plants, animal, and humans (Pope, 1999).

Carbofuran is a broad-spectrum systemic carbamate insecticide, widely used in agricultural practice around the world. Although carbofuran can break down rapidly in the environment, nontarget aquatic biota may be exposed to sublethal concentrations of these pesticides (Bretaud et al., 2002)

As for other carbamate and organophosphate insecticides, toxicity of carbofuran has been correlated with is inhibitory effect on acetylcholinesterase

activity at central cholinergic synapses and at neuromuscular junctions (Bretaud et al., 2000). The difference is related to the manner of inhibition, since carbamate promotes a reversible inhibition and organophosphates act as irreversible inhibitors of cholinesterase activity (Shore and Douben, 1994).

Such inhibition leads to accumulation of acetylcholine at nerve endings, with possible consequences to locomotion and equilibrium in exposed fish (Saglio et al., 1996). Due to the specificity of this neurochemical effect, inhibition of AchE has been used as an indicator of exposure of nontarget species to organophosphate and carbamate insecticides (Gruber and Munn, 1998).

Mollusks as bioindicators of contamination

Snails also satisfy the main requirements for a good sentinel organism (Gomot, 2000). They are exposed to pollutants through the ingestion of both contaminated plants and soil (dietary intake), by contact and diffusion through the skin (cutaneous uptake and by breath) (Coerdassier et al., 2000).

There are very few standardized tests for the evaluation of soil contamination or for the determination of the toxicity of chemicals on species in the soil fauna (Gomot, 2000). As the current standard tests use only animals involved in decomposition, the use of snails has been proposed (Gastropoda, Pulmonata) for the development of sublethal toxicity tests, as these terrestrial invertebrates are primary consumers of green plants and saprophytes at the soil surface (Gomot, 2000). The fact that the biological cycle is fully controllable (Gomot, 1994) facilities the study of the toxic effects of agents on any of the essential phases of their life cycle (Gomot, 1997).

The ChE activity in *H. aspersa* is strongly inhibited by the ingestion of the pesticide dimethoate used in numerous countries. This marker, therefore, seems to be particularly useful for the detection of environmental contamination by organophosphorus compounds, which would enable the risks of this type of pollution to be anticipated and, thus, avoided. The land snail *H. aspersa* has the biological characteristics required for use as a bioindicator of terrestrial pollution by organic xenobiotics (Coeurdassier et al., 2001).

To understand the mechanism of the interaction between the purinergic and cholinergic systems and the differences observed in vertebrates and invertebrates with respect to the effects of pesticides on these system, it is interesting to develop novel approaches in order to investigate the action of pesticides, in the commercial and pure form, on the energetic metabolism and neurotransmission in *Helix aspersa.* Therefore, the analysis of the effects of different pollutants, such as pesticides and heavy metals, on biochemical parameters in mollusks could be an interesting tool in order to evaluate the level of environmental contamination promoted by these compounds.

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Capítulo 2:

Dahm, K. C. S, Rückert, C,Tonial, E. Mbonan, C. D Effect of carbamate and organophosphate pesticides on nucleotidase activities from digestive gland of *Helix aspersa.* (Submetido ao periódico *Toxicology in vitro*).

Effect of carbamate and organophosphate pesticides on nucleotidase activities from digestive gland of *Helix aspersa*

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Abstract

ATP can be coreleased with as acetylcholine, acting as a neurotransmitter or modulator of cholinergic synapses. Hydrolysis of extracellular ATP to adenosine is catalyzed by NTPDases (nucleoside triphosphate diphosphohydrolase) and 5´-nucleotidase. Furthermore, acetylcholine inactivation is mediated by acetylcholinesterase, which has been used as indicator of exposure to carbamates and organophosphate pesticides. Here we demonstrated the effect *in vitro* of pure and commercial pesticides on the ATPase and cholinesterase activities in the digestive gland of *H.aspersa*. There were no changes in AMP hydrolysis in the presence of malathion and carbofuran. Futhermore, malathion did not alter ATP hydrolysis in any concentration tested. In the presence of Malatol 500CE® (5-20 μM), the results have shown a significant activation of ATP hydrolysis, but not in AMP hydrolysis. Carbofuran, at 1000μM, promoted an inhibitory effect on ATP hydrolysis. In contrast, it was found an activation of AMP hydrolysis in the presence of 32 nM Furadan 350S® and an inhibitory effect on ATP hydrolysis at all concentrations tested. There were no changes in ChE activity in presence of pesticides in the commercial and pure form. These findings showed a different sensitivity between pure and commercial pesticides on nucleotide hydrolysis and suggest that the purinergic neurotransmssion can be target to the pesticides toxicity.

Keywords: mollusks, *Helix aspersa*, nucleotidases, digestive gland, pesticides, cholinesterase

1. Introduction

The successful evolution of mollusks is a consequence of their extraordinary adaptive capacity. The brown garden snail *Helix aspersa* (Müller,1774) (Mollusca, Gastropoda, Helicidae) is a terrestrial mollusk, which has been disseminated into many parts of the world intentionally as a food delicacy and accidentally by the movement of plants, and by hobbyists who collect snails. The snail was introduced to California in the 1850s as a source of escargot and has been considered to be troublesome as a pest of crops and ornamental plants (Capinera, 2001). Many investigators have attempted to develop methods of slug control, but only few advances have been made (Panigrahi and Raut, 1993; Hollingsworth et al., 2002).

Herbicides such as organophosphates are used in both agricultural and non-agricultural areas (Tate at al., 1997, Bondarenko et al., 2004). Organophosphorus compounds have been used for a wide variety of applications, such as therapeutic agents, agricultural chemicals, plasticizers and lubricant fuel additives. However, their most widespread utilization is as insecticides for the control of pests on plants, animals and humans (Pope, 1999). Carbofuran is a broad-spectrum systemic carbamate insecticide, widely used in agricultural practice around the world. Although carbofuran can break down rapidly in the environment, nontarget aquatic biota may be exposed to sublethal concentrations of these pesticides (Bretaud et al., 2002). 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 nontarget species (Dembele et al., 2000; Roex et al., 2003). However, there is some difficulty in classifying cholinesterase from invertebrates, since these enzymes have apparent affinity for any choline ester, suggesting that they should be classified as cholinesterases and not as acetylcholinesterases or butyrylcholinesterases (Bocquené et al., 1997; Mora et al., 1999).

Acetylcholine and ATP are co-released in the nervous terminals, where ATP acts as a neurotransmitter or modulator of cholinergic synapses (Cunha and Ribeiro, 2000; Burnstock, 2000). The hydrolysis of extracelluar ATP to AMP is catalyzed mainly by a family of ectonucleotidases named NTPDases (nucleoside triphosphate diphosphohydrolase). The nucleotide AMP is hydrolyzed to adenosine, an important neuromodulator, by the action of an ecto-5'-nucleotidase (Zimmermann,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), 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).

The effects of nucleotides and nucleosides have been investigated in mollusks, but the effects of agonists and antagonists indicated that the purinoceptors from invertebrates are distinct from those vertebrates (Hoyle and Greenberg, 1988; Knight et al., 1992a, 1992b). Recently, Borges et al. (2004)

demonstrated an ATPase activity in nervous ganglia and digestive gland of *H. aspersa* with different kinetic characteristics than those previously described (Plesner, 1995; Da Silva et al., 2002).

Considering that the molluscan digestive gland perform multiple functions in the physiology of the animal and the interaction between the purinergic and cholinergic systems, here we verify the effect *in vitro* of the pesticides in the commercial and pure form commonly used in agriculture on the Ca^{2+}-activated ATPase and ChE activities in the digestive gland of *H.aspersa*.

2. Material and Methods

2.1. Experimental model

Adult *H. aspersa* snails were collected all year long from gardens (pesticide free) of metropolitan region of Porto Alegre, RS, Brazil. Animals were maintained in plastic boxes (68 x 60 x 22 cm) at 25±5ºC in a photoperiod of 12h light/12h dark for least 7 days before experiments. Snails were fed with lettuce (*Latuca sativa*) *ad libitum*. The Ethics Committee from Pontifícia Universidade Católica do Rio Grande do Sul approved the protocol under number 0401979.

2.2. Chemicals

Malatol 500CE® (95%, BioCarb- Industria Química LTDA) and Furadan 350S® (FMC do Brasil Industria e Comércio S.A) were purchased from commercial suppliers. The pure compounds were kindly donated by FMC Química do Brasil LTDA (CAS Number 1563-66-2, 96% carbofuran; 2,3- Dihydro-2,2-dimethyl-7-

benzofuranol methylcarbamate), and Indol do Brasil (CAS Number 121755, malathion; [(Dimethoxyphosphinothioyl)thio] butanedioic acid diethyl ester).ATP and Trizma base were purchased from Sigma (St. Louis, MO, USA). Coomassie Blue G, bovine serum albumin, calcium and magnesium chloride were purchased from Sigma (St. Louis, MO, USA). The kit for ChE activity was obtained from Wiener Lab. All other reagents used were of analytical grade. The concentration used for commercial compounds is close to the concentrations recommended for the dilution of technical products, when used in the agricultural area.

