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EFEITO DE PESTICIDAS E DE METAIS PESADOS NA ATIVIDADE DE ECTONUCLEOTIDASES EM SISTEMA NERVOSO CENTRAL DE ZEBRAFISH (*Danio rerio*)

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Toda a nossa ciência, comparada com a realidade,

é primitiva e infantil – e, no entanto,

é a coisa mais preciosa que temos.

Albert Einstein

A palavra do bioquímico pode não ser a última na descrição da vida,

mas sem sua ajuda, a última palavra nunca será dita.

Frederick G. Hopkins

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Sumário

Resumo

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 contaminação de efluentes hídricos por metais pesados e pesticidas tornaram-se inevitáveis e, conseqüentemente, efeitos adversos ocorrem em seres humanos, plantas e animais, onde um dos grupos mais atingidos são os peixes. O zebrafish (*Danio rerio*) é um modelo experimental consolidado em diversas áreas da ciência, como a neurociências e toxicologia. O genoma desta espécie já esta quase todo seqüenciado e estudos demonstraram que muitos genes deste peixe são similares ao de mamíferos, incluindo a espécie humana. Evidências acumuladas por muitas décadas demonstram que nucleotídeos e nucleosídeos, principalmente o ATP e a adenosina exercem diversos efeitos sinalizadores no espaço extracelular. No sistema nervoso, o neurotransmissor ATP é armazenado de forma vesicular e liberado na fenda sináptica onde pode agir sobre receptores específicos localizados membrana celular, denominados receptores purinérgicos do tipo P2. Estes receptores são subdivididos em receptores ionotrópicos P2X e receptores metabotrópicos P2Y. A inativação do sinal mediado pelo ATP extracelular é mediada por uma família de enzimas denominadas ectonucleotidases. Entre este grupo de enzimas destacam-se a NTPDase (nucleosídeo trifosfato difosfoidrolase) e a ecto-5'-nucleotidase. Após sofrer catabolismo pelas ectonucleotidases, o neurotransmissor ATP é hidrolisado ao neuromodulador adenosina. Este nucleosídeo exerce seus efeitos através dos receptores metabotrópicos denominados purinoceptores P1. Estudos do nosso laboratório demonstraram a presença de ectonucleotidases como a NTPDase e a ecto-5'-nucleotidase no SNC de zebrafish. Além disso já foram citados na literatura que esta espécie apresenta purinoceptores do tipo P2X e P2Y. Neste estudo, nós investigamos o efeito *in vitro* e *in vivo* de metais pesados na atividade de ectonucleotidases em SNC de zebrafish. A exposição *in vitro* ao mercúrio inibiu a hidrólise de ATP, ADP de uma forma acompetitiva e a hidrólise do AMP de um modo não-competitivo. O chumbo inibiu in vitro a hidrólise somente do ATP de um modo acompetitivo. A exposição *in vivo* ao mercúrio ou chumbo (20 μg/L, por 24h, 96h ou 30 dias) causou um efeito diferencial na hidrólise dos nucleotídeos. A exposição ao mercúrio durante 96h causou uma inibição significativa na hidrólise do ATP, ADP e AMP. Interessantemente, após 30 dias de exposição ao mercúrio a hidrólise do ATP voltou aos níveis do controle, a hidrólise do ADP aumentou, e a hidrólise do AMP continuou inibida, quando comparada ao controle. Após a exposição ao chumbo durante 96h, apenas a hidrólise do ATP foi inibida. Após a exposição crônica durante 30 dias ao chumbo, uma inibição significativa na hidrólise de ATP, ADP e AMP foi observada. Nós também investigamos o efeito de carbamatos e organofosforados sobre as atividades ectonucleotidásicas em SNC de zebrafish. Após a exposição *in vivo* ao carbofuran por sete dias na concentração 50 e 500 μg/L, foi observada uma diminuição significativa somente na hidrólise de ADP em ambas concentrações testadas. O malathion, na concentração de 500 μg/L, foi capaz de inibir a hidrólise de ADP e AMP. Para verificar se o carbofuran e o malathion agem diretamente nas enzimas, foram realizados experimentos *in vitro* com estes pesticidas. O carbofuran e o malathion inibiram a hidrólise de ATP e ADP, mas não tiveram efeito na hidrólise de AMP. O carbofuran inibiu a hidrólise do ATP e ADP de uma forma acompetitiva. O malathion inibiu a hidrólise do ATP de uma forma não competitiva e a hidrólise do ADP de uma forma acompetitiva. Nossos resultados demonstram que tanto metais pesados quanto pesticidas afetam a atividade das ectonucleotidases em SNC de zebrafish, sugerindo que o sistema purinérgico pode ser um alvo relacionado à neurotoxicidade destes contaminantes ambientais.

Abstract

Currently, considering that our economy depends on industrialized products and productive harvests, industrials and agricultural dejects have become a relevant problem for human and environmental health. The contamination of hidric effluents by heavy metals and pesticides has become eminent and, consequently, adverse effects are inevitable in humans, plants and animals, which fish are one of the most affected groups. Zebrafish (*Danio rerio*) is a consolidated model system in many research areas, including neuroscience and toxicology. The genome of this specie is almost sequenced and studies have shown that many genes of this specie are similar to mammals, including the human specie. Evidence has shown that nucleotides and nucleosides, mainly ATP and adenosine, exert extracellular signaling effects. In the nervous system, the neurotransmitter ATP is stored in vesicles and released in the synaptic cleft, where can act on specific cellular membrane receptors called purinergic P2 receptors. These receptors are subdivided on ionotropic P2X receptors and metabotropic P2Y receptors. The inactivation of ATP extracellular signaling is mediated by a family of enzymes named ectonucleotidases. This group of enzymes includes NTPDase (nucleoside triphosphate diphosphohydrolase) and ecto-5'-nucleotidase. After the catabolism promoted by ectonucleotidases, the neurotransmitter ATP is hydrolyzed to the neuromodulator adenosine. This nucleoside exerts its effects by the activation of P1 metabotropic receptors. Studies from our laboratory have demonstrated the presence of NTPDase and ecto-5'-nucleotidase in CNS of zebrafish. Furthermore, P2X and P2Y purinoceptors already are described in this specie. Here we investigated the *in vitro* and *in vivo* effect of heavy metals on ectonucleotidases in CNS of zebrafish. The *in vitro* exposure to mercury inhibited ATP and ADP hydrolysis by an uncompetitive manner and AMP hydrolysis by noncompetitive manner. Lead inhibited only the *in vitro* ATP hydrolysis by a uncompetitive manner. *In vivo* exposure to mercury and lead (20 μg/L, for 24h, 96h or30 days) caused a differential effect on nucleotide hydrolysis. Mercury exposure during 96h caused a significant inhibition on ATP, ADP and AMP hydrolysis. Interestingly, after 30 days of mercury exposure, the ATP hydrolysis returned to control levels, ADP hydrolysis strongly increased and AMP hydrolysis remaining inhibited, when compared to control group. After 96h of lead exposure, only the ATP hydrolysis was inhibited. After the chronic lead exposure during 30 days, a significant inhibition in ATP, ADP and AMP was observed. We also investigated the effect of carbamates and organophosphate pesticides on ectonucleotidase activities in CNS of zebrafish. After the *in vivo* exposure to carbofuran during seven days to 50 and 500 μg/L, only an inhibition of ADP hydrolysis was observed in both concentrations tested. Malathion, in the concentration of 500 μg/L, was able to inhibit ADP and AMP hydrolysis. In order to verify if carbofuran and malathion act directly in the enzymes, *in vitro* experiments were performed with the pesticides. Carbofuran and malathion inhibited ATP and ADP hydrolysis, but no effect on AMP hydrolysis was observed. Carbofuran inhibited ATP and ADP by an uncompetitive manner. Malathion inhibited ATP hydrolysis by a competitive manner and ADP hydrolysis by an uncompetitive manner. Our results have shown that heavy metals and pesticides affect the ectonucleotidase activities in CNS of zebrafish, suggesting that the purinergic system can be a target related to the neurotoxic effects of these environmental contaminants.

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Lista de Abreviaturas

ADP - adenosina 5'-difosfato

AMP - adenosina 5'-monofosfato

AMPc- adenosina 3',5'- monofosfato cíclico

ATP - adenosina 5'-trifosfato

CD39 - antígeno de ativação celular linfóide

CD73 - proteína de superficie de linfócitos

DAG - diacilglicerol

ESTs - "expressed sequence tags" (genes expressos)

GABA - acido γ- aminobutírico

IP3 - inositol 1,4,5-trifosfato

5'-NC - 5'-nucleotidase

NTPDase -nucleosídeo trifosfato difosfoidrolase

PKA- proteína quinase A (proteína quinase dependente de AMP cíclico)

SNC - sistema nervoso central

1. INTRODUÇÃO

1.1 Problemática Ambiental

Devemos aceitar que a população humana na Terra continuará a aumentar e que este aumento terá conseqüências danosas para o meio ambiente. A população humana atual está consumindo os recursos mais rapidamente do que eles são regenerados pela biosfera e, ao mesmo tempo, despejando tantos dejetos que a qualidade do meio ambiente se deteriora numa taxa alarmante (RICKLEFS, 1996). 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). A contaminação dos alimentos, água e ar com estes poluentes tornaram-se inevitáveis e, conseqüentemente, efeitos adversos ocorrem em seres humanos, plantas e animais, onde um dos grupos mais atingidos são os peixes (MACHADO et al. 2002).

1.2. Metais Pesados

A exposição aos metais pesados vem ocorrendo ao longo da história de nossa civilização, a qual tem se ampliado muito com a atividade agrícola e industrial. Os metais pesados possuem diversos mecanismos de ação tóxica, sendo o principal a sua ação sobre 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 dano de organelas celulares como mitocôndrias, lisossomas e microtúbulos (FERRER, 2003).

Devido ao amplo espectro de 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). Neste grupo de compostos, o chumbo e o mercúrio são os que apresentam os efeitos mais tóxicos ao sistema nervoso central (DENNY & ATCHINSON, 1996; ANTONIO et al., 2003; ZHENG et al., 2003).

A neurotoxicidade dos metais pesados, chumbo e mercúrio, está relacionada, pelo menos em parte, à sua interferência na homeostasia do cálcio intracelular e na dinâmica de neurotransmissores em sinapses centrais e periféricas (COOPER & MANALIS, 1983; COOPER et al., 1984; SUSZKIW et al., 1984; ERDELYI et al., 1998). Além da neurotransmissão, os metais podem alterar a expressão gênica, causar danos via radicais livres e alterar membranas celulares (JEHAN & MOTLAG, 1995; ADONAYLO & OTEIZA, 1999; CARVAN III et al., 2000a; BERNTSSEN et al., 2003).

Diversos sistemas de neurotransmissão podem ser afetados pelo chumbo e mercúrio, tais como: colinérgico (SCHIMD et al., 1974; LUO & BERMAN, 1997; MIRZOIAN & LUETJE, 2002; ANTONIO et al., 2003), dopaminérgico

(KOMULAINEN & TUOMISTO, 1982; RADEMACHER et al., 2001; FARO et al., 2001), serotoninérgico (OUDAR ET AL., 1989; KALA & JADHAV, 1995), glutamatérgico (BROOKES, 1992; ALBRECHT & MATYJA, 1996; NIHEI & GUILARTE, 2001; SOARES et al., 2003), noradrenégico (RONNBACK & HANSSON, 1992) e gabaérgico (BONDY & AGRAWAL, 1980; BRAGA et al., 1999; LASLEY & GILBERT, 2002).

Existem poucos estudos avaliando a toxicidade do chumbo e mercúrio sobre o sistema purinérgico em sistema nervoso central. OLIVEIRA e colaboradores (1994) verificaram o efeito *in vitro* e *in vivo* do cloreto de mercúrio na atividade da NTPDase, enzima envolvida na degradação de nucleotídeos extracelulares, em sinaptossomas de córtex cerebral de ratos em desenvolvimento. O estudo demonstrou uma inibição significativa *in vitro* do cloreto de mercúrio, não observando alterações nesta atividade enzimática após tratamento *in vivo*. MORETTO e colaboradores (2004) avaliaram o efeito do tratamento subcrônico *in vivo* com cloreto de mercúrio na atividade da NTPDase, 5'-nucleotidase e acetilcolinesterase de córtex cerebral de ratos. Os dados mostraram um aumento significativo na atividade da NTPDase e acetilcolinesterase, mas não foram observadas mudanças significativas na atividade da 5'-nucleotidase.

1.3. Pesticidas

Pesticidas são substâncias intencionalmente introduzidas no ambiente para controlar pragas como insetos, nematódeos e ácaros. Estas substâncias se tornaram muito difundidas na agricultura moderna com intuito de 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; SAGLIO et al., 1996; STEINBERG et al., 1995). Os pesticidas carbamatos inibem a atividade da acetilcolinesterase reversivelmente, já o mecanismo de inibição dos organofosforados é irreversível (WALKER, 2001). A inibição da acetilcolinesterase vem sendo utilizada como um marcador específico para a indicação da exposição de espécies não-alvos a carbamatos e organofosforados (GUILHERMINO et al., 1998; GRUBER & MUNN, 1998). Entretanto, outros efeitos necessitam ser avaliados para completamente compreender os efeitos neurotóxicos destes contaminantes (SALVI et al., 2003).

Interessantemente, poucos estudos relacionando à toxicidade de pesticidas em sistemas de neurotransmissão não-colinérgicos foram realizados até o momento. GUPTA e colaboradores (1984) examinaram o efeito tóxico de múltiplas doses de carbofuran e metil-paration nas concentrações de GABA, adrenalina, noradrenalina, dopamina e serotonina no cérebro. O tratamento com pesticidas provocou um aumento significativo nos níveis destes neurotransmissores, sugerindo um efeito não-específico da toxicidade mediada pelos pesticidas testados. BRETRAUD e colaboradores (2002) verificaram o efeito bioquímico e comportamental do carbofuran em sistema nervoso central de

goldfish. Este estudo avaliou os níveis de catecolaminas, os quais aumentaram significativamente após a exposição ao carbofuran. Alterações comportamentais também foram sensíveis à ação do carbofuran.

Recentemente, nosso laboratório desenvolveu um estudo avaliando o efeito de carbamatos e organofosforados sobre o sistema de neurotransmissão purinérgica. 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*. Interessantemente, apenas as formas comercias, e não as formas puras, inibiram significativamente a atividade das duas enzimas.

1.4. Zebrafish ou Peixe-Zebra (*Danio rerio*)

O Zebrafish ou peixe-zebra (*Danio rerio*) é um pequeno teleósteo (3-4 cm) da família Cyprinidae, sendo uma espécie bastante conhecida pelos aquariofilistas (Fig 1). Este peixe possui muitas características que o tornam um modelo experimental bastante atrativo em muitas áreas científicas, das quais podemos citar: pequeno custo e espaço requerido para manutenção, rápido desenvolvimento e ciclo biológico, grande prole, embriões translúcidos e suscetíveis à manipulação e microinjeção (LELE & KRONE, 1996). Foi criada uma rede de informações na web sobre o zebrafish (http://zfin.org), na qual laboratórios do mundo inteiro podem depositar informações sobre esta espécie (SPRAGUE et al., 2003). Além do mais, existe um excelente, compreensivo e freqüentemente atualizado manual de manutenção e controle das condições em laboratórios sobre este peixe (WESTERFIELD, 2000).

Fig.1: Zebrafish (*Danio rerio*)

O pioneiro a estudar esta espécie foi George Streisinger que, no final da década de 60, utilizou técnicas de análise mutacional para estudar o desenvolvimento embrionário do zebrafish (GRUNWALD & EISEN, 2002). Atualmente, este peixe é um modelo experimental consolidado em diversas áreas da ciência, tais como: genética e genômica, desenvolvimento, teratologia, comportamento, toxicologia e neurociências (VASCOTTO et al., 1997). O interesse pela espécie pode ser observado pelo número crescente de laboratórios que tem utilizado este teleósteo como um modelo experimental em suas pesquisas (SPRAGUE et al., 2001).

Nos últimos anos, houve um progresso considerável na genética e genômica do zebrafish. Em 2001, o Instituto Sanger começou o seqüenciamento do genoma total desta espécie, com previsão de término do projeto para 2005 (VOGEL, 2000; STERN & ZON 2003). Concomitante com o seqüenciamento do genoma total também esta ocorrendo o seqüenciamento de ESTs. A seqüência do genoma mitocondrial já está completa nesta espécie e pode ser base para estudos filogenéticos (BROUGHTON et al., 2001). O estudo do genoma do zebrafish pode servir como um complemento funcional para o projeto genoma humano, o qual produz enormes quantidades de seqüências, mas carece de informações funcionais para a maioria dos genes identificados (DOOLEY & ZON,

2000). Além disso, grandes segmentos dos cromossomos do zebrafish estão em sintonia com os cromossomos humanos e de camundongo, e muitos genes apresentam um alto grau de similaridade, quando comparados em sua seqüência (BARBAZUK et al., 2000).

O zebrafish se tornou o principal modelo experimental para o estudo do desenvolvimento de vertebrados (ANDERSON & INGHAM, 2003). As características básicas de sua embriogênese são bem conhecidas, assim como o destino celular durante o seu desenvolvimento (KIMMEL & WARGA, 1988; KIMMEL, 1989). Atualmente, um amplo espectro de estudos sobre o desenvolvimento de diversos sistemas, órgãos e patologias relacionadas são realizados nesta espécie (DODD et al., 2000; ACKERMANN & PAW, 2003; NORTH & ZON, 2003; RASSETT & CURRIE, 2004; TREDE et al., 2004).

Comparando-se as seqüências do genoma humano e o do zebrafish, muitos genes como os do ciclo celular, supressores tumorais e oncogenes se mostram conservados (AMATRUDA et al., 2002). Já foram observados muitos tipos de neoplasias no zebrafish, os quais são parecidos histologicamente e geneticamente com os humanos, o que mostra que a biologia do câncer é muito similar nestes organismos (AMATRUDA et al., 2002; STERN & ZON, 2003). Além disso, animais transgênicos podem ser gerados com alterações em genes específicos envolvidos no câncer (LONG et al., 1997).

