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BIOQUÍMICA

IDENTIFICAÇÃO BIOQUÍMICA E MOLECULAR DE NUCLEOTIDASES EM FRAÇÃO SOLÚVEL E MICROSSOMAL DE TECIDO CARDÍACO DE RATOS

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"A alegria está na luta, na tentativa, no sofrimento envolvido e não na vitória propriamente dita."

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Apresentação

Conforme as normas do Programa de Pós Graduação em Ciências Biológicas:Bioquímica, esta tese está organizada em três partes principais:

Parte I – contendo os Resumos, a Lista de Abreviaturas, a Introdução e os Objetivos do trabalho;

Parte II – aborda essencialmente os Materiais e Métodos e os Resultados do trabalho, sendo apresentada na forma de capítulos constituídos por artigos científicos publicados e/ou em preparação;

Parte III – constitui-se da interpretação dos resultados (Discussão), Conclusões, Perspectivas, Referências (apenas as citadas nas Partes I e III) e da Lista de Figuras.

Sumário

PARTE I

RESUMO

Os nucleotídeos da adenina (ATP, ADP e AMP) e o nucleosídeo adenosina são importantes moléculas sinalizadoras que estão envolvidas nos mecanismos de crescimento e diferenciação celular, angiogênese, fluxo sanguíneo, condução e taxa cardíaca, e sensibilidade à estimulação adrenérgica no coração. Paralelamente a esses efeitos extracelulares, algumas evidências com fibras musculares permeabilizadas e vesículas de retículo sarcoplasmático cardíaco (chamados de microssomas) têm sugerido que estas purinas poderiam modular correntes de Ca^{2+} do retículo sarcoplasmático, ativando diretamente o canal iônico. Mudanças nos níveis de ATP, ADP, AMP e adenosina alteram suas ações, o que tem sido implicado em diversos processos patofisiológicos do coração. Consequentemente, as enzimas que metabolizam nucleotídeos, denominadas ectonucleotidases, que fazem parte da sinalização purinérgica, desempenham um componente modulatório essencial, pois controlam a quantidade e a taxa de degradação dos nucleotídeos além da formação do respectivo nucleosídeo. No presente estudo, foi analisada a presença dos membros das famílias das E-NTPDases, E-NPPs e ecto-5'-nucleotidase em frações solúvel e microssomal cardíacas de ratos. No estudo das E-NTPDases, a caracterização bioquímica das atividades de hidrólise de ATP e ADP em condições de inibição da atividade da ATPase mitocondrial e da adenilato cinase, revelou enzimas dependentes de cátions divalentes que apresentam pH ótimo de 8,0 na fração solúvel e 7,5 na fração microssomal. Os valores de K_M aparente calculados a partir do plot de Eadie-Hofstee na fração solúvel foram 131,2 e 57,1 µM e de V_{max} 71,3 e 9,3 nmol Pi/ min /mg de proteína para a hidrólise de ATP e ADP, respectivamente. Na fração microsomal, os valores de K_M calculados para ATP e ADP foram 315,2 e 131 µM, e os valores de V_{max} foram 1180,6 and 100,4 nmol Pi/ min/ mg de proteína, respectivamente. Considerando a atividade da ecto-5'-nucleotidase, utilizando AMP como substrato, os resultados mostraram que o cation e a concentração requerida para a atividade máxima nas duas frações, foi magnésio na concentração final de 1 mM. O pH ótimo para ambas frações foi 9,5. Os valores de K_M foram 59,7 µM e 134,8 µM com um valor de V_{max} calculado de 6,7 e 143,8 nmol Pi/ min/ mg de proteína para fração solúvel e microsomal, respectivamente. A análise de Western blotting para a ecto-5'-nucleotidase revelou uma proteína de 70kDa nas duas frações e uma maior densidade na fração microssomal. Usando *p*-nitrofenil-5'-timidina monofosfato (*p*-Nph-5'-TMP) como substrato para as E-NPPs, nós observamos um pH ótimo alcalino e dependência para cations divalentes. Os valores de K_M corresponderam a 118,5 e 91,9 µM e os valores do V_{max} calculado foram 2,5 e 113,8 nmol *p*-nitrofenol/ min/ mg de proteína para a fração solúvel e microsomal, respectivamente. A presença dessas enzimas no coração tem, provavelmente, uma função fisiológica na geração de adenosina. Além disso, na fração microsomal, essas enzimas poderiam exercer um papel na modulação do processo de excitação-contração do coração através do envolvimento com o influxo de Ca^{2+} para o retículo sarcoplasmático.

Com o intuito de investigar os possíveis efeitos da cafeína sobre a atividade das ectonucleotidases na fração solúvel e microsomal cardíaca, nós utilizamos a administração aguda e crônica de cafeína. Para o estudo agudo, nós administramos a cafeína por oral gavage em uma única dose de 5, 15 ou 45 mg/Kg. Os controles receberam salina e todos os animais foram mortos 1,5h após a gavage. No tratamento crônico, a cafeína (0,3 or 1,0 mg/mL) foi oralmente administrada por 14 dias na água e os animais foram mortos no 15º dia. Os resultados preliminares mostram que tanto o tratamento agudo quanto o crônico foram capazes de alterar diferentemente a hidrólise dos nucleotídeos em ambas frações. Todavia, mais dados são necessários para tentar relacionar os resultados encontrados e os conhecidos efeitos da cafeína no coração.

ABSTRACT

Adenine nucleotides (ATP, ADP, and AMP) and the nucleoside adenosine are important signaling molecules that are very much involved in the mechanisms of cell growth and differentiation, angiogenesis, coronary blood flow, cardiac conduction and heart rate, and sensitivity to adrenergic stimulation in the heart. Besides these extracellular effects, some evidences with permeabilized muscle fibers and cardiac sarcoplasmic reticulum vesicles (called microsomes) have suggested that these purines could modulate Ca^{2+} currents of the sarcoplasmic reticulum activating directly the ionic channel. Changes in the adenine nucleotides and adenosine levels alter their actions and have been implicated in diverse patho-physiological processes of the heart. Consequently, nucleotide-metabolizing enzymes (ectonucleotidases) play an essential modulatory component of the purine signaling once that controls the rate, amount and timing of nucleotide degradation and ultimately, the nucleoside formation. In the present study, we have analyzed the presence of the E-NTPDase family members, E-NPPs and ecto-5'-nucleotidase in rat cardiac soluble and microsomal fractions. In the E-NTPDase study, biochemical characterization of ATPase and ADPase activities, under conditions where mitochondrial ATPase and adenylate kinase activities were blocked, demonstrates divalent-cation dependent enzymes that presented optimum pH of 8.0 to soluble, and 7.5 to microsomal fraction. The apparent K_M values calculated from the Eadie-Hofstee plot for ATP and ADP in soluble fraction were 131.2 and 57.1 µM, respectively. V_{max} values calculated were 71.3 and 9.3 nmol Pi/ min/ mg of protein for ATP and ADP hydrolysis, respectively. In microsomal fraction, the K_M values calculated for ATP and ADP were 315.2 and 131 µM, respectively. V_{max} values in this fraction were 1180.6 and 100.4 nmol Pi/ min/ mg of protein when using ATP and ADP as substrates, respectively. Considering ecto-5'-nucleotidase activity with AMP as substrate, the results showed that the cation and the concentration required for maximal activity in the two fractions was magnesium in the final concentration of 1.0 mM. The pH optimum for both fractions was 9.5. The apparent K_M calculated were 59.7 µM and 134.8 µM with a V_{max} values calculated of 6.7 and 143.8 nmol Pi/ min/ mg of protein from soluble and microsomal fractions, respectively. Western blotting analysis to ecto-5'-nucleotidase revealed a 70kDa protein in both fractions and a major density in the microsomal fraction. Using *p*-nitrophenyl-5'-thymidine monophosphate (*p*-Nph-5'-TMP) as substrate for E-NPPs, we observed an alkaline optimum pH and divalent cation dependence. The K_M value corresponded to 118.5 and 91.9 µM and V_{max} value calculated were 2.5 and 113.8 nmol *p*-nitrophenol/ min/ mg of protein from soluble and microsomal fractions, respectively. The presence of these enzymes in the heart probably has a physiological function in the generation of signaling adenosine. Furthermore, in the microsomal fraction, they could have a role in the modulation of the excitation-contraction coupling process throughout the involvement with the Ca^{2+} influx to sarcoplasmic reticulum.

To investigate the possible effects of caffeine on ectonucleotidase activity in cardiac soluble and microsomal fractions of rats, we used acute and chronic caffeine administration. For the acute study, we administrate to the rats by gavage, a single oral dose of 5, 15 or 45 mg/Kg b.w. of caffeine. Controls received saline and all animals were euthanized 1.5h after gavage. In the chronic treatment, caffeine (0.3 or 1.0 mg/mL) was orally administered for 14 days in the drinking water and the animals were euthanized on the day 15. Preliminary results showed that acute and chronic caffeine treatment were capable of differently alter nucleotide hydrolysis in both fractions. However, more data are necessary to correlate the results founded and the known cardiac effects of caffeine.

LISTA DE ABREVIATURAS

ADK - adenosina cinase

ACR – regiões conservadas da apirase

CD39 - antígeno de ativação celular linfóide

CD73 - ecto-5'-nucleotidase

Ecto-ADA - ecto-adenosina deaminase

Ecto-ATPDase - ecto-ATP difosfoidrolase

E-NPP - ecto-nucleotídeo pirofosfatase/fosfodiesterase

E-NTPDase - ecto-nucleosídeo trifosfato difosfoidrolase

GPI - glicosil-fosfatidilinositol

mRNA - RNA mensageiro

NAD - nicotinamida adenina dinucleotídeo

NTPDase – nucleosídeo trifosfato difosfoidrolase

P2X - receptor purinérgico ionotrópico

P2Y - receptor purinérgico metabotrópico

p-nitrophenyl-5'-TMP - *p*-nitrofenil-5'-timidina monofosfato

RT-PCR – reação em cadeia da polimerase – transcriptase reversa

1. INTRODUÇÃO

1.1 Sistema Purinérgico

O sistema purinérgico constitui-se de uma importante via de sinalização celular em que purinas extracelulares como ATP, GTP e seus metabólitos, exercem seus efeitos através da interação com receptores de membrana específicos, denominados receptores purinérgicos ou purinoceptores (Burnstock, 2002).

A primeira evidência sobre as potentes ações das purinas foi publicada por Drury & Szent-Györgyi em 1929. Nesse estudo os autores demonstraram que a adenosina e o AMP eram capazes de induzir efeitos cronotrópicos negativos no coração, além de dilatação arterial, diminuição da pressão arterial sanguínea e inibição da contração intestinal. Somente quatro décadas depois surgiram evidências para o papel do nucleotídeo ATP como um neurotransmissor em nervos não-adrenérgicos e não-colinérgicos de intestino e bexiga (Burnstock *et al.*, 1970). A partir de então a palavra "purinérgico" foi criada e o conceito de um sistema de sinalização purinérgica, usando nucleotídeos purínicos e nucleosídeos como mensageiros extracelulares foi proposto (Burnstock, 1972). Ainda na mesma década houve a identificação e caracterização dos purinoceptores, enfatizando ainda mais a hipótese purinérgica (Burnstock, 1976; Burnstock, 1978).

Atualmente a sinalização purinérgica é amplamente reconhecida como um sistema primitivo envolvido em muitos mecanismos neuronais e não-neuronais (Burnstock & Knight, 2004; Burnstock, 2006).

1.1.1 Nucleotídeos e nucleosídeos extracelulares e seus receptores

Extracelularmente os nucleotídeos e nucleosídeos purínicos (como ATP, ADP, AMP e adenosina) e pirimidínicos (como UTP e UDP) atuam como importantes moléculas sinalizadoras que induzem uma multiplicidade de efeitos em diversos sistemas biológicos (Ralevic & Burstock, 1998).

De uma maneira geral, os processos relacionados às purinas e pirimidinas incluem neurotransmissão no sistema nervoso central, contração não-colinégica e não-adrenérgica em músculo liso e interações neurônio-glia (Ralevic & Burnstock, 1998), efeitos inotrópicos, cronotrópicos e arritmogênicos no miocárdio (Vassort, 2001), funções gastrointestinais e hepáticas (Roman & Fitz, 1999), regulação da resposta de células epiteliais (Schwiebert & Zsembery, 2003), regulação do fluxo sanguíneo (Gonzalez-Alonso *et al.*, 2002), resposta imune e inflamação (Bours *et al.*, 2006) e agregação plaquetária em sítios de injúria vascular (Marcus *et al.*, 2003; Gachet, 2006). Além da sinalização de eventos agudos, as purinas e pirimidinas também apresentam papéis tróficos na proliferação e crescimento celular (Burnstock, 2006), indução de apoptose e atividade anticâncer (Bours *et al.*, 2006; White & Burnstock, 2006), formação da placa aterosclerótica (Di Virgilio & Solini, 2002), cicatrização, formação e reabsorção óssea (Hoebertz *et al.*, 2003) e alterações vasculares no diabetes (Solini *et al.*, 2004).

A liberação de nucleotídeos endógenos para o meio extracelular pode acontecer por diversos mecanismos (Figura 1) e representa um componente crítico para o início da cascata de sinalização (Yegutkin, 2008). O extravasamento massivo de nucleotídeos por lise celular é um mecanismo não-específico que está restrito à injúria de órgãos, estresse mecânico e algumas

condições inflamatórias (Bours *et al.*, 2006; Volonté & D'Ambrosi, 2009). Além disso, mecanismos não-líticos de efluxo de nucleotídeos representam uma rota distinta e importante de aparecimento dessas moléculas no meio extracelular. Vários tecidos com características celulares excitatórias e/ou secretórias tais como terminais nervosos e células cromafins (Burnstock, 2007), células acinares pancreáticas (Sorensen & Novak, 2001) e plaquetas circulantes (Marcus *et al.*, 2003; Gachet, 2006) estocam ATP e ADP em grânulos especializados e regulam a liberação dessas vesículas contendo nucleotídeos (exocitose) de uma maneira dependente de Ca^{2+}. Os nucleotídeos podem ainda atravessar a membrana plasmática em direção ao compartimento extracelular usando canais iônicos e transportadores de ATP de membrana (Yegutkin, 2008; Volonté & D'Ambrosi, 2009). Razões adicionais pelas quais as purinas podem estar presentes no lado externo da célula são a sua pequena massa molecular, a alta taxa de difusão no meio extracelular e o alto gradiente formado entre os ambientes intra e extracelulares (Volonté & D'Ambrosi, 2009).
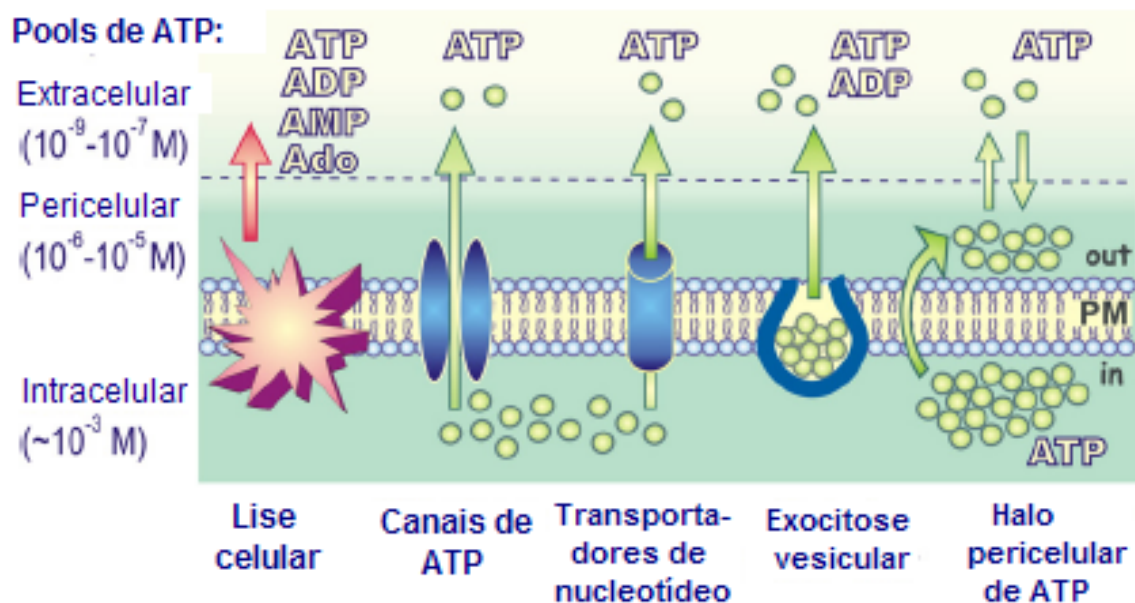

Figura 1. Vias de liberação das purinas para o espaço extracelular. Adaptado de *G. Yegutkin (2008).*

Uma vez no espaço extracelular, estas purinas servem como ligantes para um grande número de distintos receptores de superfície celular, os purinoceptores (Figura 2), os quais compreendem os receptores P1, ativados por adenosina, e os receptores P2, ativados por ATP, ADP, UTP e UDP (Burnstock, 1978; Erlinge & Burnstock, 2008).

Os receptores P1 se subdividem em A_1, A_{2A}, A_{2B} e A_3 e são ativados por adenosina, com o potencial agonista na ordem adenosina > AMP > ADP> ATP. Enquanto os receptores A_1 e A_3 são acoplados a proteína G_i e inibem a adenilato ciclase, ambos os receptores A_{2A} e A_{2B} são acoplados à proteína G_s e estimulam a adenilato ciclase (Ralevic & Burnstock, 1998; Czajkowski & Baranska, 2002).

A classificação dos receptores P2 foi inicialmente baseada em critérios farmacológicos (Burnstock & Kennedy, 1985), e posteriormente reforçada através de técnicas de clonagem e expressão em sistemas heterólogos (Evans *et al.*, 1995). Estes receptores podem ser classificados em duas famílias: receptores P2X e P2Y. Os receptores P2X atuam como canais ionotrópicos ativados por ATP e estão divididos em sete subtipos ($P2X_{1-7}$). Os receptores metabotrópicos P2Y são acoplados a proteínas G e estão divididos nos subtipos $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$ (Ralevic & Burnstock, 1998; Communi *et al.*, 2001; Hollopeter *et al.*, 2001; Erlinge & Burnstock, 2008; Yegutkin, 2008). Os receptores purinérgicos apresentam uma ampla distribuição tecidual e, como conseqüência dos seus ligantes, estão implicados em um grande número de funções.
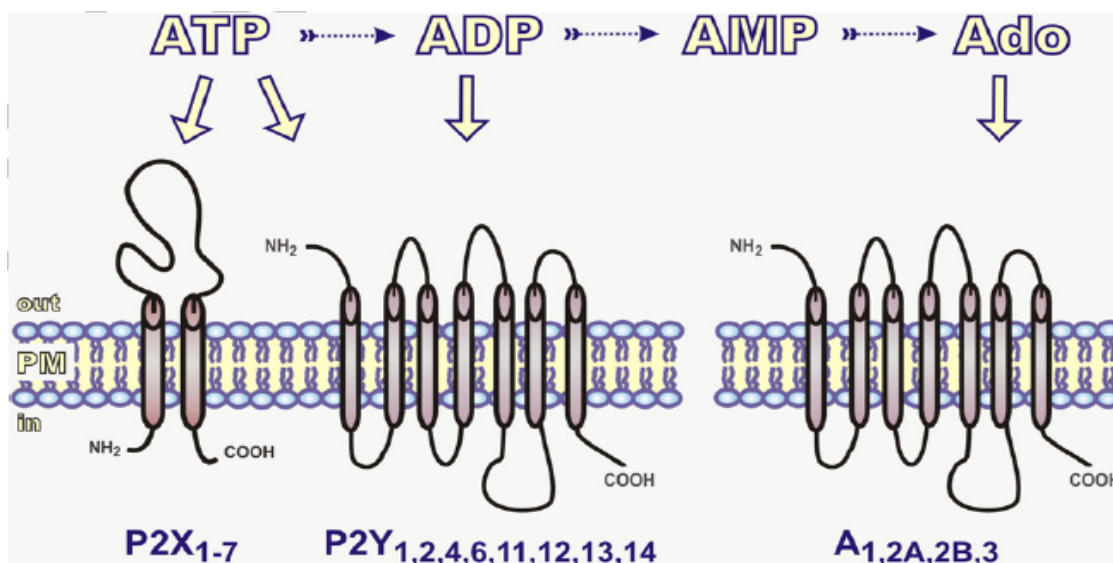
Figura 2. Vias de sinalização purinérgica. Sinalização mediada por nucleotídeos através de receptores ionotrópicos P2X e metabotrópicos P2Y. O nucleosídeo adenosina age em quatro receptores próprios acoplados à proteína G. *Obtido de G. Yegutkin (2008).*

1.1.2 Os nucleotídeos ATP e ADP no sistema cardiovascular

Os nucleotídeos ATP e ADP influenciam fortemente o sistema cardiovascular exercendo potentes efeitos em diversos processos fisiológicos e patológicos (Ralevic & Burnstock, 1991; Ralevic & Burnstock, 2003; Burnstock, 2006; Yegutkin, 2008). Múltiplos fatores são importantes para determinar a resposta provocada por estas purinas, entre eles destaca-se a natureza do subtipo de purinoceptor envolvido e a sua localização (Ralevic & Burnstock, 1991). Todas as células do sistema cardiovascular expressam um ou mais subtipos de purinoceptores (Ralevic & Burnstock, 2003), dependendo da célula e do receptor, essas purinas podem desempenhar numerosos efeitos que estão principalmente relacionados a cardioproteção.

O nucleotídeo ATP é capaz de atuar com efeitos opostos dependendo da concentração, da célula e do receptor. O ATP liberado como co-transmissor de nervos simpáticos é capaz de contrair a musculatura vascular lisa via receptores

P2X, enquanto o ATP liberado dos nervos motores sensoriais durante a atividade do "arco-reflexo" pode dilatar os vasos via receptores P2Y. Além disso, o ATP liberado das células endoteliais durante mudanças do fluxo sangüíneo (shear stress) ou durante hipóxia, é capaz de agir em receptores P2Y nestas células e liberar óxido nítrico (NO), resultando em relaxamento (Kunapuli & Daniel, 1998; Burnstock, 2002). O ATP também pode exercer influência sobre o sistema vascular por interferir no processo de agregação plaquetária e promover proliferação de células musculares lisas e células endoteliais (Soslau & Youngprapakorn, 1997; Ralevic & Burnstock, 2003).

Além dos efeitos na vasculatura cardiovascular, o ATP apresenta efeitos cronotrópicos (ritmo), dromotrópicos (condutividade) e inotrópicos (contratilidade) em preparações de coração isolado (Rongen *et al.*, 1997). O ATP geralmente é responsável por efeitos inotrópicos positivos e sua rápida aplicação em células induz várias formas de arritmia. Além disso, estudos recentes têm demonstrado que esse nucleotídeo é capaz de modular correntes de Ca^{2+}, Na^+, K^+ and Cl^- em cardiomiócitos (Vassort, 2001). Outros possíveis papéis do ATP no coração incluem a hipertrofia, precondicionamento e apoptose (Webb, 1996; Kunapuli & Daniel, 1998; Vassort, 2001; Ralevic & Burnstock, 2003).

Em relação ao nucleotídeo ADP, o principal efeito funcional dessa molécula é a estimulação da agregação plaquetária por meio da interação com os receptores $P2Y_{12}$ das plaquetas (Gachet, 2001). Por esse motivo, o controle dos níveis extracelulares de ADP torna-se fundamental para a regulação de processos trombóticos e/ou hemorrágicos. Em adição, o ADP pode atuar nos receptores $P2Y_1$ das células endoteliais e musculares lisas, causando dilatação

(Ralevick e Burnstock, 2003), sendo que esta também pode ocorrer pela liberação de NO que o ADP é capaz de estimular (Kunapuli & Daniel, 1998). O AMP, por sua vez, trata-se de um agente anti-agregador plaquetário e de extrema importância como substrato para formação da adenosina.

Paralelamente a estes efeitos extracelulares, algumas evidências com fibras musculares permeabilizadas e vesículas de retículo sarcoplasmático cardíaco (chamados de microssomas) têm sugerido que os nucleotídeos da adenina poderiam modular correntes de Ca^{2+} do retículo sarcoplasmático, ativando diretamente o canal iônico (Meissner, 1984; Meissner & Henderson, 1987; Duke & Steele, 1998).