2.3. Membrane preparation of the digestive gland

To isolate the digestive gland, the shell was removed (n=5). The membrane preparations was made according to Barnes et al.(1993). Briefly, the digestive gland was homogenized in 5 vol. (w/v) in a solution of NaCl (0.65%) containing a protease inhibitor (0.1mM PMSF). The homogenate was centrifuged at 1000 x *g* for 10 min, the pellet discarded, and the supernatant centrifuged for 20 min at 40000 x *g*. The pellet was frozen in liquid nitrogen for 10 s, thawed, resuspended twice and centrifuged for 20 min at 40000 x *g*. The membrane was prepared fresh daily and maintained at 4ºC throughout the preparation.

2.4. Enzyme assays

Enzyme activity was assayed in standard reaction medium containing 50 mM Tris- HCl, pH 7.2, 5mM $CaCl_2$ or $MgCl_2$ in a final volume of 200μl. Membranes of digestive gland of *H. aspersa* (2.5-5μg protein) were added to reaction medium

and pre-incubated with the different agricultural chemical concentrations for 10min at 30ºC. The pure compounds used were the OP carbamate carbofuran (96%) at the concentrations 10, 100, 500 and 1000 μM and OP malathion (95%) at the concentrations 2,5, 5, 10, and 20 μM. The commercial compounds used were Malatol 500CE® (malathion) at 2,5, 5, 10 and 20 μM and Furadan 350S® (carbofuran) at 0.8, 1.6, 8, 16, 32 nM. The reaction was initiated by the addition of substrate (ATP or AMP) at final concentration of 1 mM, incubated for 20 min at 30ºC and stopped by addition of 200μl 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 min, and the inorganic phosphate (P_i) released was measured according to 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 TCA were used to correct for non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol of Pi released min^{-1} mg^{-1} of protein. The experiments were performed in triplicate.

2.5. ChE assays

Fractions of the digestive gland of *H. aspersa* were pre-incubated for 10 min with pesticides in the same concentrations used for ATPase assays, in a final volume of 100μl. ChE activity was measured using 7 mM S-butyrylthiocholine iodide as substrate, 50mM phosphate buffer, pH 7.7 and 0.25 mM 5,5'-dithiobis-2-nitrobenzoic (DTNB) (Ellman et al., 1961). The reaction was initiated by the addition of aliquots with 1-3 μg of protein. Protein concentrations and incubation

time were chosen to assure the linearity of the reaction. Specific activity is expressed as μmol of thiocholine released h^{-1} mg^{-1} of protein. The experiments were performed in triplicate.

2.6. Protein determination

Protein was determined by Comassie Blue method using bovine serum albumin as a standard (Bradford,1976).

2.7 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan test, considering a level of significance of 5%. All analysis was performed using the Statistical Package for Social Science (SPSS) software program.

3. Results

In this study, we have demonstrated the effect *in vitro* of commercial and pure pesticides on the ATPase and AMPase activities of the membrane fractions from the digestive gland of *H. aspersa.* There were no significant changes in ATP and AMP hydrolysis in any concentration of malathion (2,5 - 20μM) tested (Fig.1A). Carbofuran, at 10 -1000μM, did not alter AMP hydrolysis in the concentrations tested (Fig.1B). However, it was found an inhibition (35%) on ATP hydrolysis in presence of 1000 μM carbofuran (Fig. 1B).

It has been observed a different sensitivity between pure and commercial pesticides in relation to the effects on nucleotide hydrolysis. The results have

shown a significant activation of ATP hydrolysis in the presence of 5, 10 and 20 μM (38%, 40 % and 35%, respectively) in the presence of Malatol 500CE® (Fig.2A). In contrast, malatol 500CE® (2,5- 20μM) did not promote significant changes in AMP hydrolysis in any concentrations tested (Fig.2A). Another interesting difference between these enzymes in relation to the commercial compounds can be observed when Furadan 350S® was tested. It was observed an activation of AMP hydrolysis (35%) in the presence of 32 nM Furadan 350S® (Fig. 2B). However Furadan 350S®, at 0,8 - 32 nM, promoted an inhibitory effect on ATP hydrolysis in the range of 28-40% (Fig. 2B). There were no significant changes in ChE in any concentrations tested of pesticides in the commercial and pure form (Fig.3) .

4. Discussion

The present study has shown a differential sensitivity of ATPase and AMPase activities in the presence of pure and commercial pesticides in membrane preparations of digestive gland of *H. aspersa*. Furthermore, our results have demonstrated the insensitivity of ChE to carbamates and organophosphates, widely used in the agriculture These results are suprising, since the inhibition of ChE activity is the first toxic action of carbamates and OPs, and this effect is one the most important biomarkers (Shore and Douben, 1994). Terrestrial invertebrates, including gastropod mollusks, have been considered useful biomarkers of environmental contamination (Coeurdassier et al., 2001). In agreement with our results, there are some reports questioning the utilization of this parameter for invertebrates, since that a large number of animals demonstrate

ChE insensitivity to carbamates and OPs. The existence of many isoenzymes with different levels of inhibition by these compounds can impair the utilization of this enzyme as a biomarker in invertebrates (Bocquené at al., 1997). Bocquené et al. (1997) have shown the presence of two isoenzymes of ChE in the gills of the oyster. *Crassosterea gigas*, one sensitive and the other resistant to carbamates and OPs. Furthermore, there are many chemicals that inhibit this enzyme, such as detergents and metallic compounds (Guilhermino et al. 1998). The ChE activity from the hemolymph of the mussel, *Mytilus galloprovincialis*, is inhibited by detergents dodecyl benzyl sulfonate and sodium dodecyl sulfate (Guillhermino et al., 1998).

It is known that ATPase activity can be considered an important index of cellular activity and also as a toxicological tool (Rahman et al., 2000). Barber et al. (2001) have showed that Ca^{2+}-stimulated ATPase of synaptossomes of hen brain was inhibited by OP compounds, such as chlorpyrifos, chlorpyrifos-oxon and phenyl saligenin phosphate, but not parathion and paraoxon. Furthermore, Rahman et al. (2000) demonstrated an inhibition of AChase, 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. However, there are few investigations regarding the effect of OPs and carbamates on the ATPase activity in invertebrates. Da Siva et al. (2003) evaluated the effects *in vitro* of pure and commercial pesticides on Ca^{2+}-activated ATPase and ChE activities in the nervous system of the slug *Phyllocaulis soleiformis*. Both enzymes were insensitive to pure carbofuran (0,01-1 mM), glyphosate (0,01-1mM) and malathion (7.5 - 120μM).

However, carbofuran, in the commercial formulation Furadan 350S®, inhibited ATPase and ChE activities and glyphosate, when used in the commercial of Gliz 480 CS®, inhibited ATPase and increased cholinesterase activity. The commercial formulation Malatol 500 CE® did not alter the enzyme activities (Da Silva et al., 2003). In agreement with our findings, these authors have shown that the ChE present in this slug nervous tissue is insensitive to pesticides in the commercial and pure forms. The insensitivity of ChE activity to pesticides in *H. aspersa* and in *P. solleiformis* reinforces the idea that this enzyme present a different behavior between vertebrates and invertebrates.

In our study, the Ca^{2+}-activated ATPase of the digestive gland of H. aspersa was insensitive to malathion and presented an inhibitory just in the highest concentration tested (1000 μM). However, when compared to equivalent pure compounds, the significant activation promoted by Malatol 500 CE® and the dose-dependent inhibition exerted by Furadan 350S® suggest a possible effect of excipient substances present in the commercial formulations on nucleotide hydrolysis.

In regard to AMP hydrolysis, we observed a slight activation only at 32 nM, the highest concentration tested, which lead us to propose that AMP hydrolysis may be resistant to this chemical aggression. The AMP hydrolysis is catalyzed by an enzyme named 5´-nucleotidase, which is already characterized in several species, such as bacteria, protists, mollusks and vertebrates in general (Bianchi and Spychala, 2003; Tasca et al., 2003; Senger et al., 2004). This enzyme is responsible by the generation of adenosine, a homeostatic regulator and an

important neuromodulator (Cunha et al., 2005). The administration of agonists of purinoceptors, such as adenosine, AMP, ADP and ATP induced different responses in isolated heart, in rectum and esophagus of *H. aspersa*, suggesting that these receptors are important to the physiological function of this mollusk (Knight et al., 1992a; Knight et al., 1992b). However, the effects of agonists to purinoceptors in invertebrates were varied when compared to the effects promoted by these agonists in vertebrates, presenting only some similar effects (Hoyle and Greenberg, 1988). Therefore, considering the importance of AMP hydrolysis for adenosine production and for modulation to the agonist levels for purinoceptors, the insensitivity of AMP hydrolysis could suggest a protective mechanism in order to preserve the physiological functions of this mollusk when exposed to the pesticides.