Recentemente, estudos avaliando características comportamentais do zebrafish foram desenvolvidos (GUO, 2004). A maioria dos trabalhos avaliou o efeito de pesticidas, drogas e xenobióticos na atividade comportamental desta espécie (STEINBERG et al., 1995; LEVIN & CHEN, 2004; LEVIN et al. 2004; SWAIN et al., 2004). Alguns estudos também observaram a importância do comportamento inato e

adquirido em modelos de agressão, sociabilidade e sua preferência por ambientes claros ou escuros (SERRA et al., 1999).

Devido às vantagens de se usar o zebrafish como modelo experimental, o efeito agudo e crônico de diversas substâncias tóxicas pode ser avaliado facilmente. Devido ao pequeno espaço requerido por estes animais, uma quantidade menor de toxinas é empregada nos testes toxicológicos. Além disso, o efeito e a acumulação de diversas substâncias químicas vêm sendo testados no zebrafish desde o final dos anos 70 (LELE & Krone, 1996). Muitos compostos, tais como pesticidas, metais pesados, fenóis e misturas complexas de compostos já foram avaliados em diversos órgãos deste peixe, o que indica o crescente interesse nesta espécie como um modelo de toxicologia ambiental e bioindicação (CARVAN et al., 2000; YAMAZAKI et al., 2002).

Atualmente, muitos estudos são realizados nesta espécie para estudar as bases moleculares da neurobiologia, identificando genes envolvidos na formação de circuitos neuronais, no comportamento e nos mecanismos envolvidos na neuropatogênese (VASCOTTO et al., 1997; FETCHO & LIU, 1998; GUO, 2004). Muitos sistemas de neurotransmissão já foram identificados no zebrafish tais como: glutamatérgico (EDWARDS & MICHEL, 2002; TODD et al., 2004), colinérgico (BEHRA et al., 2002; CLEMENTE et al., 2004), dopaminérgico (RINK & WULLIMANN, 2002; BOEHMLER et al., 2004), serotoninérgico (RINK & GUO, 2004), histaminérgico (KASLIN & PANULA, 2001), gabaérgico (KIM et al., 2004) e purinérgico (KUCENAS et al., 2003; RICO et al., 2003).

1.5. ATP

O papel do trifosfato de adenosina (ATP) no espaço intracelular como molécula energética, doando grupos fosfato de alta energia e acoplando reações exoergônicas a reações endoergônicas já esta bem estabelecido na literatura. No espaço extracelular esta molécula pode ser liberada a partir de vesículas por um mecanismo voltagem-dependente, indicando que ela age como um transmissor. O trabalho pioneiro propondo o papel de molécula transmissora do ATP foi feito por HOLTON & HOLTON (1953), estudando o sistema nervoso sensorial. Entretanto, sua ação como neurotransmissor só foi reconhecida pelos estudos realizados por Geoffrey Burnstock, que desenvolveu a hipótese purinérgica (BURNSTOCK et al., 1970; BURNSTOCK, 1972). Atualmente, se propõe que o ATP é uma molécula sinalizadora primitiva e ubíqua, a qual foi conservada como um cotransmissor em quase todos tipos celulares, desempenhando importantes papéis, tanto em estados fisiológicos, quanto em patológicos (CHOW et al., 1997; LAMBRECHT, 2000; BURNSTOCK, 2002; BURNSTOCK, 2004).

No sistema nervoso central e periférico, o ATP age como neurotransmissor excitatório e possivelmente como neuromodulador (CUNHA, 2000; SALGADO et al., 2000). Além da sinalização entre neurônios, o neurotransmissor ATP pode atuar na comunicação neurônio-glia, na comunicação glia-glia e também como um fator trófico (NEARY et al., 1996; COTRINA et al., 2000). O ATP pode ser armazenado e liberado juntamente com diversos outros neurotransmissores, tais como: acetilcolina, glutamato, norarenalina, serotonina e gaba (BURNSTOCK, 1999; BURNOSTOCK, 2004).

Na fenda sinaptica, receptores de superfície celular podem propagar sinais a partir de sua ligação com o ATP. Estes receptores são denominados purinoceptores P2 e são

divididos em duas subclasses, os receptores ionotrópicos P2X, que são canais iônicos dependentes de ligantes, e os receptores metabotrópicos P2Y, que são acoplados a proteína G (Tabela 1). Estas duas subclasses de receptores operam em diferentes escalas de tempo e distância (KHAKH, 2001). Os receptores P2X agem em milisegundos abrindo canais e propagando impulsos de curta distância. Os receptores P2X possuem baixa afinidade por ATP (na faixa de micromolar) (BURNSTOCK, 2004b). Os receptores P2Y são ativados por baixas concentrações de ATP (na faixa nanomolar) e a sua ligação com o ATP desencadeia cascatas de segundos mensageiros que amplificam e prolongam a duração do sinal a centenas de milisegundos a até segundos (NORTH & BARNARD, 1997, ILLES & RIBEIRO, 2004b). Membros de ambos tipos de receptores são amplamente distribuídos no sistema nervoso central e periférico e estão envolvidos em uma miríade de funções (BARNARD et al., 1997, BOEHM, 2003, BURNSTOCK & KNIGTH, 2004).

Os receptores P2X apresentam pelo menos sete subtipos ($P2X_1$-$P2X_7$), podendo estes ocorrer na forma de homo ou heteroligomêros (ROBERTSON et al., 2001). Estes subtipos possuem dois domínios transmembrana e uma grande região ou alça extracelular, contendo o sítio de ligação do ATP, bem como caudas N e C terminais intracelulares (KHAKH, 2001). Estes receptores são relacionados a canais ligantes de sódio FMRamida (canais FNaC) e canais de potássio, mas diferem substancialmente dos receptores nicotínicos e canais iônicos glutamatérgicos (BURNSTOCK, 1997; WILLIAMS & JARVIS, 2000), Os receptores P2X apresentam permeabilidade substancial ao cálcio, mas não são seletivos quanto à sua permeabilidade à demais cátions (RALEVIC & BURNSTOCK, 1998; WILLIAMS & JARVIS, 2000).

A clonagem e caracterização molecular dos subtipos dos receptores P2X do zebrafish já foram realizadas (DIAZ-HERNANDEZ et al., 2002; BOUÉ-GRABOT et al., 2000; EGAN et al., 2000; NORTON et al., 2000). A análise da seqüência de nove genes sugere que cinco deles são ortólogos a genes dos receptores P2X de mamíferos, dois são parálogos e um ainda precisa ser devidamente classificado (KUCENAS et al., 2003). Todos os subtipos de receptores P2X do zebrafish contêm resíduos altamente conservados, os quais são encontrados nas subunidades de mamíferos.

Até o momento, na família de receptores P2Y foram identificados quatorze subtipos, mas somente oito são entidades moleculares que possuem respostas funcionais (RALEVIC & BURNSTOCK, 1998; LAZAROWSKI et al., 2003. ILLES & RIBEIRO, 2004). Assim como outros receptores acoplados à proteína G, estes receptores possuem a arquitetura típica de sete domínios hidrofóbicos transmembrana, com sítios de ligação para o ATP no sexto e sétimo domínio transmenbrana (BURNSTOCK, 1997). Os sistemas de segundos mensageiros modulados pela ativação destes receptores envolvem a ativação da fosfolipase C, que cliva fosfatidilinositóis de membrana, gerando os segundos mensageiros inositol-1,4,5-trifosfato (IP3) e o diacilglicerol (DAG) (BERRIDGE, 1993). O inositol-1,4,5-trifosfato promove a mobilização de cálcio do retículo endoplasmático, elevando a concentração de cálcio do citosol e o diacilglicerol ativa proteínas quinases, como a PKC (NESTLER et al., 1984). Algumas subunidades de receptores P2Y parecem estar envolvidas, tanto na estimulação (COMMUNI et al., 1997, 1999), como na inibição da adenilato ciclase (BOYER et al., 1997), assim como podem agir em canais iônicos. Também existem evidências de que os receptores P2Y estão envolvidos na transdução de

sinal mediada por proteínas ligantes de GTP e proteínas quinases como a PKC, MAP, ERK1 e 2 (COMMUNI et al., 2000; BOEYNAEMS et al., 2000).

Estudos levando em consideração a evolução estrutural de receptores P2Y demonstraram a presença de dois subtipos de receptores GPR34 (sensíveis a ADP) no zebrafish (SCHULZ & SCHÖNEBERG, 2003). Além disso, GREGORY & JAGADEESWARAN (2002) demonstraram a presença de receptores P2Y(1) em trombócitos deste teleósteo.

Tabela1. Classificação atual dos receptores P2X e P2Y

Receptor[#]	Agonistas de origem fisiológica	Sinalização
$P2X_1$-$P2X_7$	ATP	Canais catiônicos acoplados a ligantes
$P2Y_1$	ADP	G_q / PLC / Ca^{2+} + PKC
$P2Y_2$	ATP / UTP	G_q / PLC / Ca^{2+} + PKC
$P2Y_4$	UTP	G_q / PLC / Ca^{2+} + PKC
$P2Y_6$	UDP	G_q / PLC / Ca^{2+} + PKC
$P2Y_{11}$	ATP	G_q / PLC / Ca^{2+} + PKC
$P2Y_{12}$	ADP	G_i / AC / ↓ AMPc; Canais iônicos
$P2Y_{13}$	ADP	G_i / AC / ↓ AMPc; Canais iônicos
$P2Y_{14}$	UDP-glicose	G_i / AC / ↓ AMPc; Canais iônicos

Adaptado de Lazarowski et al., 2003.

#As recomendações para nomenclatura da IUPHAR para os receptores P2Y de mamíferos foram adotadas. Os produtos dos genes adicionais tentativamente denominados receptores $P2Y_5$, $P2Y_7$, $P2Y_9$, E $P2Y_{10}$ não possuem respostas funcionais a nucleotídeos. O receptor $P2Y_3$ é o ortólogo do receptor $P2Y_6$ encontrado em aves. O receptor $P2Y_8$ encontrado em *Xenopus laevis* possui uma alta homologia com os receptores $P2Y_2$ e $P2Y_4$ de mamíferos.

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. Esta degradação pode inativar a sinalização mediada

pelo ATP através dos receptores P2 e aumentar a sinalização mediada pela adenosina através dos receptores P1 (KATO et al., 2004).

1.6. Adenosina

A adenosina é um nucleosídeo que produz diversos efeitos fisiológicos em todos os tipos celulares (RIBEIRO et al., 2003). As primeiras evidências sobre as atividades fisiológicas desta substância foram obtidas através do estudo de DRURY & SZENT-GYORGYI (1929), que observaram o efeito da adenosina no coração de mamíferos.

Em condições normais, a adenosina é continuamente formada intracelularmente e extracelularmente. A produção intracelular é mediada pela ação de uma 5'-nucleotidase que desfosforila o AMP (SCHUBERT et al., 1979; ZIMMERMANN, 1996) ou pela hidrólise da S-adenosil-homocisteína, catalisada pela S-adenosil-homocisteína hidrolase (BROCH & UELAND, 1980). A adenosina gerada intracelularmente pode ser transportada ao espaço extracelular através de transportadores bidirecionais, por um mecanismo de difusão facilitada que regula os níveis intracelulares e extracelulares deste nucleosídeo (FREDHOLM et al., 2001). No espaço extracelular, a adenosina é formada principalmente pela ação da ecto-5'-nucleotidase que defosforila o AMP, no último passo da cadeia enzimática que hidrolisa os nucleotídeos da adenina. Esta rota catalítica é inteiramente finalizada em poucas centenas de milisegundos, e o passo limitante parece ser a defosforilação do AMP a adenosina pela ação da ecto-5'-nucleotidase (DUNWIDDIE et al., 1997). Outra fonte potencial de adenosina extracelular é o AMP cíclico que pode ser liberado por neurônios e ser convertido a AMP por uma ecto-

fosfodiesterase e, conseqüentemente, dando origem a adenosina (BRUNDEGE et al., 1997).

No espaço extracelular, a adenosina exerce seus efeitos modulatórios através da ativação de receptores específicos de superfície celular, denominados receptores P1 (BRUNDEGE & DUNWIDDIE, 1997). Até o momento, quatro subtipos de receptores P1 foram farmacologicamente definidos e clonados: A_1, A_{2A}, A_{2B} e A_3, sendo todos eles acoplados a proteína G (RALEVIC & BURNSTOCK, 1998; FREDHOLM et al., 2001) (Tabela 2).

Tabela 2. Classificação atual dos receptores de adenosina

Receptor	Agonistas de origem fisiológica	Sinalização
A_1	adenosina	$G_{i/o}$ / ↓AMPc, ↑IP_3, ↑K^+, ↓Ca^{2+}
A_{2A}	adenosina	G_s / ↑AMPc
A_{2B}	adenosina	G_s G_q / ↑AMPc, ↑IP_3
A_3	adenosina / inosina	G_i G_q / ↓AMPc, ↑IP_3

Adaptado de Ralevic & Burnstock, 1998.

Os receptores A_1 são os receptores de adenosina mais abundantes no sistema nervoso central e com a mais alta afinidade pela adenosina.. Estes receptores são acoplados a proteína $G_{i/o}$ e inibem a atividade da adenilato ciclase, diminuindo a formação de AMP cíclico (SCHULTE & FREDHOLM, 2003). Estes receptores também podem ser acoplados a fosfolipase C aumentando os níveis de trifosfato de inositol e diacilglicerol (FREDHOLM et al., 2001). Entre as ações mediadas pelos receptores A_1 pré-sinapticos, está a capacidade de inibir a liberação de diversos neurotransmissores tais como acetilcolina, glutamato, dopamina e noradrenalina (FREDHOLM & DUNWIDDIE, 1988; BRUNDEGE & DUNWIDDIE, 1997; FREDHOM et al., 2001; RIBEIRO et al.,

2003). Além disso, estes receptores promovem a inativação de canais de cálcio do tipo N, P e Q, bem como a inativação de diversos tipos de canais de potássio, promovendo a hiperpolarização da célula (FREDHOLM et al., 2001).

Embora os receptores A_1 controlem a maioria dos efeitos fisiológicos conhecidos mediados pela adenosina no cérebro, muitos estudos demonstraram que os receptores A_2 desempenham importantes papéis modulatórios em algumas áreas do cérebro. Os receptores A_2 estimulam a atividade da adenilato ciclase e, conseqüentemente, aumentam os níveis AMP cíclico (Tabela 2). Os receptores A_2 são subdivididos em A_{2A} e A_{2B}, apresentando alta e baixa afinidade pela adenosina, respectivamente (BRUNDEGE & DUNWIDDIE, 1997; BRUNS et al., 1986). Os receptores A_{2A} já foram caracterizados, através de estudos com ligantes e técnicas genético-moleculares, no estriado e outras estruturas dos gânglios basais, no hipocampo e no córtex cerebral (SEBASTIAO & RIBEIRO, 1996). Os receptores A_{2B} já foram identificados em células gliais e em neurônios e parecem ter uma distribuição mais ampla no cérebro que os receptores A_{2A} (HAAS & SELBACH, 2000). Os receptores A_{2B} são acoplados também a fosfolipase C (RALEVIC & BURNSTOCK, 1998).

Os receptores adenosinérgicos do subtipo A_3 possuem seus efeitos fisiológicos pouco conhecidos. Estudos sugerem que estes receptores inibem a adenilato ciclase e podem ativar a fosfolipase C (BRUNDEGE & DUNWIDDIE, 1997; SUH et al., 2001).

Os receptores de adenosina do subtipo A_1 possuem uma ampla distribuição filogenética, sendo encontrados no sistema nervoso central de muitos vertebrados (SIEBENALLER & MURRAY, 1986). Muitos estudos foram realizados em peixes, demonstrando a caracterização farmacológica e a expressão destes receptores (LUCCHI

ET AL., 1992; LUCCHI et al., 1994; POLI et al., 1999; BERAUDI et al., 2003). Além disso, foi demonstrado que os receptores A_1 podem modular a liberação de neurotransmissores, como acetilcolina e glutamato em teleósteos (SATOH et al., 1997; ZHANG & SCHMDT, 1999; POLI et al., 2001). Os receptores A_2 também foram encontrados em peixes (REY & BURNSIDE, 1999; SATOH et al., 1997; SUNDIN & NILSSON, 1996). Entretanto, ROSATI e colaboradores (1995), através de evidências farmacológicas, demonstraram a presença de receptores A_1, mas não de receptores A_2 em cérebro do goldfish.

1.7. Ectonucleotidases

A sinalização mediada por nucleotídeos extracelulares necessita de mecanismos eficientes para a inativação de seu sinal. Até o momento, não foram identificados transportadores responsáveis pela sua captação celular, e estes, devido à alta densidade de suas cargas, não passam facilmente pelas membranas celulares. Muitos trabalhos realizados evidenciaram a presença de uma variedade de enzimas localizadas na superfície celular denominadas ectonucleotidases, que são capazes de hidrolisar nucleotídeos, e assim inativar a sinalização mediada por eles (ZIMMERMANN, 1994; 2001) (Figura 2). O produto final das reações de hidrólise é o nucleosídeo, que pode agir sobre seus próprios receptores e, após ser captado pela célula, participar da rota de salvação do metabolismo das purinas.