1.1.3 O nucleosídeo adenosina

A adenosina, um precursor ou um metabólito dos nucleotídeos da adenina, é uma molécula presente em todas as células do sistema biológico (Kitakase *et al.*, 1991). Desde o clássico trabalho de Drury & Szent-Györgyi em 1929, no qual as propriedades antiarrítmicas e vasodilatadoras deste nucleosídeo foram pela primeira vez demonstradas, tem se tornado claro que a adenosina desempenha não somente um importante papel no metabolismo celular, mas também um importante papel fisiológico no sistema cardiovascular (Mubagwa *et al.*, 1996; Balas, 2002).

A formação de adenosina intracelular pode se dar por duas vias principais que são: a clivagem da S-adenosil-homocisteína pela enzima S-adenosil-homocisteína hidrolase, e pela degradação de AMP por ação de uma 5'-nucleotidase citosólica (Patel & Tudball, 1986). Depois de formada, a adenosina pode passar através da membrana plasmática por difusão facilitada,

através de transportadores de nucleosídeos. Estes transportadores são bidirecionais e equilibram os níveis intracelulares e extracelulares de adenosina (Dunwiddie & Masino, 2001). Além de ser liberada como tal para o meio extracelular, a adenosina também pode ser formada no espaço extracelular, através da hidrólise do AMP extracelular por ação de uma ecto-5'-nucleotidase (Zimmermann, 1992; Dunwiddie & Masino, 2001).

O coração e os vasos sangüíneos apresentam diversos mecanismos para proteção a estímulos capazes de causar algum tipo de injúria, dentre eles pode-se destacar o efeito da adenosina via receptores adenosinérgicos ou receptores P1 (A_1, A_2, A_{2B} e A_3) (Willems *et al.*, 2005). Em 1997, Rongen *et al.* mostraram que a formação de adenosina é aumentada durante o processo de isquemia, causando vasodilatação via ações dependentes e independentes do endotélio, aumentando o fluxo sangüíneo e restaurando o suprimento de oxigênio ao tecido cardíaco (Ralevic & Burnstock, 2003). Sabe-se que a vasodilatação induzida pela adenosina é resultado da ativação dos receptores A_2 que são expressos em praticamente todo o sistema vascular de mamíferos (Ralevic & Burnstock, 2003). Recentes trabalhos mostram a diferença entre os efeitos agudos da adenosina (Peart & Headrick, 2003; Flood *et al.*, 2003) e os efeitos de pré-condicionamento adenosinérgico (Peart & Headrick, 2003), mostrando múltiplas vias de proteção deste nucleosídeo (Willems *et al.*, 2005).

Os efeitos da adenosina também envolvem a atenuação pré-sináptica da liberação de norepinefrina em terminais nervosos simpáticos em adição aos seus efeitos pós-sinápticos (Richardt *et al.*, 1987). A adenosina é ainda capaz de antagonizar os efeitos inotrópicos positivos e arritmogênicos dos agonistas β-adrenérgicos no miocárdio ventricular através da interação com receptores A_1

(Mubagwa *et al.,* 1996). Em termos de alvos celulares, a adenosina parece proteger diretamente cardiomiócitos ou tecido miocárdico (provavelmente via receptores A_1 e A_3) (Roscoe *et al.*, 2000) e, além disso, protege por limitar a inflamação em tecido miocárdico e vascular (Vinten-Johansen *et al.*,1999). A adenosina também é capaz de atuar como um inibidor da agregação plaquetária, sendo por este motivo, também conhecida como uma molécula antitrombogênica (Kitakaze *et al.*, 1991; Kawashima *et al.*, 2000).

Em virtude das ações descritas até o momento, a adenosina tem tido na clínica uma importante aplicação terapêutica no tratamento de isquemia e outras disfunções circulatórias. No entanto, quando em altas concentrações, a adenosina pode também levar a efeitos antagônicos, com ação pró-arrítmica, podendo originar a pausa ou o bloqueio do nodo sino-atrial e átrio-ventricular (Mubagwa & Flameng, 2001).

Após interagir com receptores específicos, a ação da adenosina pode ser finalizada através da enzima ecto-adenosina deaminase (ecto-ADA), ou ainda através de fosforilação até 5'-AMP catalisada pela enzima adenosina cinase (ADK) (Dunwiddie & Masino, 2001; Yegutkin, 2008).

Tendo em vista a evidente participação das purinas em diferentes processos envolvidos na funcionalidade cardiovascular normal, deve-se atentar para o fato de que distúrbios ou prejuízos na sinalização purinérgica podem estar relacionados ao desenvolvimento de patologias vasculares. Desta maneira, a manutenção da sinalização purinérgica normal, incluindo desde as moléculas sinalizadoras, até seus receptores e suas enzimas de degradação, têm se mostrado um importante alvo para o tratamento de doenças cardiovasculares (Ralevick & Burnstock, 2003; Erlinge & Burnstock, 2008).

1.2 A família das Ectonucleotidases

Após a liberação no meio extracelular, os nucleotídeos da adenina exercem seus efeitos através da interação com os purinoceptores localizados na membrana celular e posteriormente são metabolizados através da ação de ecto-enzimas que fazem a conversão destes nucleotídeos até adenosina.

Diversos trabalhos têm demonstrado que o ATP extracelular é degradado por um conjunto de enzimas que constituem a "via das ectonucleotidases". Estas enzimas apresentam tanto uma sobreposição de distribuição tecidual como especificidades por substratos. Desta forma, os nucleosídeos di e trifosfatados podem ser hidrolisados principalmente por membros das famílias E-NTPDase (ecto-nucleosídeo trifosfato difosfoidrolase), E-NPP (ecto-nucleotídeo pirofosfatase/fosfodiesterase) e fosfatases alcalinas. Os nucleosídeos monofosfatados estão sujeitos à hidrólise pela ecto-5'-nucleotidase e também pelas fosfatases alcalinas (Zimmermann, 2001; Robson *et al.*, 2006). Esta via pode resultar na inativação da sinalização mediada pelo ATP via receptores P2 e contribuir para a sinalização mediada pela adenosina, através dos receptores P1 (Richardson *et al.*, 1987; Sebastião *et al.*, 1999) (Figura 3). Desta forma, as ectonucleotidases constituem um eficiente mecanismo de controle dos níveis de nucleotídeos e nucleosídeos no espaço extracelular (Zimmermann, 1996; Zimmermann, 2001).
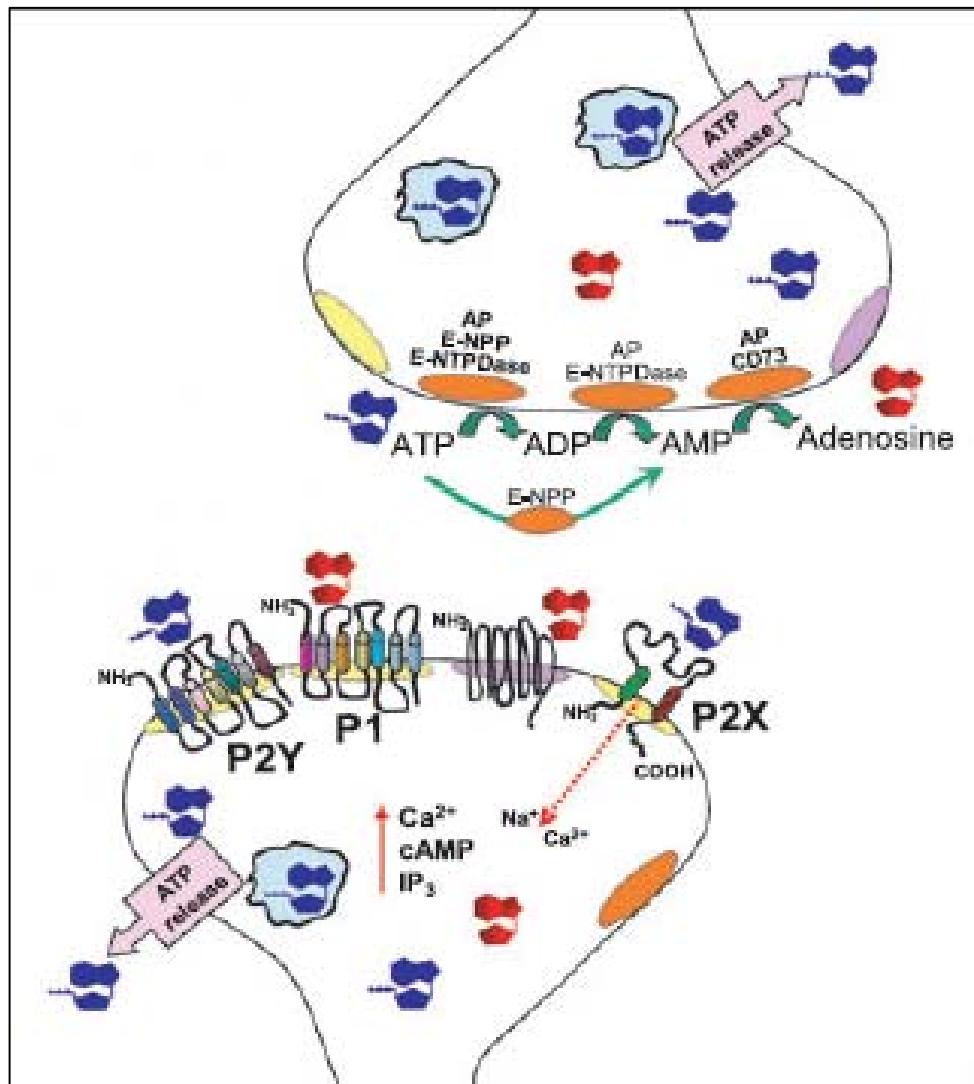
Figura 3. Representação da maquinaria protéica envolvida na sinalização purinérgica. *Adaptado de Volonté & D'Ambrosi, 2009.*

1.2.1 Ecto-nucleosídeo trifosfato difosfoidrolases (E-NTPDases)

As enzimas da família das E-NTPDases hidrolisam especificamente nucleotídeos na superfície de muitos tipos celulares e a sua ação tem sido intensamente reportada. Na literatura mais antiga, informações sobre estas enzimas podem ser encontradas sob diferentes nomes, incluindo ecto-ATPase, ATP difosfoidrolase, apirase, ATPDase, nucleosídeo difosfatase, etc (Plesner, 1995; Zimmermann, 1996). O nome ATP difosfoidrolase ou apirase (EC 3.6.1.5), foi primeiramente proposto por Meyerhof em 1945, como uma designação geral para enzimas que hidrolisam todos os di- e

trifosfonucleosídeos até seus respectivos nucleosídeos monofosfatos e fosfato inorgânico (Pi), liberando 2 mol de Pi por mol de nucleosídeo trifosfatado e 1 mol de Pi por nucleosídeo difosfatado. A partir de 1945, mas principalmente nas duas últimas décadas, ATP difosfoidrolases tem sido descritas em diferentes fontes: em tecidos vegetais (Krishnan, 1949; Valenzuela *et al.*, 1989), insetos (Ribeiro *et al.*, 1989; Sarkis *et al.*, 1996), aves (Carl & Kirley, 1997) e tecidos de mamíferos, como preparações em sistema nervoso central (SNC) e periférico (Battastini *et al.*, 1991; Sarkis & Saltò, 1991), aorta bovina (Coté *et al.*, 1992), secreções seminais (Rosenberg *et al.*, 1988), vasos umbilicais humanos (Yagi *et al.*, 1992), pulmão bovino (Picher *et al.*, 1993), plaquetas e soro de ratos (Frasseto *et al.*, 1993; Oses *et al.*, 2004), plaquetas humanas (Pilla *et al.*, 1996), células neoplásicas humanas (Dzhandzhugazyan *et al.*, 1998), células de sertoli de ratos (Casali *et al.*, 2001), linfócitos humanos (Leal *et al.*, 2005), glândulas submandibulares de ratos (Henz *et al.*, 2006), células de tumor de Walker 256 (Buffon *et al.*, 2007), dentre outros.

Em 1994, Malisweski e colaboradores identificaram molecularmente o primeiro membro da família E-NTPDase. Um protótipo da enzima foi clonado, seqüenciado e identificado como um antígeno de ativação celular, CD39, principalmente expressa em linfócitos ativados (Malisweski *et al.*, 1994) e inicialmente descrita como proteína marcadora de células B transformadas pelo vírus Epstein Barr (Wang & Guidotti, 1996). Experimentos subseqüentes com apirases solúveis e clonadas de batata (Handa & Guidotti, 1996) e de diferentes tecidos de mamíferos (Kaczmarek *et al.*, 1996) confirmaram a homologia desta enzima ao CD39 humano.

Como características comuns, as enzimas da família das E-NTPDases são capazes de hidrolisar nucleosídeos tri e ou difosfatos, mas não os monofosfatos, necessitam concentrações milimolares de cátions como Ca^{2+} e Mg^{2+} para atingirem a atividade máxima e são insensíveis a inibidores específicos de ATPases do tipo P (ex. $Na^+K^+ATPase$), tipo V (ex. bomba de prótons vacuolar) e tipo F (ex. ATPase mitocondrial) (Zimmermann *et al.*, 1998; Yegutkin, 2008). Além disso, após a análise das sequências de diversas ecto-ATPDases e ecto-ATPases, foi demonstrado que estas enzimas compartilham cinco regiões altamente conservadas chamadas de "regiões conservadas da apirase", ACR (ACR 1 - 5 apyrase conserved regions) (Handa & Guidotti, 1996; Schulte *et al.*, 1999). A existência desses sítios conservados pode estar relacionada com a formação do sítio catalítico das enzimas e/ou com a integridade estrutural das E-NTPDases (Grinthal & Guidotti, 2000).

Atualmente, baseado em sua estrutura e propriedades catalíticas, particularmente na relação de hidrólise ATP/ADP, a família das NTPDases em mamíferos está constituída de oito membros clonados e funcionalmente caracterizados: NTPDase 1 (CD39), NTPDase 2 (CD39L1), NTPDase 3 (CD39L3, HB6), NTPDase 4 (UDPase), NTPDase 5 (CD39L4) e NTPDase 6 (CD39L2), NTPDase 7 e NTPDase 8.

Dentre os oito tipos de genes clonados e caracterizados, as NTPDases 1, 2, 3 e 8 estão localizadas na superfície celular com sítios catalíticos voltados para o meio extracelular, as NTPDases 5 e 6 apresentam localização intracelular e sofrem secreção depois de expressão heteróloga. Já as NTPDases 4 e 7 têm localização totalmente intracelular com o sítio ativo voltado para o lúmem das organelas intracelulares (Zimmermann, 2001; Robson *et al.*, 2006) (Figura 4).
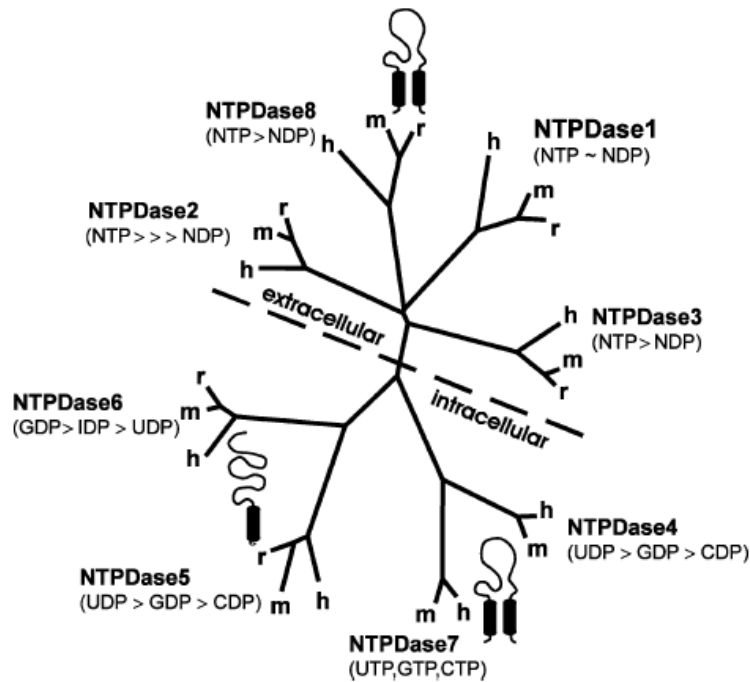
Figura 4: Árvore filogenética hipotética para os membros da família das NTPDases (NTPDase 1 a 8) de rato (r), humano (h) e camundongo (m). O comprimento das linhas indica as diferenças entre as seqüências de aminoácidos. O gráfico representa a separação entre NTPDases localizadas na superfície (superior) e intracelular (inferior). A preferência por substrato para cada subtipo e a topografia na membrana para cada grupo de enzimas está também representada (um ou dois domínios transmembrana, indicados por cilindros). *Obtido de Robson et al. 2006.*

Os subtipos de NTPDases, além de diferirem na localização celular, apresentam diferentes propriedades funcionais. Enquanto todos os membros da família catalisam a hidrólise de ambos nucleosídeos trifosfato (NTP) e nucleosídeos difosfatos (NDP), as razões de hidrólise (NTP:NDP) variam significativamente para essas reações, resultando em enzimas que hidrolisam preferencialmente NTPs (NTPDase2), preferencialmente NDPs (NTPDase 5 e 6) ou ambos nucleotídeos (NTPDase 1, 3 e 8) (Zimmermann, 2001; Grinthal & Guidotti, 2002; Robson *et al.*, 2006) (Figura 4). Diferenças na seqüência e também nas estruturas secundárias, terciárias e quaternárias podem estar relacionadas às diferentes propriedades catalíticas encontradas entre os diferentes tipos de NTPDases (Heine, 2001; Grinthal & Guidotti, 2004).

A NTPDase1, que apresenta a mesma preferência pela hidrólise do ATP e do ADP (NTP/NDP 1:1), tem sido a mais estudada dos membros da família da E-NTPDases. A NTPDase 1 tem sido extensivamente estudada em células endoteliais e em plaquetas, possuindo um papel bem descrito na regulação do fluxo sanguíneo e trombogênese. Essa enzima, em associação com a ecto-5'-nucleotidase presente na superfície das células endoteliais e plaquetas, converte o ADP, pró-agregante plaquetário, no anti-agregante adenosina, limitando a extensão da agregação plaquetária intravascular (Frassetto *et al.*, 1993; Pilla *et al.*, 1996; Kaczmarek *et al.*, 1996; Marcus *et al.*, 1997; Imai *et al.*, 1999; Koziak *et al.*, 1999). Em concordância, NTPDase1 solúvel recombinante, é capaz de bloquear a agregação plaquetária induzida *in vitro* por ADP (Gayle *et al.*, 1998).

Estudos prévios demonstraram que ratos deficientes de CD39/ATP difosfoidrolase apresentam problemas relacionados com hemostasia e trombogênese (Enjyoji *et al.*, 1999). Além disso, nos terminais nervosos simpáticos, onde noradrenalina e ATP são co-liberados, NTPDase1 tem sido associada à degradação do ATP até adenosina, apresentando um possível papel na modulação da exocitose de norepinefrina, via receptores P2X ou P2Y. A liberação excessiva de norepinefrina é a principal causa de arritmias e disfunção vascular coronariana na isquemia miocárdica (Benedict *et al.*, 1996; Machida *et al.*, 2005). Assim, formas solúveis e ligadas à membrana da NTPDase1/CD39 são potenciais agentes terapêuticos para a inibição de processos trombogênicos (Gayle *et al.*, 1998) e arrítmicos (Machida *et al.*, 2005) e podem representar uma nova geração de moléculas cardioprotetoras (Marcus *et al.*, 2005).

Diferentemente da NTPDase 1 e dos outros dois membros relacionados, NTPDases 3 e 8, a característica que mais distingue a NTPDase2 é a sua clara preferência pelos nucleosídeos trifosfatados. A enzima hidrolisa os nucleosídeos difosfatados apenas marginalmente, tendo uma preferência de até 30 vezes pelo ATP em relação ao ADP como substrato (Kegel *et al.*, 1997).

A NTPDase2 é particularmente associada com eventos na superfície das adventícias de vasos da musculatura onde, em contraste à NTPDase1, pode promover a agregação plaquetária pela presença de ADP (Sevigny *et al.*, 2002). A enzima também é descrita em cultura de astrócitos, células de Schwan e outras células gliais do sistema nervoso central e periférico (Wink *et al.*, 2006; Langer *et al.*, 2007). As propriedades bioquímicas e a localização específica da NTPDase2 sugerem que a enzima possa regular funções cardíacas como a regulação da ativação de receptores P2X ou P2Y, sendo capaz de promover a degradação do ATP, que exerce efeitos inotrópicos e cronotrópicos, além de poder participar da modulação da liberação de norepinefrina (Sevigny *et al.*, 2002, Marcus *et al.*, 2005).

A NTPDase 3 (CD39L3) é considerada um intermediário funcional entre a NTPDase 1 e a NTPDase 2, pois hidrolisa o ATP e o ADP em uma razão de 3:1. Muito do conhecimento sobre a NTPDase 3 está relacionado a sua estrutura, entretanto muito pouco é sabido a respeito do seu papel fisiológico, bem como sua distribuição na natureza. A expressão da NTPDase3 tem sido principalmente associada com estruturas neuronais no cérebro, onde pode agir como regulador dos níveis de ATP na pré-sinapse, além de coordenar muitos eventos homeostáticos (Belcher *et al.*, 2006). A sua exata contribuição e relação com

NTPDase 1, 2 e 8 permanece a ser elucidada nos diversos tecidos onde há sobreposição de suas funções.

As NTPDases 4 (UDPases), embora possuam a mesma estrutura geral das NTPDases 1 a 3, diferem principalmente em relação à localização celular. NTPDases 4 de humanos estão localizadas no complexo de Golgi (NTPDase 4β) ou em vacúolos lisossomais (NTPDase 4α) (Wang & Guidotti, 1998). Ambas enzimas hidrolisam nucleosídeos di e trifosfatados, mas possuem uma baixa preferência por ATP e ADP. Elas diferem em sua preferência por nucleotídeos e também na dependência de cátions divalentes. A NTPDase 4α tem alta preferência por UTP e TTP, enquanto que CTP e UDP são os melhores substratos da NTPDase 4β.

Já as NTPDases 5 (CD39L4) e NTPDases 6 (CD39L2) encontram-se ancoradas na membrana celular somente pela porção NH_2 terminal e possuem uma larga região COOH terminal extracelular. Ambas enzimas são ativadas por cátions divalentes e apresentam uma maior preferência por nucleotídeos difosfatados. A NTPDase 5 tem maior preferência para hidrolisar principalmente GDP e UDP, enquanto a NTPDase 6 hidrolisa preferencialmente GDP e IDP. Acredita-se que estas enzimas sejam liberadas da membrana e então secretadas para o meio extracelular, indicando tratar-se de enzimas na forma solúvel.

A NTPDase 5 está localizada no retículo endoplasmático, enquanto a NTPDase 6, encontra-se no complexo de Golgi (Zimmermann, 2001). A identificação e caracterização bioquímica da NTPDase 5 (Mulero *et al.*, 2000) não permitiu ainda um completo entendimento da sua função fisiológica. Devido a sua clara preferência por nucleosídeos difosfatados, acredita-se que o seu papel fisiológico seja reduzir os níveis circulantes do ADP e não do ATP.

Já a NTPDase6, embora a expressão tenha sido demonstrada em tecidos de diversos órgãos (cérebro, fígado, rim, pulmão, placenta, músculo esquelético, pâncreas, etc) (Chadwick & Frischauf, 1998), é consenso que a sua maior expressão é em coração, principalmente em células do músculo cardíaco e em células endoteliais capilares (Hicks-Berger *et al.*, 2000; Braun *et al.*, 2000, Yeung *et al.*, 2000).

As NTPDases7 e 8 preferem como substratos nucleosídeos trifosfatados. Entretanto, a NTPDase 7 está localizada em vesículas intracelulares, enquanto que a NTPDase 8 foi a última a ser clonada, caracterizada e descrita como uma ecto-enzima de membrana expressa em fígado, rins e intestino de camundongos com uma razão de hidrólise de aproximadamente 2:1 (Bigonnesse *et al.*, 2004; Zimmermann, 2001). Recentemente, uma NTPDase 8 também foi clonada e caracterizada em fígado humano, e uma forma solúvel desta enzima foi gerada por expressão de seu domínio extracelular em células embrionárias de rim humano (HEK293) (Knowles & Li, 2006). A expressão desta enzima também foi encontrada em canalículos hepáticos, sendo sugerido um papel regulatório na secreção biliar e/ou no salvamento de nucleosídeos (Fausther *et al.*, 2007). Assim, os dados encontrados na literatura, até então, sugerem um forte papel desta enzima principalmente em eventos relacionados ao sistema hepático.