Our investigation evaluated the relationship between agricultural chemicals, recognized or not as anticholinesterasic agents, and the enzymes responsible for the hydrolysis of the neurotransmitters ATP and acetylcholine in digestive gland of *H. aspersa*. In the present work, the differences between commercial and pure pesticides also open a new question about the contribution of the excipient from the commercial formulation for the toxicity of pesticides.

Acknowledgements

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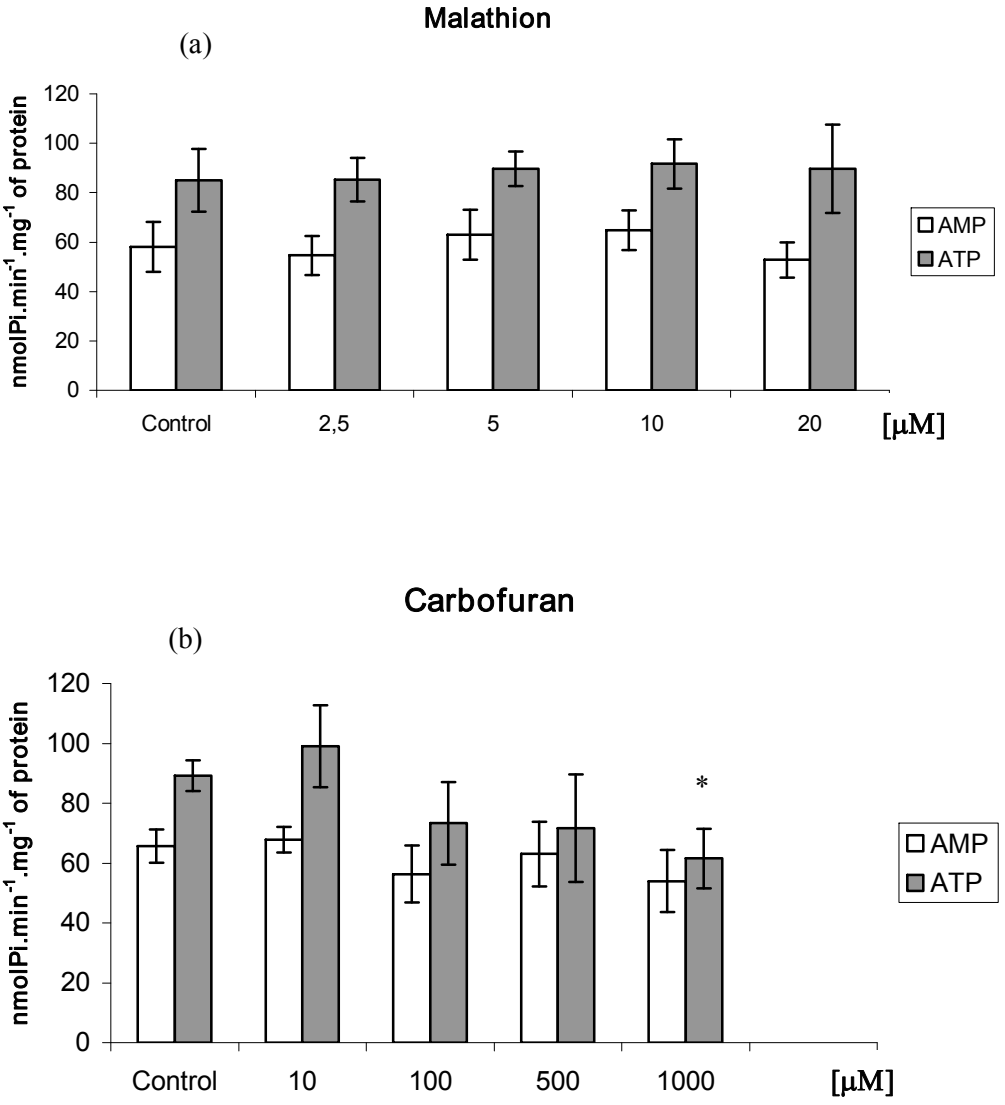
LEGENDS TO FIGURES

Fig.1. *In vitro* effects of pure pesticides (a) Malation and (b) Carbofuran on the Ca^{2+}- activated ATPase and Mg^{2+}- AMP hydrolysis in the digestive gland of *H. aspersa*. Bars represent mean ± S.D. of at least three experiments, each in triplicate. *represents statistical difference by one-way ANOVA (P<0.05, Duncan´s test).

Fig.2. *In vitro* effects of commercial pesticides (a) Malatol 500CE® and (b) Furadan 350S® on the Ca^{2+}- activated ATPase and Mg^{2+}-AMP hydrolysis in the digestive gland of *H. aspersa*. Bars represent mean ± S.D. of at least three experiments, each in triplicate. *represents statistical difference by one-way ANOVA (P<0.05, Duncan´s test).

Fig.3: *In vitro* effects of pure pesticides on the cholinesterase activity in the digestive gland of *H. aspers*:(a)marathon(95%) (b)Malatol (c)carbofuran(96%) and (d)Furadan. Bars represent mean ± S.D. of at least three experiments, each in triplicate. In the control group, chemicals were not added.

Fig.1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100
80
60
40
20
0
Control
2,5
5
10
20
[µM]
AMP
ATP
Carbofuran
(b)
nmolPi.min-1.mg-1 of protein
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100
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60
40
20
0
Control
10
100
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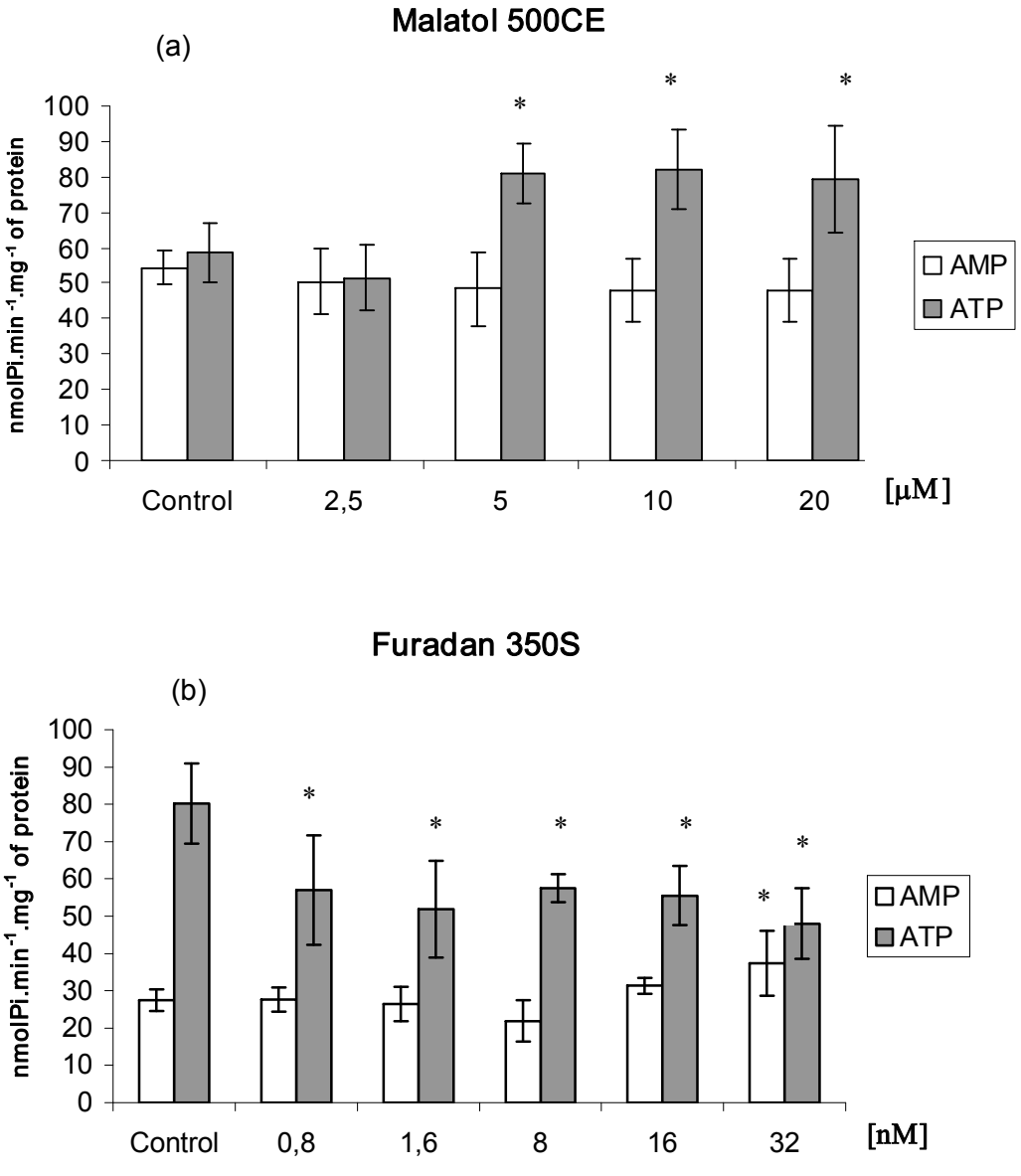
[µM]
*
AMP
ATP