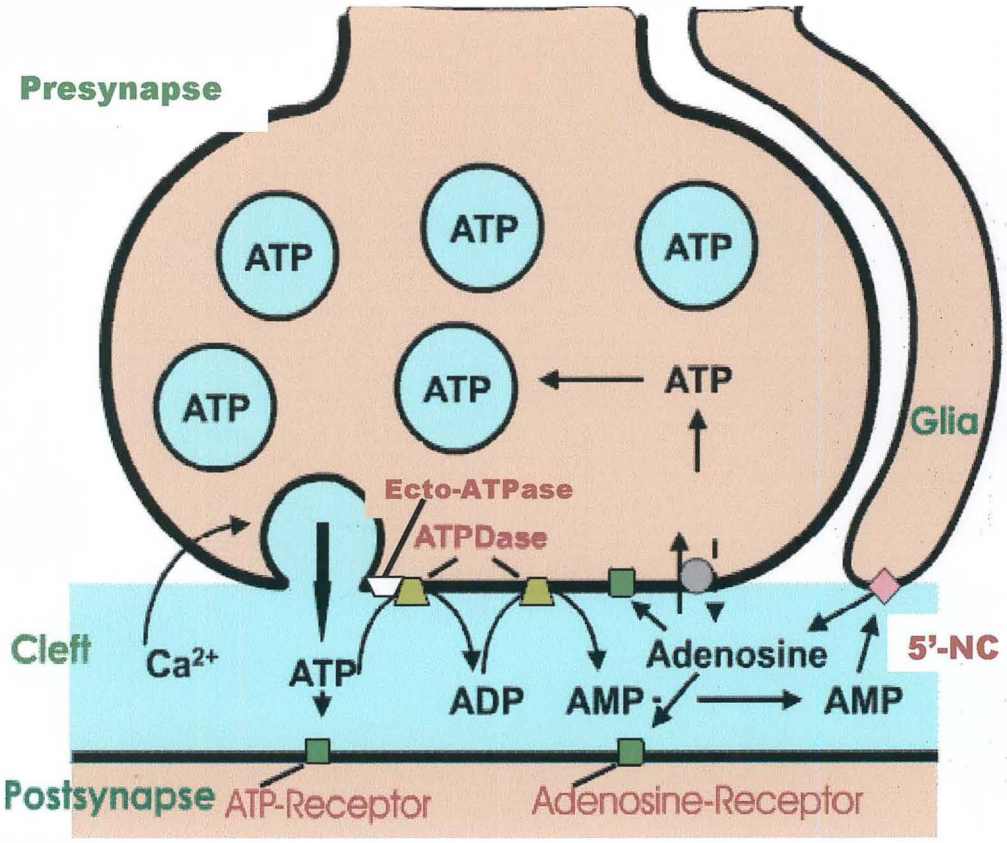

Figura 2. Modelo esquemático de uma sinapse purinégica. O ATP pode ser liberado através de vesículas na fenda sináptica, onde pode agir nos receptores P2 e/ou ser hidrolisado à adenosina, que por sua vez pode agir nos receptores P1.
(Modificado de www.biozentrum.uni-frankfurt.de/prof/zimmermann)

As ectonucleotidases desempenham um importante papel controlando a disponibilidade de ligantes para os purinoceptores. Elas hidrolisam os nucleotídeos, controlando a disponibilidade e a extensão da ativação dos receptores (ZIMMERMANN, 1996a). Além disso, estas enzimas possuem outros papéis relevantes como o

envolvimento em interações célula-célula e célula-matriz, particularmente durante o desenvolvimento (ZIMMERMANN, 1996b).

Estudos realizados recentemente demonstraram que membros de muitas famílias de ectonucleotidases podem contribuir para a hidrólise de nucleotídeos extracelulares. Estas enzimas não se sobrepõem apenas em sua distribuição tecidual, mas também em sua especificidade de substrato (KEGEL et al., 1997). Nucleosídeos 5'-tri e -difosfatados podem ser hidrolisados por membros da família das E-NTPDases (ecto-nucleosídeo trifosfato difosfoidrolase, ATP-difosfoidrolase, apirase e ecto-ATPase), família das E-NPP (ectonucleotídeo pirofosfatase /fosfodiesterase) e pelas fosfatases alcalinas (ZIMMERMANN, 1999; GODING et al., 2003). Nucleosídeos 5'-monofosfatados podem sofrer hidrólise pela ação da ecto-5'-nucleotidase, fosfatases alcalinas e possivelmente por alguns membros da família das E-NPPs (ZIMMERMANN, 2001). Além disso, ectoenzimas capazes catalisar a interconversão de nucleotídeos, como a nucleosideo difosfoquinase e a mioquinase já foram descritas (LU & INOUYE, 1996).

Na família das NTPDases, já foram descritos até o momento oito membros em mamíferos que foram clonados e caracterizados (ZIMMERMANN, 2001; BIGONNESSE et al., 2004). Esta família de enzimas é encontrada em outras espécies de vertebrados, invertebrados, plantas, fungos, e protozoários (HANDA & GUIDOTTI, 1996; VASCONCELOS et al., 1996; SMITH et al., 1997; ZIMMERMANN, 1999; ZIMMERMANN & BRAUN, 1999).

Entre as NTPDases, os membros 1, 2, 3 e 8 possuem seus sítios catalíticos voltados para o meio extracelular. Já os outros membros (NTPDase 4-7) possuem seu sítio catalítico voltados para o lúmen de organelas, como o complexo de Golgi, o retículo

endoplasmático e/ou vacúolos lisossomais/autofágicos. As NTPDases 5 e 6 também podem ser encontradas na membrana plasmática e possivelmente secretadas por clivagem proteolítica (MULERO et al., 1999).

Esta família de enzimas possuem uma topologia de membrana comum com dois domínios transmembrana e uma alça extracelular, contendo cinco domínios denominados ACRs (regiões conservadas da apirase). Estas enzimas possuem uma ampla especificidade de substrato, hidrolisando nucleotídeos púricos e pirimídicos. Para a sua atividade catalítica máxima, estas enzimas necessitam de cátions divalentes, como cálcio e magnésio e um pH alcalino (ZIMMERMANN, 2000). Na maioria dos casos, os valores de K_M para ATP e ADP estão na ordem micromolar (PLESNER, 1995).

A expressão heteróloga das quatro ecto-NTPDases revelaram que estas enzimas possuem diferenças consideráveis na sua especificidade por ATP e ADP. A NTPDase 1 (Ecto-apirase, ecto-ATP difosfoidrolase, CD39) hidrolisa ATP e ADP quase na mesma razão (KACZMAREK et al., 1996; WANG & GUIDOTTI, 1996; HEINE et al., 1999). A NTPDase 2 (ecto-ATPase, CD39L1) possui uma preferência de 30 vezes para a hidrólise do ATP comparado com o ADP (KEGEL et al., 1997; KIRLEY, 1997; MATEO et al., 1999). A NTPDase 3 hidrolisa o ATP três vezes mais eficientemente que o ADP (SMITH & KIRLEY, 1998). A NTPDase 8 possui uma preferência de duas vezes para a hidrólise de ATP comparado com o ADP (BIGNONESSE et al., 2004).

A ecto-5'-nucleotidase, também conhecida como a proteína linfocitária CD73 hidrolisa nucleotídeos 5'-monofosfatatos púricos e pirimídicos até o respectivo nucleosídeo. Esta enzima é amplamente distribuída em tecidos de vertebrados (GRONDAL & ZIMMERMANN, 1987; ZIMMERMANN, 1992). Geralmente, o AMP é

o nucleotídeo hidrolisado com maior eficiência, sendo que os valores de K_M para esta substância estão na faixa de micromolar (ZIMMERMANN, 1992). Esta atividade enzimática é dependente de cátions divalentes, como cálcio e magnésio. Os nucleotídeos ATP e ADP são inibidores competitivos da 5'-nucleotidase com valores de K_i na faixa micromolar (ZIMMERMANN, 1996). A ecto-5'-nucleotidase é uma enzima ancorada à membrana plasmática por glicosil-fosfatidilinositol (GPI), sendo que formas solúveis da enzima podem ser originadas mediante a ação de uma fosfolipase específica. No sistema nervoso central, a ecto-5'-nucleotidase é encontrada principalmente em células gliais, mas estudos também demonstraram esta atividade associada a neurônios (ZIMMERMANN, 1996; ZIMMERMANN et al., 1998).

Muitos estudos demonstraram a presença de ectonucleotidases como a NTPDase (SARKIS & SALTÓ, 1991; SCHETINGER et al., 2001) e 5'-nucleotidase (VOGEL et al., 1992; VOLKNANDT, 1991) em teleósteos. Em zebrafish, estudos do nosso laboratório demonstraram a presença de uma NTPDase e uma ecto-5'-nucleotidase em membranas cerebrais (RICO et al., 2003; SENGER et al., 2004). Estas enzimas possuem características cinéticas similares às ectonucleotidases já descritas em mamíferos, tais como: 1) dependência a cátions divalentes; 2) pH ótimo para sua atividade na faixa de 7.0 a 8.0; 3)K_M na faixa de micromolar; 4) ampla especificidade a nucleotídeos.

1.8. OBJETIVOS

O objetivo principal deste estudo é avaliar o efeito de poluentes amplamente encontrados no ambiente aquático, como metais pesados e pesticidas, na atividade de enzimas envolvidas na hidrólise do neurotransmissor ATP até o neuromodulador adenosina.

Considerando que: (1) o zebrafish é um importante e consolidado modelo experimental em estudos toxicológicos; (2) o sistema purinérgico exerce um importante papel na sinalização no sistema nervoso central; (3) receptores e enzimas, envolvidas neste importante sistema de neurotransmissão, já foram descritos nesta espécie - O presente projeto apresenta os seguintes objetivos específicos:

- Caracterizar a atividade da ecto-5´-nucleotidase em membranas cerebrais de zebrafish.
- Verificar o efeito *in vitro* dos metais pesados (acetato de chumbo e cloreto de mercúrio) na atividade ATPásica, ADPásica, AMPásica em membranas cerebrais de zebrafish.
- Determinar o tipo de inibição exercido pelos metais pesados (acetato de chumbo e cloreto de mercúrio) na atividade ATPásica, ADPásica, AMPásica em membranas cerebrais de zebrafish.
- Verificar o efeito *in vivo* da exposição aguda e crônica aos metais pesados (acetato de chumbo e cloreto de mercúrio) nas atividades ATPásica, ADPásica, AMPásica em membranas cerebrais de zebrafish.

- Verificar o efeito *in vitro* de pesticidas (malathion e carbofuran) sobre a hidrólise de ATP, ADP e AMP em membranas cerebrais de zebrafish.
- Determinar o tipo de inibição que os pesticidas (malathion e carbofuran) exercem sobre a hidrólise de ATP, ADP e AMP em membranas cerebrais de zebrafish.
- Avaliar o efeito *in vivo* da exposição crônica (7 dias) a diferentes pesticidas (malation e carbamatos) sobre a hidrólise de ATP, ADP e AMP em membranas cerebrais de zebrafish.

2. Artigos científicos

2. 1. Capítulo 1- SENGER, M. R., RICO, E. P., DIAS, R. D., BOGO, M. R., BONAN, C. D. 2004. Ecto-5′-Nucleotidase activity in brain membranes of zebrafish (*Danio rerio*). Comp. Biochem. Physiol. 139B, 203-207.

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Ecto-5′-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*)

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Abstract

Adenosine, a well-known neuromodulator, may be formed intracellularly in the CNS from degradation of AMP and then exit via bi-directional nucleoside transporters, or extracellularly by the metabolism of released nucleotides. This study reports the enzymatic properties of an ecto-5′-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*). This enzyme was cation-dependent, with a maximal rate for AMP hydrolysis in a pH range of 7.0–7.5 in the presence of Mg^{2+}. The enzyme presented a maximal activity for AMP hydrolysis at 37 °C. The apparent K_m and V_{max} values for Mg^{2+}-AMP were 135.3±16 μM and 29±4.2 nmol Pi·min^{-1}·mg^{-1} protein, respectively. The enzyme was able to hydrolyze both purine and pyrimidine monophosphate nucleotides, such as UMP, GMP and CMP. Levamisole and tetramisole (1 mM), specific inhibitors of alkaline phosphatases, did not alter the enzymatic activity. However, a significant inhibition of AMP hydrolysis (42%) was observed in the presence of 100 μM α,β-methylene-ADP, a known inhibitor of ecto-5′-nucleotidase. Since 5′-nucleotidase represents the major enzyme responsible for the formation of extracellular adenosine, the enzymatic characterization is important to understand its role in purinergic systems and the involvement of adenosine in the regulation of neurotransmitter release.

Keywords: Zebrafish; Ecto-5′-nucleotidase; Adenosine; Ectonucleotidases; Extracellular nucleotides; ATP; Fish; *Danio rerio*

1. Introduction

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; Magalhães-Cardoso et al., 2003). In neurons, ATP can be stored in axon terminals and co-released during depolarization in the synaptic cleft with several neurotransmitters (Rathbone et al., 1999; Bodin and Burnstock, 2001). 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).

After its release, the neurotransmitter ATP can be converted into adenosine by a family of surface-located enzymes called ectonucleotidases (Bonan et al., 2001; Cunha, 2001). The ectonucleotidase pathway comprises several enzymes able to degrade nucleoside triphosphates and diphosphates, which belong to the E-NTPDase family (ectonucleoside triphosphate diphosphohydrolase) and the ectophosphodiesterase/nucleotide pyrophosphatase (E-PNPP) family (Zimmermann and Braun, 1999; Zimmermann, 2001). These enzymes, together with an ecto-5′-nucleotidase, are involved in the removal of released ATP, which acts as a signaling molecule, but also in the production of another important messenger, adenosine.

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5′-Nucleotidase activity is widely distributed in vertebrate tissues (Grondal and Zimmermann, 1987; Zimmermann, 1992) and catalyzes the hydrolysis of a variety of nucleoside 5′-monophosphates, such as AMP, CMP, UMP, IMP and GMP (Bianchi and Spychala, 2003). At least seven different 5′-nucleotidase activities have been described with different biochemical and molecular properties (Bianchi and Spychala, 2003). Beyond its enzymatic properties, 5′-nucleotidase is a lymphocyte surface protein CD73 and may be involved in cell adhesion (Zimmermann, 1992, 1996; Bianchi and Spychala, 2003). Adenosine, a product of AMP catabolism by the action of 5′-nucleotidase, is a well-known neuromodulator that can also reach the extracellular space by its release through bi-directional non-concentrative adenosine transporters (Deckert et al., 1988). Adenosine modifies cell functioning by operating G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3), which can inhibit (A_1) or enhance (A_2) neuronal communication (Dunwiddie and Masino, 2001; Fredholm et al., 2001).

Zebrafish is a consolidated model system in many research areas, including neuroscience (Lele and Krone, 1996; Vascotto et al., 1997; Ivetac et al., 2000) and it has been shown that its genome shares similarities with the human genome (Barbazuk et al., 2000). There is evidence indicating the role of ATP as a neurotransmitter in this specie, since P2X purinoceptors have been cloned and characterized in zebrafish (Boué-Grabot et al., 2000; Egan et al., 2000; Norton et al., 2000; Diaz-Hernandez et al., 2002). Furthermore, studies in teleost brain have shown that adenosine A_1 receptors appeared similar to that found in mammals (Lucchi et al., 1992, 1994; Beraudi et al., 2003; Kucenas et al., 2003). Recently, studies have demonstrated the presence of NTPDase activity in brain membranes of zebrafish (Rico et al., 2003), which could play an important role in the modulation of the nucleotide and nucleoside levels. Since there is evidence about purinoceptors in the brain of lower vertebrates and that 5′-nucleotidase represents the major enzyme responsible for the formation of extracellular adenosine, this study aims to investigate an ecto-5′-nucleotidase activity in brain membranes of zebrafish.

2. Materials and methods

2.1. Materials

Trizma Base, ammonium molybdate, polyvinyl alcohol, Malachite Green, AMP, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin and magnesium chloride were purchased from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.2. Experimental animals

Zebrafish (*Danio rerio*) were obtained from commercial suppliers. The animals weighting 0.250–0.450 g were kept in 50-l aquarium at 25±5 °C for at least 7 days before the experiments. Fish were fed once daily with a commercial fish pellet and kept under a natural light–dark photoperiod. The procedure for maintenance and use of the animals were according to Colégio Brasileiro de Experimentação Animal (COBEA).

2.3. Brain membranes

Brain membranes were prepared as described by Barnes et al. (1993). Briefly, whole zebrafish brains were homogenized in 60 volumes (v/w) of chilled Tris–citrate buffer (50 mM Tris–citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4, adjusted with citric acid) in a motor driven Teflon-glass homogenizer. The homogenate was centrifuged at 1000×*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×*g*. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris–citrate buffer and recentrifuged for 20 min at 40,000×*g*. This fresh–thaw–wash procedure was used to ensure lysis of the membranes. The final pellet, containing a mixture of intracellular and extracellular brain membranes, was resuspended and used in the enzyme assays. The material was maintained at 2–4 °C throughout preparation.

2.4. Enzyme activity

After membrane preparation, the optimum conditions for AMP hydrolysis were determined. Brain membranes of zebrafish (3–5 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (or other cation as indicated) 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 (AMP or other, as indicated) 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. Samples (0.4 ml) were removed and added to 1 ml of reagent used for determination of inorganic phosphate (Pi), composed of 5.7% ammonium molybdate, 2.3% polyvinyl alcohol and 0.08% Malachite Green and prepared as described previously (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 nmol of Pi released $\cdot$ min^{-1} $\cdot$ mg $protein^{-1}$. All enzyme assays were run in duplicate.

2.5. Protein determination

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

2.6. Statistical analysis

Data were expressed as means±S.D. and analyzed by Student's *t*-test or one-way analysis of variance (ANOVA), considering $P<0.05$ as significant.

3. Results and discussion

An enzyme with characteristics of an ecto-5′-nucleotidase was detected in brain membranes of zebrafish. Previous experiments demonstrated that the time course of AMP hydrolysis is linear up to 30 min in the presence of Mg^{2+} (Fig. 1A). Mg^{2+}-AMP hydrolysis increased as a function of protein concentration and the product formation was linear in the range of 3–10 μg protein in the incubation medium (Fig. 1B).