1.2.2 Ecto-nucleotídeo pirofosfatases/fosfodiesterases (E-NPPs)

A família das E-NPPs consiste em sete membros estruturalmente relacionados (NPP1 a NPP7) que foram numerados de acordo com a sua ordem de descobrimento. Os membros desta família multigênica possuem uma

ampla especificidade de substratos e são capazes de hidrolisar ligações pirofosfato e fosfodiester em (di) nucleotídeos, ácidos nucléicos, nucleotídeos açúcares, assim como ésteres de fosfato de colina e lisofosfolipídeos (Stefan *et al.*, 2005; Yegutkin, 2008). O *p*-nitrofenil-5'-timidina-monofosfato (*p*-nitrofenil-5'-TMP) tem sido usado como um substrato artificial, específico para as E-NPPs (Sakura *et al.*, 1998).

Os membros da família E-NPPs possuem uma ampla distribuição tecidual e incluem a NPP1(PC-1), NPP2 (PD-Iα, autotaxina), NPP3 (PD-Iβ, B10, gp130^{RB13-6}), NPP4, NPP5, NPP6 e NPP7 (esfingomielinase alcalina). Exceto para a NPP2, que é secretada no meio extracelular, todos os demais membros são ligados à membrana por um único domínio transmembrana N-terminal e apresentam um domínio para clivagem proteolítica, sugerindo que possam ocorrer como enzimas solúveis (Zimmermann, 2001; Stefan *et al.*, 2005; Stefan *et al.*, 2006). No entanto, somente as NPP1-NPP3, que possuem um ancestral comum, têm sido responsabilizadas pela hidrólise de nucleotídeos (Stefan *et al.*, 2006).

NPP1-NPP3, sozinhas ou combinadas, têm sido expressas em vários tipos celulares estudados (Bollen *et al.*, 2000), apesar de isoformas individuais estarem confinadas a subestruturas e/ou tipos celulares específicos (Narita *et al.*, 1994; Harahap & Goding, 1988; Johnson *et al.*, 2001; Blass-Kampmann *et al.*, 1997; Goding *et al.*, 2003). NPP1 foi originalmente descoberta na superfície de linfócitos B de camundongo como um antígeno de diferenciação celular plasmático (PC-1) (Takahashi *et al.*, 1970). Em humanos é altamente expressa em ossos e cartilagens, tendo expressão intermediária em coração, fígado, placenta e testículos (Goding *et al.*, 2003). Camundongos deficientes em NPP1

revelaram uma produção excessiva de tecido ósseo, sugerindo que esta enzima desempenha um papel essencial no controle da mineralização óssea, pela produção de PP$_i$ (Stefan *et al.*, 2005; Stefan *et al.*, 2006). Além disso, esta enzima também está presente nos rins, ductos de glândulas salivares, cérebro e epidídimo (Harahap *et al.*, 1988; Yano *et al.*, 1985). Stefan e colaboradores, em 1999, verificaram a presença de um híbrido NPPγ-cDna em tecidos de ratos, que estavam principalmente presentes em fígado, rins e coração. Em muitos estudos, a NPP1 também tem sido correlacionada com a sinalização da insulina e na etiologia da resistência à insulina (Gijsbers *et al.*, 2003; Goding *et al.*, 2003; Stefan *et al.*, 2005).

A NPP2 foi descoberta como um fator de motilidade autócrino (autotaxina, NPP2α) (Stracke *et al.*, 1992) e é expressa em diversos tipos celulares (Bollen *et al.*, 2000; Bächner *et al.*, 1999), o que indica que esta enzima possui capacidades multifuncionais (Stefan *et al.*, 2005). A NPP2 é uma proteína secretada que se acumula em fluidos corporais, tais como plasma e fluido cerebroespinhal. Esta enzima tem sido correlacionada com estímulo da proliferação, contração e migração celular (Stefan *et al.*, 2005; Stefan *et al.*, 2006), além de participar da formação de vasos sanguíneos e progressão do câncer (Koike *et al.*, 2006). Alguns estudos demonstram a expressão desta enzima em cérebro, pulmão, duodeno e glândulas adrenais (Stefan *et al.*, 1999) e existem evidências para a sua presença também em coração humano (Bollen *et al.*, 2000).

NPP3 (gp130^{RB13-6} or B10) foi inicialmente reconhecida pelo anticorpo monoclonal RB13-6 como uma glicoproteína de células precursoras gliais de cérebro de ratos (Deissler *et al.*, 1995; Deissler *et al.*, 1999). A NPP3 possui um

papel importante em alergias, sendo definida como um marcador de ambos basófilos e mastócitos (Stefan *et al.*, 2005). Foram encontrados poucos transcritos de NPPβ (NPP3) em cérebro e glândulas adrenais de ratos, mas abundantes em fígado, rins e coração (Stefan *et al.*, 1999).

Muitas vezes se torna difícil a distinção entre os membros das famílias das E-NPPs e E-NTPDases , devido sua co-expressão em tecidos de mamíferos e por possuírem similaridades nas suas especificidades por substratos. O que se sabe é que muitas vezes estas enzimas trabalham em conjunto ou consecutivamente, como já foi proposto em trabalhos do nosso grupo (Fürstenau *et al.*, 2006; Henz *et al.*, 2007; Cognato *et al.*, 2008) (Figura 5).
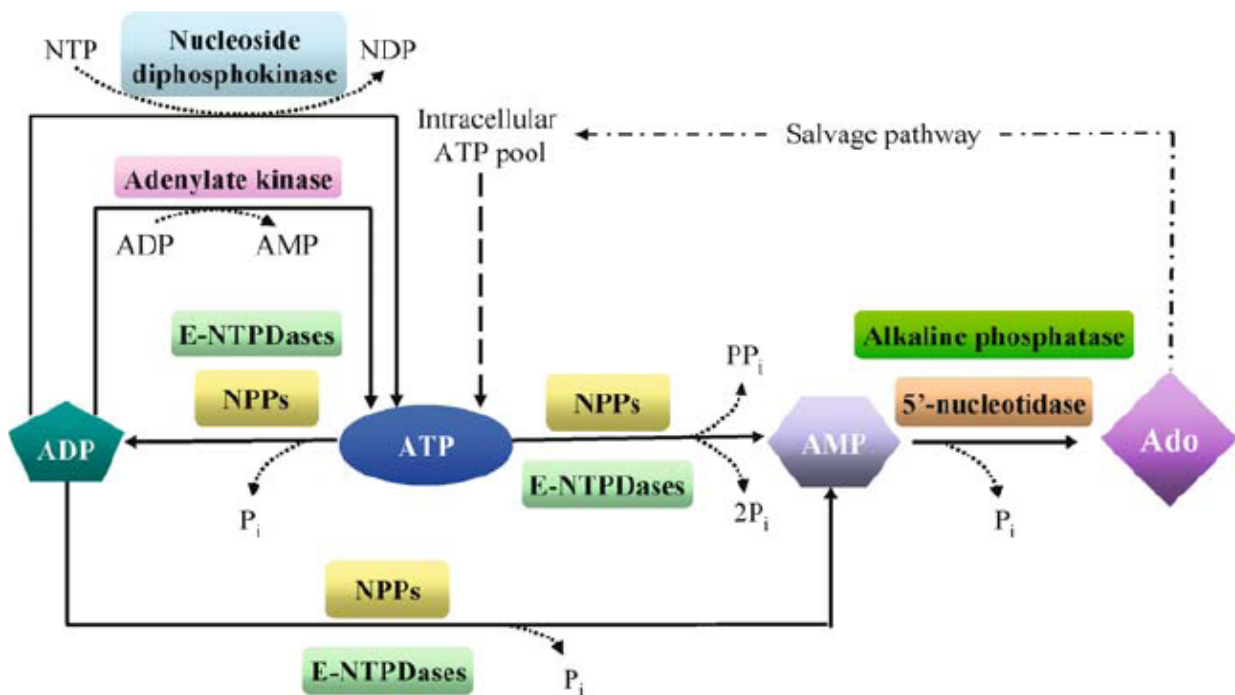
Figura 5: E-NPPs participando da rede de metabolismo de nucleotídeos extracelulares. A concentração de nucleotídeos no meio extracelular é resultado da liberação de nucleotídeos a partir de células, síntese por nucleosídeos difosfocinases e adenilato cinases e hidrólise por ectonucleotidases. *Adaptado de Stefan et al., 2006.*

1.2.3 Ecto- 5'-nucleotidase

A ecto-5'-nucleotidase (CD73) também participa do metabolismo dos nucleotídeos da adenina, atuando em conjunto com as E-NTPDases ou E-NPPs (Figuras 3 e 5). A ecto-5'-nucleotidase é uma enzima ancorada à membrana plasmática por glicosil-fosfatidilinositol (GPI), que representa um marcador de maturação para os linfócitos T e B, sendo ausente nas células imaturas (Airas *et al.*, 1997). O ancoramento da enzima pode ser clivado por uma fosfolipase C específica para GPI, dando origem às formas solúveis da enzima (Zimmermann, 1992). A ecto-5'-nucleotidase pode exercer uma ampla variedade de funções dependendo de sua expressão tecidual e celular. Ela encontra-se presente na maioria dos tecidos e sua principal função é a hidrólise de nucleosídeos monofosfatados extracelulares, tais como AMP, GMP ou UMP, a seus respectivos nucleosídeos (Sträter, 2006). Sua presença tem sido descrita em cólon, rins, cérebro, fígado, pulmão e coração (Zimmermann, 1992; Zimmermann, 1996; Moriwaki *et al.*, 1999). Na vasculatura, a ecto-5'-nucleotidase está predominantemente associada com o endotélio vascular de grandes vasos como a aorta, carótida e artéria coronária (Koszalka *et al.*, 2004). O principal papel fisiológico atribuído à ecto-5'-nucleotidase, é a formação de adenosina a partir do AMP extracelular e a subsequente ativação dos receptores P1, que em sistema nervoso resulta principalmente na inibição da liberação de neurotransmissores excitatórios (Brundege & Dunwidie, 1997), enquanto que em sistema vascular, resulta em vasodilatação e na inibição da agregação plaquetária (Kawashima *et al.*, 2000).

1.3 Cafeína

1.3.1 Conceito e consumo

A cafeína (1,3,7 trimetilxantina) é um alcalóide natural que está entre as substâncias psico-ativas mais consumidas no mundo. Existem mais de 60 espécies de plantas que fornecem a cafeína, sendo as mais conhecidas o café, o chá, o cacau, a erva-mate e o guaraná. O café é a principal fonte de cafeína e conforme o tipo de grão e preparo, pode fornecer de 40 a 180 mg de cafeína por 150 mL de bebida. O chá contém de 24 a 50 mg de cafeína para cada 150 mL e bebidas a base de cola contêm aproximadamente 40 mg por 350 mL. Nas chamadas "bebidas energéticas", encontra-se cerca de 80 mg de cafeína por 250 mL e no chimarrão, em torno de 0,93 mg/mL (Martín *et al.*, 2007).

O consumo mundial de cafeína é estimado em 70 a 76 mg/pessoa/dia, sendo que nos Estados Unidos e Canadá é de 210 a 238 mg/dia e pode chegar a mais de 400 mg/pessoa/dia em países como Suécia e Finlândia (Barone & Roberts, 1996; Daly & Fredholm, 1998). A dose letal de cafeína está em torno de 200 mg/Kg, o que equivale a 80-100 copos médios de café (Fredholm, 1999).

1.3.2 Metabolismo

A cafeína é completamente absorvida pelo trato gastrointestinal após 45 minutos de sua ingestão. A meia-vida varia entre as faixas etárias, na gravidez, em combinação com alguns medicamentos e com a integridade hepática. Em adultos saudáveis a meia-vida é de aproximadamente 3-4 horas, enquanto que

em ratos fica em torno de 1 hora. Em mulheres que tomam anticoncepcionais é de 5-10 horas e naquelas em gestação, de 9-11 horas. Em recém-nascidos a meia-vida é de 30 horas. Nos indivíduos com doença hepática, a meia-vida da cafeína pode chegar a 96 horas (Fredholm, 1999).

Por ser uma molécula hidrofóbica a cafeína tem sua passagem facilitada em todas as membranas biológicas, ultrapassando inclusive a barreira hemato-encefálica (Lachance *et al.*, 1983; Tanaka *et al.*, 1984). Em ratos adultos, por exemplo, a concentração de cafeína plasmática é semelhante à encontrada no fluído cérebro-espinhal (Liu *et al.*, 2006).

A cafeína é metabolizada principalmente no fígado pelas enzimas do sistema citocromo P450 em dimetilxantinas, como a paraxantina, teobromina e teofilina. Cada um destes metabólitos tem suas funções no organismo, sendo excretados na urina após metabolizados. Nos humanos, a paraxantina é o metabólito predominante (72 a 80 %), enquanto em roedores, apesar da paraxantina ser o metabólito plasmático dominante, os níveis de teofilina também estão elevados.

Geralmente, uma dosagem de 10 mg/Kg em ratos, equivale aos efeitos de 3,5 mg/Kg nos humanos, o que corresponde de 2 a 3 copos de café (Fredholm *et al.*, 1999).

1.3.3 Mecanismo de ação e efeitos no sistema cardiovascular

Embora existam vários mecanismos de ação da cafeína, o único mecanismo conhecido que é significativamente afetado por doses relevantes de cafeína é a interação com os receptores de adenosina (A_1 e A_{2A}), antagonizando as ações deste agonista (Daly & Fredholm, 1998; Fredholm *et*

al., 1999). Portanto, a cafeína, além de inibir alguns efeitos da adenosina, é capaz de desempenhar funções contrárias às do nucleosídeo.

Os efeitos da cafeína no sistema cardiovascular envolvem modificações diretas da contratilidade da musculatura do coração e dos vasos sanguíneos, assim como influência sobre a neurotransmissão central e periférica (Fredholm, 1984). Potenciais efeitos tóxicos da cafeína variam de um aumento moderado na taxa cardíaca até mais severas arritmias cardíacas causadas por atraso na condutividade miocárdica (Donnerstein *et al.*, 1998; Nawrot *et al.*, 2003). Embora algumas evidências suportem a idéia de que a elevação da pressão sanguínea causada pela cafeína possa contribuir para a mortalidade relacionada aos problemas cardiovasculares (James, 2004), os dados a respeito de variações da pressão sanguínea induzidas pelo consumo agudo de cafeína são conflitantes (Karatzis *et al.*, 2005). Além disso, estudos epidemiológicos da ingestão de cafeína não têm sido hábeis a responder se é a cafeína *per se* ou outros componentes do café que são responsáveis pelos efeitos associados à doença cardiovascular (Nawrot *et al.*, 2003).

Na cardiologia experimental, cafeína em altas concentrações (10-20 mM) tem sido amplamente utilizada em estudos com cardiomiócitos isolados para causar rápida liberação de Ca^{2+} do retículo sarcoplasmático. Em baixas concentrações, a cafeína reduz o fluxo de Ca^{2+} nas células miocárdicas (Fabiato, 1982; Kapelko *et al.*, 1994). Esta propriedade da cafeína explica seus efeitos inotrópicos negativos, os quais são mais pronunciados em corações isolados de ratos (Kapelko *et al.*, 1994). A performance cardíaca com ingestão prolongada de cafeína tem sido muito pouco explorada e concentra-se em efeitos durante a gravidez e lactação (Temples *et al.*, 1987). Tendo em vista

essa diversidade de efeitos da cafeína sobre o sistema cardiovascular, torna-se muito interessante a investigação de outros possíveis mecanismos de ação dessa molécula sobre o sistema cardiovascular.

2. OBJETIVOS

Objetivo Geral: Identificar através de ferramentas bioquímicas e moleculares a presença de nucleotidases em fração solúvel e microssomal de tecido cardíaco de ratos, além de avaliar um possível envolvimento nas alterações cardiovasculares relacionadas à cafeína.

Objetivos Específicos:

Capítulo I – Definir as propriedades cinéticas e bioquímicas da enzima ecto-5'-nucleotidase em fração solúvel e microssomal cardíaca de ratos, além de identificar a presença dessa enzima por imunodetecção nas duas frações.

Capítulo II – Identificar os possíveis membros da família das E-NTPDases solúveis e em microssomas de ventrículos cardíacos de ratos adultos por meio da caracterização de suas propriedades bioquímicas e imunodetecção.

Capítulo III – Investigar a presença de enzimas da família das E-NPPs em fração solúvel e microssomal cardíaca de ratos, através da caracterização das propriedades cinéticas e bioquímicas dessa atividade.

Anexo I – Constitui-se de dados preliminares de um manuscrito em preparação, onde o objetivo é investigar uma possível relação entre os efeitos que a cafeína exerce sobre o coração e as ectonucleotidases caracterizadas em fração solúvel e microssomal de ventrículos cardíacos.

PARTE II

CAPÍTULO I

AMP hydrolysis in soluble and microsomal rat cardiac cell fractions: kinetic characterization and molecular identification of 5'-nucleotidase

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AMP hydrolysis in soluble and microsomal rat cardiac cell fractions: kinetic characterization and molecular identification of 5′-nucleotidase

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Synopsis

The present study describes the enzymatic properties and molecular identification of 5′-nucleotidase in soluble and microsomal fractions from rat cardiac ventricles. Using AMP as a substrate, the results showed that the cation and the concentration required for maximal activity in the two fractions was magnesium at a final concentration of 1 mM. The pH optimum for both fractions was 9.5. The apparent K_m (Michaelis constant) values calculated from the Eadie–Hofstee plot were 59.7 ± 10.4 μM and 134.8 ± 32.1 μM, with V_{max} values of 6.7 ± 0.4 and 143.8 ± 23.8 nmol P_i/min/mg of protein (means $\pm$ S.D., $n = 4$) from soluble and microsomal fractions respectively. Western blotting analysis of ecto-5′-nucleotidase revealed a 70 kDa protein in both fractions, with the major proportion present in the microsomal fraction. The presence of these enzymes in the heart probably has a physiological function in adenosine signalling. Furthermore, the presence of ecto-5′-nucleotidase in the microsomal fraction could have a role in the modulation of the excitation–contraction-coupling process through involvement of the Ca^{2+} influx into the sarcoplasmic reticulum. The measurement of maximal enzyme activities in the two fractions highlights the potential capacity of the different pathways of purine metabolism in the heart.

Key words: adenosine, AMP hydrolysis, ecto-5′-nucleotidase, heart, microsome, soluble 5′-nucleotidase

INTRODUCTION

Adenosine and adenine nucleotides (ATP, ADP and AMP) are signalling molecules that exert multiple biological actions in the heart and other tissues [1–5]. The first study on the effects of these purines on the cardiovascular system demonstrated that adenosine and AMP induced a decrease in heart rate, arterial dilatation, lowering of blood pressure and inhibition of intestinal contraction [6]. It is now clear that purines exert potent and diverse effects on the cardiovascular system and are involved, principally, in cardioprotection [7,8]. In the heart, adenosine not only plays a role in regulating growth and differentiation, angiogenesis, coronary blood flow, cardiac conduction and heart rate, substrate metabolism and sensitivity to adrenergic stimulation, but may also play a role as an endogenous determinant of ischaemic

tolerance [7–10]. From a therapeutic viewpoint, adenosine-based therapies protect against ischaemic injury in a variety of animal models as well as in human cardiac tissue [2,8,9]. The effects of adenosine are mediated through the binding to P1 receptors that are expressed in the plasma membrane of cells in a variety of tissues [2,5]. Different pathophysiological processes increase the level of adenosine in the extracellular medium, and one of the major pathways of adenosine formation is the enzymatic dephosphorylation of AMP by 5′-nucleotidase both intracellularly and extracellularly [2,3,11,12].

5′-Nucleotidases are a group of enzymes that catalyse, with different specifities, the dephosphorylation of extracellular monophosphate nucleotides, resulting in the release of their corresponding nucleosides. Widely distributed in bacteria, plant cells and vertebrate tissues, 5′-nucleotidases are classified according to their cellular location and biochemical properties: five

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35

cytoplasmic forms, one in the mitochondrial matrix and a membrane-anchored ecto-5′-nucleotidase (also known as CD73, EC 3.1.3.5), [13]. All members of this family (except for ecto-5′-nucleotidase) are absolutely dependent on Mg^{2+} for their maximal activity [13]. The molecular mass, apparent K_m and pH of 5′-nucleotidases are variable and depend on the type of 5′-nucleotidase [11,13].

The physiological function of 5′-nucleotidases probably differs in various organisms and tissues, and possibly extends beyond its catalytic activity. Ecto-5′-nucleotidase anchored to the plasma membrane by GPI (glycosylphosphatidylinositol) is involved in cell–cell and cell–matrix interactions and in transmembrane signalling, as well as in the control of cell growth, maturation and differentiation processes [11,13]. Acting with the E-NTPDases (ecto-NTP diphosphohydrolases), which catalyse ATP and ADP hydrolysis, the activity of 5′-nucleotidases controls the intracellular and extracellular levels of AMP and adenosine. Seven human 5′-nucleotidases with different subcellular localization have been cloned, suggesting that these enzymes perform important metabolic functions [13]. Furthermore, alterations in 5′-nucleotidase levels have been observed in a considerable number of diseases [11,13].

Although the actions of adenosine and adenine nucleotides have been characterized in great detail, the contribution of the different subcellular fragments to cardiac adenosine production are not well understood to date. A study by Kiviluoma et al. [14] on heart muscle demonstrated that cytosol, lysosomes and plasma membrane were particularly rich in 5′-nucleotidase activity, whereas mitochondria and sarcoplasmic reticulum were devoid of activity. A mitochondrial 5′-nucleotidase has also been cloned and characterized previously [13].

In the present study, the aim was to demonstrate 5′-nucleotidase activity in the microsomal fraction from rat cardiac cells, which is derived from sarcoplasmic reticulum. Furthermore, we describe the characteristics of 5′-nucleotidase in a soluble fraction, investigate the biochemical and kinetic properties and demonstrate the presence of this enzyme in both fractions.

MATERIALS AND METHODS

Chemicals
AMP, EDTA, Trizma base, levamisole, tetramisole, aprotinin, leupeptin and pepstatin were obtained from Sigma–Aldrich. PMSF was obtained from Gibco. Nitrocellulose membrane was from Bio-Rad. A polyclonal antibody against ecto-5′-nucleotidase (SC-14684) was purchased from Santa Cruz Biotechnology. A horseradish-peroxidase-conjugated goat anti-IgG secondary antibody was from Jackson ImmunoResearch. ECL® (enhanced chemiluminescence) detection reagents were from Amersham Biosciences. X-ray film (T-MAT G/RA Film) was purchased from Kodak. All other reagents were of analytical grade.

Animals
Male Wistar rats weighing 200–280 g were used in the present study. All the animals were housed in cages with food and

water available *ad libitum*. They were maintained under a 12 h light/12 h dark cycle at a constant temperature of $23 \pm 2°C$. Procedures for the care and use of animals were performed according to the regulations of COBEA (Colégio Brasileiro de Experimentação Animal), based on the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Isolation of cardiac soluble and microsomal fractions
Rats were killed by decapitation, the hearts were carefully removed and the ventricles were isolated. The fractions were prepared as described previously [15], with minor modifications. Briefly, cardiac ventricles of two animals were minced and homogenized for 3 min in 1:23 (w/v) of 0.25 M sucrose/10 mM Tris/HCl (pH 7.4), using a tissue homogenizer (Sorvall Omni-Mixer 17105, setting 4). The homogenate was centrifuged at $10\,000\,g$ for 30 min. The pellet was discarded and the supernatant was centrifuged at 21 400 rev./min for 60 min (Hitachi P28S rotor). The supernatant obtained represented the soluble fraction, whereas the pellet, resuspended in homogenization buffer, represented the microsomal fraction. The soluble and microsomal fractions were prepared fresh daily and kept at 4°C throughout the process.