Fig.2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70
60
50
40
30
20
10
0
Control
2,5
5
10
20
[μM]
AMP
ATP
*
Furadan 350S
(b)
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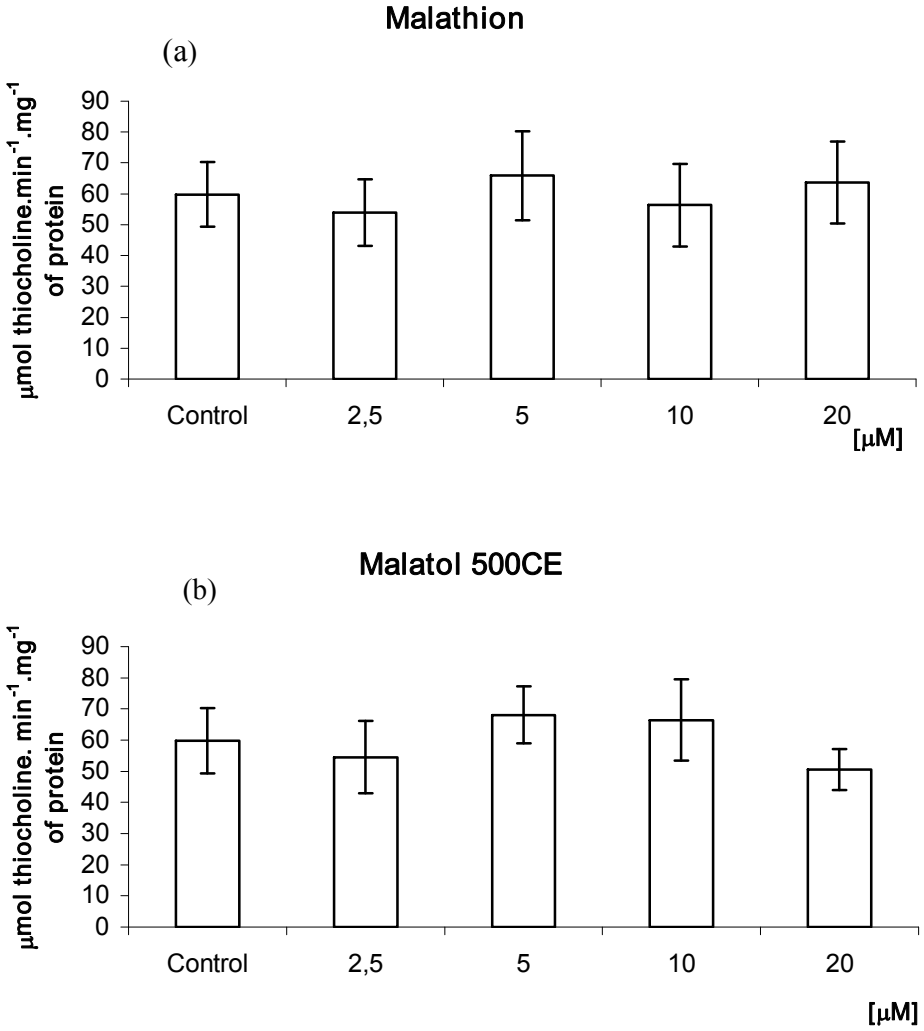
8
16
32
[nM]
AMP
ATP
*

Fig.3.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(a)
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80
70
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Control
2,5
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20
[µM]
Malatol 500CE
(b)
µmol thiocholine. min-1.mg-1 of protein
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20
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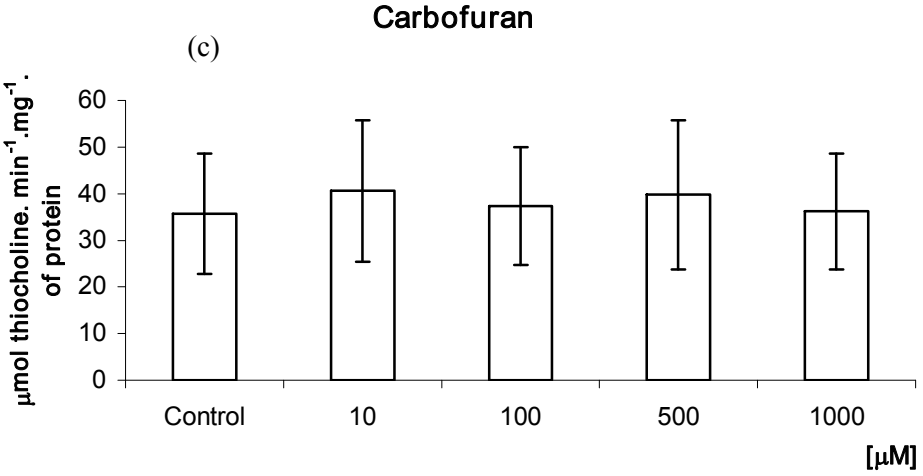
(c)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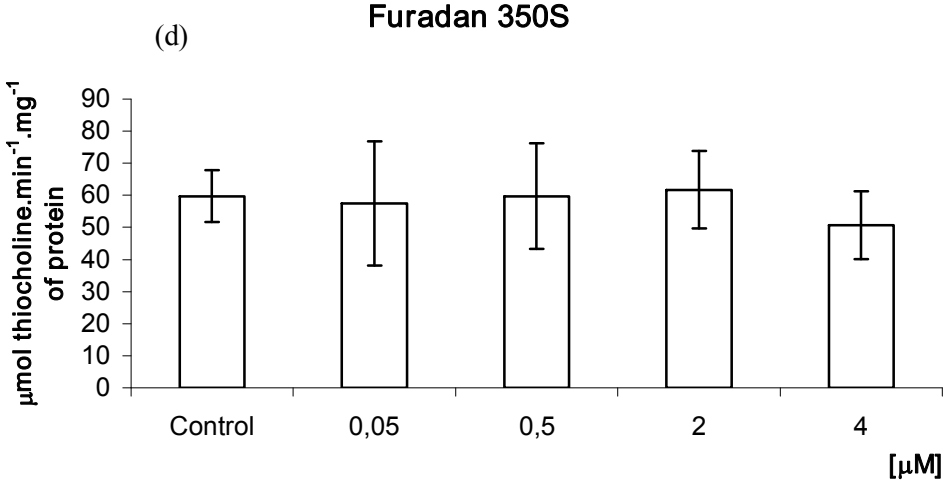
[µM]

(d)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80
70
60
50
40
30
20
10
0
Control
0,05
0,5
2
4
[µM]

Capítulo 3:

Dahm, K. C. S, Rückert, C.,Tonial, E. M.,Bonan, C. D Influence of heavy metals on nucleotidase and cholinesterase activities from digestive gland of *Helix aspersa* (Submetido para o periódico *Comparative Biochemistry and Phisiology Part C: Toxicology and Pharmacology*).

Influence of heavy metals on nucleotidase and cholinesterase activities from digestive gland of Helix aspersa

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Running title: Heavy metals and nucleotidases in mollusks

Abstract

Zinc, copper and cadmium are important environmental contaminants and studies have shown the differences in purinergic and cholinergic systems of invertebrates, such as mollusks. Here we evaluate the effect in vitro of these metals on the ATPase, 5´-nucleotidase and cholinesterase (ChE) activities in the digestive gland of Helix aspersa. Zinc (500 and 1000 μM) promoted a significant decrease on 5´-nucleotidase activity. However, it was unable to promote changes on ATP hydrolysis. Cupper (25 and 50 μM), inhibited significantly the ATPase activity, but did not alter 5´-nucleotidase when compared to control (no metal added). In relation to effects of cadmium, it was possible to observe an inhibitory effect on ATP hydrolysis in the concentrations of 100, 500 and 1000μM and a similar decrease of AMP hydrolysis was observed at 500 and 1000μM . However, there are no significant changes in ChE activity from homogenates of digestive gland of H. aspersa for all metals tested. This study demonstrated that zinc, cadmium and copper affect ATPase and 5´-nucleotidase in digestive gland, but not ChE, suggesting that purinergic system can be a target related to toxicity induced by these metals and a possible indicator of biological impact of exposure to heavy metal contaminants.

Keywords: mollusks, Helix aspersa, nucleotidases, digestive gland, heavy metals, cholinesterase

1.Introduction

Metals occur naturally in the environment, but since the industrial revolution the amount of metals has increased significantly (Hopkin, 1989). There are several anthropogenic sources of metal pollution, such as mining activities, smelting, combustion of fossil fuels, and certain agricultural activities (Bakir et al 1973; Soon 1981; Hopkin 1989; Gummow et al 1991). Traffic pollutants include potentially toxic metals for health, such as cadmium (Cd) and zinc (Zn) (ATSDR, 1994, 1999a,b; Caussy et al., 2003). The dispersion of contaminants is influenced by meteorological conditions, like wind (Piron- Frenet et al., 1994), rainfall, profiles (Bennouma, 1988) or traffic intensity (Garcia and Millan, 1998). The concentrations of metals in the roadside soil are influenced by the same factors (Garcia et al, 1996; Othman et al, 1997; Garcia and Millan, 1998) and by soil parameters.