The 5′-nucleotidase activity was divalent cation-dependent and its sensitivity to Mg^{2+}, Ca^{2+} and Zn^{2+} is demonstrated in Fig. 2. Mg^{2+} and Ca^{2+} were activators of AMP hydrolysis in brain membranes of zebrafish (Fig. 2A). AMP hydrolysis in the presence of Mg^{2+} was dose-dependent, increasing at 1 and 5 mM, but presented a decrease at 10 mM $MgCl_2$. AMP hydrolysis was activated by Ca^{2+}, but this effect was not concentration-dependent. Cation dependency was confirmed by a decrease in AMP hydrolysis in the absence of added cation. However, interestingly, when 5 mM EDTA was added to the incubation medium in the presence of 5 mM Ca^{2+} or Mg^{2+}, we did not observe a significant decrease in the enzyme activity. For this reason, we tested different

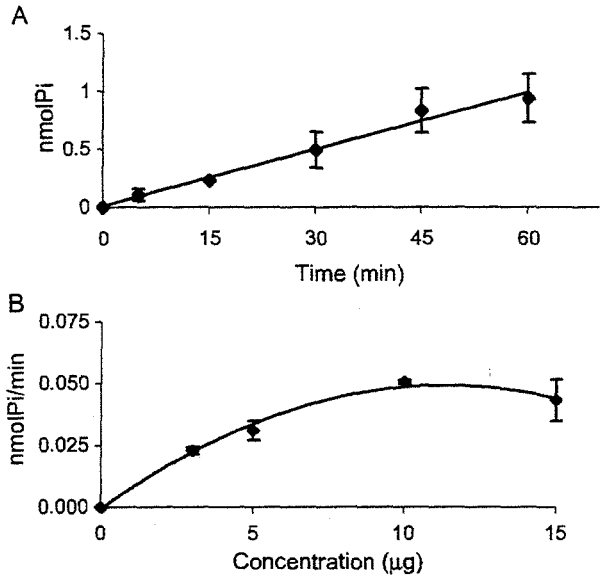

Fig. 1. (A) Time course for Mg^{2+}-AMP hydrolysis in 3–5 μg fractions of zebrafish (*D. rerio*) brain membranes. (B) Effect of different protein concentrations on Mg^{2+}-AMP hydrolysis in zebrafish brain membranes. Data represents means±S.D. of three different experiments, each in duplicate.

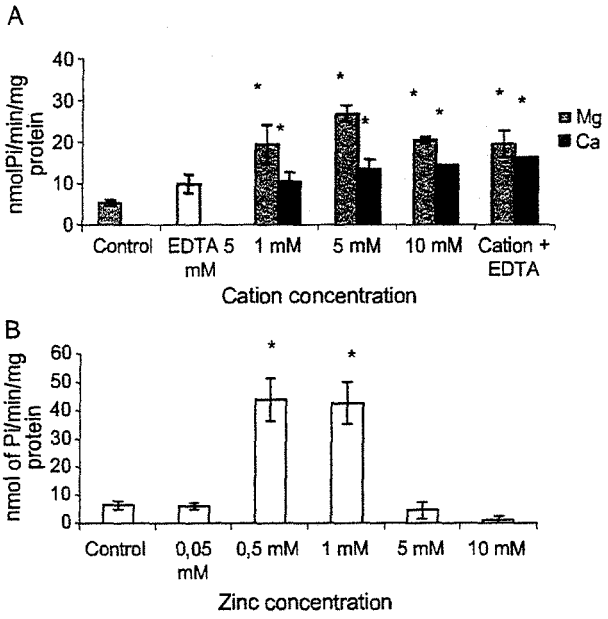

Fig. 2. (A) Effect of different concentrations of $MgCl_2$ and $CaCl_2$ on AMP hydrolysis in brain membranes of zebrafish (*D. rerio*). Control group was incubated without addition of cation. Cation+EDTA was incubated in the presence of 5 mM $MgCl_2$ or 5 mM $CaCl_2$ plus 5 mM EDTA. (B) Effect of different concentrations of $ZnCl_2$ on AMP hydrolysis in brain membranes of zebrafish. Control group was incubated without addition of $ZnCl_2$. Enzyme assays were carried out as described in Section 2. Data represent means±S.D. of four different experiments, each in duplicate. *Indicates difference when compared to the control group. Data were analyzed statistically by one-way ANOVA followed by Duncan test as a post-hoc test, considering a $P<0.05$ significant.

concentrations of EDTA (1, 5, 10 and 20 mM) on AMP hydrolysis in the presence of 5 mM $MgCl_2$. EDTA did not promote changes in AMP hydrolysis and a substantial activity was measurable in the presence of different EDTA concentrations tested (data not shown). Therefore, it is possible to suggest that, after the chelation induced by EDTA, there still remained an amount of cation able to activate the enzyme activity. Considering that the ecto-5′-nucleotidase contains catalytically important Zn^{2+} in the active site (Knöfel and Sträter, 1999; McMillen et al., 2003; Bianchi and Spychala, 2003), we evaluated the effect of different Zn^{2+} concentrations (0.05–10 mM) on the AMP hydrolysis in brain membranes of zebrafish (Fig. 2B). Although EDTA produced no recognizable inhibition, which could be consistent with a possible lack of requirement by the enzyme for divalent cation, addition of $MgCl_2$ (1–10 mM), $CaCl_2$ (1–10 mM) or $ZnCl_2$ (0.5–1 mM) proved to be stimulatory (Fig. 2A,B). However, it is important to observe that a significant augmentation of activity was brought about by the addition of low concentrations of Zn^{2+} and, at higher concentrations, activity decreased strongly (Fig. 2B). The lack of inhibitory effect of EDTA suggests that, if the enzyme requires Mg^{2+} or Zn^{2+}, these divalent cations probably are tightly bound to it. Considering that AMPase activity exhibited a stable

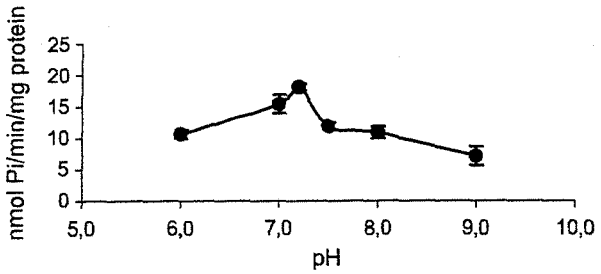

Fig. 3. Effect of pH on Mg^{2+}-AMP hydrolysis in brain membranes of zebrafish (*D. rerio*). Enzyme activity was described in Section 2, using the following buffers: 50 mM Tris–histidine, pH 6.0, 7.0, 7.2, 8.0, 9.0. Data represent means±S.D. of three different experiments, each in duplicate.

activation in the presence of $MgCl_2$, which is a classical activator of 5′-nucleotidase (Zimmermann, 1992), we used 5 mM $MgCl_2$ for subsequent enzyme assays.

Mg^{2+}-AMP hydrolysis was evaluated by measuring pH dependence on enzyme activity. In a medium containing 50 mM Tris–histidine (pH 6.0, 7.0, 7.2, 8.0 and 9.0), the maximal rate for AMP hydrolysis was observed at pH 7.2, similar to other 5′-nucleotidases described in the literature (Zimmermann, 1992, 1996) (Fig. 3). Mg^{2+}-hydrolysis was also assayed at different temperatures (10, 20, 37 and 45 °C). In the temperatures tested, the results showed that the enzyme displayed the highest activity at 37 °C (data not shown).

Mg^{2+}-AMP hydrolysis was determined at substrate concentrations in the range of 100–2000 μM. Enzyme activity increased with increasing concentrations of the nucleotide (Mg^{2+} fixed at 5 mM with variable concentrations of AMP) (Fig. 4). K_m and V_{max} for AMP hydrolysis in brain membranes of zebrafish were estimated from the Lineweaver-Burk plot with three different enzyme preparations. The apparent K_m and V_{max} values for Mg^{2+}-AMP were 135.3±16 μM (mean±S.D.) and 29±4.2 nmol $Pi \cdot min^{-1} \cdot mg^{-1}$ of protein (mean±S.D.), respectively.

Ecto-5′-nucleotidase has been described as an enzyme with a broad substrate specificity (Zimmermann, 1996). Our results showed that brain membranes of zebrafish were able

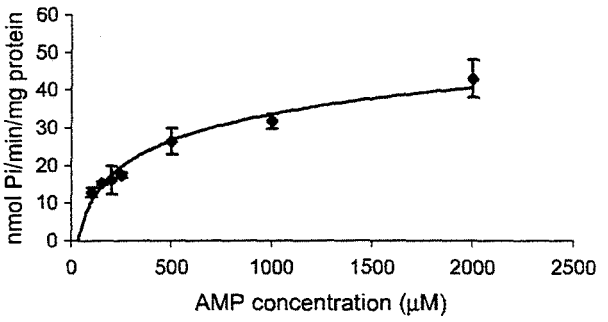

Fig. 4. Effect of different concentrations of substrate (100–2000 μM) on AMP hydrolysis in brain membranes of zebrafish (*D. rerio*). All experiments used fixed 5 mM Mg^{2+} with variable concentrations of nucleotide. Data represent means±S.D. of three different experiments, each in triplicate.

Table 1
Substrate specificity of 5′-nucleotidase in brain membranes of zebrafish (*D. rerio*)

Substrate	% Control activity
AMP	100±6
UMP	97±11
GMP	107±5
CMP	49±11

Results represent mean±S.D. of at least three experiments. Data are expressed as percentage of control activity (AMP hydrolysis—100%). Control Mg^{2+}-AMPase activity was 19.94±1.1 nmol $Pi \cdot min^{-1} \cdot mg^{-1}$. All substrates were used at 1 mM.

to hydrolyze all nucleoside monophosphates tested, presenting a relevant rate of AMP, GMP, UMP and CMP hydrolysis (Table 1).

To avoid the influence of other enzymes, such as alkaline phosphatase, in the AMP hydrolysis, inhibitors of this enzyme activity were tested. Levamisole and tetramisole, specific alkaline phosphatase inhibitors, had no effect upon AMP hydrolysis in brain membranes of zebrafish (Table 2). Conversely, the known 5′-nucleotidase inhibitor, adenosine 5′-[α,β-methylene]diphosphate (AMPCP), was also tested and inhibited enzyme activity significantly (42%, Table 2).

In this family of enzymes, the ecto-5′-nucleotidase (e-N), responsible for extracellular degradation of AMP, is the best-characterized enzymatic source of adenosine. It exhibits a K_m for AMP in the micromolar range and it is inhibited by AMPCP. Soluble 5′-nucleotidase with high K_m for AMP (millimolar range) is found in the cytosolic fraction (cN-I) and another form of 5′-nucleotidase activity (e-Ns), with a low K_m for AMP (K_m=15 μM in rat brain), has been observed in soluble fractions from rat liver, kidney and brain (Orford and Saggerson, 1996). Therefore, our results showed an enzyme in brain membranes of zebrafish that shares kinetic properties with an ecto-5′-nucleotidase.

Neurotransmitter signalling pathways require effective mechanisms for removing or metabolizing extracellular signalling molecules and, in the case of extracellular nucleotide signalling, a broad range of nucleotide-degrading and interconverting ecto- or extracellular enzymes have been identified (Zimmermann, 2001). The activity of the ectonucleotidase pathway appears critical to define the pattern of formation of extracellular ATP-derived adenosine to allow the activation of either inhibitory A_1 or facilitatory

Table 2
Effects of inhibitors on AMP hydrolysis in brain membranes of zebrafish (*D. rerio*)

Inhibitor	Concentration (mM)	% Control enzyme activity
AMPCP	0.1	57.90±0.86*
Tetramisole	1	95.61±9.54
Levamisole	1	81.58±12.4

Results are expressed as percentage of control activity (100%). Control AMPase activity was 22.64±2.64 nmol $Pi \cdot min^{-1} \cdot mg^{-1}$. AMPCP, adenosine 5′-[α,β-methylene]diphosphate. Data represent mean±S.D. of at least three experiments. *Significant difference from control activity (100%) by Student's *t*-test ($P<0.05$).

A_{2A} adenosine receptors (Magalhães-Cardoso et al., 2003). In summary, considering the previous identification of purinoreceptors and NTPDase in central nervous system of zebrafish, the presence of an ecto-5′-nucleotidase activity may be a key component in the control of this signalling pathway in this species.

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2.2. Capítulo 2- SENGER, M. R., RICO, E. P., ARIZI, M.B., DIAS, R. D., BOGO, M. R., BONAN, C. D. 2005. Effects of Hg^{2+} and Pb^{2+} on NTPDase and ecto-5'-nucleotidase activity in central nervous system of zebrafish (*Danio rerio*).

Effects of Hg^{2+} and Pb^{2+} on NTPDase and ecto-5'-nucleotidase activity in central nervous system of zebrafish (*Danio rerio*)

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Abstract

Heavy metals, such as mercury and lead, are important environmental contaminants, presenting risk to the aquatic biota and humans. Many cellular processes, such as neurotransmission, are affected by exposure to mercury and lead. ATP is a primitive signaling molecule that can be inactivated by ectonucleotidases. Here we investigated the *in vitro* and *in vivo* effects of mercury chloride ($HgCl_2$) and lead acetate (Pb(CH3COO)2) on NTPDase and ecto-5'-nucleotidase activity in zebrafish brain membranes. In vitro exposure to $HgCl_2$ promoted significant decrease on nucleotide hydrolysis. Pb(CH3COO)2 significantly inhibited ATP hydrolysis, but not ADP and AMP hydrolysis. Double-reciprocal plots indicated that $HgCl_2$ inhibited ATP and ADP hydrolysis in an uncompetitive manner and AMP hydrolysis in a non-competitive manner. Pb(CH3COO)2 inhibited the ATP breakdown in an uncompetitive manner. In vivo exposure of zebrafish to $HgCl_2$ or Pb(CH3COO)2 (20 μg/L, during 24 h, 96 h and 30 days) caused differential effect on nucleotide hydrolysis. Exposure to $HgCl_2$ during 96h caused significant inhibition of nucleotide hydrolysis. Interestingly, after 30 days of exposure to $HgCl_2$, ATP hydrolysis return to the control levels, ADP hydrolysis was strongly increased and AMP hydrolysis remained inhibited when compared to the control. After 96 h of exposure to Pb^{2+}, it is possible to observe significant decrease only on ATP hydrolysis. Chronic exposure to Pb(CH3COO)2 during 30 days also promoted significant inhibition of ATP, ADP and AMP hydrolysis. Thus, this study demonstrated that mercury and lead affect ectonucleotidase activities, suggesting that purinergic system can be a target related to neurotoxicity and a possible indicator of exposure to heavy metal contaminants.

Keywords: NTPDase, ecto-5'-nucleotidase, nucleotidase, zebrafish, heavy metals, mercury, lead.

1. Introduction

Heavy metals, such as mercury and lead are important environmental contaminants, which can reach aquatic systems derived from effluents of industrial, urban and mining sources. These substances present severe risk to the aquatic biota and humans, even at sublethal concentrations. Animals exposed to Hg^{2+} and Pb^{2+} have adverse developmental, reproductive, neurological and behavioral effects (Clarkson et al., 1985; Semczuk and Semczuk-Sicora, 2001; Berntssen et al., 2003; Rademacher et al., 2003). Many cellular processes are affected by exposure to Hg^{2+} and Pb^{2+} and the correct function of central nervous system can be impaired by neurochemical changes. Studies have demonstrated that the synaptic transmission can be altered after exposure to these heavy metals. Changes in the release (Busselberg et al., 1991; Cooper & Manalis, 1983; Erdelyi et al., 1998), extracellular metabolism and/or uptake (Antonio et al. 2003; Rademacher et al. 2003) and expression (Cheung & Verity, 1985) of components of neurotransmitter systems, have been related.

ATP is a primitive signaling molecule that has been retained as a cotransmitter in every nerve type in both peripheral and central nervous system (Burnstock, 2004). This molecule is released to the synaptic cleft in a calcium-dependent manner, where it can act as a fast neurotransmitter or as a modulator, regulating the activity of other transmitter substances (Burnstock, 1997; Cunha and Ribeiro, 2000). ATP exerts its effects through purinoceptors, divided in two major classes, ionotropic P2X and metabotropic P2Y receptors (Ralevic & Burnstock, 1998; Khakh, 2001; Illes & Ribeiro, 2004). At the synapse, ATP can be catabolized by a group of enzymes called ectonucleotidases, which

includes NTPDase family (Nucleoside triphosphate diphosphohydrolase) and ecto-5′-nucleotidase. The final product of this enzyme cascade is the nucleoside adenosine, an important neuromodulator that acts on G-protein-coupled receptors, named A_1, A_{2A}, A_{2B} and A_3 (Ribeiro et al., 2003).

Ectonucleotidases are ubiquitous enzymes with a broad phylogenetic distribution, occurring in many vertebrate tissues (Zimmermann, 1996). NTPDases present the ability to hydrolyze diphosphate and triphosphate nucleotides. NTPDases 1-3 and 8 are extracellular enzymes that can be classified according the ATP/ADP preference (Zimmermann, 2001; Bigonnesse et al., 2004). NTPDase 1 (CD39, ecto-apyrase, ecto-ATP diphosphohydrolase) hydrolyzes ATP and ADP almost equally well. NTPDase 2 (CD39L1, ecto-ATPase) has a 30-fold preference for the hydrolysis of ATP over ADP. NTPDase3 (HB6) is a functional intermediate and hydrolyzes ATP approximately three times more rapid than ADP. NTPDase 8 slightly prefers ATP over ADP by a ratio of about 2. The product of NTPDases is the nucleotide AMP that can be hydrolyzed by the action of an ecto-5'-nucleotidase. The ecto-5'-nucleotidase or CD73 have a pivotal role together with the NTPDases in regulating the concentration of extracellular nucleotides to the purinoceptores (Cunha, 2001; Zimmermann, 2001).