5′-Nucleotidase assay
Unless otherwise stated, enzyme activity was determined in incubation medium [50 mM glycine buffer (pH 9.5) and 1 mM $MgCl_2$] in a final volume of 200 μl. Soluble and microsomal proteins (90 μg and 50 μg respectively) were added to the reaction medium and pre-incubated for 10 min at 37°C. The reaction was started by the addition of AMP to a final concentration of 2 mM. After 40 min incubation (soluble fraction) or 10 min incubation (microsomal fraction), the reactions were stopped by the addition of 200 μl of 10% (v/v) TCA (trichloroacetic acid). The samples were chilled on ice and the P_i released was measured following the method of Chan et al. [16]. For all enzyme assays, incubation times and protein concentrations were chosen to ensure the linearity of the reactions. All experiments were performed in triplicate. Controls with the enzyme preparation added after mixing with TCA were used to correct for non-enzymatic substrate hydrolysis. Enzyme activity is expressed as nmol P_i released/min/mg of protein. For analysis of pH dependence, AMP hydrolysis was determined as described above, except that the glycine buffer was substituted for a buffer solution reaction mixture containing 50 mM Tris, 50 mM Hepes and 50 mM glycine buffers (pH 6–10.5).

Western blotting analysis
After isolation of cardiac subcellular fractions, a solution containing protease inhibitors (5 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin and 100 mM PMSF) was added (1:30 dilution) to maintain the sample integrity. Total protein (75 μg) was resolved by SDS/PAGE (15% gels), and the resolved proteins were transferred on to a nitrocellulose membrane. The membrane was stained with Ponceau solution to demonstrate that the protein

36

concentration was similar in the different lanes. The membrane was then washed with TBST [Tris-buffered saline with Tween 20; 50 mM Tris/HCl (pH 7.5), 150 mM NaCl and 2% (v/v) Tween 20] for 20 min at room temperature (23 ± 2°C). After this, the membrane was incubated at 37°C for 2 h with a polyclonal anti-ecto-5′-nucleotidase antibody (1:500 dilution) in TBST with 1% non-fat dried skimmed milk powder. After washing the membrane, a horseradish-peroxidase-conjugated-goat anti-IgG antibody (1:1000 dilution) was incubated with the membrane for 1 h at room temperature. The membrane was washed again with TBST and incubated with ECL detection reagents, which produced a chemiluminescence signal that was detected by exposure to X-ray film. The protein bands were quantified by densitometry and the band density was then calculated and expressed as arbitrary units (Gel Pro Imager; Media Cybernetics).

Protein determination

Protein concentrations were measured by the Coomassie Blue method of Bradford [17], using bovine serum albumin as a standard.

RESULTS

Time course and protein concentration

We first investigated AMP hydrolysis in soluble and microsomal fractions from rat cardiac cells as a function of time and protein concentration in order to determine the optimum assay conditions. The fractions were incubated as described in the Materials and methods section. The results indicated that the time course of AMP hydrolysis was linear up to 50 min (for the soluble fraction) and up to 15 min (for the microsomal fraction) (results not shown). In order to ensure that the incubation time was within the linear portion of the reaction, we choose 40 min and 10 min as the assay times for the soluble and microsomal fractions respectively.

With regards to protein concentration, the results demonstrated that AMP hydrolysis was linear up to 110 μg (for the soluble fraction) and up to 70 μg (for the microsomal fraction) (results not shown). Thus in the subsequent experiments we used 90 μg of the soluble protein fraction and 50 μg of the microsomal protein fraction.

Cation dependence

To further optimize assay conditions, the effects of different Ca^{2+} and Mg^{2+} concentrations on AMP hydrolysis were investigated. In both fractions, the results showed a gradual decrease in enzyme activity in the presence of different concentrations of Ca^{2+} when compared with the respective control groups [3.51 ± 0.446 nmol P_i/min/mg of protein (mean $\pm$ S.D., $n = 4$) from the soluble fraction and 105.29 ± 8.43 nmol P_i/min/mg of protein (mean $\pm$ S.D., $n = 3$) from the microsomal fraction]. A significant increase in AMP hydrolysis was observed with all concentrations of Mg^{2+}
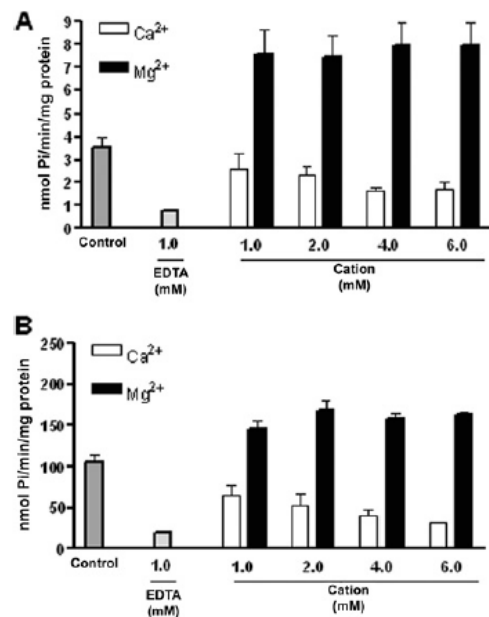
**Figure 1 Dependence of AMP hydrolysis on bivalent cations**
Hydrolysis of AMP by soluble (A) and microsomal (B) rat cardiac cell fractions was analysed in the absence of cations (Control), in the presence of EDTA (1 mM) and in the presence of 1–6 mM Ca^{2+} (white bars) or Mg^{2+} (black bars). Results are expressed as nmol P_i/min/mg of protein and are means $\pm$ S.D. ($n \geqslant 3$).

tested when compared with the respective control groups, and the pattern was similar for both fractions (Figure 1). In subsequent experiments, a final concentration of 1 mM MgCl₂ was used.

An activity of approx. 46% and 72% of the total measurable activity in the presence of 1 mM of Mg^{2+} from soluble and microsomal fractions respectively, was still seen in the absence of added bivalent cations. However, this endogenous activity could be removed almost completely by the addition of 1 mM EDTA. This result indicates the presence of endogenous bivalent cations in the soluble and microsomal fractions and the dependence on cations for enzyme activity in both fractions.

Effect of pH

The optimum pH value for AMP hydrolysis was determined in a mixture containing 50 mM Tris, 50 mM Hepes and 50 mM glycine buffers (pH 6–10.5). A parallel profile was obtained for both fractions and the maximal rate of AMP hydrolysis was observed at pH 9.5 (Figure 2). In parallel experiments, we used 1 mM levamisole or tetramisole, classical alkaline phosphatase inhibitors, to exclude the effect of alkaline phosphatase on AMP hydrolysis. The inhibitors studied did not have any effect on AMP hydrolysis, excluding the participation

37
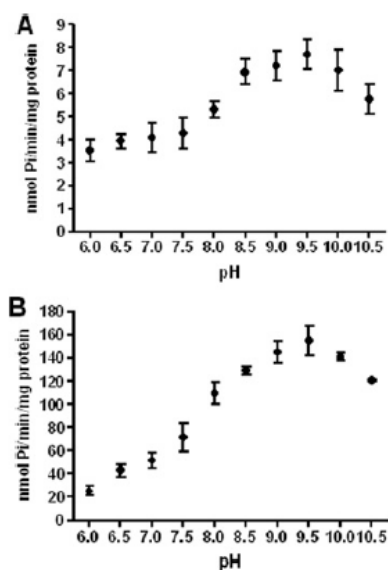
Figure 2 Effect of pH on AMP hydrolysis in soluble (A) and microsomal (B) rat cardiac cell fractions

Enzyme activity was determined as described in the Materials and methods section using a mixture of Tris, Hepes and glycine buffers (pH 6–10.5). Results are expressed as nmol P_i/min/mg of protein and are means $\pm$ S.D. ($n = 3$).

of alkaline phosphatase in substrate hydrolysis (results not shown).

Kinetic constants

AMP hydrolysis in the cardiac soluble fraction was determined at different concentrations of AMP (10–2000 μM). For the microsomal fraction, AMP hydrolysis was determined at various concentrations of AMP (75–2000 μM). The results (insets in Figure 3) indicated that enzyme activity increased with increasing concentrations of nucleotide. The Michaelis constant (K_m) and V_{max} values were estimated from the Eadie–Hofstee plot (Figure 3). The apparent K_m values calculated from this plot were 59.7 ± 10.4 μM and 134.8 ± 32.1 μM, with V_{max} values of 6.7 ± 0.4 nmol P_i/min/mg of protein and 143.8 ± 23.8 nmol P_i/min/mg of protein for soluble and microsomal fractions respectively (means $\pm$ S.D., $n = 4$).

Western blotting analysis

A polyclonal antibody against ecto-5′-nucleotidase was used for Western blotting analysis. As seen in Figure 4, the antibody reacted specifically with a protein with a molecular mass of approx. 70 kDa in both soluble and microsomal rat cardiac cell fractions. The band densities were quantified by densitometry and expressed as arbitrary units. The microsomal fraction contains a large quantity of ecto-5′-nucleotidase (approx. 143.72%) in comparison with the soluble fraction.

DISCUSSION

Adenine nucleotides and adenosine are continually present in variable amounts in both the intracellular and extracellular spaces of the heart. Adenosine is the final product of the breakdown of ATP, ADP and AMP by the ectonucleotidase cascade. This nucleoside has an important role in the modulation of cardiac functions and its possible cardioprotective effects have been emphasized [1–3,5,7,9]. In the present study, we demonstrate the kinetic and biochemical properties of a 5′-nucleotidase with respect to AMP hydrolysis in soluble and microsomal fractions from rat cardiac cells. At the same time we identified and quantified the presence of ecto-5′-nucleotidase in these fractions.

It is known that Mg^{2+} optimizes assay conditions for a great number of 5′-nucleotidases [11,13] and, in the present study, both the soluble and microsomal fractions presented an elevation in AMP hydrolysis in the presence of this bivalent cation. The Mg^{2+} concentration required for maximal activity was near to the millimolar range for the two fractions. On the other hand, the addition of calcium decreased AMP hydrolysis when compared with the control group without cation addition. When EDTA (1 mM), a metal chelator, was added to the reaction mixture, this resulted in a decrease in the 5′-nucleotidase activity in both fractions, demonstrating that these enzymes are dependent on bivalent cations for activity.

The optimum pH for 5′-nucleotidases is variable and depends on the location of the enzyme. Generally, this pH is in the range of pH 6–8 [11]. In the present study, using a mixture of Tris, Hepes and glycine buffers, we observed the greatest enzyme activity at pH 9.5 for both fractions. A study by Naito and Lowenstein [18] with purified soluble 5′-nucleotidase from rat heart demonstrated the complex behaviour of this enzyme in the presence of various buffers with a pH above pH 7.5. They suggest that some buffers, such as glycine buffers, interact with the enzyme, possibly by interacting with a metal ion bound to the enzyme. Furthermore, they demonstrated that Mg^{2+} ions modify this behaviour, since they activate the enzyme above pH 8 in the presence of some buffers. They also observed that in the absence of Mg^{2+} ions, glycine buffer yields an optimum pH at 9.5 and the activity of 5′-nucleotidase in this buffer was increased approx. twice by the addition of 10 mM Mg^{2+} ions. The optimum pH values observed in the results of the present study with a buffering system containing glycine in the presence of Mg^{2+} are in accordance with the previous study, which described pH values for heart purified 5′-nucleotidase [18]. Furthermore, we excluded the participation of alkaline phosphatases because the classical alkaline phosphatase inhibitors, levamisole and tetramisole, did not have any effect on AMP hydrolysis at pH 9.5 (results not shown).

The comparison of enzyme activities in cardiac soluble and microsomal fractions showed different kinetic properties. The 5′-nucleotidase from the soluble fraction presented a low K_m in comparison with the enzyme from the microsomal fraction, but in both fractions the values were in the micromolar range. In relation to the V_{max} values, the enzyme from the soluble fraction demonstrated a value approx. 21 times lower when compared
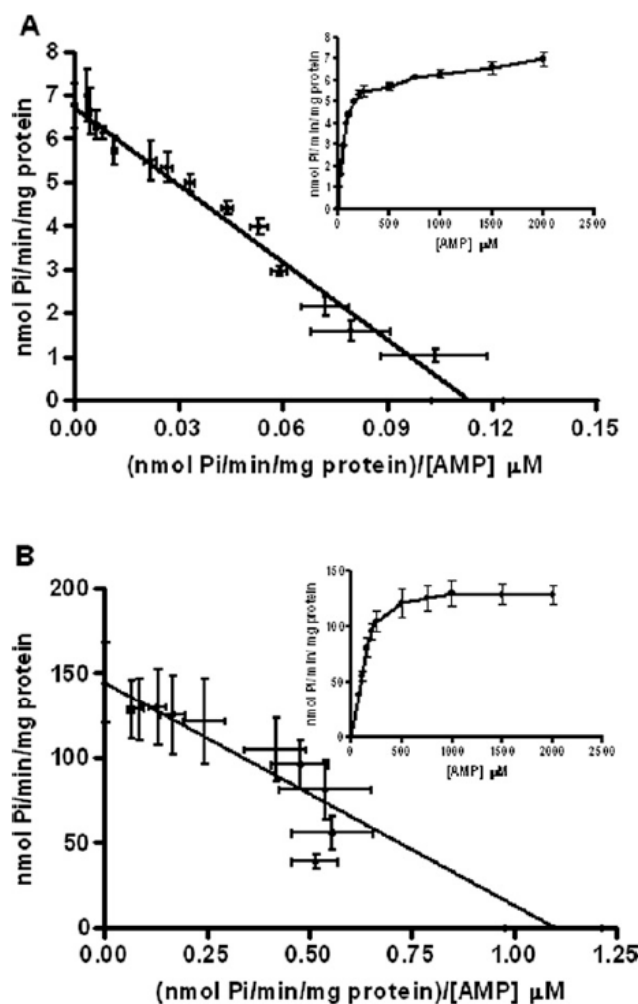
**Figure 3   Eadie–Hofstee plot for AMP hydrolysis**
Nucleotide hydrolysis as a function of substrate concentration from soluble (A) and microsomal (B) rat cardiac cell fractions is shown (insets). The mean K_m values calculated for AMP hydrolysis were 59.7 ± 10.4 μM and 134.8 ± 32.1 μM with V_{max} values of 6.7 ± 0.4 nmol P_i/min/mg of protein and 143.8 ± 23.8 nmol P_i/min/mg of protein for soluble and microsomal fractions respectively. Results are means $\pm$ S.D. ($n = 4$).

with that observed for the microsomal fraction enzyme. These differences are related in the literature and can be attributed to the type of 5′-nucleotidase and the location of the enzyme [11,13].

As well as the biochemical and kinetic analysis, Western blotting was used to quantify the levels of ecto-5′-nucleotidase present in the two fractions. The method used revealed a protein of approx. 70 kDa in both fractions. The microsomal fraction showed a strong signal, indicating that the presence of ecto-5′-

nucleotidase is greater in this fraction compared with the soluble fraction. The presence of an ecto-enzyme in the soluble fraction is not surprising. Zimmermann [11] previously described that up to 50% of the enzyme may be associated with intracellular membranes and released during homogenization, forming a soluble protein. The detection of individual nucleotidases by enzymatic assays in cell lysates is problematic because different nucleotidases can be co-expressed in the same tissue or cell type. For this reason, we cannot exclude the presence of a soluble

39

SF   MF

~ 70kD
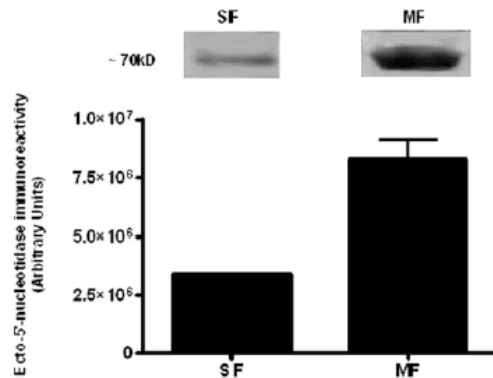
Figure 4 Ecto-5′-nucleotidase identification in soluble (SF) and microsomal (MF) rat cardiac cell fractions measured by Western blotting

The protein bands were quantified by densitometry and the density was calculated and expressed as arbitrary units. The image is representative of three experiments and results are means $\pm$ S.D. ($n = 3$). kD, mass in kDa.

5′-nucleotidase since different kinetic properties were found in both fractions. The characteristics of soluble 5′-nucleotidases derived from various tissues have been demonstrated in a great number of studies, principally using the purified enzyme [11,13,18,19]. In heart muscle, the expression of these enzymes has a physiological function in the generation of adenosine signalling during ischaemia and in the regulation of nucleotide pools [13].

The presence of 5′-nucleotidase producing adenosine in the subcellular fragments of the heart may have other important roles beyond those well established roles. The microsomal fraction is derived from the sarcoplasmic reticulum. This is the major intracellular organelle that sequesters intracellular Ca^{2+} and regulates the relaxation and tension development of the myocardium. Previous studies have demonstrated that adenine nucleotides and adenosine affect the release of Ca^{2+} from the sarcoplasmic reticulum both in skeletal muscle and in cardiac fibres [7,20–23]. Furthermore, the work of Duke and Steele [22] utilizing frog skeletal-muscle fibres suggests that adenosine could exert opposite effects to adenine nucleotides in this mechanism. Therefore the presence of a 5′-nucleotidase in the microsomal fraction controlling AMP hydrolysis and adenosine levels could be important for the regulation of Ca^{2+} influx throughout the sarcoplasmic reticulum membrane, modulating the excitation–contraction coupling process.

In conclusion, the great variety of actions of adenosine emphasizes the importance of studying the enzymes involved in its production, and the aim of this work was to characterize AMP hydrolysis in a soluble cellular fraction and to compare this activity with that in a microsomal fraction in order to try and determine the contribution of each fraction to adenosine production in the heart.

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41

CAPÍTULO II

Kinetic and molecular analysis of NTPDase in rat cardiac

soluble and microsomal fractions

Artigo em preparação para ser submetido ao periódico *Molecular and Cellular*

Biochemistry

Kinetic and molecular analysis of NTPDase in rat cardiac soluble and microsomal fractions

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Abstract

Alterations in the adenine nucleotides levels have been implicated in diverse patho-physiological processes of the heart. Nucleotide-metabolizing enzymes play an important role in the regulation of nucleotide levels and consequently in their actions. In the present study, we describe biochemical characterization of ATPase and ADPase activities in rat cardiac soluble and microsomal fractions, under conditions where mitochondrial ATPase and adenylate kinase activities were blocked. Based on the cation-dependence, optimum pH obtained and sensitivity to inhibitors, we can suggest that these activities probably belong to the NTPDase family of enzymes. The apparent K_M values calculated for ATP and ADP in soluble fraction were 131.2 ± 16.7 and 57.1 ± 6.9 µM, respectively. V_{max} values calculated were 71.3 ± 12.8 and 9.3 ± 1.1 nmol Pi/min/mg protein for ATP and ADP hydrolysis, respectively. In microsomal fraction, the K_M values calculated for ATP and ADP were 315.2 ± 58.8 and 131 ± 30.7 µM, respectively. V_{max} values in microsomal fraction were 1180.6 ± 150 and 100.4 ± 13.2 nmol Pi/min/mg protein when using ATP and ADP as substrates, respectively. In addition, Western blot analysis revealed immunoreactivity for NTPDase5 in soluble fraction and NTPDase2 in microsomal fraction. The presence of NTPDase activity in cardiac soluble and microsomal fractions can contribute to the better understanding of the purinergic signaling in the heart.

Introduction

In the cardiovascular system, adenine nucleotides exert multiple physiological and pathological roles, and are very much involved in the mechanisms underlying local control of vessel tone as well as cell migration, proliferation, differentiation, and death during angiogenesis, atherosclerosis and restenosis [1-4]. In the heart, adenosine triphosphate (ATP) generally elicits positive or negative inotropic and chronotropic effects [2,5]. Furthermore, this nucleotide has been demonstrated to modulate Ca^{2+}, Na^+, K^+ and Cl^- currents in cardiomyocytes and to induce acidosis, cell depolarization and arrhythmia in the heart [5]. Other possible roles for ATP concern aspects such as hypertrophy, preconditioning and apoptosis [2,5,6]. The extracellular effects of ATP are mediated by interaction with cell-surface receptors called P2 receptors that are expressed with some selectivity on different types of cells in the cardiovascular system [1-4,6]. Besides these extracellular effects, some evidences with permeabilized muscle fibers and cardiac sarcoplasmic reticulum vesicles (called microsomes) have suggested that adenine nucleotides could modulate the Ca^{2+} currents of the sarcoplasmic reticulum activating directly the ionic channel [7-9].

The enzymatic breakdown of ATP constitutes an important modulatory component of the purinergic signaling, since it regulates ATP actions at P2 receptors and can evokes opposite effects by the action of the ATP degradation products, adenosine diphosphate (ADP) and adenosine [2-4,10]. Members of several enzyme families are capable of hydrolyzing extracellular ATP and ADP, and have also been allocated to the cardiovascular system. These include the family of E-NTPDases (ecto-nucleoside triphosphate diphosphohydrolases), along with the E-NPP family (ecto-nucleotide pyrophosphatase/phosphodiesterase) and the alkaline phosphatases [3,4,10].

E-NTPDase describes a family of enzymes that hydrolyze nucleoside tri- and diphosphates with substantial differences in specify. Eight different members of the NTPDase protein family that share five apyrase-conserved regions (ACRs) have been

identified. The individual subtypes differ in cellular location and functional properties. Four of the NTPDases are typical cell surface-located enzymes with an extracellularly facing catalytic site (NTPDase1,2,3,8). NTPDase5 and 6 exhibit intracellular localization and undergo secretion after heterologous expression. NTPDase4 and 7 are entirely intracellularly located, facing the lumen of cytoplasmic organelles. All NTPDases require Ca^{2+} and/or Mg^{2+} ions in the millimolar range for maximal activity and are inactive in their absence. The four cell surfaced-located forms (NTPDase1,2,3,8) can be differentiated according to substrate preference, divalent cation usage and product formation. The functional roles of these enzymes in diverse systems are strongly related to the different physiological effects of the nucleotides and their metabolic products [3,4,10,11].

Based on the wide distribution of NTPDases in various tissues, cells and subcellular fragments [4,10,11], and considering the variety of effects of adenine nucleotides in the cardiovascular system, mainly in the heart [2,3], the aim of the present study was to characterize the kinetic and biochemical properties of NTPDase activity in rat cardiac fractions, one soluble and other enriched in vesicles derived from sarcoplasmic reticulum (called microsomal fraction).

Materials and Methods

Chemicals

Nucleotides, oligomycin, sodium azide, ouabain, orthovanadate, N-ethylmaleimide (NEM), lanthanum, levamisole, suramin, Evans blue, gadolinium chloride, Trizma Base and EDTA were obtained from Sigma-Aldrich (St Louis, MO, USA). Bicinchoninic acid (Pierce, São Paulo, Brasil). PVDF membrane (Immobilon P, Millipore, Bedford, MA, USA). ECL kit (Amersham, São Paulo, Brasil). All other reagents were also of analytical grade.

Animals

Male *Wistar* rats weighing 200 - 280 g were used in this study. All animals were housed in cages with food and water available *ad libitum*. They were maintained under a 12-h light/dark cycle at a constant temperature of 23 ± 2 °C. Procedures for the care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

Isolation of cardiac soluble and microsomal fractions

Rats were killed by decapitation, hearts were carefully removed and the ventricles were isolated. The fractions were prepared as described by Floreani et al. 2003 [12], with minor modifications. Briefly, both left and right cardiac ventricles of two animals were minced and homogenized in 1:23 (w/v) of 0.25 M sucrose-10 mM Tris (pH 7.4), using a tissue homogenizer (Sorvall Omni-Mixer, 17105) for 3 min at setting 4. The homogenate was centrifuged for 30 min at 10,000 x g. The pellet (P1) was discarded and the supernatant (S1) was centrifuged for 60 min at 105,000 x g. The supernatant (S2) obtained represented the soluble fraction, whereas the pellet (P2), resuspended in the homogenization buffer, represented the microsomal fraction. Protein was measured by the Coomassie Blue method using bovine serum albumin as standard [13]. Both fractions were prepared fresh daily and kept at 4 °C throughout the process.

Measurement of ATP and ADP hydrolysis

Unless otherwise stated, ATP and ADP hydrolysis in soluble fraction were determined in a reaction medium containing 50 mM Tris-HCl buffer (pH 8.0), 4.0 mM $CaCl_2$ and 2.0 µg/mL oligomycin in a final volume of 200 µL. For ADP hydrolysis, 1.0 mM NEM was added to the reaction medium. About 90 µg of protein were added to the reaction medium and preincubated for 10 min at 37 °C. The reaction was started by the

addition of ATP or ADP to a final concentration of 2.0 mM. After 6 min of incubation for ATP and 25 min of incubation for ADP, the reactions were stopped by the addition of 200 µL 10% trichloroacetic acid (TCA). The samples were chilled on ice and the inorganic phosphate (Pi) released was measured according to Chan et al. 1986 [14].