Due their high potential for accumulation of pollutants (Coughtrey & Martin, 1976; Hopkin, 1989; Jones, 1991), snails and slugs may provide important links in transfer of chemicals from vegetation or plant litter to carnivores. Such transfer along food chains is one of the most important aspects of ecotoxicology. They are able to accumulate bioavailable metals in their organs and they present an important organotropism for the digestive gland and the kidney (Brooks et al, 1992; Berger and Dallinger, 1993; Pihan, 2001).

Van Straalen et al. (1987) suggested that the main differences in the ecophysiology of metals are due to their essentiality versus nonessentiality to organisms. Nutritional metals, like zinc (Zn^{2+}) and copper (Cu^{2+}), are regulated, and xenobiotics, like cadmiun (Cd^{2+}), are accumulated. However, Zn^{2+}, Cu^{2+} and Cd^{2+}

can be potentially toxic to organisms if it occurs at concentrations high enough to induce toxic effects (Harris, 1991; Beyer and Storm, 1995). This rule does not seem to apply to animals with haemocyanin in their oxygen-carrying system, such as isopods and mollusks, which accumulate Cu over a broad range of environmental concentrations (Moser & Wieser, 1979; Hughes et al, 1980, Hopkin, 1990). This situation is complicated even more by the fact that metals can be deposited in intracellular granules. These granules may be insoluble in the gut of predators and may be voided largely intact in the faeces. The metals are no then accumulated in the tissues of the predator (Hopkin, 1989, Nott & Nicolaidou, 1990). In snails, as suggested by Beeby and Richmond(1989), some metals may be deposited additionally in shells. This would mean that the consequences of metal pollution in snails are significantly different for animals feeding on their soft tissue only (e.g. carabids, shrews) from those eating their shells also(e.g. birds).

Several cell types, including neurons and glial cells, can release nucleotides and nucleosides into the extracellular space that can play important roles in physiological and/or pathological conditions (Lucchi et al, 1992; Burnstock, 2004) ATP is the principal agonist of P2 receptors, which are subdivided in two major classes: ionotropic P2X receptors and metabotropic P2Y receptors (Ralevic and Burnstock, 1998). Acetylcholine and ATP are co-released in the nervous terminals, where ATP acts as a co-transmitter or modulator of cholinergic synapses. The inactivation of extracellular ATP to AMP is mediated mainly by a family of ectonucleotidases named NTPDases (nucleoside triphosphate diphosphohydrolase), which are ubiquitous enzymes with a broad phylogenetic

distribution (Zimmermann, 1996). The nucleotide AMP is hydrolyzed to adenosine, an important neuromodulator, by the action of a 5'-nucleotidase (CD73, EC 3.6.1.5), which has an important role together NTPDases in regulating the concentration of extracellular nucleotide and nucleosides to purinoceptors, such as ATP and adenosine (Zimmermann,1996; 2001). Recently, Borges et al. (2004) demonstrated an ATPase activity in nervous ganglia and digestive gland of Helix aspersa with different kinetic characteristics than those previously described (Plesner, 1995; Sarkis et al., 1995; Da Silva et al., 2002).

There is evidence that adenosine can modulate acetylcholine release through inhibitory A_1 or facilitatory A_{2A} receptors (Rebola et al., 2002; Magalhães-Cardoso et al., 2003). The actions of neurotransmitter acetylcholine can be inactivated by the action of an acetylcholinesterase activity (Patocka et al., 2004; Aldunate et al., 2004). However, some authors have shown difficulty in classifying cholinesterase from invertebrates, since these enzymes have apparent affinity for any choline ester, suggesting that they should be classified generally as cholinesterases (ChE), and not as acetylcholinesterases or butyrylcholinesterases (Bocquené et al., 1997; Mora et al., 1999).

Considering that zinc, copper and cadmium are important environmental contaminants and previous studies have shown the differences in purinergic and cholinergic systems of invertebrates, such as mollusks, here we evaluate the effect in vitro of zinc chloride, copper sulfate and cadmium acetate on the ATPase, 5´-nucleotidase and ChE activities in the digestive gland of Helix aspersa.

2. Material and methods

2.1.Experimental model

Adult H. aspersa snails were collected all year long from gardens of metropolitan region of Porto Alegre, RS, Brazil. Animals were maintained in plastic boxes (68x60x22 cm) at 25± 5º C, in a photoperiod of 12h light/12h dark for least 7 days before experiments. Snails were fed ad libitum with lettuce (Latuca sativa) .

2.2 Chemicals

ATP and Trizma base were purchased from Sigma (St. Louis, MO, USA). The kit for ChE activity was obtained from Wiener Lab. The metals Cadmium acetate [$Cd(CH_3COO)_2$; CAS number 5743-04-4], Zinc chloride ($ZnCl_2$, CAS number 7646-85-7) and copper sulfate ($CuSO_4$, CAS number 7758-98-7) were purchased from Merck. All the others reagents were of the highest purity available.

2.3 Membrane preparation of the digestive gland

To isolate the digestive gland, the shell was removed (n=5). The membrane preparations was made according to Barnes et al.(1993). Briefly, the digestive gland was homogenized in 5 volumes (w/v) in a solution of NaCl (0.65%) containing a protease inhibitor (0.1mM PMSF). The homogenate was centrifuged at 1000 X *g* for 10 min, the pellet discarded, and the supernatant centrifuged for 20 min at 40000 X *g*. The pellet was frozen in liquid nitrogen for 10 s, thawed, ressuspended twice and centrifuged for 20 min at 40000 X *g*. The membrane was

prepared fresh daily and maintained at 4ºC throughout the preparation and experiment.

2.4 Enzyme assays

Enzyme activity was assayed in standard reaction medium containing 50 mM Tris- HCl, pH 7.2, 5mM $CaCl_2$ or $MgCl_2$ in a final volume of 200μl. Membranes of digestive gland of H. aspersa (2.5-5μg protein) was added to reaction medium and pre-incubated with the different heavy metals concentrations for 10min at 30ºC. The concentrations tested were: Zn (0,5, 10, 50, and 500μM), Cu (0,5, 1, 10, 25 and 50 μM) and Cd (1, 50, 100, 500 and 1000μM). The reaction was initiated by the addition of substrate (ATP or AMP) at final concentration of 1mM, incubated for 20 min at 30ºC and stopped by addition of 200μl 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 min, and the inorganic phosphate (P_i) released was measured according to 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 TCA were used to correct for non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol of Pi released min^{-1} mg^{-1} of protein. We performed at least four different experiments, each in triplicate.

2.5 ChE assays

Digestive glands were weighted and gently homogenized in 50 volumes (w/v) of a solution of NaCl (0.65%) containing a protease inhibitor (0.1mM PMSF).

The homogenates were centrifuged during 5 min at 1000 X g. The supernatant was pre-incubated for 10 min with metals in the same concentrations used for ATPase assays, in a final volume of 100μl. ChE activity was measured using 7 mM S-butyrylthiocholine iodide as substrate, 50mM phosphate buffer, pH 7.7 and 0.25 mM 5,5'-dithiobis-2-nitrobenzoic (DTNB) (Ellman et al., 1961). The reaction was initiated by the addition of aliquots with 1-3μg of protein. Protein concentrations and incubation time were chosen to assure the linearity of the reaction. Specific activity is expressed as μmol of thiocholine released h^{-1} mg^{-1} of protein. We performed at least four different experiments, each in triplicate.

2.6 Protein determination

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

2.7 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan test, considering a level of significance of 5%. All analyses were performed using the Statistical Package for Social Science (SPSS) software program.

3. Results

The in vitro effects of zinc chloride, copper sulfate and cadmium acetate on ATPase, 5´-nucleotidase and ChE activities from digestive gland of Helix aspersa were evaluated. Zinc chloride promoted a significant decrease of 51% and 53% on 5´-nucleotidase activity at 500 and 1000 μM, respectively (Fig. 1A). However, zinc

chloride was unable to promote changes on ATPase and ChE activity in all concentrations tested (Fig. 1A and 1B).

Cupper sulfate, at 25 and 50 μM, inhibited significantly the ATPase activity (37% and 52%, respectively) of membrane preparations from digestive gland of Helix aspersa (Fig.2A). In contrast, this metal did not alter 5´-nucleotidase and ChE activities at all concentrations tested, when compared to control (no metal added) (Fig. 2A and 2B).

In relation to in vitro effects of cadmium, it was possible to observe an inhibitory effect on ATP hydrolysis in the concentrations of 100, 500 and 1000μM cadmium acetate (41%, 44% and 42%, respectively) (Fig.3A). A similar decrease of AMP hydrolysis was observed at 500 and 1000μM cadmium acetate (36% and 40%, respectively) (Fig. 3A). However, there are no significant changes in ChE activity from homogenate of digestive gland of H. aspersa at all concentrations analyzed (Fig. 3B).