At this moment, there are few studies demonstrating the effect of heavy metals on ectonucleotidases. Oliveira et al. (1994) investigated the effect in vitro and in vivo of $HgCl_2$ on synaptosomal ATP diphosphohydrolase from cerebral cortex of developing rats. Furthermore, Moretto et al. (2004) verified the subcronic (0,1 mg/Kg; 30 doses/30 days) effect of $HgCl_2$ on NTPDase and 5'-nucleotidase activity of adult rats, showing a significant increase on NTPDase activity, but not on 5'-nucleotidase activity.

Zebrafish is a consolidated model system in neuroscience and toxicological studies (Linney et al., 2004). The genome project of this animal is almost complete and studies have demonstrated regions of syntenic relationship between zebrafish and human genomes (Barbazuk et al. 2000; Vogel, 2000; Stern & Zon 2003). ATP receptors were already identified in this teleost (Kucenas et al., 2003) and recently, we characterized the presence of a NTPDase and an ecto-5'-nucleotidase activity in brain membranes of zebrafish (Rico et al., 2003; Senger et al., 2004).

Considering that mercury and lead are important environmental contaminants and previous studies have demonstrated the presence of purinergic receptors and enzyme activities involved in extracellular catabolism of nucleotides in zebrafish brain, the aim of present study was to investigate the effect of mercury chloride and lead acetate on NTPDase and ecto-5'-nucleotidase activity in central nervous system of zebrafish.

2. Material and methods

2.1. Animals

Zebrafish were obtained from commercial suppliers and maintained in a 50-L aquarium. The fish of both sexes were kept in distilled water at 25±2 °C under a natural light-dark photoperiod. Feeding and maintenance of fishes were done according to Westerfield (2000).

2.2. Treatments

For the *in vitro* experiments, mercury chloride or lead acetate at the final concentrations of 0,05-1 mM were added to reaction medium, preincubated with the brain membranes and maintained throughout the enzyme assay.

For the *in vivo* treatments, animals were introduced to the test aquarium containing solutions of mercury or lead at the final concentration of 20 μg/L. The animals were maintained in the aquarium test for different exposure periods: 24 h, 96h or 30 days.

2.3. 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 1,000 *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 *g*. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris-citrate buffer, and recentrifuged for 20 min at 40,000 *g*. This fresh-thaw-wash procedure was used to ensure lysis of the membranes. The final pellet was resuspended and used in the enzyme assays. The material was maintained at 2-4°C throughout preparation.

2.4. Enzyme assays

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 (Pi) (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 nmol of Pi released.min^{-1}.mg of $protein^{-1}$. All enzyme assays were run at least in triplicate.

2.5. Protein determination

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

2.6. 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

The *in vitro* effects of mercury chloride and lead acetate on NTPDase activity (ATP and ADP hydrolysis) and 5'-nucleotidase (AMP hydrolysis) were verified in brain membranes of zebrafish. Mercury chloride promoted a significant decrease in ATP hydrolysis in all concentrations tested (at the range 0,05 to 1 mM) and the inhibitory effect varied from 31 to 85 %, respectively (Fig. 1). Mercury chloride also had an inhibitory effect in the concentrations of 0,25 to 1 mM for ADP (25 to 72% inhibition) and AMP hydrolysis (30 to 63%), respectively (Fig.1). Lead acetate significantly inhibited ATP hydrolysis in brain membranes of zebrafish at the concentrations of 0,25 to 1 mM, but not altered ADP and AMP hydrolysis (Fig.2).

The kinetic of the interaction of these metals with NTPDase or 5´-nucleotidase in brain membranes of zebrafish was determined. The Lineweaver-Burk double reciprocal plot was analyzed over a range of substrates (0,1 to 0,25 mM) in the absence and in the presence of the mercury chloride (Fig. 3) or lead acetate (Fig. 4). The data indicated that mercury inhibited ATP and ADP hydrolysis in an uncompetitive manner, decreasing both K_M and V_{max} (Fig. 3A and 3B, respectively). The inhibition promoted by mercury chloride in the AMP hydrolysis was noncompetitive, because the K_M values did not change, while maximal AMPase velocity decreased with increasing mercury concentration (Fig. 3C). In relation to the inhibitory effect promoted by lead acetate, the data showed an uncompetitive inhibition (Fig. 4).

Studies have reported that mercury and lead can reach 20 μg/L in the aquatic environment. (Berzas Nevado et al., 2003; Jha et al., 2003). To evaluate the effect *in vivo*

of these heavy metals in the purinergic system, animals were exposed to a treatment with mercury chloride and lead acetate, at 20 μg/L during 24 h, 96 h and 30 days. Exposure of fishes to Hg^{2+} or Pb^{2+} caused differential effect on nucleotide hydrolysis in brain membranes of zebrafish. After 24 h of exposure to mercury chloride, there are no significant changes on nucleotide hydrolysis (Fig. 5). The exposure to mercury chloride during 96h caused a significant inhibition of ATP (37%), ADP (42%) and AMP (39%) hydrolysis (Fig. 5). Interestingly, after 30 days of exposure to mercury chloride, ATP hydrolysis return to the control level and ADP hydrolysis was strongly increased (118%) when compared to the control values. AMP hydrolysis at this time of exposure remaining inhibited (32%).

In relation to the treatment with lead acetate, there are no significant changes on nucleotide hydrolysis after exposure to lead acetate during 24 h. After 96 h of exposure to lead acetate, it is possible to observe a significant decrease on ATP hydrolysis (17%), but not on ADP and AMP hydrolysis (Fig.6). The chronic exposure to lead acetate during 30 days also promoted a significant inhibition of ATP (33%), ADP (37%) and AMP (40%) hydrolysis in brain membranes of zebrafish.

4. Discussion

This study reveals that *in vitro* and *in vivo* of exposure to mercury chloride and lead acetate promoted significant changes on nucleotide hydrolysis in zebrafish brain membranes. Experiments *in vitro* have shown that mercury chloride inhibited ATP, ADP and AMP hydrolysis in dose-dependent effect. The results observed in the double-

reciprocal plot indicated that mercury chloride inhibited ATP and ADP hydrolysis in an uncompetitive manner. The data related to the kinetic interaction of mercury chloride and ecto-5′-nucleotidase indicated that this metal inhibited the enzyme in a non-competitive manner. Lead acetate, in a dose-dependent response, inhibited only the ATP breakdown in an uncompetitive manner. For the *in vivo* treatments, we have tested an environmental low concentration of mercury chloride and lead acetate since there is evidence that these metals can reach the 20 μg/L in the aquatic environment. (Berzas Nevado et al., 2003; Jha et al., 2003). After 96 h of exposure, mercury chloride caused an inhibition on ectonucleotidase activities. However, after chronic treatment (30 days), ATP hydrolysis returned to the control level, but ADP hydrolysis presented a dramatic increase in brain membranes of zebrafish and AMP hydrolysis remaining inhibited. This process can be due to an overcompensation response of the NTPDase to these pollutants (Calabrese & Baldwin, 2003). Thus, the exposure to low concentrations of this metal in the aquatic enviroment interferes with the purinergic system differing with the time and level of exposure. Moretto et al.(2004) have showed that treatment with subcutaneous injections of low doses of mercury chloride (01.mg/kg; 30 doses/30 days) promote activation of NTPDase and acetylcholinesterase, but not on 5′-nucleotidase in synaptosomes from cerebral cortex. According these authors, their results could be related to hormetic-like biphasic effect (Calabrese & Baldwin, 2001). Considering that our study has tested a low concentration of these metals, it is possible to suggest that a similar hormetic effect could influence the response of NTPDase to these pollutants.

Previous studies have showed that environmental pollutants, including heavy metals, are known to upregulate protective measures, such as induction of xenobiotic-

metabolizing enzymes, signal transduction and the oxidative stress response, via transcriptional activation of important particular genes in these pathways (Carvan III et al., 2000; Nihei et al., 2001). Furthermore, the activation of transcription factors in zebrafish cell cultures by environmental pollutants have been described (Carvan III et al., 2000). Therefore, the significant activation of NTPDase after 30 days of treatment could be related to changes in the expression levels of NTPDase after long-term exposure to mercury chloride. Further studies are in progress in our laboratory in order to investigate possible transcriptional changes in NTPDase induced by heavy metals.

In the literature, the evaluation of erythrocyte pyrimidine 5′-nucleotidase has been used as a toxicological parameter to occupational exposure to lead (Kim et al., 2002a, 2002b). However, until now there are no studies about the effect of lead acetate on the extracellular ATP- metabolizing enzymes, such as NTPDase and ecto-5′-nucleotidase. Our study presents the first evidence about the inhibitory effect of this heavy metal on ectonucleotidase pathway in zebrafish after long-term exposure.

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 effect that nucleotides have on cells depends on the extracellular catabolism mediated by ectonucleotidases, 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 ectonucleotidase 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, alterations in other neurotransmission systems can explain some neurotoxicological characteristics of the heavy metals. These substances affect many transmission systems, like glutamatergic (Nihei & Guilarte, 2001; Soares et al., 2003), gabaergic (Braga et al., 1999; Lasley & Gilbert, 2002), serotoninergic (Oudar, et al., 1989; Kala & Jadhav, 1995), dopaminergic (Rademacher et al., 2001; Faro, et al., 2001) and cholinergic (Luo & Berman, 1997; Mirzoian & Luetje, 2002) systems. Many studies have demonstrated the coliberation and reciprocal modulation between ATP and other neurotransmitters systems (Burnstock, 2004).

Based on the data presented herein, this study demonstrated that mercury and lead affect the NTPDase and ecto-5'-nucleotidase activities, suggesting that purinergic system can be a target related to neurotoxicity induced by lead and mercury and a possible indicator of the biological impact of exposure to heavy metal contaminants.

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Figure legends:

Fig. 1: *In vitro* effect of varying concentrations of Hg^{2+} on ATP, ADP and AMP hydrolysis in zebrafish brain membranes. Bars represent mean ± S.D. of at least four independent experiments. The control specific activities for ATP, ADP and AMP hydrolysis were 567.5 ± 83.8, 161.1 ± 8.8 and 23.4 ± 3.9 nmol Pi. min^{-1}. mg^{-1} protein, respectively. * Significantly different from control group (without metal added) ($P < 0.05$).

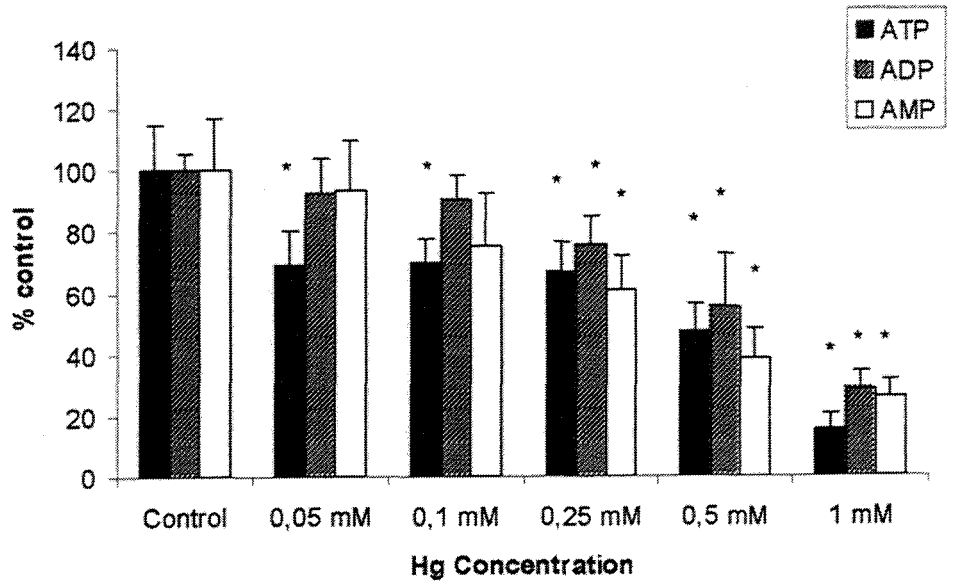
Fig. 2: *In vitro* effect of varying concentrations of Pb^{2+} on ATP, ADP and AMP hydrolysis in zebrafish brain membranes. Bars indicate mean ± S.D of at least four independent experiments. Control specific activities of ATP, ADP and AMP hydrolysis were 605.8 ± 28.4, 141 ± 7.7 and 19.9 ± 3.2 nmol Pi. min^{-1}. mg^{-1} protein, respectively.
* Significantly different from control (without metal added) ($P < 0.05$).

Fig. 3: Lineweaver-Burk plot of ATP (A), ADP (B) and AMP (C) hydrolysis and their interaction with Hg^{2+}.The concentration of the nucleotides varied from 0.1 to 0.250 mM in absence (+) and in the presence of 0,5 mM (◊) and 1 mM (□) of Hg^{2+}. All experiments were repeated at six to eight times and similar results were obtained. The data showed represent a typical experiment

Fig. 4: Lineweaver-Burk plot of ATP hydrolysis and its interaction with Pb^{2+}. The concentration of the nucleotides varied from 0.1 to 0.250 mM in absence (+) and in the presence of 0,5 mM (◊) and 1 mM (□) of Pb^{2+}. The experiment was repeated six times and similar results were obtained. The data showed represents a typical experiment

Fig. 5: *In vivo* effect of Hg^{2+}on nucleotide hydrolysis in zebrafish brain membranes. Bars represent mean ± SD of four independent experiments. Control specific activities for ATP, ADP and AMP hydrolysis were 619.2 ± 59.6, 146.4 ± 15.3 and 23.5 ± 3.5 nmol Pi. min^{-1}. mg^{-1} protein, respectively. * Significantly different from control (without metal added) ($P < 0.05$). # indicates statistical difference in relation to control and group submitted to 96h of exposure.

Fig. 6*:* *In vivo* effect of Pb^{2+} on nucleotide hydrolysis in CNS of zebrafish. Bars represent mean ± SD of four independent experiments. Control specific activities for ATP, ADP and AMP hydrolysis were 598 ± 35.7, 143.3 ± 14.8 and 22.4 ± 3.4 nmol Pi. min^{-1}. mg^{-1} protein, respectively. * Significantly different from control (without metal added) ($P < 0.05$). # indicates statistical difference in relation to control and group submitted to 96h of exposure.


ATP
ADP
AMP
140
120
100
80
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% control
Control
0,05 mM
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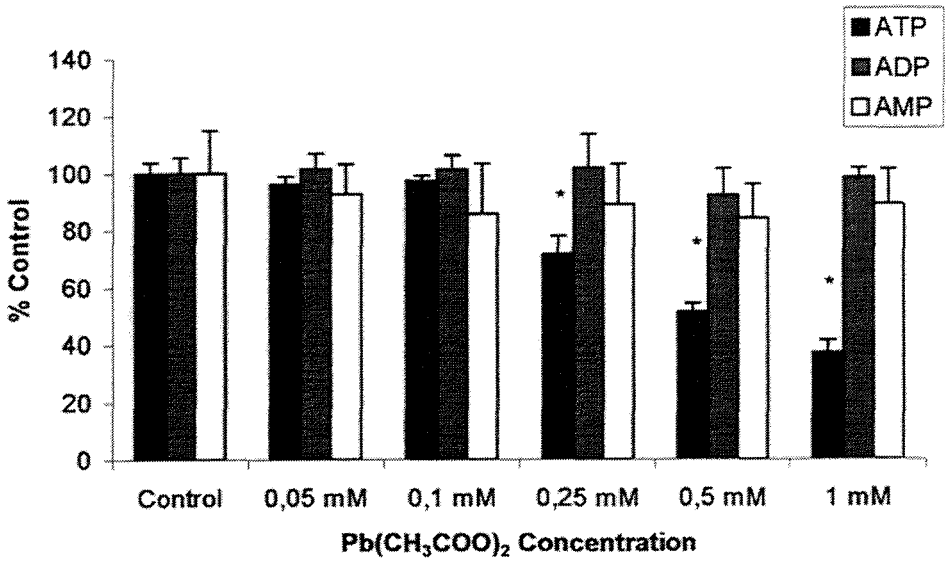
0,5 mM
1 mM
Hg Concentration

Fig.1


ATP
ADP
AMP
140
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0,05 mM
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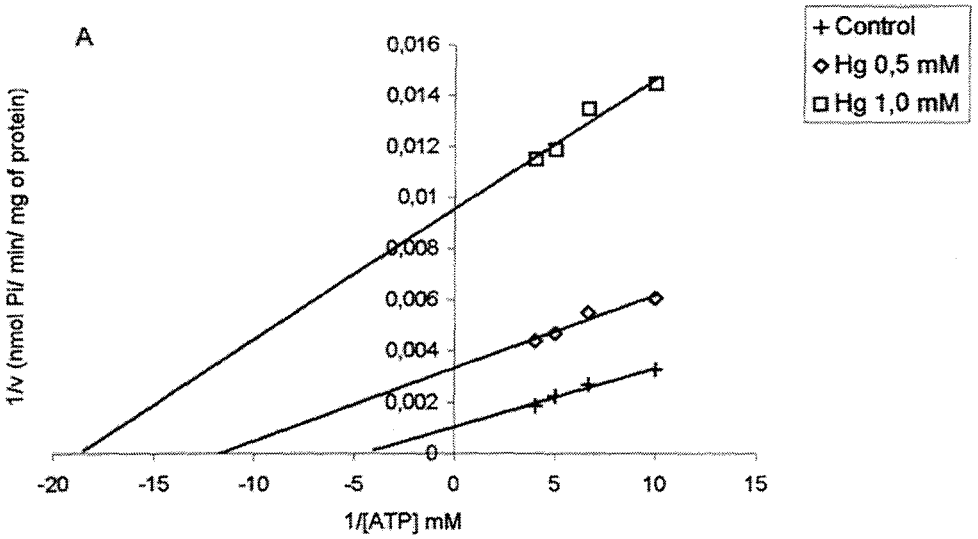
0,5 mM
1 mM
Pb(CH3COO)2 Concentration