In microsomal fraction, ATP and ADP hydrolysis were determined in a reaction medium containing 50 mM Tris-HCl buffer (pH 7.5), 4.0 mM $CaCl_2$ and 2.0 µg/mL oligomycin in a final volume of 200 µL. In the reaction mixture used to determine for ADP hydrolysis, 4.0 mM NEM was added. Microsomal protein (20 µg for ATP and 50 µg for ADP hydrolysis) was added to the reaction medium and preincubated for 10 min at 37 °C. The reaction was started by the addition of ATP or ADP to a final concentration of 2.0 mM. After 2 min of incubation for ATP and 10 min of incubation for ADP, the reactions were stopped by the addition of 200 µL 10% trichloroacetic acid (TCA). The samples were chilled on ice and the inorganic phosphate (Pi) released was measured how described above.

For all enzyme assays, incubation times and protein concentration were chosen in order to ensure the linearity of the reactions. All samples were run in triplicate. Controls with the addition of the enzyme preparation after mixing with TCA were used to correct for non-enzymatic substrate hydrolysis. Enzyme activity was expressed as nmol of phosphate (Pi) released per minute per milligram of protein.

Inhibitor studies

In order to test the effects of classical ATPase inhibitors and P2 receptors antagonists on ATP and ADP hydrolysis, both cardiac fractions were preincubated for 10 min in the presence or absence of 1.0 mM ouabain, 0.1 mM orthovanadate, 1.0 mM NEM, 0.1 mM lanthanum, 1.0 mM levamisole, 0.25 mM suramin, 0.1 mM Evans blue, 2.0 µg/mL oligomycin, sodium azide (0.1, 5.0, 10 and 20 mM), gadolinium chloride (0.1, 0.2, 0.3 and 0.5 mM). Incubation times, protein and substrate concentrations were

used as described above. Results are expressed as percentage of control enzyme activity.

Adenylate kinase assay

Adenylate kinase activity was determined in a reaction medium containing 50 mM Tris-HCl buffer (pH 8.0 for soluble and pH 7.5 for microsomal fraction), 4.0 mM CaCl$_2$, 10 mM glucose, 0.2 mM NADP$^+$, 10 units of Hexokinase (EC 2.7.1.1), 5 units of glucose-6-phosphate dehydrogenase (EC 1.1.149) and 90 µg of soluble protein or 50 µg of microsomal protein. Reaction was started by the addition of 1.0 mM ATP or 2.0 mM ADP and the final volume was 1.0 mL. In some experiments 1.0 mM NEM was also present. Reduction of NADP$^+$ was monitored spectrophotometrically at 340 nm [15].

Antibodies

The following rabbit polyclonal antibodies were used to identify the E-NTPDase family members in rat cardiac soluble and microsomal fractions: antibody C10F, raised against mouse NTPDase1 [16]; BZ3-4F, raised against rat NTPDase2 [17-19]; KHL 7, anti-peptide antibodies against the rat/mouse NTPDase3 C-terminal sequence [20]. Anti-human NTPDase5 and NTPDase6 were generated by immunization of rabbits with synthetic peptides corresponding to amino acids 109-122 [21] and PEP002A [22], respectively, obtained from published sequences of the N-terminal region of these NTPDases.

Western blot

Soluble and microsomal fractions prepared as described were dissolved in the following buffer (100 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 100 mM sodium fluoride, 0.5 ug/ml aprotinin, 1 ug/ml leupeptin and 1 mM phenylmethylsulfonyl

fluoride, 20 mM Tris, pH 7.5). Protein content was determined by using Bicinchoninic acid assay using bovine serum albumin (BSA) as standard. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was performed loading a total of 80 ug of protein on a 4–10% polyacrylamide gel (40 ul/well) under non-reducing conditions followed by transfer to nitrocellulose membrane by electroblotting. After blocking with 5% milk in Tris–saline buffer containing 0.05% Tween 20, membranes were probed with an appropriate primary antibody (NTPDases 1,2,3,5 and 6 diluted 1:1000) overnight, at 4 °C and visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:3000), followed by enhanced chemiluminescence assay with ECL kit.

Results

Determining time course and optimal protein concentration

We first investigate ATP and ADP hydrolysis in rat cardiac soluble and microsomal fractions as a function of time and protein concentration in order to determine the best assay conditions. The fractions were incubated as described in Material and Methods. The results indicated that the time course of ATP and ADP hydrolysis for soluble fraction was linear up to 10 min and up to 35 min, respectively (Fig. 1A). In microsomal fraction, the time course was linear up to 3 min for ATP and up to 15 min for ADP (Fig. 1C). In order to ensure that the incubation time was within the linearity of the reaction, we choose 6 min and 25 min (ATP and ADP, respectively) as the assay times for soluble fraction, and 2 min and 10 min (ATP and ADP, respectively) as the assay times for microsomal fraction in the subsequent experiments.

Concerning to protein concentration, the results demonstrated that ATP and ADP hydrolysis were linear up to 110 μg for soluble and up to 70 μg for microsomal fraction (Fig. 1B and 1D, respectively). Thus, in the subsequent experiments, we used 90 μg protein of soluble fraction for both substrates, and 20 μg protein of microsomal fraction for ATP and 50 μg protein for ADP hydrolysis.

Cation dependence

To further optimize assay conditions, we evaluated ATP and ADP hydrolysis in the absence or presence of divalent cations added, or EDTA. As shown in Fig. 2A and 2B, in the presence of 0.25 mM EDTA, ATP and ADP hydrolysis were practically negligible when compared with respective controls (without the addition of divalent cations) in the soluble fraction. In the microsomal fraction (Fig. 2C and 2D), ATP and ADP hydrolysis founded in the control group were completely removed by the addition of 0.25 mM EDTA. On the other hand, the hydrolysis of the two nucleotides in both fractions was increased by the presence of different concentrations of Ca^{2+} or Mg^{2+}. These data clearly indicate the cation dependence for both enzyme activities.

In both fractions, some differences were observed in enzymatic activation by different cations at different concentrations tested. ATP and ADP hydrolysis were more activated by Mg^{2+} compared to Ca^{2+}, in some concentrations. Despite these differences, in the subsequent experiments we used a final concentration of 4.0 mM $CaCl_2$ for ATP and ADP hydrolysis in both fractions.

Effect of pH

The optimum pH values for ATP and ADP hydrolysis were determined in a medium containing a mixture of 50 mM Tris and 50 mM HEPES buffer (pH varying from 6.0 to 9.0). Using ATP as substrate, the pH curves showed the highest activity at pH 8.0-8.5 and pH 7.5-8.5 for soluble and microsomal fractions, respectively (Fig. 3A and 3C). For ADP hydrolysis, the same pattern was observed in both fractions and the maximal rate of hydrolysis was observed at pH 7.5 (Fig. 3B and 3D). For the subsequent experiments, the pH 8.0 was chosen for the hydrolysis of ATP and ADP in soluble fraction and the pH 7.5 was used for the measurement of the hydrolysis of both substrates in microsomal fraction.

Kinetic constants

ATP and ADP hydrolysis in cardiac soluble and microsomal fractions were determined at different substrate concentrations ranging from 75 to 2000 µM (Fig. 4). The results (insets in Fig. 4) indicated that enzyme activities increased with increasing concentrations of nucleotides. Michaelis constant (K_M) and V_{max} values were estimated from the Eadie-Hofstee plot. The apparent K_M calculated from this plot for ATP and ADP in soluble fraction were 131.2 ± 16.7 and 57.1 ± 6.9 µM, respectively. V_{max} values calculated were 71.3 ± 12.8 and 9.3 ± 1.1 nmol Pi/min/mg protein for ATP and ADP, respectively (Fig. 4A and 4B). In the microsomal fraction, the K_M calculated for ATP and ADP were 315.2 ± 58.8 and 131 ± 30.7 µM, respectively. V_{max} values in microsomal fraction were 1180.6 ± 150 and 100.4 ± 13.2 nmol Pi/min/mg protein for ATP and ADP, respectively (Fig 4C and 4D).

Effect of inhibitors on ATP and ADP hydrolysis

In order to evaluate the sensivity of the enzymatic activities for ATP and ADP hydrolysis or the presence of different nucleotide-metabolizing enzymes in the soluble and microsomal fractions, we have tested the effects of distinct compounds that were reported to affect nucleotide hydrolysis. Table 1 summarizes effects of the some ATP and ADP hydrolysis inhibitors in both fractions.

Ouabain, a specific Na^+/K^+-ATPase inhibitor [23]; orthovanadate, an inhibitor of transport ATPases, acid phosphatases, alkaline phosphatases, phosphotyrosine phosphatases and Na^+/K^+-ATPase [24,25]; levamisole, a specific alkaline phosphatase inhibitor [26]; and lanthanum, the Ca^{2+}, Mg^{2+}-ATPase inhibitor [27], did not affected the ATP and ADP hydrolysis in both fractions, excluding these enzymes as a contaminants. NEM, another Ca^{2+}, Mg^{2+}-ATPase inhibitor and also adenylate kinase inhibitor [28,29] did not alter ATP hydrolysis in both fractions, but decreased ADP hydrolysis by 65% and 22% in soluble and microsomal fractions, respectively, suggesting the presence of adenylate kinase activity in both preparations. The

presence of adenylate kinase in both fractions was confirmed with a protocol that uses a coupled reaction able to detect any ATP formation when the reaction starts with ADP (data not shown). To exclude the influence of this enzyme in our assay, all incubations with ADP as substrate were performed in the presence of NEM. Based on inhibition curves (data not shown), we choose to use 1.0 mM and 4.0 mM of NEM for the assay of soluble and microsomal fractions, respectively.

The mitochondrial ATPase inhibitor, oligomycin [30], inhibited ATP and ADP hydrolysis in soluble fraction by 41% and 39%, respectively. In microsomal fraction, this inhibitor significantly decreased only ATP hydrolysis by 33%. Oligomycin was then added from a concentrated ethanol solution resulting in a final concentration of 1% ethanol in the incubation medium. The addition of ethanol alone did not arrest the enzyme activities in the absence of inhibitor (data not shown). Sodium azide, another mitochondrial ATPase inhibitor at low concentrations and a NTPDase inhibitor at high concentrations [31,32], showed a different profile of inhibition in each preparation studied. In soluble fraction, sodium azide inhibited ATP and ADP hydrolysis in all concentrations used. With respect to microsomal fraction, our results showed that sodium azide tested at low concentration (0.1 mM) did not altered ATP and ADP hydrolysis. On the other hand, high concentrations of this inhibitor (5.0, 10 and 20 mM) decreased ATP hydrolysis (26%, 34% and 39%, respectively) and ADP hydrolysis (36%, 47% and 61%, respectively). To exclude the participation of mitochondrial ATPase in our assays, all incubations using ATP and ADP as substrates were performed in the presence of 2.0 µg/mL oligomycin.

Gadolinium chloride has been demonstrated to inhibit E-NTPDases from several sources [33-36]. Our results showed that this compound tested at different concentrations had no effect on ATP and ADP hydrolysis in the soluble fraction. On the other hand, gadolinium chloride in the final concentration of 0.2, 0.3 and 0.5 mM inhibited ATP hydrolysis (21%, 21% and 30%, respectively) and ADP hydrolysis (29%, 23% and 41%, respectively) in the microsomal fraction. The antagonists of P2

receptors, suramin and evans blue, that have been used as ectonucleotidases inhibitors [34,36,37], caused inhibition on ATP and ADP hydrolysis in both soluble and microsomal fractions. Suramin inhibited ATP and ADP hydrolysis by 90% and 84%, respectively in the soluble fraction; in the microsomal fraction this inhibition was 87% for ATP and 44% for ADP hydrolysis. Evans blue inhibited ATP and ADP hydrolysis by 89% and 92%, respectively in the soluble fraction, and, in the microsomal fraction, this compound practically abolished the ATP and ADP hydrolysis (98% and 99% of inhibition, respectively).

Western blot analysis

To identify the NTPDase family members presented in cardiac soluble and microsomal fractions we performed Western blot analysis of the samples under non-reducing conditions. The antibodies against NTPDase1,2,3,5 and 6 were tested and revealed immunoreactivity only for NTPDase5 in soluble fraction and in microsomal fraction only NTPDase2 was detected (Fig. 5). The other antibodies tested not revealed immunoreactivity.

Discussion

NTPDase family has become the subject of intensive research within the past decade mainly because the important role of these enzymes on the modulation of the purinergic signaling. Members of this family have been identified under physiological and pathological conditions in many cells and tissues, including the heart. Furthermore, their use for therapeutic purposes have been suggested [4,11,38,39]. In this paper we present the kinetic and biochemical analysis of ATP and ADP hydrolysis in rat cardiac soluble and microsomal fractions under conditions where mitochondrial ATPase and adenylate kinase were blocked. The results presented here support our proposal that these activities correspond to enzymes belonging to the NTPDase family.

Firstly, the involvement of NTPDase activity in the hydrolysis of ATP and ADP in soluble and microsomal fractions was evidenced by the cation dependence, since there was a decrease of both hydrolysis in the presence of EDTA [4,10,11,27,28,32,33]. In addition, high activity in the presence of both Ca^{2+} and Mg^{2+} that are known by optimizing assay conditions for NTPDase activity, was observed [4,10,11,35,36,40,41]. In some concentrations tested, Mg^{2+} was more effective than Ca^{2+} in activating ATP and ADP hydrolysis. However, we choose to use $CaCl_2$ in all experiments in order to exclude the significant interference of Mg^{2+}-dependent enzymes, such as adenylate kinase and Na^+/ K^+- ATPase [40]. Besides, using a buffering system, the best enzyme activity for ATP and ADP hydrolysis in both fractions was reached between pH 7.5 and 8.5. These pH values are in accordance with those previously described for NTPDases [35,36,40,41].

The exclusion of some enzymatic associations was performed using different enzyme inhibitors such as ouabain, orthovanadate, lanthanum and levamisole, which did not affected ATP or ADP hydrolysis in both fractions. The contribution of mitochondrial ATPase for ATP hydrolysis was verified with the use of oligomycin, which inhibited the hydrolysis of this substrate in soluble and microsomal fractions. However, oligomycin also decreased ADP hydrolysis in the two fractions indicating a possible enzymatic combination between mitochondrial ATPase and adenylate kinase that could mimic NTPDase activity. The possibility that ADP hydrolysis occurs by prior conversion to ATP, catalyzed by adenylate kinase and later hydrolysis by mitochondrial ATPase was investigate with the use of two criteria. The first was the evaluation of the rate of ADP hydrolysis in the absence and in the presence of an adenylate kinase inhibitor (NEM) [29]. The second criteria consisted of a protocol that was previously used by us and by others to detect any ATP formation when the reaction starts with ADP [15,23,27]. Both procedures indicated the presence of adenylate kinase in soluble and microsomal fractions. To exclude this enzymatic association (mitochondrial ATPase

plus adenylate kinase) and to ensure that ATP and ADP hydrolysis were carried out by an NTPDase, we always added oligomycin and NEM in the reaction medium.

Interestingly, sodium azide, another compound known to inhibit mitochondrial ATPase at 0.1 mM [31], also decreased ATP and ADP hydrolysis in the soluble fraction, suggesting the occurrence of the same enzymatic combination between mitochondrial ATPase and adenylate kinase described above. On the other hand, in the microsomal fraction 0.1 mM of sodium azide did not alter the hydrolysis of ATP or ADP. Although sodium azide inhibits mitochondrial ATPase as oligomycin, its mechanism of action is distinct and may promote different effects according to the sample. In fact, it was observed that both inhibitors had different effects in the mitochondrial ATPase activity, in which sodium azide decreased ATP hydrolysis whereas oligomycin did not affect the hydrolysis of this substrate in digestive gland of *Helix aspersa* [42]. When we tested sodium azide at concentrations able to inhibit NTPDase activity, a parallel inhibition of ATP and ADP hydrolysis both in the soluble and in the microsomal fractions occurred. Similar effects were obtained by other studies that reported a significant inhibition of NTPDase activity from several sources at high concentrations of sodium azide [34-36,41]. In agreement with previous reports, the most potent inhibition of the hydrolysis of ATP and ADP was found when testing the antagonists of P2 receptors, suramin and evans blue [34,36].

Although the mode of action of gadolinium chloride remains unclear, this compound has been described as a potent inhibitor for both membrane-bound [34-36] and soluble forms of NTPDases [33]. In this study, we demonstrate that gadolinium chloride did not affect ATP and ADP hydrolysis in soluble fraction but it was capable of inhibits the hydrolysis of both substrates in the microsomal fraction. This different pattern of inhibition by gadolinium chloride suggests that this compound did not seem to be a specific inhibitor of NTPDase activity.

With respect to the kinetic properties, our results demonstrate that K_M values calculated from the Eadie-Hofstee plot with ATP and ADP as substrates were in the

micromolar range for both fractions, which is in accordance with the literature about NTPDases. In relation to V_{max}, the ratio of ATP/ADP hydrolysis was about 8 in the soluble and about 12 in the microsomal fraction, suggesting a preferential ATPase activity in both fractions.

The presence of enzymes with ATPase activity in cardiac tissue has been demonstrated by immunohistochemical and biochemical analysis of murine, porcine and human hearts [17,43-45]. In addition, a significant mRNA expression of *Entpd*2 in murine and human hearts, suggest that this enzyme would be a likely candidate responsible for such activity [36,46-48]. However, the activity of other members of the NTPDase family can not be excluded in the cardiac tissue. NTPDase1 have been demonstrate by immunohistochemistry and Western blot analysis of human heart [49]. In addition, the mRNA expression of *Entpd3* in rat cardiomyocytes suggests another potential enzyme that could modulate extracellular nucleotide levels in the heart [48]. Recently, our group identified by RT-PCR the mRNA expression of *Entpd*1,2,3,5 and 6 in rat heart left ventricle [36]. Interestingly, Western blot analysis for NTPDase (1, 2, 3, 5 and 6) of soluble fraction, under non-reducing condition, revealed only immunodetection of NTPDase5. This result is in agreement with previous reports that described the secretion of this intracellular protein to the soluble medium, forming a soluble NTPDase [10]. On the other hand, NTPDase5 isoform is described to hydrolyze preferentially diphosphate nucleotides [10], which was different from our kinetic analysis where this enzyme hydrolyzed triphosphate nucleotides preferentially. Based on these results, we could suppose that were changes on the conformation of NTPDase5 protein after its secretion to the extracellular medium, and this could be responsible for the preference for triphosphate nucleotides. In the microsomal fraction, only NTPDase2 could be detected, corroborating with the hydrolysis ratio found in our kinetic analysis.

Soluble nucleotidases have been detected in serum or plasma of humans and other species [50-53]; however, the origin of these enzymes is poorly understood and

generally is attributed to their release after different cellular stimulus [24,54-56]. Up to the present moment, the physiological role of soluble nucleotidases is not clear, but recent studies have considered the administration of these enzymes for antiplatelet therapy under experimental prothrombotic conditions [57-59]. In our study, the homogenization of the cardiac tissue disrupts cell membrane and probably releases diverse NTPDases isoforms to the soluble medium. Nevertheless, the presence of a "true" soluble enzyme can not be discarded. As well as in other tissues, soluble NTPDases could represent an important auxiliary effector system for local inactivation of acutely elevated nucleotides, especially at sites of injury and inflammation in the heart. However, further studies are required to understand the pertinence of these activities.

The presence of NTPDase activity hydrolyzing adenine nucleotides has been described in microsomes of various tissues [60-62] and probably may have other important roles beyond that well established. The microsomal fraction is enriched by vesicles derived from sarcoplasmic reticulum, that is the major intracellular organelle that sequesters and releases Ca^{2+}, regulating relaxation and tension development by the myocardium [7,8]. High intracellular Ca^{2+} concentrations trigger a rapidly pumped of the ion into the sarcoplasmic reticulum by a Ca^{2+}-ATPase, while depolarization of the surface membrane open Ca^{2+} channels of the reticulum releasing Ca^{2+} to the cytosol. Evidences suggest that abnormal release of Ca^{2+} from these channels has been associated with certain pathological states, including arrhythmias and heart failure [63,64]. Previous studies have demonstrated that adenine nucleotides, adenosine and caffeine (an adenosine receptor antagonist) affect differently Ca^{2+} release from sarcoplasmic reticulum both in skeletal muscle and in cardiac fibers [7-9,65-66]. The modulation of these effects can occur indirectly through signaling at cell surface purine receptors [64,67]; however, direct activation of the Ca^{2+} channel have been observed in studies with isolated channels or sarcoplasmic reticulum vesicles (microsomes) [7,8]. Additional evidence about the influence of purinergic signaling on Ca^{2+} release from

sarcoplasmic reticulum can be visualized in a recent report in which adenosine was described to modulate Ca^{2+} release through binding at A_{2A} receptors in the sarcoplasmic reticulum [68]. Supported by the results discussed above, we are capable of suggest that NTPDase activity founded in microsomal fraction could modulate Ca^{2+} release from sarcoplasmic reticulum and consequently could modulate the cardiac excitation-contraction coupling process.

In summary, the great variety of actions of adenine nucleotides on the cardiovascular system emphasizes the importance of the study of the enzymes that modulate their levels. Our results demonstrate ATP and ADP hydrolysis in rat cardiac soluble and microsomal fractions. Based on the cation-dependence, optimum pH obtained and sensitivity to inhibitors, we can suggest that these activities probably correspond to NTPDase enzymes. The presence of NTPDase activity in the soluble fraction can contribute to the better understanding of the extracellular purinergic signaling in the heart. In addition, the characterization of NTPDase activity in the cardiac sarcoplasmic reticulum can add new information about the intracellular role of these enzymes.

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Table

Table 1: Effect of distinct compounds on ATPase and ADPase activities from rat cardiac soluble and microsomal fractions

Compounds	Concentration (mM)	% of control enzyme activity			
		Soluble		Microsomal	
		ATP	ADP	ATP	ADP
Ouabain	1.0	92.7 ± 21.5	92.9 ± 11.8	104.7 ± 4.1	94.4 ± 8.3
Orthovanadate	0.1	88.9 ± 11.0	83.1 ± 8.7	102.4 ± 2.2	95.5 ± 10.7
NEM	1.0	96.6 ± 13.9	35.1 ± 10.1***	95.8 ± 6.9	77.6 ± 9.3*
Lanthanum	0.1	93.3 ± 16.2	98.7 ± 5.1	98.6 ± 8.2	90.6 ± 10.6
Levamisole	1.0	91.6 ± 14.6	83.2 ± 6.8	95.6 ± 4.7	97.0 ± 13.1
Suramin	0.25	9.7 ± 1.9***	15.7 ± 8.9***	13.3 ± 2.5***	56.1 ± 10.8***
Evans blue	0.1	10.4 ± 4.4***	8.1 ± 3.9***	2.3 ± 0.5***	0.7 ± 0.25***
Oligomicyn	2 µg/mL	58.9 ± 5.5*	61.2 ± 1.5*	67.1 ± 10.2***	82.5 ± 8.8
Sodium azide	0.1	62.3 ± 1.9*	71.1 ± 5.8**	91.8 ± 6.7	90.9 ± 9.9
	5.0	39.6 ± 3.6***	47.5 ± 6.0***	74.1 ± 0.9***	64.0 ± 10.1***
	10	35.8 ± 3.7***	45.2 ± 5.9***	66.0 ± 1.5***	52.7 ± 4.4***
	20	30.9 ± 3.4***	37.2 ± 10.6***	61.0 ± 3.5***	38.5 ± 5.7***
Gadolinium chloride	0.1	96.9 ± 19.7	100.7 ± 3.1	85.1 ± 8.4	79.3 ± 11.5
	0.2	89.9 ± 17.3	100.6 ± 4.4	79.1 ± 7.6*	70.8 ± 7.2**
	0.3	82.9 ± 17.3	102.1 ± 3.1	78.6 ± 8.1**	76.8 ± 6.3*
	0.5	85.9 ± 7.3	103.5 ± 8.3	70.0 ± 4.0***	59.1 ± 9.6***

ATPase and ADPase activities were assayed as described in Material and Methods and are expressed as percentage of the respective control activity. Results are expressed as mean ± S.D. of three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-HSD test. Difference from control enzyme activity (100%): * $P<0.05$; ** $P<0.01$; *** $P<0.001$.
The 100% values correspond to 90.8 ± 9.3 and 18.1 ± 2.4 nmol Pi/min/mg protein for ATP and ADP hydrolysis, respectively in the soluble fraction; 2392.5 ± 92.1 and 239.9 ± 23.5 nmol Pi/min/mg protein for ATP and ADP hydrolysis, respectively in the microsomal fraction.