4. Discussion

This study have shown that the in vitro exposure to metals, such as zinc, cooper and cadmium promoted significant changes nucleotidase activities from digestive gland of H. aspersa, but not alter ChE activity. Furthermore, it is important to mention that metals significantly inhibited nucleotidase activities in a dose-dependent manner. The in vitro studies do not reproduce all the complexity of what it happens in nature, but they do give short-term indications on the impact of

the contaminants on key species, which have an important function in the ecosystems due to their biology and distribution, but also to the ease with which they can be bred for experiments (Vaufleury and Pihan,, 2000).

Terrestrial mollusks have been widely used as indicators of environmental pollution (Pihan and de Vaufleury, 2000; Snyman et al., 2002). It is well known that heavy metals are accumulated to very high concentrations in, especially, digestive gland of mollusks (Berger et al., 1993; Marigonez et al., 1998; Blasco and Puppo, 1999, Vaufleury and Pihan, 2000). The effect of accumulated heavy metals and other pollutants on the molluscan digestive gland cell structure, and the possible use of such cellular changes as biomarkers of exposure to xenobiotics, have been investigated (Vega et al., 1989, Marigomez et al., 1998, Etxeberria et al., 1994). In the case of Helix, the literature is scarce, since very few researchers have analyzed the digestive gland separately from the other soft tissues (Moser and Wieser, 1979; Gomot and Pihan, 1997). Snyman et al.(2005) demonstrated that in snails exposed to 80 and 240 $\mu g.g^{-1}$ copper oxychloride, copper was strongly accumulated in digestive gland of individuals to levels far greater than added to the food. After 6 weeks of exposure to copper oxychloride, the digestive gland has significantly higher copper concentrations that the other parts of the body (Snyman et al., 2005). Furthermore, measurable changes in epithelium cell height and area of digestive gland occur as a result of copper accumulation and subsequent detoxification process (Snyman et al.,2005).

Dallinger et al. (1993) considered snails as macroconcentrators of Zn and Cd. However, Laskowski and Hopkin (1996) have shown that Zn was not

accumulated in snail tissues to concentrations exceeding its levels in food, but Cu and Cd were clearly concentrated in soft tissues in comparison to their concentrations in food. Studies have shown that cadmium is nonessential metal and is ten times more toxic than zinc, a essential metal involved in important metabolic process (Walker et al., 1986; Jackson, 1989; Depledge et al., 1994). In our experiments, cadmium, zinc and copper promoted differential effects on ATPase and/or 5´-nucleotidase activities in digestive gland of H.aspersa. Considering the essentiality and non-essentiality of these metals and the diversity of morphological and metabolic changes induced by these compounds, these findings suggest that nucleotidase pathway can be a sensitive bioindicator to detect these pollutants. However, ChE activity is not altered by any metal at all concentrations tested. Studies have shown that ChE is widely sensitive to many chemicals that inhibit this enzyme, such as detergents and metallic compounds (Guilhermino et al. 1998, Perez et al, 2004). In agreement with our results, there are some reports questioning the utilization of this parameter for invertebrates, since that a large number of animals demonstrate ChE insensitivity to agricultural chemicals. The existence of many isoenzymes with different levels of inhibition by these compounds can impair the utilization of this enzyme as a biomarker in invertebrates (Bocquené at al., 1997).

Extracellular nucleotides are important messenger both in physiological as well in pathological conditions. After its release in the synaptic cleft, ATP can be degraded to ADP, AMP and adenosine. Adenosine has a strong neuroprotective effect, contrasting with the excitatory effect triggered by ATP (Di virgilio, 2000; Kato et al., 2004). Studies have demonstrated that purines can induce cytotoxic effect

(Chow et al., 1997; Inoue, 2002). The administration of agonists of purinoceptors, such as adenosine, AMP, ADP and ATP induced different responses in isolated heart, in rectum and esophagus of H. aspersa, suggesting that these receptors are important to the physiological function of this mollusk (Knight et al., 1992a; Knight et al., 1992b). However, the effects of agonists to purinoceptors in invertebrates were varied when compared to the effects promoted by these agonists in vertebrates, presenting only some similar effects (Hoyle and Greenberg, 1988). The effect that nucleotides have on cells depends on the extracellular catabolism mediated by nucleotidases, which regulate the concentration of ATP/adenosine and the response mediated by P2/P1 receptors, respectively. It is possible to hypothesize that changes in the nucleotidase activities induced by the exposure to heavy metals can promote alterations in the extracellular nucleotide concentrations. The impairment in the control of nucleotide levels may evoke an imbalance of purinergic neurotransmission, affecting nucleotide-mediated signal transduction, which could contribute to neurotoxic effects promoted by these toxicants. Furthermore, the measurement of biochemical parameters, such ATPase and 5´-nucleotidase activities in digestive gland of H. aspersa could be used as biomarkers of exposure to these metals.

Based on the data presented herein, this study demonstrated that zinc, cadmium and copper affect ATPase and 5´-nucleotidase in digestive gland, but not ChE, suggesting that purinergic system can be a target related to toxicity induced by these metals and a possible indicator of biological impact of exposure to heavy metal contaminants.

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Legends to Figures:

Fig.1: In vitro effects of zinc (a) on ATP, AMP hydrolysis and (b) cholinesterase activity in the digestive gland of H. aspersa. Bars represent mean ± S.D. of at least three experiments, each in triplicate. *represents statistical difference by one-way ANOVA (P<0.05, Duncan´s test). In the control group, chemicals were not added.

Fig.2: In vitro effects of copper (a) on ATP, AMP hydrolysis and (b) cholinesterase activity in the digestive gland of H. aspersa. Bars represent mean ± S.D. of at least three experiments, each in triplicate. *represents statistical difference by one-way ANOVA (P<0.05, Duncan´s test). In the control group, chemicals were not added.

Fig.3: In vitro effects of cadmium (a) on ATP, AMP hydrolysis and (b) the cholinesterase activity in the digestive gland of H. aspersa. Bars represent mean ± S.D. of at least three experiments, each in triplicate. *represents statistical difference by one-way ANOVA (P<0.05, Duncan´s test). In the control group, chemicals were not added.

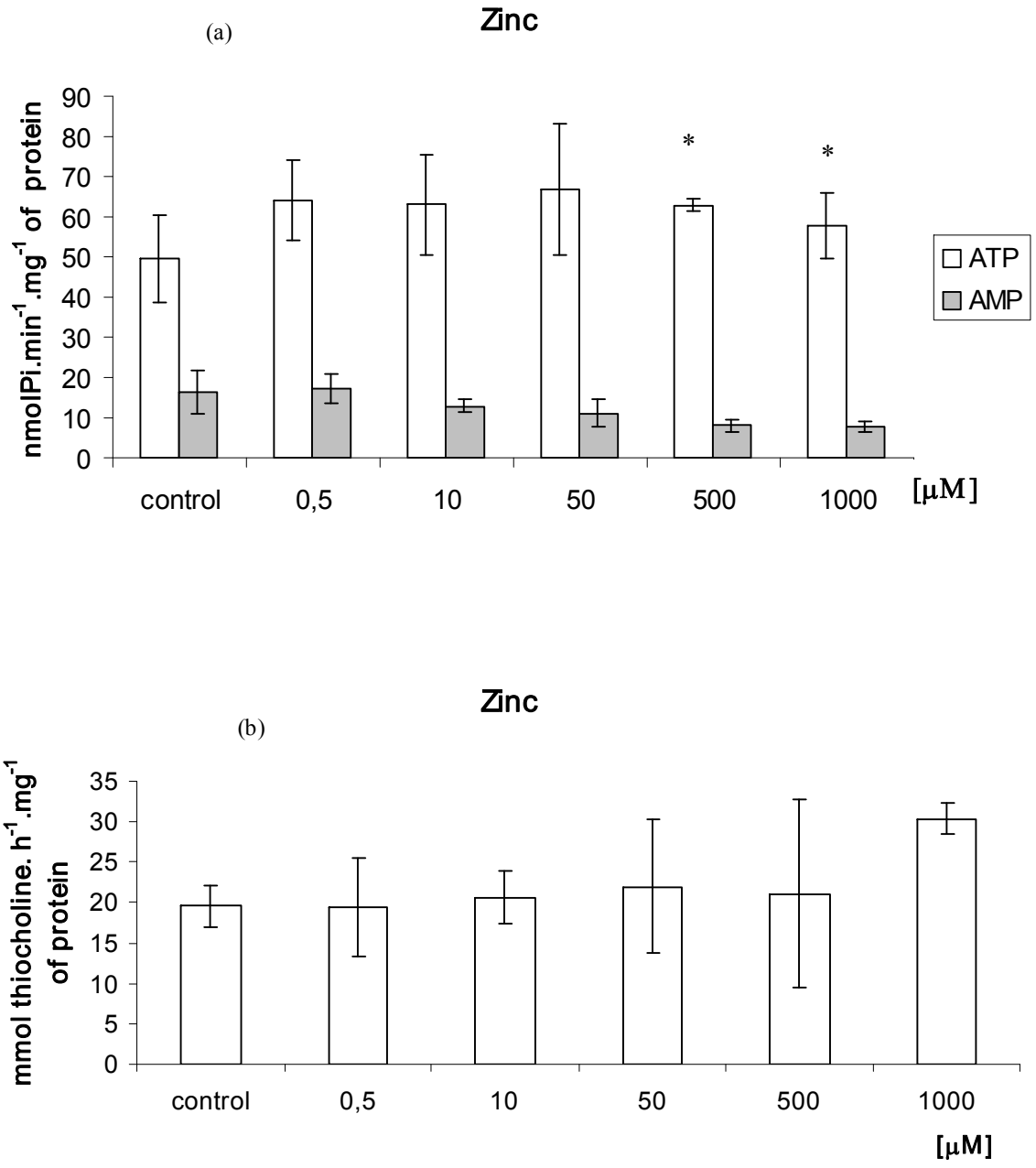
(a)
Zinc
nmolPi.min-1.mg-1 of protein
90
80
70
60
50
40
30
20
10
0
control
0,5
10
50
500
1000
[μM]
*
*
ATP
AMP
(b)
Zinc
mmol thiocholine. h-1.mg-1 of protein
35
30
25
20
15
10
5
0
control
0,5
10
50
500
1000
[μM]