Fig.2


A
+ Control
◇ Hg 0,5 mM
□ Hg 1,0 mM
1/v (nmol Pi/ min/ mg of protein)
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0,002
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-20
-15
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0
5
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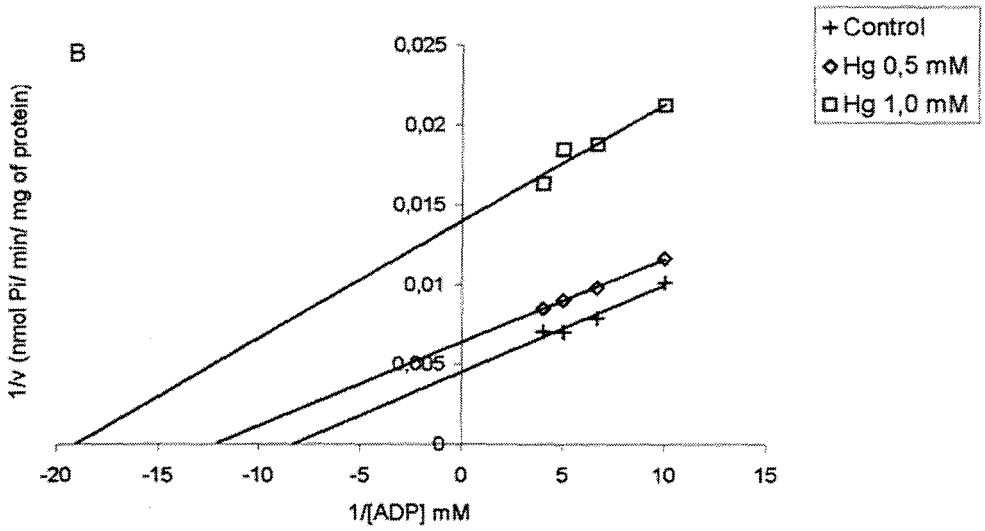
15
1/[ATP] mM


B
+ Control
◇ Hg 0,5 mM
□ Hg 1,0 mM
1/v (nmol Pi/ min/ mg of protein)
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0,02
0,015
0,01
0,005
0
-20
-15
-10
-5
0
5
10
15
1/[ADP] mM

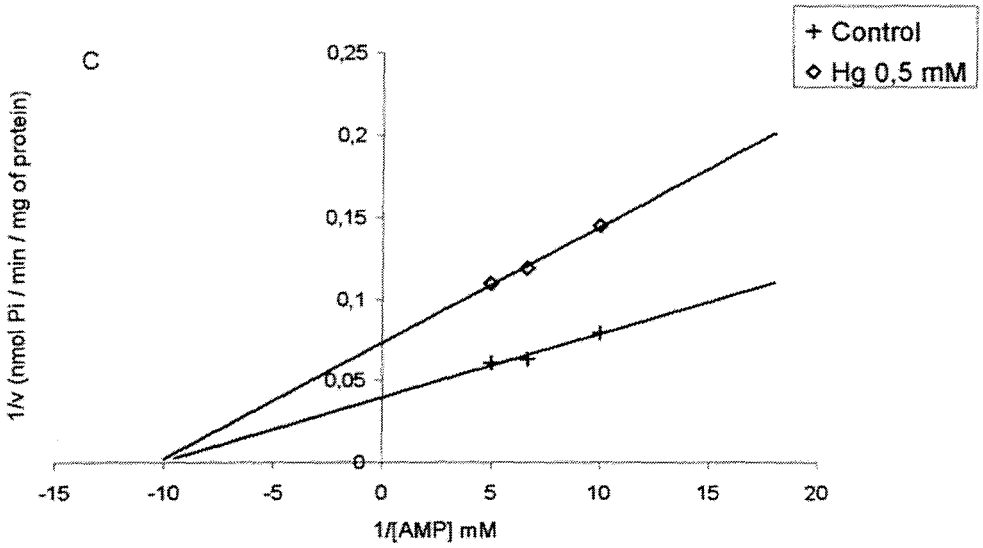
C
+ Control
◇ Hg 0,5 mM
1/v (nmol Pi / min / mg of protein)
0,25
0,2
0,15
0,1
0,05
0
-15
-10
-5
0
5
10
15
20
1/[AMP] mM

Fig.3

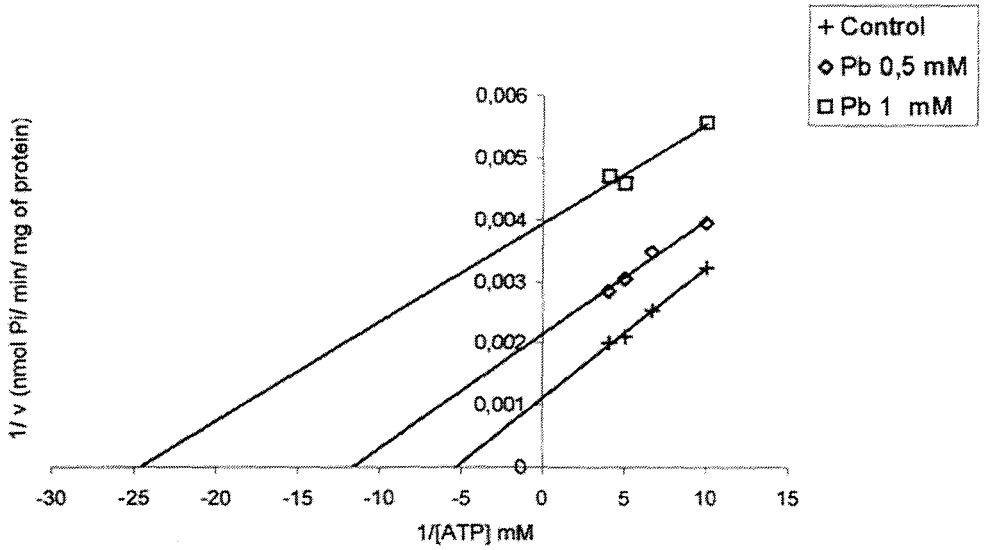
+ Control
◇ Pb 0,5 mM
□ Pb 1 mM
1/ v (nmol Pi/ min/ mg of protein)
0,006
0,005
0,004
0,003
0,002
0,001
0
-30
-25
-20
-15
-10
-5
0
5
10
15
1/[ATP] mM

Fig.4

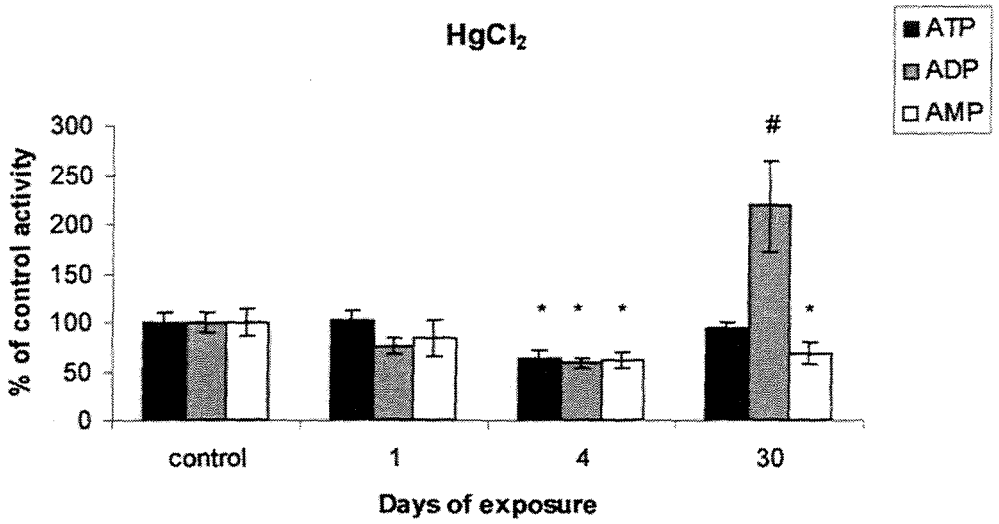
HgCl2
ATP
ADP
AMP
% of control activity
300
250
200
150
100
50
0
#
*
control
1
4
30
Days of exposure

Fig.5

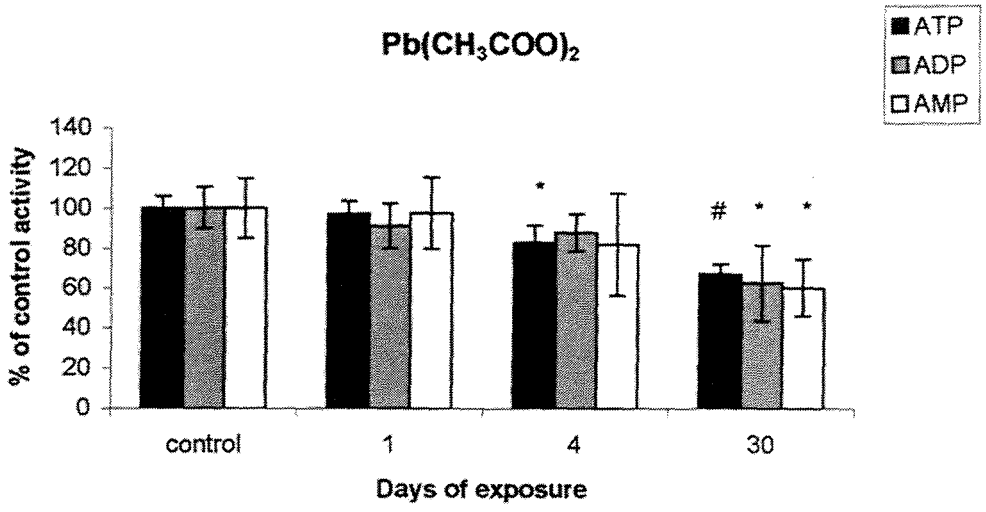
Pb(CH3COO)2
ATP
ADP
AMP
% of control activity
140
120
100
80
60
40
20
0
*
#
control
1
4
30
Days of exposure

Fig.6

2.3. Capítulo 3- SENGER, M. R., RICO, E. P., ARIZI, M.B., ROSENBERG, D. B., DIAS, R. D., BOGO, M. R., BONAN, C. D. 2005. Carbofuran and malathion inhibit nucleotide hydrolysis in zebrafish (*Danio rerio*) brain membranes. (submetido ao periódico Toxicology)

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

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Abstract

Carbofuran and malathion are broad spectrum pesticides widely used in agricultural practice throughout the world. Toxicity of these pesticides has been correlated with its inhibitory effect 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 includes NTPDase (nucleoside triphosphate diphosphohydrolase) 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 vivo* and *in vitro* to carbofuran and malathion on ectonucleotidase activities from brain membranes of zebrafish. After exposure to carbofuran (50 and 500 μg/L) during seven days, ADP hydrolysis was significantly decreased in both concentrations tested (19% and 24.5%, respectively). Malathion, at 500 μg/L, was able to inhibit ADP and AMP hydrolysis (28% and 58.5%, respectively). 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 varying from 0.25 to 5 mM of pesticides. 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. This study has shown that ectonucleotidases from brain membranes of zebrafish can be a potential target for pesticide neurotoxicity.

Keywords: carbofuran, malathion, ectonucleotidases, NTPDase, ecto-5'-nucleotidase, zebrafish.

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 & 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, other effects remain to be considered to completely estimate the neurotoxicity of these contaminants.

Nucleotides are ubiquitous extracellular signaling molecules which induces a wide spectrum of biological effects (Burnstock, 1997; Ralevic & 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 & 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 &

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 NTPDases (nucleoside triphosphate diphosphohydrolase). 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).

It has been shown the co-transmission of acetylcholine and ATP at the nerve endings (Burnstock, 2004). There is evidence that adenosine can modulate the acetylcholine release (Ribeiro et al., 1996), as well 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 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 are already identified in this specie (Kucenas et al., 2003; Schultz & Schöneberg, 2003). Recently, we characterized the presence of a NTPDase and an ecto-5'-nucleotidase activity in brain membranes of zebrafish (Rico et al., 2003; Senger et al., 2004).

Considering 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±2 °C under a natural light-dark photoperiod. Feeding and maintenance of fish were done according to Westerfield (2000).

2.2. Chemicals

Carbofuran (95.8%; 2,3-Dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate) and Malathion (95%; [(Dimethoxyphosphinothioyl)thio] butanedioic acid diethyl ester) 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 the enzyme assays at the final concentrations of 0.25 - 5 mM. Selected concentrations of each compound were added to reaction medium before pre-incubation with the enzyme and maintained throughout the enzyme assay.

For the *in vivo* treatments, zebrafish were introduced to the test aquarium ten minutes after the addition of solutions containing carbofuran or malathion diluted in a 70% DMSO. The animals were maintained in the aquarium test for seven days in the concentration of 50 μg/L 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 1,000 *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 *g*. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris-citrate buffer, and recentrifuged for 20 min at 40,000 *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. 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 (Pi) (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 nmol of Pi released.min^{-1}.mg of $protein^{-1}$. 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

In order to evaluate possible influence of the vehicle DMSO on our results, zebrafish exposed to the DMSO 70% (25 μl/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.

Zebrafish were exposed to concentrations of carbofuran and malathion (50 μg/L and 500 μg/L), previously tested in ciprinids (Ansari & Kumar, 1984; Bretaud et al., 2002). After exposure to carbofuran (50 μg/L and 500 μg/L) during seven days, there are no significant changes in ATP and AMP hydrolysis in brain membranes of carbofuran-treated zebrafish, when compared to control group (Fig. 1A). However, ADP hydrolysis was significantly decreased after exposure to carbofuran at 50 and 500 μg/L (19% and 24.5%, respectively) (Fig. 1A). After exposure to malathion (50 μg/L), during seven days, there are no changes on ATP, ADP and AMP hydrolysis in brain membranes of zebrafish when compared to unexposed group (Fig. 1B) Malathion, at 500 μg/L, was able to inhibit ADP and AMP hydrolysis (28% and 58.5%, respectively) (Fig. 1B).

To verify if carbofuran and malathion have direct inhibitory effects on NTPDase and 5′-nucleotidase activities, we have tested *in vitro* concentrations varying from 0.25 to 5 mM of each pesticide. Controls adding DMSO in a final concentration of 14% (used to achieve the most concentrated solution of pesticides tested in vitro) did not alter significantly the ectonucleotidase activities. Carbofuran inhibited in vitro ATP hydrolysis in the concentration ranged from 1 to 5 mM (28% to 59%), but ADP hydrolysis was only

inhibited at 3 and 5 mM carbofuran (35% and 45%, respectively (Fig. 2A). There are no significant changes on AMP hydrolysis in the presence of carbofuran in all concentrations tested (Fig. 2B). Malathion, at the range 0.25 to 5 mM, inhibited *in vitro* both ATP (24% to 48%, respectively) and ADP hydrolysis (31 to 59%, respectively) (Fig. 3A). There were no significant changes on AMP hydrolysis in all concentrations tested (Fig. 3B).

The kinetics of the interaction of carbofuran and malathion with NTPDase activity in zebrafish brain membranes were also determined (Fig. 4 and Fig. 5). Lineaweaver-Burk double reciprocal plot was 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. 4) and malathion (Fig. 5). The data indicated that the inhibition of ATP (Fig. 4A) and ADP (Fig. 4B) 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 is increased and the V_{max} did not change significantly (Fig. 5A). Malathion inhibited ADP hydrolysis in an uncompetitive manner (Fig. 5B).

4. Discussion

In the present investigation, we have shown that carbofuran and malathion can inhibit *in vivo* and *in vitro* ectonucleotidase activities in zebrafish brain membranes. Interestingly, the exposure of zebrafish to carbofuran (50 μg/L and 500 μg/L) inhibited only ADP hydrolysis in brain membranes when compared to unexposed group, but ATP and AMP hydrolysis are not modified by this treatment. The exposure to malathion

inhibited significantly ADP and AMP in the concentration of 500 μg/L after treatment during seven days. Carbofuran, when directly added in the enzyme assays (in vitro study) produced an inhibition in ATP and ADP hydrolysis in an uncompetitive manner. Malathion inhibited in vitro ATP and ADP hydrolysis in a competitive and in an uncompetitive manner, respectively.

Studies have demonstrated that exposure *in vivo* to malathion caused a dose-dependent inhibition of acetylcholinesterase in the zebrafish brain (Ansari & Kumar, 1984). The authors tested concentrations ranged from 500 μg/L to 1100 μg/L during seven 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 (1.1 mg/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 acetylcholinesterase, could be considered as potential useful indicators of environmental contamination by organic compounds.

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 increases in the levels of these neurotransmitters. The effect of carbofuran on brain catecholamines of goldfish was investigated. The exposure to carbofuran 50 μg/L or 500 μg/L during 24 and 48h 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 (Bigonesse 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 effect in vitro 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 pure CB and OP compounds tested inhibited ATPase activity. In regard to this effect of pesticides upon 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, 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.

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Figure legends

Fig. 1: *In vivo* effect of carbofuran (A) and malathion (B) on nucleotide hydrolysis in zebrafish brain membranes. Bars represent the mean ± of four different experiments. The control ATPase, ADPase and AMPase activities (with no pesticide) were 689.92 ± 67; 163 ± 18.68 and 24 ± 1.75 nmol $Pi.min^{-1}.mg^{-1}$ of protein, respectively. * Significantly different from control group ($P \leq 0.05$).