Figure Legends

Figure 1: Time course and protein concentration curves for ATP and ADP hydrolysis. Time course in soluble fraction was performed with 90 µg of protein for both nucleotides; in microsomal fraction were used 20 µg of protein for ATP and 50 µg of protein for ADP. In relation to protein concentration curves were used 6 min and 25 min for ATP and ADP hydrolysis in soluble fraction, respectively. In microsomal fraction, protein curves were performed for 2 min to ATP and 10 min to ADP hydrolysis. The plots are representative of three independent experiments for each nucleotide and fraction.

Figure 2: Divalent cations dependence on ATP and ADP hydrolysis. Hydrolysis of ATP and ADP by rat cardiac soluble (A and B, respectively) and microsomal (C and D, respectively) fractions were analyzed in the absence of cations (Control), in the presence of 0.25 mM EDTA and in the presence of 1.0-6.0 mM Ca^{2+} or Mg^{2+}. Bars represent means ± S.D. of four independent experiments. Results are expressed as nmol Pi/min/mg of protein.

Figure 3: Effect of pH variation on ATP and ADP hydrolysis from rat cardiac soluble (A and B, respectively) and microsomal (C and D, respectively) fractions. Enzyme activities were determined using a mixture of 50 mM Tris and 50 mM HEPES (pH range 6.0 to 9.0). Data represent means ± S.D. of three different experiments. Results are expressed as nmol Pi/min/mg of protein.

Figure 4: Eadie-Hofstee plot for ATP and ADP hydrolysis in rat cardiac soluble (A and B, respectively) and microsomal (C and D, respectively) fractions. The substrate concentrations tested ranged from 75 to 2000 µM (insets). The K_M and V_{max} values calculated for ATP and ADP hydrolysis in the soluble fraction were 131.2 ± 16.7 µM and 71.3 ± 12.8 nmol Pi/min/mg protein (mean ± S.D., n=6), and 57.1 ± 6.9 µM and 9.3

± 1.1 nmol Pi/min/mg protein (mean ± S.D., n=4), respectively. In the microsomal fraction, the K_M and V_{max} values calculated for ATP and ADP hydrolysis were 315.2 ± 58.8 µM and 1180.6 ± 150 nmol Pi/min/mg protein (mean ± S.D., n=5), and 131 ± 30.7 µM and 100.4 ± 13.2 nmol Pi/min/mg protein (mean ± S.D., n=4), respectively. Data represents a typical experiment.

Figure 5: Representative Western blot analysis of NTPDases family members by cardiac soluble (SF) and microsomal fraction (MF) prepared under non-reducing conditions. Immunodetection demonstrate the presence of NTPDase5 in soluble fraction and NTPDase2 in microsomal fraction. Positions of molecular size markers are shown in kDa.

Fig. 1

Soluble Fraction

Microsomal Fraction

Fig. 2

Soluble Fraction

Microsomal Fraction

A

B

C

D

ATP hydrolysis

ADP hydrolysis

Fig. 3

Soluble Fraction

Microsomal Fraction

ATP hydrolysis

ADP hydrolysis

Fig. 4

Fig. 5

NTPDase 5   NTPDase 2
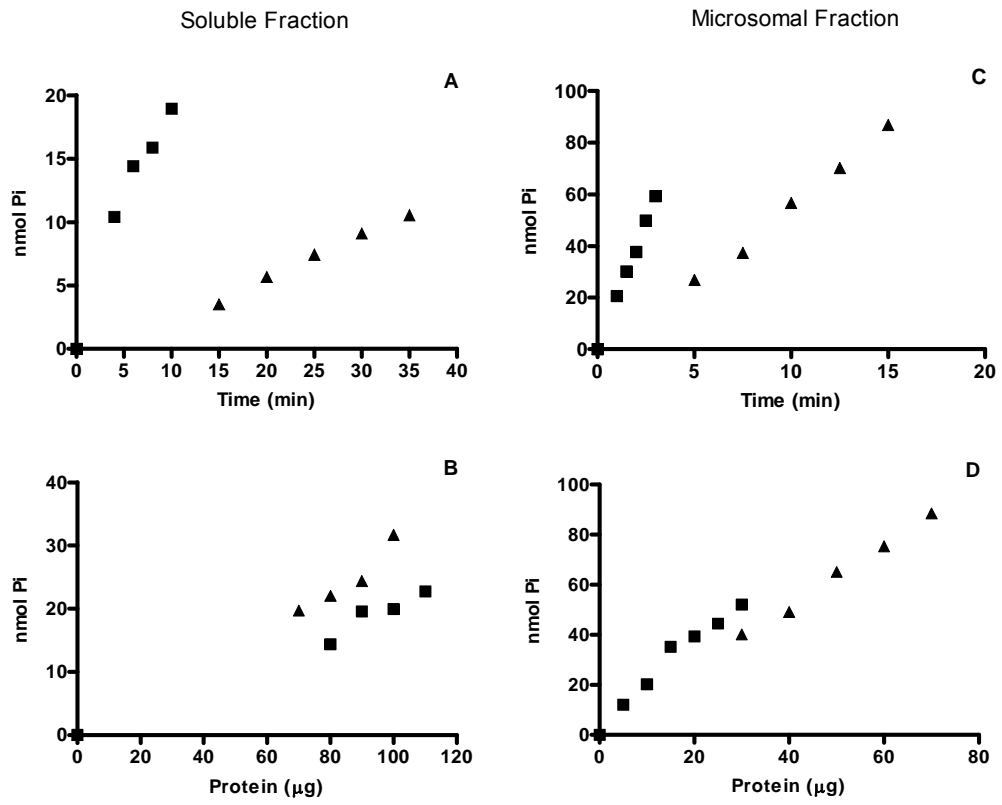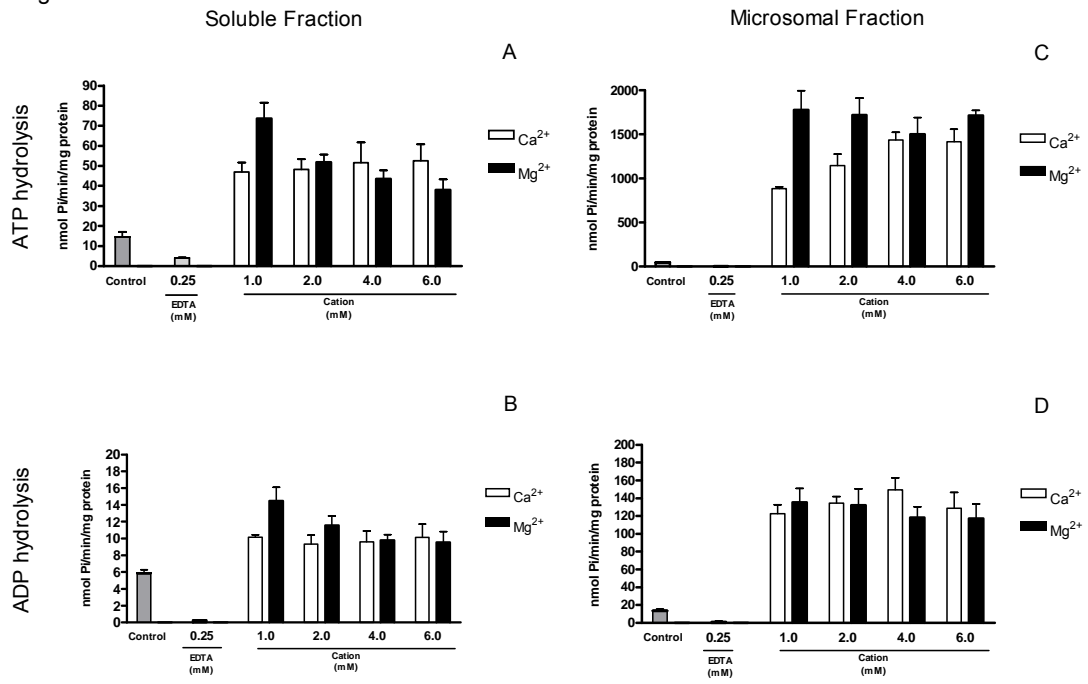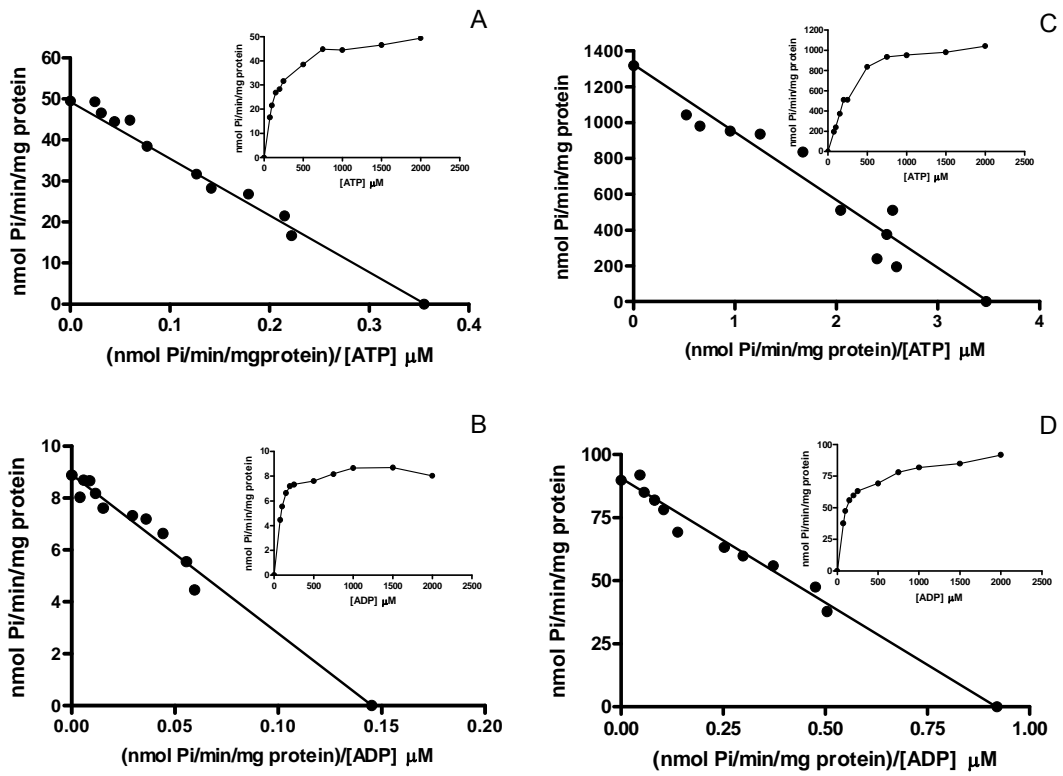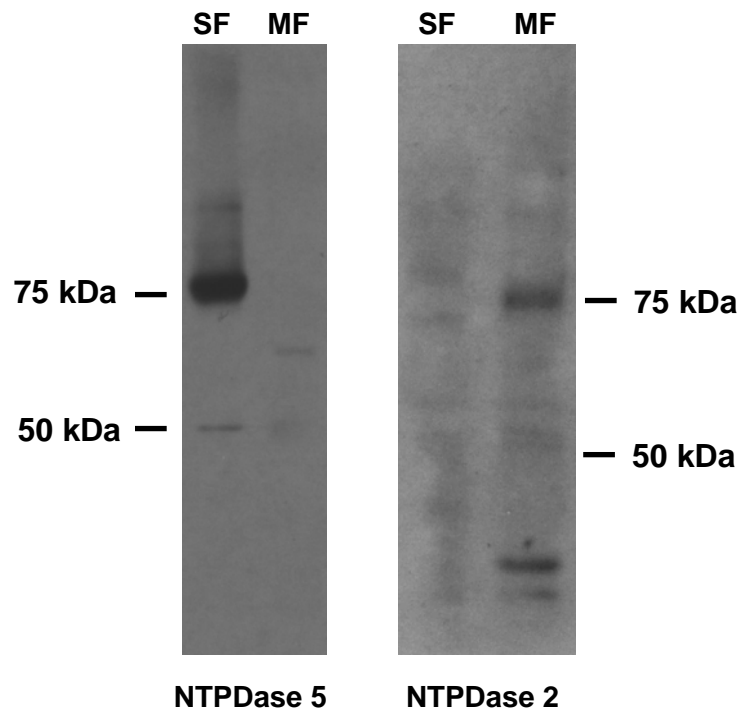
Capítulo III

Biochemical characterization of an ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP, E.C. 3.1.4.1) from rat cardiac soluble and microsomal fractions

Artigo em preparação

Biochemical characterization of an ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP, E.C. 3.1.4.1) from rat cardiac soluble and microsomal fractions

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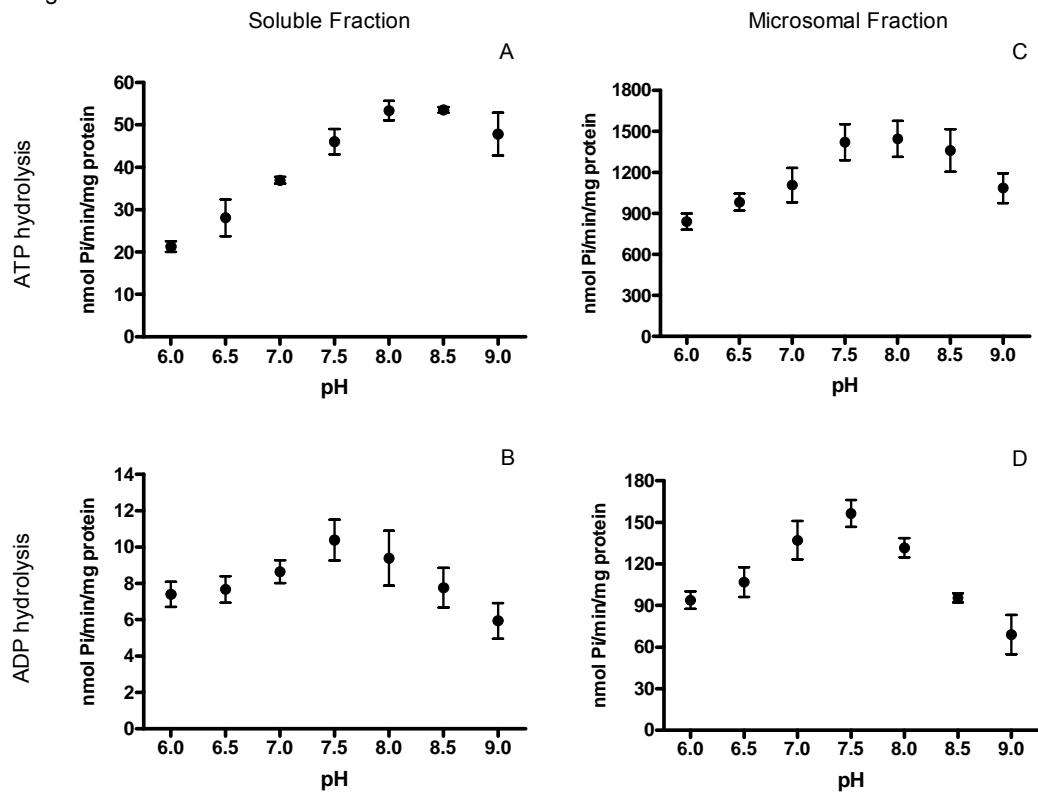Universidade Federal do Rio Grande do Sul,
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Abstract

In the present study we reported the kinetic and biochemical characterization of NPP activity in rat cardiac fractions, one soluble and other enriched in vesicles derived from sarcoplasmic reticulum (called microsomal fraction). The fractions demonstrated ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activities, which could be observed by extracellular hydrolysis of p-Nph-5'-TMP and other biochemical characteristics such as dependence of metal ions and inactivation by a metal ion chelator. The K_M value for the hydrolysis of p-Nph-5'-TMP in soluble and microsomal fraction, were 118.53 ± 27.28 uM and 91.92 ± 12.49 uM, respectively (mean ± S.D., n = 7). The V_{max} values calculated were 2.52 ± 0.15 and 113.87 ± 21.09 nmol p-nitrophenol/min/mg in soluble and microsomal fraction, respectively (mean ± S.D., n = 7). Between the different compounds tested to evaluate the activity of other enzymes on p-Nph-5'-TMP hydrolysis, only suramin (0.25 mM) was capable to produce a significative inhibition of substrate hydrolysis. Thus, our results strongly suggest the presence of E-NPP enzymes in rat heart. These activities could contribute with the modulation of nucleotide levels that are describe to be involve in several physiological and pathological processes in heart.

Keywords: NPPs; adenine nucleotides; microsomes; rat heart

Introduction

Extracellular nucleotides can be hydrolyzed by a variety of enzymes that are located on the cell surface or may also be soluble in the interstitial medium or within body fluids (Zimmermann, 2001). These enzymatic activities can regulate the extracellular concentration of these nucleotides and nucleosides modulating their local effects. In heart, the nucleotides ATP, ADP, AMP and the nucleoside adenosine are known to regulate several activities, including vascular tone, cardiac function and haemostasis (Burnstock, 1990; Kunapuli and Daniel, 1998; Ralevic and Burnstock, 2003). These effects generally occur by interaction with cell-surface receptors called purinoceptors; however, beyond these extracellular actions, some evidences with permeabilized muscle fibers and cardiac sarcoplasmic reticulum vesicles (called microsomes) have suggested that adenine nucleotides and adenosine could exerce intracellular effects (Meissner, 1984; Meissner and Henderson, 1987; Duke and Steele, 1998).

Members of several enzyme families are capable of hydrolyzing extracellular ATP and other nucleotides, and have also been allocated to the cardiovascular system. These include the family of E-NTPDases (ecto-nucleoside triphosphate diphosphohydrolases), along with the E-NPP family (ecto-nucleotide pyrophosphatase/phosphodiesterase), alkaline phosphatases and ecto-5'-nucleotidase (Zimmermann, 2001; Burnstock, 2006; Yegutkin, 2008).

The family of E-NPPs consists of seven structurally related enzymes that are located at the cell surface, either expressed as transmembrane proteins or as secreted enzymes (Stefan *et al.*, 2005). In addition, NPP members have been localized in different cellular compartments suggesting specific physiological functions (Goding *et al.*, 2003). Three of the seven members of the NPP family, namely NPP1-3, are known to hydrolyze nucleotides and have been detected in almost all tissues. The enzymatic action of NPP1-3 (in)directly results in the termination of nucleotide signaling, the salvage of nucleotides and/or the generation of new messengers like ADP, adenosine

or pyrophosphate (Stefan *et al.,* 2006). Recently, our group described the biochemical characteristics of this enzyme activity in synaptosomes from rat heart left ventricle and identified the enzyme expression, of the E-NPP family members in cardiac tissue (Rücker *et al.*, 2007). In this work the co-expression of NPPs and NTPDases in cardiac synaptosomes was suggested as a multiple system for extracellular hydrolysis control.

Catalysis by NPPs affects processes as cell proliferation and motility, angiogenesis, bone mineralization and digestion. The NPPs are also implicated in the pathophysiology of cancer, insulin resistance and calcification diseases (Stefan *et al.*, 2005). The NPP1 and NPP3 are type II transmembrane glycoproteins comprising two identical disulfide-bounded subunits. Mutations in NPP1 gene have been associated with generalized arterial calcification in infancy, ossification of the posterior longitudinal ligament of the spine, and insulin resistance (Ruf *et al.,* 2005; Terkeltaub, 2006). NPP3 (gp130[RB13-6]) was initially recognized by the monoclonal antibody RB13-6 as a 130-kD glycoprotein, on a specific subset of rat brain glial precursor cells (Deissler *et al.*, 1995; Deissler *et al.*, 1999). NPP2 (autotaxin, PD-1*a*) is synthesized as a pro-enzyme and further processed to be a secretory molecule. This enzyme exert a variety of biologically significant effects in cell growth, differentiation, adhesion, and migration, translated into functional effects in angiogenesis, tumor metastasis, and embryonic development (Stefan *et al.*, 2005).

Considering that adenine nucleotides and adenosine regulate a variety of intra- and extracellular physiological responses in the cardiovascular system, investigations about the enzymes responsible by its concentrations are very important. Furthermore, data concerning the possible roles of NPPs on purinergic signaling of the heart are limited and are focused on the extracellular actions. Thus, in the present study, we have demonstrate NPP activity in rat cardiac fractions, one soluble and other enriched in vesicles derived from sarcoplasmic reticulum (called microsomal fraction), in order to contribute with the understanding about the control of the nucleotide levels in cardiac system.

Materials and Methods

Animals

Male Wistar rats weighing 200 - 280 g were used in this study. All animals were housed in cages with food and water available ad libitum. They were maintained under a 12-h light/dark cycle at a constant temperature of 23 ± 2 °C. Procedures for the care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

Isolation of cardiac soluble and microsomal fractions

Rats were killed by decapitation, hearts were carefully removed and the ventricles were isolated. The fractions were prepared as described by Floreani *et al.* (2003), with minor modifications. Briefly, both left and right cardiac ventricles of two animals were minced and homogenized in 1:23 (w/v) of 0.25 M sucrose-10 mM Tris (pH 7.4), using a tissue homogenizer (Sorvall Omni-Mixer, 17105) for 3 min at setting 4. The homogenate was centrifuged for 30 min at 10,000 x *g*. The pellet (P1) was discarded and the supernatant (S1) was centrifuged for 60 min at 105,000 x *g*. The supernatant (S2) obtained represented the soluble fraction, whereas the pellet (P2), resuspended in the homogenization buffer, represented the microsomal fraction. Both fractions were prepared fresh daily and kept at 4 °C throughout the process.

Protein determination

Protein was measured by the Comassie Blue method, according to Bradford (1976), using bovine serum albumin as standard.

Assay of ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) activity

Unless otherwise stated, enzyme activity was determined in the following incubation medium: 50 mM Tris-HCl, pH 8.9, and 6.0 mM $MgCl_2$ in a final volume of

200 µL. Soluble and microsomal protein (90 µg and 30 µg, respectively) were added to the reaction medium and preincubated for 10 min at 37 °C. The reaction was started by the addition of p-Nph-5'-TMP to a final concentration of 0.5 mM. After 40 min of incubation for soluble and 6 min of incubation for microsomal fraction, the reactions were stopped by addition of 200 µL NaOH 0.2 N. For all enzyme assays, incubation times and protein concentration were chosen to ensure the linearity of the reactions. The amount of p-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8 x 10^{-3}/M/cm. Controls to correct for non-enzymatic substrate hydrolysis were performed by adding enzyme preparation after the reaction had been stopped with NaOH as described above. All samples were performed in duplicate. Enzyme activities were generally expressed as nmol p-nitrophenol released per minute per milligram of protein.

Inhibitor studies

The effects of the following compounds on p-Nph-5'-TMP hydrolysis from rat cardiac soluble and microsomal fraction were analyzed: 1.0 mM levamisole, 0.25 mM suramin, 10 and 20 mM sodium azide, 0.3 and 0.5 mM gadolinium chloride. Incubation times, protein and substrate concentrations were used as described above. Both fractions were preincubated in the presence of each inhibitor for 10 min at 37 °C followed by the addition of substrate. Results are expressed as percentage of control enzyme activity.

Results

Determining time course and optimal protein concentration

We first investigated the p-Nph-5'-TMP hydrolysis in rat cardiac soluble and microsomal fractions as a function of time and protein concentration in order to determine the best assay conditions. The fractions were incubated as described in Material and Methods. The results indicated that the time course of p-Nph-5'-TMP

hydrolysis for soluble fraction was linear up to 70 min and up to 10 min, in microsomal fraction (data not shown). In order to ensure that the incubation time was within the linearity of the reaction, we choose 40 min as the assay times for soluble fraction, and 6 min for microsomal fraction in the subsequent experiments.

Concerning to protein concentration, the results demonstrated that p-Nph-5'-TMP hydrolysis were linear up to 110 µg for soluble and up to 70 µg for microsomal fraction (data not shown). Thus, in the subsequent experiments, we used 90 µg protein of soluble fraction and 30 µg protein of microsomal fraction.