Fig. 1

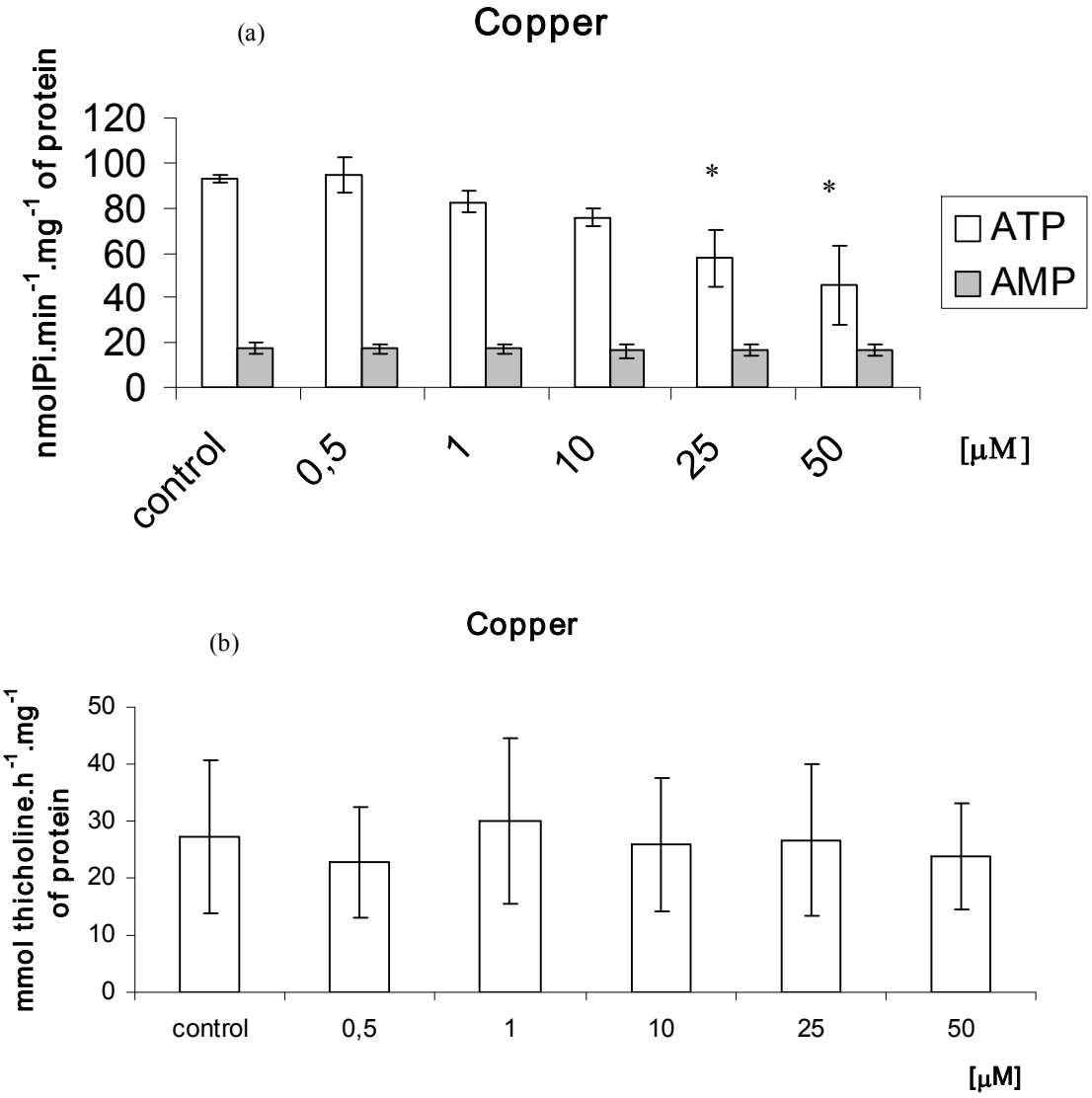
(a)
Copper
nmolPi.min-1.mg-1 of protein
120
100
80
60
40
20
0
*
*
ATP
AMP
control
0,5
1
10
25
50
[µM]
(b)
Copper
mmol thicholine.h-1.mg-1 of protein
50
40
30
20
10
0
control
0,5
1
10
25
50
[µM]

Fig. 2

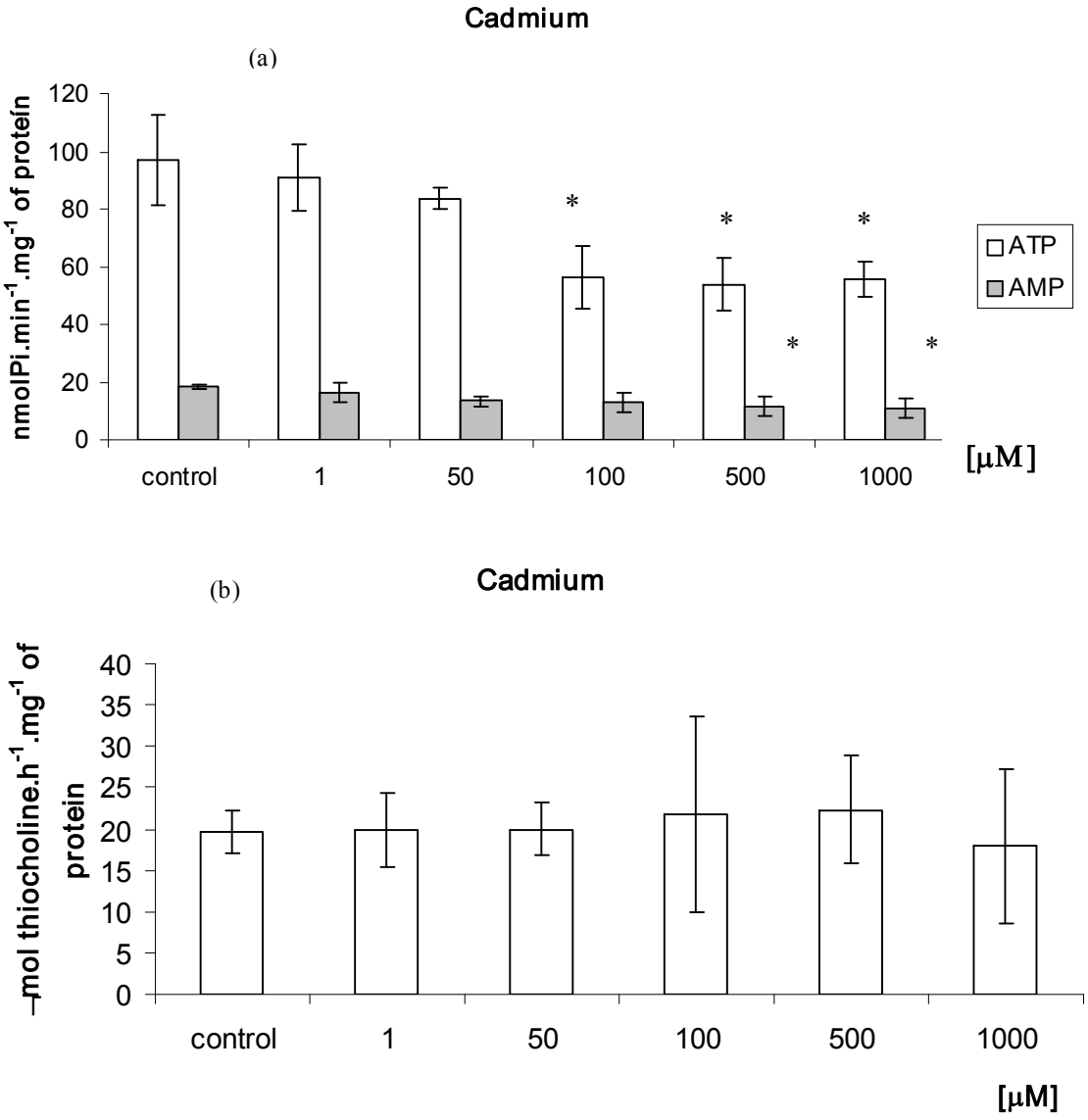
Cadmium
(a)
nmolPi.min-1.mg-1 of protein
120
100
80
60
40
20
0
control
1
50
100
500
1000
[µM]
ATP
AMP
*
(b)
Cadmium
µmol thiocholine.h-1.mg-1 of protein
40
35
30
25
20
15
10
5
0
control
1
50
100
500
1000
[µM]