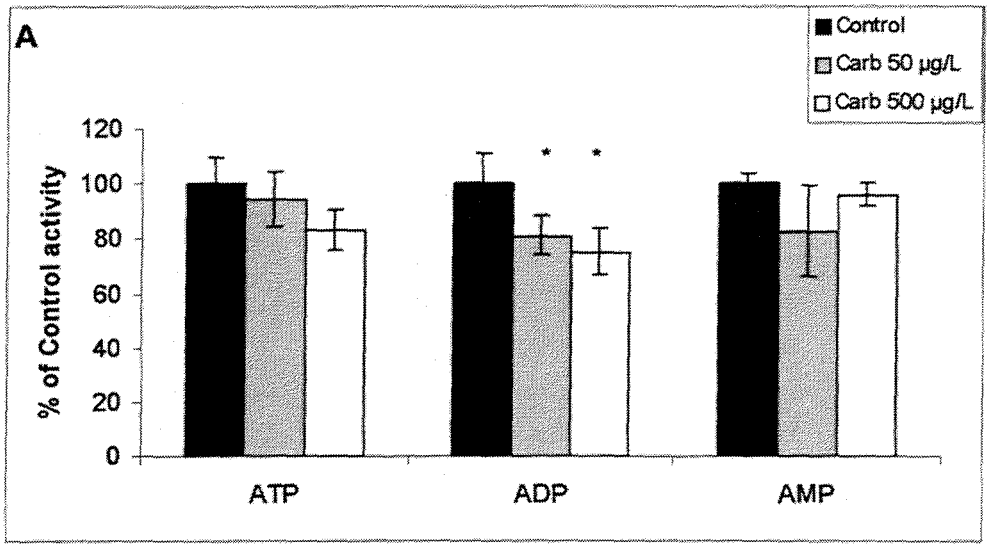
Fig. 2: *In vitro* effect of varying concentrations of carbofuran on NTPDase (A) and ecto-5′-nucleotidase (B) in zebrafish brain membranes. Bars represent the mean ± of at least four different experiments. The control ATPase, ADPase and AMPase activities (with no carbofuran) were 668.3 ± 84.7; 141 ± 23.2 and 29 ± 4.6 nmol $Pi.min^{-1}.mg^{-1}$of protein, respectively. * Significantly different from control group ($P \leq 0.05$).

Fig. 3: *In vitro* effect of varying concentrations of malathion on NTPDase (A) and ecto-5′-nucleotidase (B) in zebrafish brain membranes. Bars represent the mean ± of at least

four different experiments. The control ATPase, ADPase and AMPase activities (with no malathion) were 622.23 ± 58.8; 143.3 ± 17.8 and 22 ± 2.24 nmol $Pi.min^{-1}.mg^{-1}$ of protein, respectively. * Significantly different from control group ($P \leq 0.05$).

Fig.4: 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 (x) and in the presence of 3mM (□) and 5 mM of carbofuran (Δ). All experiments were repeated at least five times and similar results were obtained. Data represents an individual experiment.

Fig.5: 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 (x) and in the presence of 3mM (□) and 5 mM of malathion (Δ). All experiments were repeated at least five times and similar results were obtained. Data represents an individual experiment.


A
Control
Carb 50 µg/L
Carb 500 µg/L
% of Control activity
120
100
80
60
40
20
0
*
*
ATP
ADP
AMP

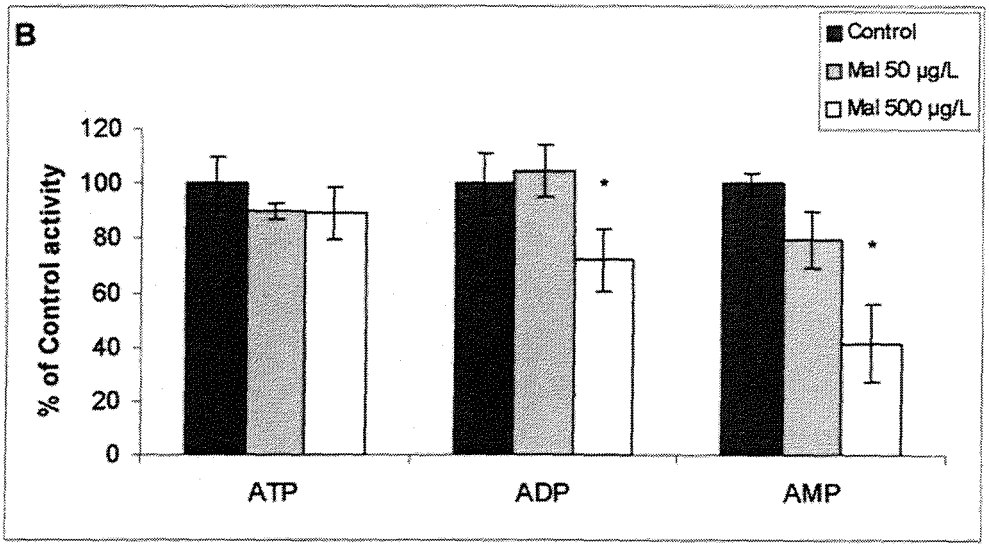
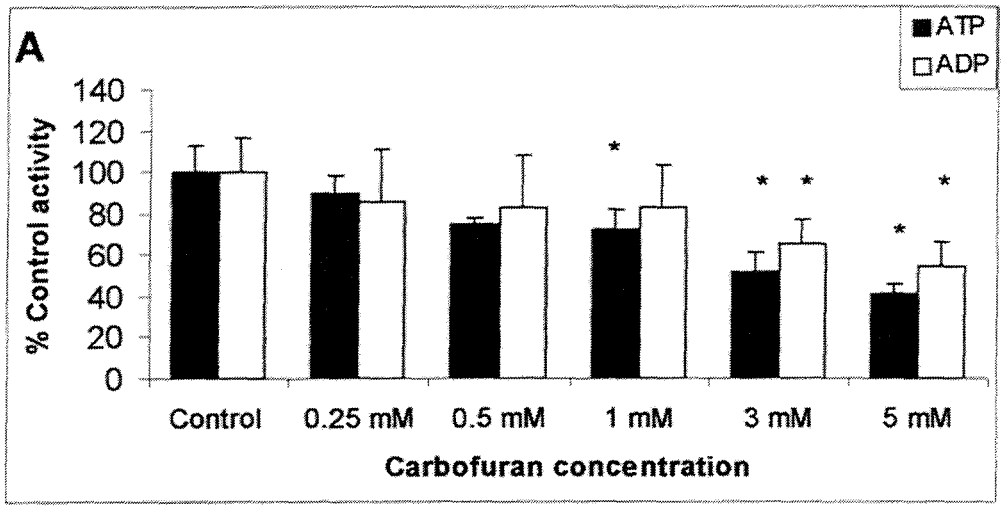

B
Control
Mal 50 µg/L
Mal 500 µg/L
% of Control activity
120
100
80
60
40
20
0
*
*
ATP
ADP
AMP

Fig.1


A
ATP
ADP
% Control activity
140
120
100
80
60
40
20
0
Control
0.25 mM
0.5 mM
1 mM
3 mM
5 mM
Carbofuran concentration

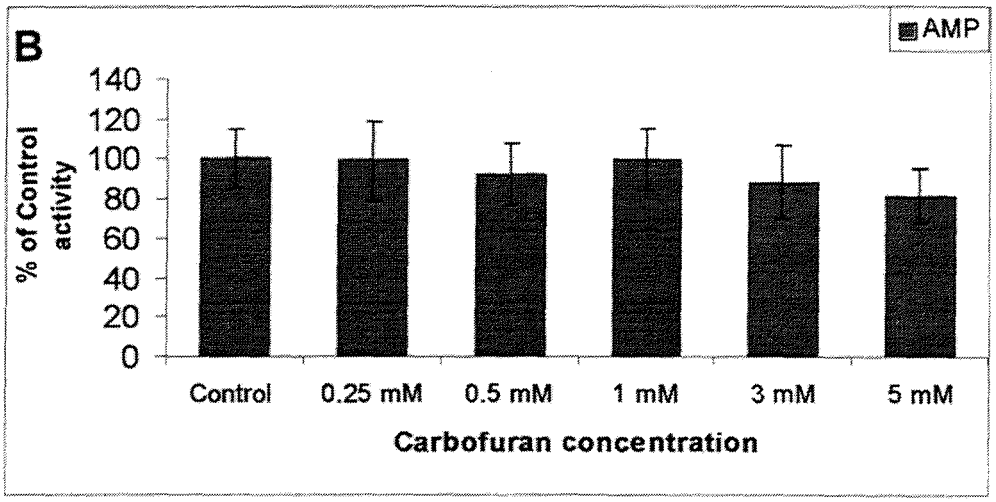
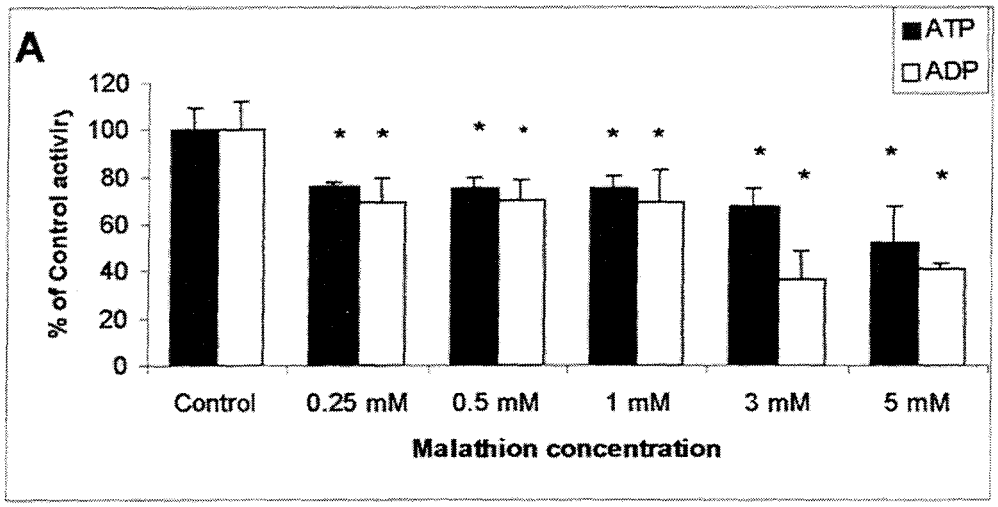

B
AMP
% of Control activity
140
120
100
80
60
40
20
0
Control
0.25 mM
0.5 mM
1 mM
3 mM
5 mM
Carbofuran concentration

Fig. 2



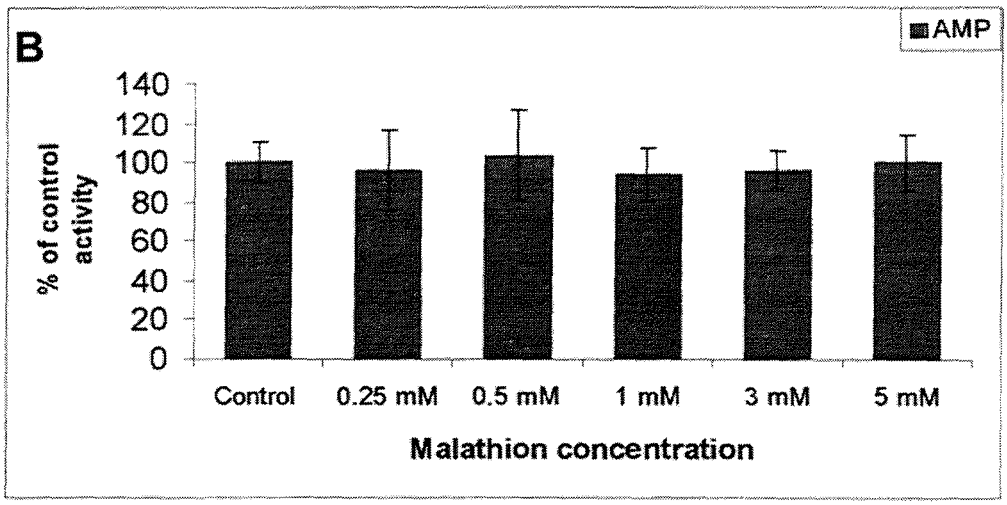
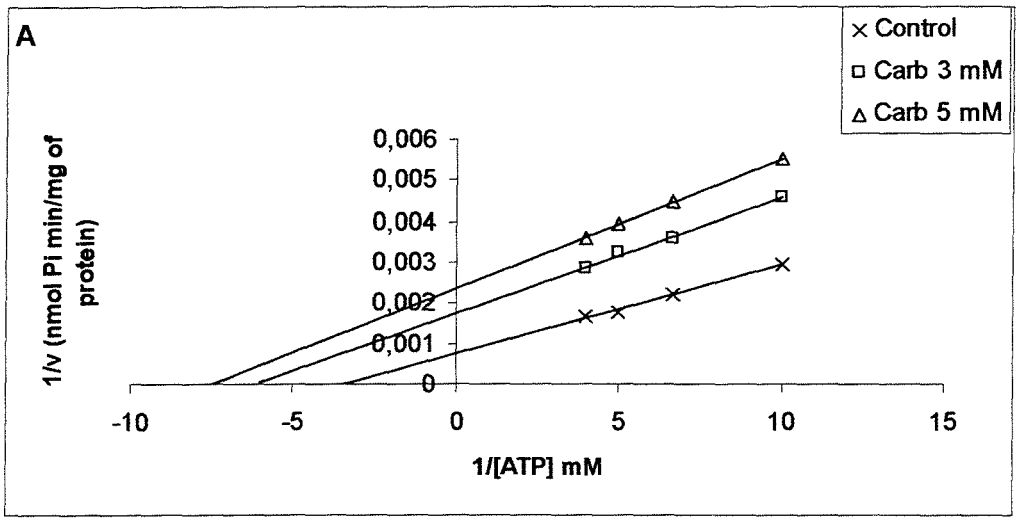


Fig.3


A
× Control
□ Carb 3 mM
△ Carb 5 mM
1/v (nmol Pi min/mg of protein)
0,006
0,005
0,004
0,003
0,002
0,001
0
-10
-5
0
5
10
15
1/[ATP] mM

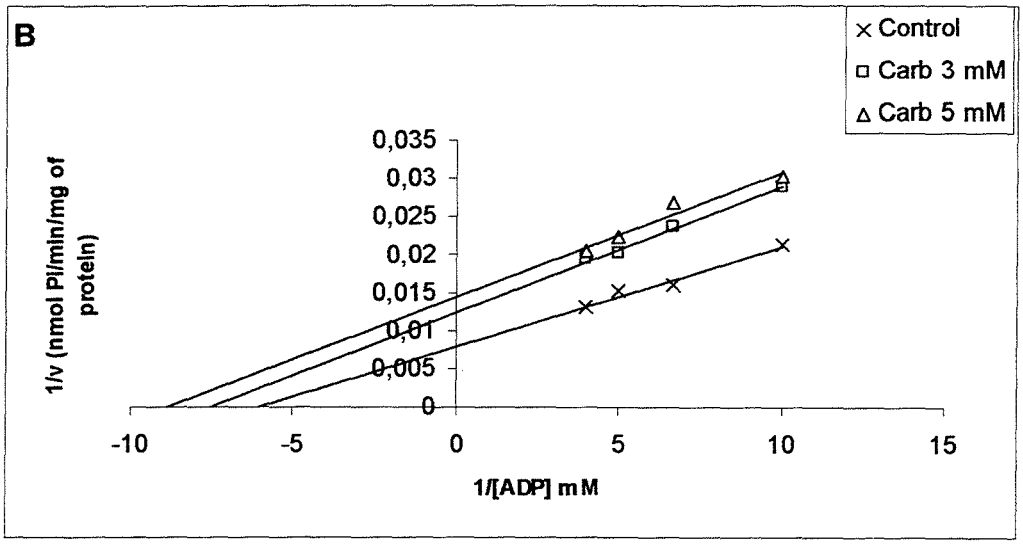
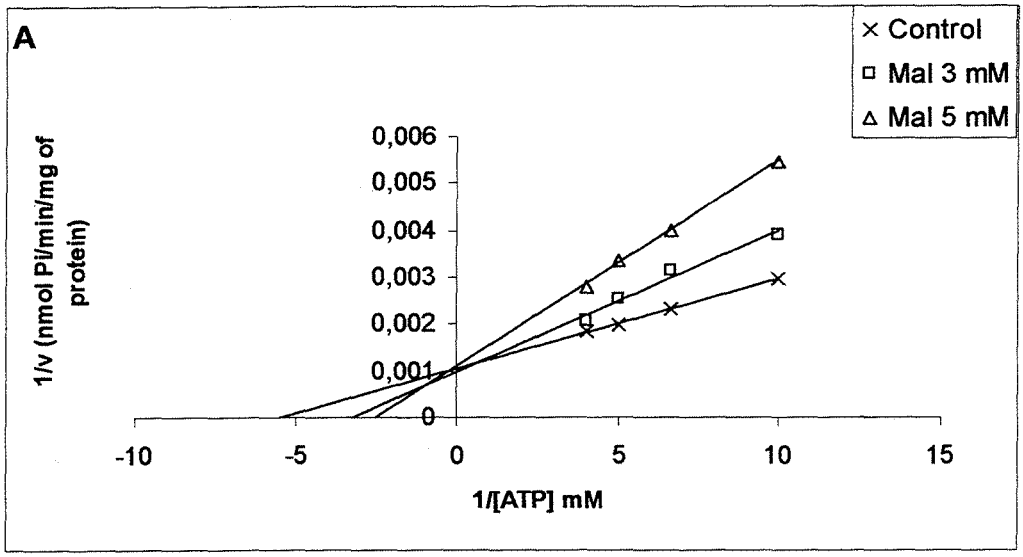

B
× Control
□ Carb 3 mM
△ Carb 5 mM
1/v (nmol Pi/min/mg of protein)
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
-10
-5
0
5
10
15
1/[ADP] mM

Fig. 4


A
Control
Mal 3 mM
Mal 5 mM
1/v (nmol Pi/min/mg of protein)
0,006
0,005
0,004
0,003
0,002
0,001
0
-10
-5
0
5
10
15
1/[ATP] mM

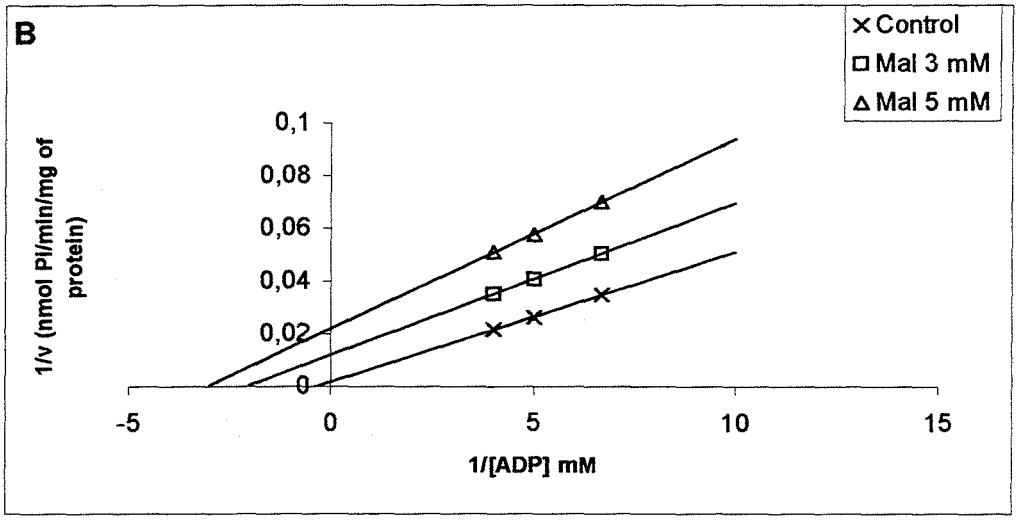

B
Control
Mal 3 mM
Mal 5 mM
1/v (nmol Pi/min/mg of protein)
0,1
0,08
0,06
0,04
0,02
0
-5
0
5
10
15
1/[ADP] mM

Fig. 5

3. Discussão geral

3.1.Considerações gerais

Nucleotídeos, principalmente o ATP, são importantes moléculas sinalizadoras no espaço extracelular e desempenham importantes papéis em condições fisiológicas e patológicas. A degradação destas moléculas é catalisada por uma família de enzimas denominadas ectonucleotidases, entre as quais se destacam a NTPDase e a ecto-5'-nucleotidase. Estas enzimas são muito importantes nas respostas mediadas pelos nucleotídeos, já que controlam os níveis extracelulares destas substâncias e a possível ativação dos receptores purinérgicos (RALEVIC & BURNSTOCK, 1998; ZIMMERMANN, 2001).