Cation dependence

In order to investigate the possibility of cation dependence for the rat cardiac soluble and microsomal fractions, we tested the hydrolysis rate for the p-Nph-5'-TMP in the presence or absence of divalent cations or EDTA (cation chelator). Calcium and magnesium concentrations were tested in the range of 2.0 – 8.0 mM. As shown in Fig. 1A, in the soluble fraction, in the presence of 0.025 mM EDTA, p-Nph-5'-TMP hydrolysis were practically negligible when compared with respective control (without the addition of divalent cations). In the microsomal fraction (Fig. 1B), p-Nph-5'-TMP hydrolysis founded in the control group were completely removed by the addition of 0.025 mM EDTA. On the other hand, the hydrolysis of p-Nph-5'-TMP in both fractions was increased by the presence of different concentrations of Ca^{2+} or Mg^{2+}. These data clearly indicate the cation dependence for both enzyme activities.

In both fractions, some differences were observed in enzymatic activation by different cations at different concentrations tested. p-Nph-5'-TMP hydrolysis was more activated by Mg^{2+} compared to Ca^{2+}, in some concentrations. Despite these differences, in the subsequent experiments we used a final concentration of 6.0 mM $MgCl_2$ for p-Nph-5'-TMP hydrolysis in both fractions.

Effect of pH

The optimum pH values for *p*-Nph-5'-TMP hydrolysis were determined in a medium containing a mixture of 50 mM Tris and 50 mM Glycine buffer (pH varying from 8.0 to 10.0). The pH curves showed the highest activity at pH 8.9 for soluble and pH 9.3 for microsomal fraction (Fig. 2A and 2B). For the subsequent experiments, the pH 8.9 was chosen for the hydrolysis of *p*-Nph-5'-TMP in soluble and microsomal fraction.

Kinetic parameters

Michaelis constant K_M and V_{max} values were calculated from the Eadie –Hofstee plot (Fig. 3) with *p*-Nph-5'-TMP as substrate. Substrate concentrations tested ranged from 25 to 750 µM. The K_M value for the hydrolysis of *p*-Nph-5'-TMP in soluble and microsomal fraction, corresponded to 118.5 ± 27.2 uM and 91.9 ± 12.4 uM, respectively (mean ± S.D., n = 7), and V_{max} value calculated in soluble and microsomal fraction, were 2.52 ± 0.1 and 113.87 ± 21.1 nmol *p*-nitrophenol/min/mg, respectively (mean ± S.D., n = 7).

Effect of inhibitors on p-Nph-5'-TMP hydrolysis

In order to exclude possible enzymatic associations in the *p*-Nph-5'-TMP hydrolysis, we tested some compounds that were reported to affect nucleotide hydrolysis. As shown in Table 1, the classical alkaline phosphatase inhibitor, levamisole (1.0 mM) (Constantoupolus *et al.,* 1977), was ineffective as inhibitor of *p*-Nph-5'-TMP hydrolysis in soluble and microsomal fraction. On the other hand, suramin (0.25 mM), a P2 receptor antagonist and an inhibitor of E-NTPDase (Leal *et al.*, 2005) and E-NPP activities (Grobben *et al.*, 2000), strongly reduced the hydrolysis of *p*-Nph-5'-TMP in both preparations. Sodium azide (10 and 20 mM) that is known to inhibit E-NTPDases at high-concentration, had no effects on substrate hydrolysis. Gadolinium chloride (0.3 and 0.5 mM), a lanthanide that interacts with different pathways of intracellular and extracellular ATP action and that has been considered the most potent

inhibitor for both soluble and membrane-bound NTPDases (Fürstenau et al., 2006; Escalada et al., 2004) had no effects on p-Nph-5'-TMP hydrolysis.

Discussion

Extracellular nucleotides/nucleosides are known to regulate several physiological responses, including vascular tone, cardiac function, and hemostasis (Burnstock, 1991; Kunapuli & Daniel, 1998; Ralevic & Burnstock, 2003). The ATP, ADP and AMP hydrolysis by ecto-enzymes (ecto-ATPase, NTPDases and 50-nucleotidase) was thought to be a very simple process during a long time. Nowadays it is well known that this is a complex process involving several families of ectonucleotidases (Goding et al., 2003). The relative contribution of the distinct ectonucleotidases species to the modulation of purinergic signaling may depend on differential tissue and cell distribution, regulation of expression, targeting to specific membrane domains, but also on substrate availability and substrate preference (Stefan et al., 2006). One of the four structurally unrelated families of ectonucleotidases is represented by E-NPPs.

In this study the enzyme activity obtained from rat cardiac soluble and microsomal fractions shares the major biochemical properties already described for E-NPPs. The pH curve showed a maximal enzymatic activity at alkaline pH in the range 8.9–9.3. These pH values are in accordance with those previously described for E-NPPs (Bollen et al., 2000). The hydrolysis of p-Nph-5'-TMP by NPPs was determined in the presence of divalent cations (Ca^{2+} or Mg^{2+}) and the results indicates that the enzyme is cation-dependent, considering that increasing concentrations of EDTA greatly reduced the catalytic activity. It has been demonstrated that E-NPPs are metalloenzymes as their activity is blocked by metal chelators (Bollen et al., 2000).

Members of E-NPP family generally have wider substrate specificity than intracellular pyrophosphatases and phosphodiesterases. The p-nitrophenyl ester of TMP is used routinely for the in vitro assay of E-NPPs. Then, Michaelis constant (K_M) and V_{max} values were calculated from an Eadie–Hofstee plot with p-Nph-5'-TMP as

substrate. The K_M value corresponded to 118.5 ± 27.2 uM and 91.9 ± 12.4 uM, in soluble and microsomal fractions, respectively (mean ± S.D., n = 7), and V_{max} value calculated in soluble and microsomal fraction, were 2.52 ± 0.1 and 113.87 ± 21.1 nmol *p*-nitrophenol/min/mg, respectively (mean ± S.D., n = 7). This is in accordance with the NPP reaction that is characterized by a K_M of 50 to 500 µM and a V_{max} of 5–300 µmol/min/mg enzyme (Kelly et al., 1975; Hosoda et al., 1999; Vollmayer *et al.*, 2003). In the sense to eliminate the possible participation of other enzymes, we tested compounds like levamisole, suramin, sodium azide, and gadolinium chloride. The results could suggest that in the conditions tested, we worked with a predominant E-NPP activity in our system incubation, since only suramin, an inhibitor of E-NPP activities (Grobben *et al.*, 2000; Fürstenau *et al.*, 2006), strongly reduced the hydrolysis of *p*-Nph-5'-TMP.

NPP expression in cardiac tissue have been demonstrate in some works (Stefan *et al.*, 1999; Bollen *et al.*, 2000); however, the exact participation of this enzyme activity on nucleotide hydrolysis is poorly understood. In a recent study, Rücker *et al.* (2007) investigate the expression of NPP1-3 in cardiac left ventricle. They observed only NPP2 and NPP3 expression in that tissue, in addition, they characterize the biochemical properties of NPP activity in cardiac synaptosomes and suggesting the possible involvement of this enzymes with the control of nucleotides and nucleoside levels in sympathetic nerve end. In our study, NPP activity identified in soluble and microsomal fractions from heart can be act as a new important way to the control of cardiac functions, since previous studies have demonstrated the participation of adenine nucleotides and adenosine in the control of cardiac excitation-contraction coupling process by direct effects on the Ca^{2+} release from sarcoplasmic reticulum (Meissner, 1984; Smith et al., 1986; Meissner and Henderson, 1987; Rousseau et al., 1988; Duke and Steele, 1998; Butanda-Ochoa et al., 2003; Hleihel et al., 2006).

Acknowledgements

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Table 1: Effect of distinct compounds on *p*-nitrophenyl-5'-TMP hydrolysis from rat cardiac soluble and microsomal fraction

Compounds	Concentration (mM)	% of control enzyme activity	
		Soluble	Microsomal
Levamisole	1.0	99.8 ± 11.4	100.4 ± 8.2
Suramin	0.25	47.2 ± 9.6*	46.8 ± 13.9*
Sodium azide	10	102.7 ± 6.6	109.1 ± 3.2
	20	106.3 ± 2.1	106.3 ± 4.9
Gadolinium chloride	0.3	100.7 ± 9.3	93.3 ± 4.3
	0.5	97.8 ± 2.5	90.5 ± 8.6

NPP activities were assayed as described in Material and Methods and expressed as percentage of respective control activity (in the absence of inhibitor). Results are expressed as mean ± SD of three experiments. Data were analyzed by Student's *t*-test. * Represents difference from control enzyme activity (100%) ($P<0.003$). The 100% values correspond to 2.3 ± 0.1 and 110.7 ± 2.6 nmol ρ-nitrophenol/min/mg protein for ρ-nitrophenyl-5'-TMP hydrolysis, in soluble and microsomal fraction respectively.

Figure Legends

Figure 1. Divalent cations dependence on p-Nph-5'-TMP hydrolysis. Hydrolysis of p-Nph-5'-TMP by rat cardiac soluble (A) and microsomal (B) fractions were analyzed in the absence of cations (Control), in the presence of 0.025, 0.05 and 1.0 mM EDTA and in the presence of 2.0-8.0 mM Ca^{2+} or Mg^{2+}. Bars represent means ± S.D. of four independent experiments. Results are expressed as nmol p-nitrophenol/min/mg of protein.

Figure 2. Effect of pH on p-Nph-5'-TMP hydrolysis in rat cardiac soluble (A) and microsomal (B) fractions. Enzyme activity was determined as described in Material and Methods using a mixture of the following buffers: Tris and Glycine (pH range 8.0 to 10.0). Data represent means ± SD of three different experiments. Results are expressed as nmol p-nitrophenol/min/mg of protein.

Figure 3. Eadie-Hofstee plot for p-Nph-5'-TMP hydrolysis. The nucleotide hydrolysis as function of substrate concentration from rat cardiac soluble (A) and microsomal (B) fractions is shown in the insets. The mean K_M values calculated for p-Nph-5'-TMP hydrolysis were found to be 118.5 ± 27.2 uM and 91.9 ± 12.4 uM, respectively, and V_{max} value calculated in soluble and microsomal fraction, were 2.52 ± 0.1 and 113.87 ± 21.1 nmol p-nitrophenol/min/mg, respectively. Data are expressed as mean ± SD, n=4.

Figure 1

Figure 2

Figure 3
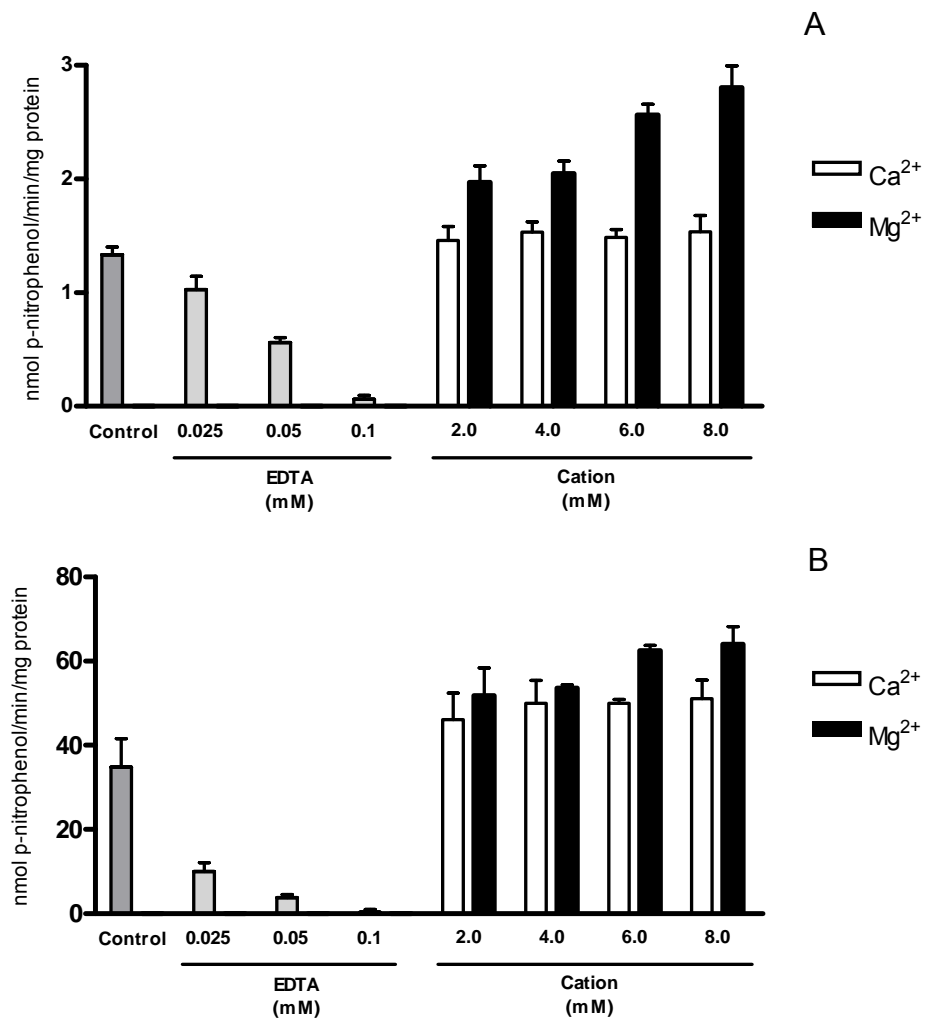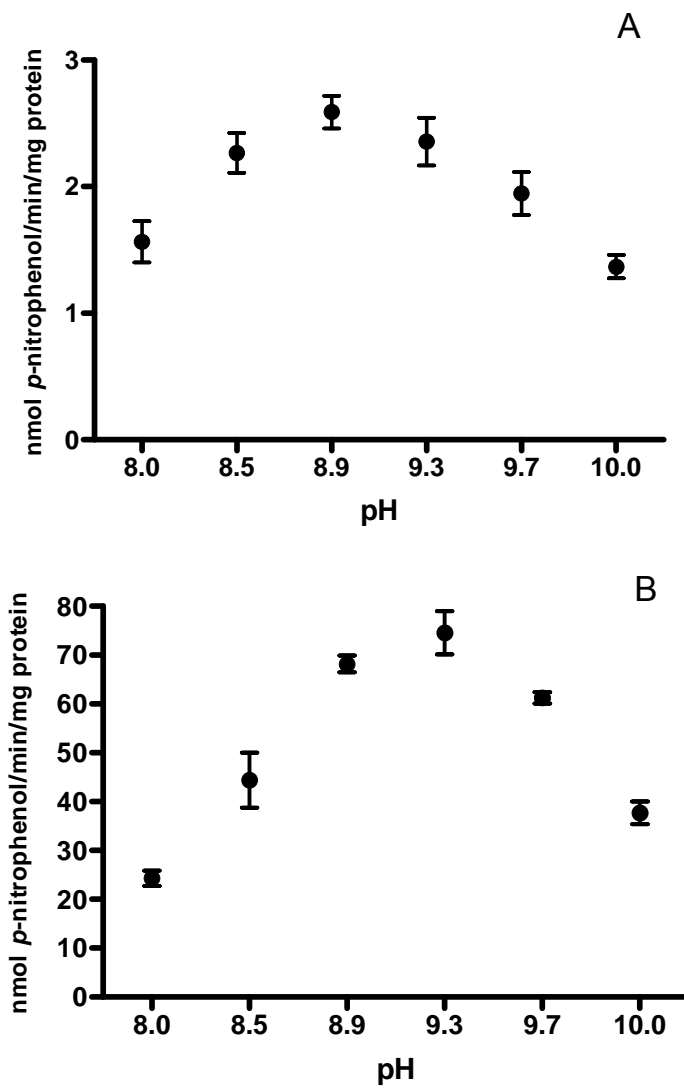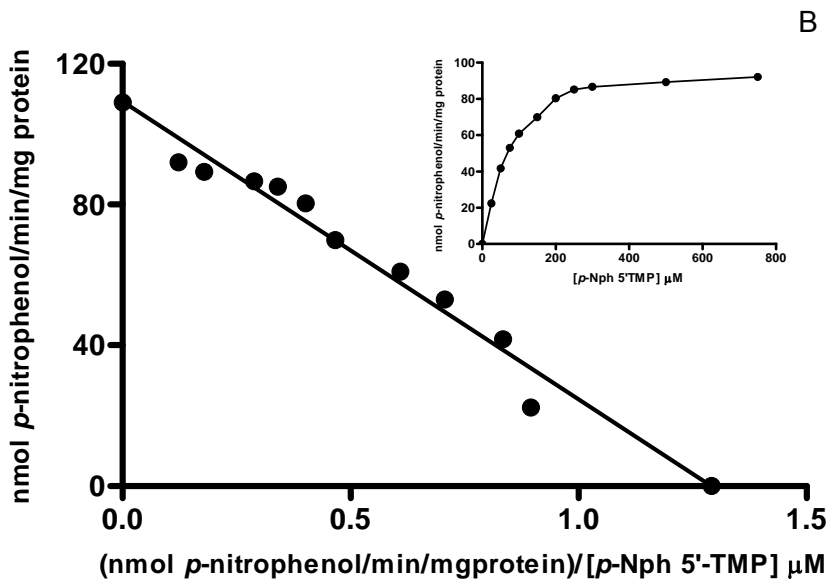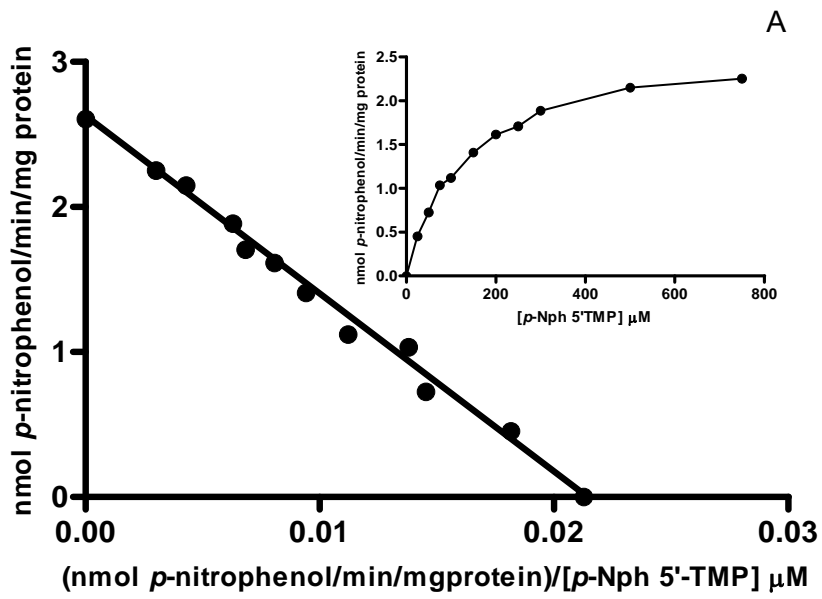
ANEXO I

Resultados obtidos durante o doutorado e que serão finalizados

após a realização de experimentos para a verificação de

parâmetros hemodinâmicos cardíacos, identificação das E-

NTPDases, além de análises de PCR em tempo real.

In vivo effect of acute and chronic administration of caffeine on ectonucleotidase activities in soluble and microsomal fractions of rat heart

Material and Methods

Chemicals

Nucleotides, *p*-Nitrophenyl thymidine 5'-monophosphate (*p*-Nph-5'-TMP), oligomycin, N-ethylmaleimide (NEM), Trizma Base and caffeine were obtained from Sigma-Aldrich (St Louis, MO, USA). All other reagents were also of analytical grade.

Animals

Male Wistar rats weighing 200 - 280 g were used in this study. Unless otherwise mentioned, all animals were housed in cages with food and water available *ad libitum*. They were maintained under a 12-h light/dark cycle at a constant temperature of 23 ± 2 °C. Procedures for the care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

Experimental Protocols

In the acute treatment, caffeine (5, 15 or 45 mg/kg of body weight, dissolved in 0.9% NaCl solution) was administered by oral gavage in a single dose at 1.5h before the sacrifice. Control animals received a single dose of NaCl.

In the chronic treatment, caffeine (0.3 or 1.0 mg/mL) was orally administered for 14 days in the drinking water. Caffeine intake was monitored throughout the experiment. Daily caffeine intake (mg/kg/day) was estimated based on the subject's fluid consumption over a 24h period and its body weight. Daily water intake in rats not

exposed to caffeine (control group) was monitored for comparison. In these experiments, the animals were euthanized on day 15.

Isolation of cardiac soluble and microsomal fractions

Rats were killed by decapitation, hearts were carefully removed and the ventricles were isolated. The fractions were prepared as described by Floreani et al. (2003), with minor modifications. Briefly, both left and right cardiac ventricles of two animals were minced and homogenized in 1:23 (w/v) of 0.25 M sucrose-10 mM Tris (pH 7.4), using a tissue homogenizer (Sorvall Omni-Mixer, 17105) for 3 min at setting 4. The homogenate was centrifuged for 30 min at 10,000 x *g*. The pellet (P1) was discarded and the supernatant (S1) was centrifuged for 60 min at 105,000 x *g*. The supernatant (S2) obtained represented the soluble fraction, whereas the pellet (P2), resuspended in the homogenization buffer, represented the microsomal fraction. Both fractions were prepared fresh daily and kept at 4 °C throughout the process.

Protein determination

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

Assays of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-5'-nucleotidase activities in soluble and microsomal fractions

ATP and ADP hydrolysis in soluble fraction were determined in a reaction medium containing 50 mM Tris-HCl buffer (pH 8.0), 4.0 mM $CaCl_2$ and 2.0 µg/mL oligomycin in a final volume of 200 µL. For ADP hydrolysis, 1.0 mM NEM was added to the reaction medium. The activity of ecto-5'-nucleotidase was determined in a reaction medium containing 50 mM Glycine buffer (pH 9.5) and 1.0 mM $MgCl_2$ in a final volume of 200 µL. About 90 µg of soluble protein were added to the reaction medium and preincubated for 10 min at 37 °C. The reaction was started by the addition of ATP, ADP

or AMP to a final concentration of 2.0 mM. After 6, 25 and 40 min of incubation for ATP, ADP and AMP, respectively, the reactions were stopped by the addition of 200 µL 10% trichloroacetic acid (TCA). The samples were chilled on ice and the inorganic phosphate (Pi) released was measured according to Chan et al. (1986).

In microsomal fraction, ATP and ADP hydrolysis were determined in a reaction medium containing 50 mM Tris-HCl buffer (pH 7.5), 4.0 mM $CaCl_2$ and 2.0 µg/mL oligomycin in a final volume of 200 µL. In the reaction mixture used to determine for ADP hydrolysis, 4.0 mM NEM was added. For ecto-5'-nucleotidase assay was used a reaction medium containing 50 mM Glycine buffer (pH 9.5) and 1.0 mM $MgCl_2$ in a final volume of 200 µL. Microsomal protein (20 µg for ATP and 50 µg for ADP and AMP hydrolysis) was added to the reaction medium and preincubated for 10 min at 37 °C. The reaction was started by the addition of ATP, ADP or AMP to a final concentration of 2.0 mM. After 2 min of incubation for ATP and 10 min of incubation for ADP and AMP, the reactions were stopped by the addition of 200 µL 10% trichloroacetic acid (TCA). The samples were chilled on ice and the inorganic phosphate (Pi) released was measured how described above.

For all enzyme assays, incubation times and protein concentration were chosen in order to ensure the linearity of the reactions. All samples were run in triplicate. Controls with the addition of the enzyme preparation after mixing with TCA were used to correct for non-enzymatic substrate hydrolysis. Enzyme activity was expressed as nmol of phosphate (Pi) released per minute per milligram of protein.

Assay of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity

The phosphodiesterase activity was assessed using *p*-Nph-5'-TMP (an artificial substrate). For assay of E-NPP activity, the reaction medium containing 50 mM Tris-HCl buffer (pH 8.9) and 6.0 mM $MgCl_2$ was preincubated for 10 minutes at 37 °C with approximately 90 µg and 50 µg of soluble and microsomal protein per tube, respectively, in a final volume of 200 µL. The enzyme reaction was started by the

addition of *p*-Nph-5'-TMP to a final concentration of 0.5 mM. After 40 min of incubation time for soluble and 6 min for microsomal fraction, 200 μL 0.2 N NaOH was added to the medium to stop the reaction. Incubation time and protein concentration were chosen in order to ensure the linearity of the reaction. The amount of *p*-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8×10^{-3}/M/cm. Controls to correct for non-enzymatic substrate hydrolysis were performed by soluble or microsomal preparations after the reaction had been stopped with NaOH. All samples were performed in triplicate. Enzyme activity was generally expressed as nmol *p*-nitrophenol released per minute per milligram of protein.