Fig. 3

4. DISCUSSÃO GERAL

Estudos que tratam da hidrólise de nucleotídeos vêm sendo realizados no Filo Mollusca. No sistema nervoso, vários nucleotídeos, entre eles o ATP e o AMP atuam como moléculas sinalizadoras extracelulares. Eles podem funcionar na neurotransmissão, na co-transmissão, na modulação e no desenvolvimento neural, atuando não só no sistema nervoso central e periférico, mas também em músculos lisos e estriados e em células gliais (RALEVIC & BURNSTOCK, 1998). O estudo da participação do sistema purinérgico nas vias de transmissão nervosa em vertebrados encontra-se bem desenvolvido, embora alguns mecanismos de modulação ainda não estejam bem caracterizados. Em invertebrados, em especial em moluscos, estudos demonstram a participação de nucleotídeos púricos principalmente na contração muscular, demonstrando diversas dificuldades em estabelecer padrões de atuação destes, bem como o tipo de receptor em que estes atuam. As primeiras evidências indicando o ATP como neurotransmissor surgiram dos estudos de HOLTON & HOLTON (1954) e HOLTON (1959), que demonstraram a liberação desta substância a partir de nervos sensoriais. Mais tarde, BURNSTOCK (1972) propôs que o ATP é o neurotransmissor liberado de nervos não-colinérgicos e não-adrenérgicos. Então, foi proposto que, além da transmissão colinérgica e noradrenérgica, existe no sistema nervoso autônomo a transmissão purinérgica, onde o ATP é o principal neurotransmissor (BURNSTOCK, 1972).

As ações de purinas em invertebrados têm sido investigadas. Em *Helix aspersa* e *Arion ater*, efeitos de nucleotídeos e nucleosídeos da adenina no coração destas espécies foram avaliados (KNIGHT et al., 1992a). Estes autores

demonstraram que a adenosina, AMP, ADP e ATP (acima de 100μM) produziram tanto excitação como inibição no coração isolado destes moluscos, sugerindo que receptores ativados por compostos purínicos neste tecido apresentam ações que não podem ser comparadas com as de vertebrados (BURNSTOCK, 1978; RALEVIC e BURNSTOCK, 1998). A administração de AMP, ADP e ATP em *Helix aspersa* produziu contrações de forma concentração-dependente no reto e esôfago, sugerindo que purinoreceptores são importantes para estas respostas fisiológicas em moluscos (KNIGHT et al., 1992b). HOYLE e GREENBERG (1988) analisaram diversas espécies pertencentes a diferentes filos de invertebrados e observaram que efeitos de agonistas e antagonistas de purinoreceptores são bastante variados, apresentando poucas similaridades com as ações observadas em vertebrados. Além disso, condições experimentais, estação do ano e idade influenciam a sensibilidade e a resposta farmacológica de órgãos musculares e músculos isolados de moluscos (CHAPMAN et al., 1984; HOLYE e GREENBERG, 1988). Recentemente, nosso laboratório demonstrou a presença de uma atividade nucleotidásica com diferentes propriedades cinéticas nos gânglios nervosos e na glândula digestiva de *Helix aspersa (*BORGES et al.,2004). Na glândula digestiva, foi observada uma atividade de hidrólise de ATP e de AMP na presença de 5 mM de cálcio e magnésio, respectivamente. Estas atividades enzimáticas apresentaram ampla especificidade de substrato, sendo capazes de hidrolisar nucleotídeos trifosfatados e monofosfatados. Além disso, esta atividade em glândula digestiva sofreu uma inibição significativa na presença de azida sódica e

N-etilmaleimida, o que sugere a possível participação da atividade NTPDásica neste tecido.

A glândula digestiva dos moluscos realiza múltiplas funções, tais como armazenamento energético e transformações metabólicas (GIARD et al., 1995). Além disso, diversos autores têm demonstrado que a glândula digestiva é o órgão mais importante para o acúmulo de poluentes em moluscos (MARIGOMEZ et al., 1998; BLASCO e PUPPO, 1999). Alterações histológicas e morfológicas na glândula digestiva foram observadas após exposição a poluentes, o que pode influenciar na capacidade de acumulação deste órgão na presença de agentes estressores ambientais. Estas mudanças na estrutura das células da glândula digestiva têm sido postuladas como possíveis bioindicadores de exposição a poluentes, como metais pesados e pesticidas (SNYMAN et al., 2005). Por esta razão, neste estudo nós avaliamos parâmetros bioquímicos relacionados ao sistema purinérgico e colinérgico após a exposição *in vitro* a metais pesados e pesticidas, com o objetivo de melhor compreender a toxicidade induzida por estes poluentes na glândula digestiva.

No primeiro capítulo da dissertação, foi realizada uma revisão sobre os principais aspectos relacionados com os sistema purinérgico e colinérgico de moluscos e sua susceptibilidade a agentes tóxicos, como carbamatos e organofosforados.

No segundo capítulo desta dissertação, avaliamos o efeito in vitro entre pesticidas amplamente utilizados na agricultura e enzimas responsáveis pela hidrólise do ATP, AMP e acetilcolina em glândula digestiva de *H. aspersa.* As diferenças encontradas entre as formas puras e comerciais destes compostos

sugerem uma contribuição do excipiente na formulação do pesticida comercial sobre a toxicidade destes químicos. De acordo com nossos resultados, é questionável a utilização da ChE como bioindicador, visto que diversos estudos têm demonstrado a insensibilidade da ChE a carbamatos e organofosforados em diversos grupos de invertebrados (BOCQUENÉ at al., 1997). Da Silva et al. (2003) demonstraram que as atividades ATPásica e colinesterásica presentes nos gânglios nervosos de *P. soleiformis* são insensíveis a agrotóxicos organofosforados, carbamatos e ao herbicida glifosato. A ação inibitória de organofosforados e carbamatos sobre a atividade colinesterásica encontra-se estabelecida para vertebrados e o efeito destes compostos sobre a atividade ATPásica tem sido pouco investigada (RAHMAN et al., 2000). A verificação da insensibilidade das atividades ATPásica e colinesterásica de *P. soleiformis* aos agrotóxicos alia-se, tanto a algumas investigações que encontraram insensibilidade em outros moluscos, como às diferenças encontradas na atuação e nos mecanismos de regulação dos níveis de purinas (DA SILVA et al., 2003). Entretanto, é possível observar que existem diferenças dentro do Filo Mollusca com relação a sensibilidade a estes compostos, desde que nossos resultados indicaram significativas alterações nas atividades nucleotidásicas tanto na presença das formas puras quanto comerciais destes pesticidas.

No terceiro capítulo, avaliamos os efeitos dos metais pesados, desde que os moluscos são bastante expostos a este grupo de poluentes. Nossos resultados demonstraram diferenças entre as atividades ATPásica e AMPásica na presença de metais pesados, como zinco, cobre e cádmio. O prejuízo no controle dos níveis dos nucleotídeos pode provocar uma alteração na neurotranmissão purinérgica,

que poderia contribuir para os efeitos tóxicos promovidos por estes compostos. Os efeitos observados reforçam a idéia de que o sistema purinérgico pode ser uma ferramenta relacionada à toxicidade induzida por contaminantes ambientais, principalmente pesticidas e metais pesados e funcionar como um possível bioindicador de contaminação ambiental.

As diferenças encontradas em nossos resultados podem ser devido à diferenciação dos receptores purinérgicos ou do sistema de degradação de nucleotídeos púricos destes animais. Certamente, estudos futuros envolvendo purificação enzimática, imunolocalização e biologia molecular contribuirão para determinar a que nível se encontra estas diferenças e, portanto, caracterizar as peculiaridades bioquímicas em moluscos.

5. CONCLUSÃO FINAL

Com os resultados apresentados nesta Dissertação, nós podemos concluir que as nucleotidases, tais como a NTPDase e a 5'- nucleotidase são enzimas sensíveis a ação de metais pesados e pesticidas e parecem ser possíveis alvos envolvidos na toxicidade mediada por estes poluentes. Além disso, é questionável a utilização da colinesterase como um biomarcador de contaminação ambiental, uma vez que diversos trabalhos têm demonstrado a insensibilidade desta enzima, em invertebrados, na presença destes agentes tóxicos.

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