O Zebrafish é um peixe ósseo da família ciprinidae muito difundido como modelo experimental em diversas áreas do conhecimento como a neurociências e a toxicologia. O seu genoma está em fase final de sequenciamento e estudos demostraram que muitos de seus genes são similares ao de mamíferos, inclusive aos genes humanos. Estudos realizados nesta espécie demonstraram a presença de receptores purinégicos do tipo P2X e P2Y (KUCENAS et al., 2003; SCHULTZ & SCHÖNENBERG et al., 2003).

Estudos prévios desenvolvidos em nosso laboratório investigaram as propriedades enzimáticas de uma NTPDase em membranas cerebrais de zebrafish (RICO et al., 2003). Esta enzima se mostrou dependente de cátions divalentes, com uma atividade máxima de hidrólise para ATP e ADP na faixa de pH entre 7.5 e 8.0 na presença de cálcio (5 mM). A temperatura ótima para hidrólise de ATP e ADP foi 37°C. A enzima foi capaz de

hidrolisar tanto nucleotídeos púricos quanto pirimídicos e se mostrou insensível a inibidores clássicos de ATPases e fosfatases alcalinas. Uma inibição significativa foi observada na presença de 20 mM de azida sódica, considerado um possível inibidor da NTPDase. Nossos resultados sugerem que a atividade NTPDásica em membranas cerebrais de zebrafish possui características cinéticas similares à NTPDase 3 de mamíferos.

3.2.Caracterização da ecto-5′-nucleotidase (SENGER et al., 2004; CAPÍTULO 1).

Considerando que nosso laboratório através de estudos prévios demonstrou a presença de uma atividade NTPDásica capaz de hidrolisar o neurotransmissor ATP a AMP e que, para a completa hidrólise do ATP ao neuromodulador adenosina ,também é necessária a ação de uma ecto-5′-nucleotidase, o primeiro objetivo desta dissertação de mestrado foi caracterizar a atividade da ecto-5′-nucleotidase em membranas cerebrais de zebrafish.

Experimentos demonstraram que a hidrólise do AMP foi linear até 30 minutos de incubação na presença de magnésio. A taxa de hidrólise do AMP aumentou em função da concentração de proteína e foi linear na faixa de 3-10 μg de proteína no meio de incubação. A atividade da 5′-nucleotidase se mostrou dependente de cátions divalentes, sendo ativada por magnésio, cálcio e zinco. A ativação da hidrólise do AMP na presença de magnésio foi dependente da concentração (1 e 5 mM), mas apresentou uma diminuição da atividade AMPásica na concentração de 10 mM deste cátion. A hidrólise do AMP foi ativada por cálcio, mas este efeito não se mostrou dependente da

concentração e menor do que o efeito promovido pelo magnésio. A dependência a cátions foi confirmada por uma diminuição acentuada na hidrólise de AMP na falta destas substâncias. Entretanto, interessantemente, quando 5 mM de EDTA foi adicionado ao meio de incubação na presença de 5 mM de magnésio ou cálcio não foi observada nenhuma diminuição significativa na atividade enzimática. Por esta razão, foram realizados experimentos testando diferentes concentrações de EDTA (1, 5, 10 e 20 mM) na presença de magnésio na concentração de 5 mM. O EDTA não promoveu nenhuma mudança na hidrólise do AMP, sugerindo que mesmo após a quelação induzida por EDTA possam restar quantidades pequenas de cátions capaz de ativar a atividade enzimática.

Considerando que a ecto-5'-nucleotidase possui zinco em seu sítio ativo (BIANCHI & SPYCHALA, 2003), nós avaliamos o efeito de diferentes concentrações deste cátion (0,05-1 mM) na hidrólise de AMP. O zinco em baixas concentrações (0,05 mM) não foi capaz de aumentar a atividade enzimática. Entretanto, nas concentrações de 0,5 e 1 mM houve um aumentou significativo da atividade enzimática, a qual foi fortemente inibida em altas concentrações (5 e 20 mM). A falta de efeito inibitório do EDTA sugere que, se cátions divalentes como o magnésio e zinco são requeridos pela enzima, estes provavelmente estão firmemente ligados a seu sítio ativo. Considerando que o magnésio foi o ativador mais estável e que este cátion é um clássico ativador da 5'-nucleotidase (ZIMMERMANN, 1992), o magnésio na concentração de 5 mM foi usado nos ensaios enzimáticos subseqüentes.

Experimentos foram realizados avaliando a dependência de pH da atividade AMPásica. A taxa máxima de hidrólise de AMP foi no pH 7.2, similar ao pH ótimo

descrito para outras 5'-nucleotidases descritas na literatura (ZIMMERMANN, 1992; 1996). A hidrólise de AMP também foi realizada em diferentes temperaturas (10, 20, 37 e 45 ^{0}C). Dentre as temperaturas testadas os resultados demonstraram que a enzima foi mais ativa na temperatura de 37°C.

A hidrólise de AMP foi determinada em concentrações de substrato, variando de 100-2000 μM. A partir deste experimento, foi possível determinar as características cinéticas da enzima como o K_M e o V_{max} que foram 135.3 ± 16 μM e 29 ± 4.2 nmol Pi. min^{-1}. mg $^{-1}$ de proteína, respectivamente.

A ecto-5'-nucleotidase é uma enzima que apresenta uma ampla especificidade a nucleotídeos monofosfatados (ZIMMERMANN, 1996). Nossos resultados demonstraram que membranas cerebrais de zebrafish são capazes de hidrolisar tanto nucleotídeos monofosfatados púricos e pirimídicos como o AMP, GMP, UMP e CMP.

Com o objetivo de descartar a interferência de outras enzimas como fosfatases alcalinas na hidrólise do AMP, inibidores específicos para estas enzimas foram adicionados ao meio de incubação. Levamisole e tetramisole, na concentraçãode 1 mM, não interferiram na hidrólise de AMP. AMPCP, um inibidor clássico da 5´-nucleotidase também foi testado, produzindo uma inibição significativa na hidrólise do AMP na concentração de 0,1 mM.

Os resultados encontrados neste trabalho demonstram que a hidrólise de nucleotídeos monofosatados em membranas cerebrais de zebrafish é realizada por uma enzima com propriedades cinéticas típicas de uma ecto-5'-nucleotidase. Considerando que estudos prévios identificaram purinoreceptores e uma atividade NTPDásica no sistema nervoso central de zebrafish, a presença de uma ecto-5'-nucleotidase no sistema

nervoso central nesta espécie parece ser um componente chave no sistema de neurotransmissão purinérgica.

3.3. Efeito do mercúrio e do chumbo na atividade das ectonucleotidases no sistema nervoso central de zebrafish (CAPÍTULO 2).

Este estudo demonstrou que a exposição *in vitro* e *in vivo* ao cloreto de mercúrio e ao acetado de chumbo promove alterações significativas na hidrólise de nucleotídeos em membranas cerebrais de zebrafish. O cloreto de mercúrio inibiu *in vitro* a hidrólise de ATP, ADP e AMP de uma forma dependente da concentração. Os resultados obtidos nos experimentos duplo-recíprocos indicaram que a inibição promovida pelo cloreto de mercúrio na hidrólise de ATP e ADP foi acompetitiva e a inibição da hidrólise do AMP foi não-competitiva .

Nos experimentos *in vivo*, peixes foram expostos (24 h, 96 h e 30 dias) a concentrações de mercúrio e chumbo encontradas no ambiente (20 μg/L) (BERZAS NEVADO, 2003; JHA et al., 2003). A exposição ao cloreto de mercúrio durante 96 h promoveu uma diminuição na atividade da NTPDase e da ecto-5'-nucleotidase. Entretanto, após o tratamento crônico (30 dias), a hidrólise do ATP retornou aos níveis do controle, a hidrólise do ADP apresentou um grande aumento, e a hidrólise do AMP continuou inibida. A exposição a baixas doses de cloreto de mercúrio no ambiente aquático interferiu no sistema purinérgico,agindo de modo diferente na hidrólise dos nucleotídeos. A ativação da NTPDase após 30 dias de tratamento pode ser um

mecanismo compensatório desta enzima frente ao cloreto de mercúrio (CALABRESE & BALDWIN, 2003). Moretto et al. (2004) demonstraram que o tratamento com baixas doses subcutâneas de cloreto de mercúrio (0.1 mg/Kg; 30 doses/30dias) promoveu a ativação da NTPDase e da acetilcolinesterase, mas não da 5′-nucleotidase em sinaptosomas do cortex cerebral de ratos. Segundo estes autores, seus resultados podem ser relacionados a um efeito bifásico do tipo hormético (CALABRESE & BALDWIN, 2001). Considerando que no nosso estudo também foram testadas doses baixas de metais, é possível sugerir que um efeito hormético similar poderia influenciar a resposta da NTPDase a estes poluentes. Muitos estudos demonstraram que poluentes ambientais podem desencadear mecanismos protetores como a indução de enzimas que metabolizam xenobióticos, fatores envolvidos na transdução de sinal e resposta ao estresse oxidativo via ativação transcricional de genes importantes nessas rotas (NIHEI et al., 2001). Além disso, a ativação de fatores de transcrição em culturas celulares de zebrafish por poluentes ambientais já foram descritos (CARVAN III et al., 2000). A ativação da NTPDase depois de 30 dias de tratamento pode ser devido a mudanças na expressão desta enzima após a longa exposição ao cloreto de mercúrio. Estudos estão sendo desenvolvidos em nosso laboratório afim de investigar possíveis mudanças transcricionais na NTPDase induzida por metais pesados.

Na literatura, a avaliação da pirimidina 5'-nucleotidase vem sendo utilizada como um parâmetro de exposição ocupacional ao chumbo (KIM et al., 2002a; 2002b). Entretanto, até este momento, não existem estudos verificando o efeito do acetato de chumbo em enzimas que metabolizam o ATP extracelular. Nosso estudo apresenta a

primeira evidência sobre o efeito inibitório deste metal pesado na rota das ectonucleotidases.

Nucleotídeos extracelulares são importantes moléculas sinalizadoras tanto em condições fisiológicas quanto patológicas. Após sua liberação na fenda sináptica, o ATP pode ser degradado a ADP, AMP e adenosina. A adenosina geralmente desencadeia um forte efeito neuroprotetor, contrastando com o efeito excitatório do ATP (DI VIRGILIO, 2000; KATO et al., 2004). Os efeitos dos nucleotídeos dependem do seu catabolismo extracelular mediado pelas ectonucleotidases, que regulam as concentrações de ATP/adenosina e as respostas mediadas por receptores P2/P1, respectivamente. É possível sugerir que a mudança na atividade das ectonucleotidases induzida pela exposição a metais pesados pode promover alterações nas concentrações extracelulares de nucleotídeos. A mudança na atividade das ectonucleotidases pode provocar um desequilíbrio na neurotransmissão purinérgica, afetando sinais de transdução mediados por nucleotídeos, os quais poderiam contribuir para os fatores neurotóxicos promovidos pelos metais pesados.

Baseado nos dados apresentados neste estudo, foi demonstrado que o mercúrio e o chumbo afetam a atividade da NTPDase e a ecto-5'-nucleotidase, sugerindo que o sistema purinérgico pode ser um alvo relacionado a neurotoxicidade induzida por estes metais pesados e um possível indicador do impacto biológico a exposição a estas substâncias.

3.4.Carbofuran e malathion inibem a hidrólise de nucleotídeos em membranas cerebrais de zebrafish (*Danio rerio*).(CAPÍTULO 3)

Este trabalho demonstrou que o carbofuran e o malathion inibem a atividade das ectonucleotidases *in vivo* e *in vitro* em membranas cerebrais de zebrafish. Interessantemente, no estudo in vivo apenas a hidrólise do ADP foi inibida pelo carbofuran em ambas as concentrações testadas (50 μg/L e 500 μg/L), mas a hidrólise do ATP e AMP não foi modificada por este tratamento. Após a exposição *in vivo,* o malathion inibiu significativamente a hidrólise de ADP e AMP apenas na concentração de 500 μg/L após os sete dias de tratamento.

No estudo *in vitro,* o carbofuran inibiu a hidrólise de ATP e ADP de uma maneira acompetitiva, sem efeito na hidrólise do AMP. O malathion inibiu *in vitro* a hidrólise do ATP e ADP, sendo a inibição da atividade ATPásica competitiva e a inibição ADPásica acompetitiva. O malathion não inibiu *in vitro* a atividade AMPásica em membranas cerebrais de zebrafish.

Estudos já demonstraram que a exposição *in vivo* ao malathion afeta a atividade da acetilcolinesterase de uma forma dose-dependente no cérebro do zebrafish (ANSARI & KUMAR, 1984). Os autores testaram concentrações variando de 0,5 a 1,1 mg/L em um período de 7 dias de exposição. A inibição ocorreu em todas concentrações testadas, variando de 67% na menor dose (500 μg/L; onde não foram observadas mortalidades), a 90% na dose mais alta testada (1100 μg/L; quando 50% dos peixes sobreviveram). Os autores sugeriram que a atividade anti-colinesterásica do malathion pode não ser a única causa de morte, porque mesmo com uma inibição de 90% da acetilcolinesterase foi

observado sobrevivência dos peixes. Nossos resultados demonstraram que após a exposição à menor dose utilizada em nosso estudo (50 μg/L), as enzimas envolvidas nos níveis de nucleotídeos extracelulares não foram significativamente alteradas. Entretanto, o malathion na concentração de 500 μg/L, inibiu a hidrólise de ADP e AMP (28% e 58.5%, respectivamente), o que nos leva a propor que estas enzimas, junto com a acetilcolinesterase, poderiam ser consideradas úteis indicadores de contaminação ambiental por pesticidas.

Na literatura, estudos já descreveram que sistemas de neurotransmissores não-colinérgicos podem também ser afetados pelos efeitos tóxicos de pesticidas. GUPTA et al., (1984) examinaram o efeito de múltiplas doses de carbofuran nas concentrações de acetilcolina, GABA, norepinefrina, dopamina e serotonina em cérebro de camundongo. O tratamento com carbofuran causou um aumento significativo nos níveis destes neurotransmissores. O efeito do carbofuran nas catecolaminas cerebrais de goldfish também foi investigado. A exposição *in vivo* ao carbofuran a 50 μg/L ou 500 μg/L durante 24 e 48h causou um aumento significativo nas catecolaminas em regiões cerebrais distintas do goldfish (BRETAUD et al., 2002). Nossos resultados demonstraram que a hidrólise do ADP foi significativamente diminuída após a exposição ao carbofuran, mas diferenças significativas não foram observadas na hidrólise do ATP e AMP. O efeito inibitório diferencial observado na hidrólise do ATP e ADP nos leva a propor que diferentes ectonucleotidases estariam envolvidas no controle da hidrólise de nucleotídeos no sistema nervoso central do zebrafish. Em mamíferos, já foram caracterizadas quatro membros de ectonucleotidaes que possuem seus sítios ativos voltados para o espaço extracelular. Futuros estudos serão necessários para identificar quais membros da família

das ectonucleotidases estão envolvidos na hidrólise de ATP e ADP no sistema nervoso central de zebrafish.Este trabalho avaliou a relação entre pesticidas, reconhecidos como agentes anticolinesterásicos, na atividade das ectonucleotidases em sistema nervoso central de zebrafish. Nosso estudo demonstrou que o sistema purinérgico pode ser um alvo potencial para neurotoxicidade induzida por pesticidas carbamatos e organofosforados.

4. Conclusão final

Com os resultados apresentados nesta Dissertação de Mestrado, nós podemos concluir que as ectonucleotidases, tais como a NTPDase e a ecto-5'-nucleotidases são encontradas no SNC do zebrafish. Estas enzimas são sensíveis a ação de metais pesados e pesticidas e parecem ser possíveis alvos envolvidos na neurotoxicidade mediada por estes poluentes.

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