Data Analysis

Results are expressed as means ± standard deviation (S.D.). The comparison among groups was made by one-way ANOVA, following by Duncan's post-hoc. Statistically significant differences between groups were considered for a $P \leq 0.05$.

Table 1. Caffeine Consumption in Chronic Treatment

Groups (14 days)	Fluid Consumption (mL/day/Kg)	Dose of Caffeine (mg/day/Kg)
Control	127.02 ± 24.7	0
0.3 mg/mL	130.98 ± 27.5	39.27 ± 8.2
1.0 mg/mL	121.86 ± 24.8	121.86 ± 24.3

Note: Values are expressed as mean ± SD.

Legend to Figures

Fig. 1 Effect of acute caffeine treatment (5, 15 and 45 mg/Kg, by gavage) on ATP, ADP, AMP and *p*-Nph-5'-TMP hydrolysis from cardiac solúvel (A) and microsomal fraction (B). Bars represent mean ± SD (n per group was 4–10 animals). In soluble fraction, control activities were 29.7 ± 2.9 for ATP, 8.5 ± 1.3 for ADP, and 7.6 ± 1.7 for AMP expressed as nmol Pi/ min/ mg of protein; for *p*-Nph-5'-TMP, control activity was 1.7 ± 0.1 expressed as nmol *p*-nitrophenol/ min/ mg of protein. In microsomal fraction, control activities were 1485.5 ± 373.2 for ATP; 243.2 ± 38.1 for ADP; 242.4 ± 28.5 for AMP expressed as nmol Pi/ min/ mg of protein; for *p*-Nph-5'-TMP, control activity was 79.1 ± 19.1 nmol *p*-nitrophenol/ min/ mg of protein. *Significantly different from the respective control group ($P < 0.05$).

Fig. 2 Effect of chronic caffeine treatment (0.3 or 1 g/L in the drinking water, during 14 days) on ATP, ADP, AMP and *p*-Nph-5'-TMP hydrolysis from cardiac solúvel (A) and microsomal fraction (B). Bars represent mean ± SD (n per group was 4–10 animals). In soluble fraction, control activities were 28.1 ± 3.8 for ATP; 7.1 ± 1.6 for ADP; 7.5 ± 1.2 for AMP expressed as nmol Pi/ min/ mg of protein; for *p*-Nph-5'-TMP, control activity was 1.4 ± 0.3 nmol *p*-nitrophenol/ min/ mg of protein. In microsomal fraction, control activities were 1711.2 ± 277.5 for ATP; 241.8 ± 34.5 for ADP; 226.1 ± 29.1 expressed as nmol Pi/ mim/ mg of protein; for *p*-Nph-5'-TMP, control activity was 59.5 ± 10.4 nmol *p*-nitrophenol/ min/ mg of protein. *Significantly different from the respective control group ($P < 0.05$).

Figure 1

A

B

Figure 2

A

B
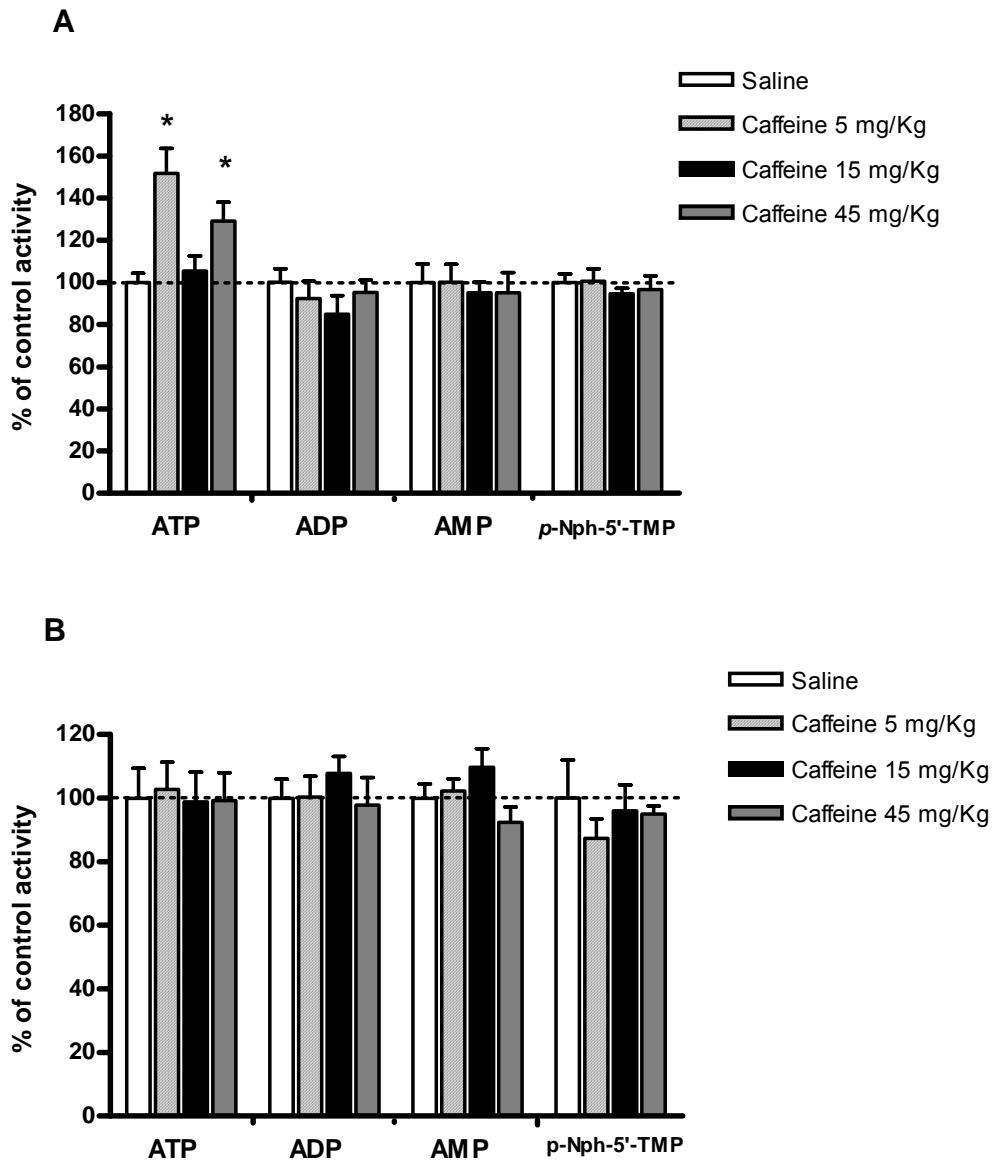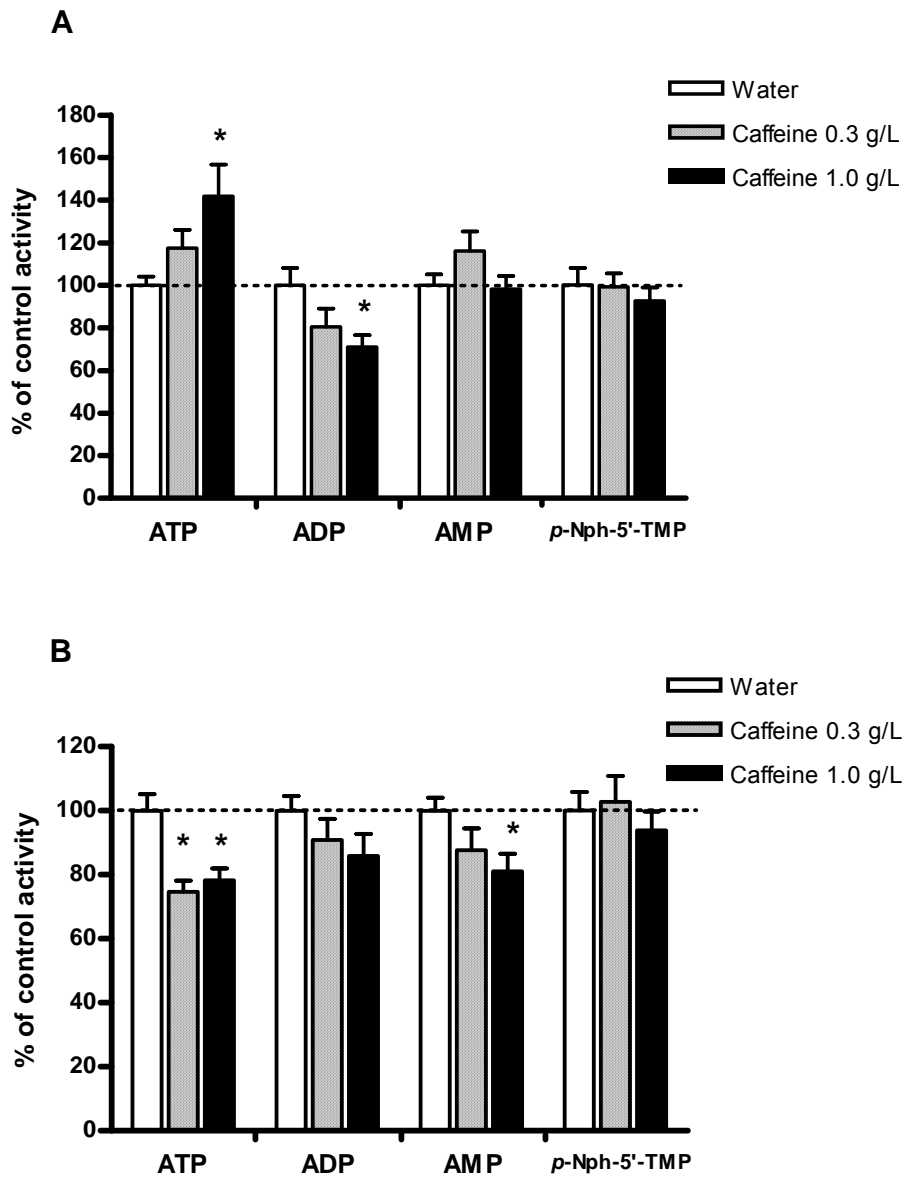
PARTE III

1. DISCUSSÃO

1.1 Propriedades da ecto-5'-nucleotidase em fração solúvel e microssomal de coração

A grande variedade de ações da adenosina enfatiza a importância do estudo das enzimas envolvidas na sua produção. No primeiro capítulo desta tese estão descritas as propriedades cinéticas e bioquímicas da atividade de hidrólise do substrato AMP, correspondendo à atividade da ecto-5'-nucleotidase. Além disso, através da análise de Western blot foi possível identificar a presença dessa enzima nas duas preparações de tecido cardíaco estudadas.

O estudo das propriedades bioquímicas da ecto-5'-nucleotidase em fração solúvel e microssomal cardíaca revelou características comuns previamente descritas para essas enzimas como dependência por cátions divalentes, enfatizada pela ausência de atividade na presença do quelante EDTA, e ativação por Mg^{2+} em concentrações na faixa de milimolar (Zimmermann, 1992; Bianchi & Spychala, 2003). O pH ótimo para a atividade dessas enzimas geralmente varia entre 6,0 e 8,0 (Zimmermann, 1992), contudo, os nossos resultados demonstraram uma melhor atividade em pH 9,5. Apesar de parecer uma faixa de pH extremamente alta para a atividade destas enzimas, um estudo anterior com 5'-nucleotidase solúvel purificada de coração de rato demonstrou que estas enzimas podem apresentar um comportamento complexo na presença de diferentes tampões em pHs acima de 7,5 (Naito & Lowenstein, 1981). Nesse estudo, os autores sugerem que alguns tampões, assim como a glicina também utilizada por nós, tem a capacidade de interagir com a enzima, possivelmente através da ligação de um íon metal, promovendo

uma alta atividade também em pH 9,5. Além disso, nesse estudo foi observado que essa atividade em tampão glicina com pH 9,5 poderia ser duplicada com a adição de Mg^{2+}, demonstrando que o cátion também é capaz de modificar o comportamento da enzima. Dessa maneira, os valores de pH ótimo observados em nossos resultados para ambas frações encontram suporte neste estudo prévio.

Para descartar que essa alta hidrólise de AMP em pH 9,5 pudesse estar acontecendo com a participação de uma fosfatase alcalina, utilizamos os inibidores clássicos dessa enzima, levamisole e tetramisole (Van Belle, 1972), os quais não apresentaram nenhum efeito sobre a hidrólise desse substrato no pH referido.

As propriedades cinéticas da ecto-5'-nucleotidase nas duas frações foram diferentes em alguns aspectos, a enzima da fração solúvel apresentou um K_M mais baixo do que a da fração microssomal, entretanto os valores nas duas preparações foram da ordem de micromolar. Em relação ao V_{max} observado, a fração microssomal apresentou um valor 21 vezes maior em comparação ao encontrado para a enzima solúvel. Essas diferentes características podem ser atribuídas ao tipo de ecto-5'-nucleotidase envolvida e à sua localização na célula (Zimmermann, 1992; Bianchi & Spychala, 2003).

Paralelamente às propriedades cinéticas e bioquímicas, a análise por Western blot identificou a ecto-5'-nucleotidase nas duas frações, porém, a quantificação dos níveis dessa proteína demonstrou uma quantidade muito maior na fração microssomal. Atualmente, sabe-se que existem pelo menos 7 tipos de 5'-nucleotidases e essa classificação está baseada principalmente na sua localização celular: uma forma ancorada à membrana plasmática (a ecto-

5'-nucleotidase), 5 formas citoplasmáticas e uma presente na matriz mitocondrial. O aparecimento da ecto-enzima na fração solúvel não nos parece surpreendente uma vez que enzimas de membrana podem sofrer clivagem, tornando-se solúveis no meio extracelular. Além disso, conforme descrito por Zimmermann em 1992, mais de 50% da enzima pode estar associada à membranas intracelulares e ser liberada durante o processo de homogeneização, formando uma proteína solúvel. Portanto, pode-se sugerir que a ecto-5'-nucleotidase identificada na fração solúvel possa ser originária de membranas intracelulares, inclusive do retículo sarcoplasmático que foi demonstrado no nosso estudo por apresentar essa forma da enzima. Em adição, não podemos excluir a presença de uma 5'-nucleotidase verdadeiramente solúvel na fração correspondente, uma vez que foram encontradas propriedades cinéticas muito diferentes nas duas frações.

1.2 Hidrólise de ATP e ADP no coração – Identificação das enzimas da família das E-NTPDases

A família da E-NTPDases tem se tornado objeto de intensiva pesquisa na última década, principalmente devido ao importante papel dessas enzimas na modulação da sinalização purinérgica. Membros dessa família têm sido identificados em condições fisiológicas e patológicas em muitas células e tecidos, incluindo o coração (Marcus *et al.*, 2005; Atkinson *et al.*, 2006; Robson *et al.*, 2006; Yegutkin, 2008). Além disso, o seu uso para propósitos terapêuticos tem sido sugerido (Marcus *et al.*, 2005; Atkinson *et al.*, 2006; Yegutkin, 2008). No segundo capítulo desta tese estão relatadas as propriedades cinéticas e bioquímicas da hidrólise de ATP e ADP na fração

solúvel e microssomal cardíaca de ratos, em condições onde a ATPase mitocondrial e a adenilato cinase foram inibidas. Os resultados apresentados suportam a nossa proposta de que esta atividade corresponde às enzimas da família das E-NTPDases.

A presença de atividade de E-NTPDases na hidrólise de ATP e ADP tanto na fração solúvel como na microssomal foi evidenciada pela ativação e dependência por cátions divalentes (Sarkis *et al.*, 1995; Zimmermann, 2001; Henz *et al.*, 2006; Robson *et al.*, 2006; Buffon *et al.*, 2007; Rücker *et al.*, 2008). O valor de pH ótimo para hidrólise de ATP e ADP em ambas frações variou entre 7,5 e 8,5, valores previamente descritos para as E-NTPDases (Sarkis *et al.*, 1995; Zimmermann, 2001; Henz *et al.*, 2006; Robson *et al.*, 2006; Buffon *et al.*, 2007; Rücker *et al.*, 2008).

A exclusão de algumas associações enzimáticas que pudessem mimetizar a atividade das E-NTPDases foi realizada usando diferentes inibidores conhecidos por atuar sobre algumas atividades enzimáticas. Assim, ouabaína, ortovanadato, lantânio e levamisole não afetaram a hidrólise de ATP ou ADP em ambas frações, descartando a participação de enzimas como Na$^+$/K$^+$-ATPase, fosfatases ácidas, fosfatases alcalinas e Ca^{2+}, Mg^{2+}-ATPase (Van Belle, 1972; LeBel *et al.*, 1980; Battastini *et al.*, 1991; Sorensen & Mahler, 1992; Cool and Blum, 1993). A contribuição da ATPase mitocondrial para a hidrólise do ATP foi verificada com o uso de oligomicina, a qual foi capaz de inibir a hidrólise deste substrato na fração solúvel e microssomal. É de conhecimento geral que o tecido cardíaco possui elevadas concentrações de ATPases mitocondriais (Sasaki *et al.*, 2001; Grover *et al.*, 2004), portanto, não foi surpresa encontrarmos uma alta inibição na hidrólise de ATP. Contudo, a

oligomicina também foi capaz de diminuir a hidrólise do ADP nas duas frações sugerindo uma possível combinação enzimática entre a ATPase mitocondrial e a adenilato cinase (enzima que converte ADP em ATP), o que poderia simular a atividade da E-NTPDase. Dois critérios foram utilizados para investigar a possibilidade de que o substrato ADP, antes de ser hidrolisado, estivesse sendo convertido à ATP pela adenilato cinase e, posteriormente, sendo hidrolisado pela ATPase mitocondrial: o primeiro critério foi a avaliação da hidrólise do ADP na ausência e na presença de um inibidor de adenilato cinase (NEM) (Russel *et al.*, 1974); o segundo critério consistiu de um protocolo previamente descrito para detectar qualquer formação de ATP quando a reação é iniciada com o substrato ADP (LeBel *et al.*, 1980; Sarkis *et al.*, 1986; Battastini *et al.*, 1991). Ambos procedimentos indicaram a presença da enzima adenilato cinase tanto na fração solúvel quanto na microssomal. Portanto, para excluir essa associação enzimática (ATPase mitocondrial e adenilato cinase) e assegurar que a hidrólise de ATP e ADP estivesse sendo realizada por uma E-NTPDase, os inibidores oligomicina e NEM foram sempre adicionados no meio de reação.

A azida de sódio, outro inibidor da ATPase mitocondrial na concentração de 0,1 mM (Plesner, 1995), reproduziu os resultados obtidos com a oligomicina na fração solúvel, causando a diminuição da hidrólise de ATP e ADP. Interessantemente, na fração microssomal essa mesma concentração de azida de sódio não foi capaz de alterar a hidrólise de ambos os substratos. Dados da literatura sugerem que essa diferença possa ser causada pelos mecanismos de ação diferenciados da azida de sódio e da oligomicina, o que promoveria distintos efeitos de acordo com a amostra utilizada (Borges *et al.*, 2004). Em

altas concentrações, a azida de sódio é capaz de inibir a atividade de E-NTPDases de várias fontes (Plesner, 1995; Knowles & Nagy, 1999; Fürstenau *et al.*, 2006; Henz *et al.*, 2006; Buffon *et al.*, 2007; Rücker *et al.*, 2008). Nossos resultados mostraram inibição das hidrólises de ATP e ADP na fração solúvel e microssomal, corroborando com a literatura prévia sobre essas enzimas. Em adição, os antagonistas de receptores P2, suramin e evans blue, também considerados inibidores de ectonucleotidases (Heine *et al.*, 1999; Fürstenau *et al.*, 2006), causaram inibição das hidrólises de ATP e ADP em ambas frações, indicando fortemente a participação de membros da família das E-NTPDases no processo de hidrólise dos nucleotídeos nas frações estudadas.

Embora ainda seja desconhecido o mecanismo pelo qual o cloreto de gadolínio atua como um potente inibidor de E-NTPDases solúveis (Escalada *et al.* 2004) e ligadas à membrana (Fürstenau *et al.*, 2006; Buffon *et al.*, 2007; Rücker *et al.*, 2008), nossos resultados demonstraram que o cloreto de gadolínio não foi capaz de afetar a hidrólise de ATP e ADP na fração solúvel, porém na fração microssomal a hidrólise de ambos substratos foi diminuída. Essas diferenças sugerem que o cloreto de gadolínio não é capaz de apresentar um padrão definido e, portanto, não parece ser um inibidor específico para a atividade das E-NTPDases.

Em relação às propriedades cinéticas, nossos resultados demonstraram que os valores de K_M calculados a partir do plote de Eadie-Hofstee utilizando ATP ou ADP como substratos foram na ordem de micromolar para ambas frações, o que está de acordo com a literatura sobre E-NTPDases. Em relação ao V_{max}, a razão de hidrólise de ATP/ADP foi cerca de 8 na fração solúvel e cerca de 12 na fração microssomal, sugerindo uma atividade preferencialmente

ATPásica em ambas frações. A presença de enzimas com atividade ATPásica no tecido cardíaco tem sido demonstrada por análises bioquímicas e de imunohistoquímica em corações de murinos, suínos e humanos (Kaczmarek *et al.*, 1996; Zinchuk *et al.*, 1999; Lemmens *et al.*, 2000; Sévigny *et al.*, 2002). Em adição, uma expressão significativa do mRNA da *Entpd*2 foi demonstrada em corações de murinos e humanos, sugerindo que esta enzima poderia ser uma candidata preferencial, responsável pela atividade encontrada (Chadwick & Frischauf, 1997, 1998; Barreto-Chaves *et al.*, 2006; Rücker *et al.*, 2008). Todavia, não se pode excluir a atividade de outros membros da família das E-NTPDases no tecido cardíaco. A NTPDase1 também tem sido demonstrada em corações humanos por análise de imunohistoquímica e Western blot (Kittel *et al.*, 2005). Além disso, a expressão do mRNA da *Entpd3* em cardiomiócitos de ratos sugere outra potencial enzima que poderia modular os níveis de nucleotídeos extracelulares no coração (Barreto-Chaves *et al.*, 2006). Recentemente, nosso grupo identificou através da técnica de RT-PCR a expressão do mRNA das *Entpd*1,2,3,5 e 6 em ventrículo esquerdo de coração de ratos (Rücker *et al.*, 2008). Com base nestas evidências, apesar dos nossos resultados demonstrarem uma atividade preferencialmente ATPásica, provavelmente atribuída a uma NTPDase2, outras enzimas da família das E-NTPDases podem estar participando da hidrólise de ATP e ADP na fração solúvel e microssomal cardíaca de ratos.

Na tentativa de identificar as específicas E-NTPDases presentes nas duas frações cardíacas, realizamos análises de Western blot. Os dados com amostras preparadas em condições não-redutoras indicaram a presença da NTPDase5 na fração solúvel, revelando uma única banda em torno de 75kDa

(Capítulo II - Figura 4). A presença da NTPDase5 na fração solúvel corrobora com a literatura que descreve que essa enzima, apesar de intracelular, pode ser secretada para o meio extracelular, tornando-se solúvel (Zimmermann, 2001). Em contrapartida, essa isoforma é descrita por ter uma preferência por substratos difosfatados e o perfil que descrevemos cineticamente caracteriza uma enzima com preferência para o nucleotídeo trifosfatado. A partir desses dados, duas hipóteses podem ser supostas: i) a enzima (no caso a NTPDase5) quando secretada, pode ter a sua conformação alterada, mudando o perfil de hidrólise dos diferentes nucleotídeos e; ii) a segunda hipótese é uma possível associação desta enzima com a atividade de outras E-NTPDases que não foram reconhecidas pelos anticorpos. Na fração microssomal, apenas a NTPDase2 foi detectada nas amostras preparadas em condições não-redutoras, revelando também uma única banda em torno de 75kDa (Capítulo II – Figura 4). A presença dessa isoforma na fração microssomal estaria de acordo com a razão de hidrólise de ATP/ADP observada nessa fração uma vez que a NTPDase2 é descrita por hidrolisar até 30 vezes mais ATP em relação ao ADP (Zimmermann, 2001). Por outro lado, se esse dado for confirmado será a primeira vez em que essa isoforma de membrana extracelular terá sido caracterizada e identificada em retículo sarcoplasmático.

Apesar de a literatura sugerir o uso desses anticorpos com amostras preparadas em condições não-redutoras, o comportamento frente às amostras preparadas em condições redutoras também foi verificado (Capítulo II – Figura 5). A análise por western blotting mostrou muitas ligações inespecíficas pelos anticorpos pois surgiam muitas bandas em ambas frações, tornando difícil a identificação das isoformas das E-NTPDases. É importante ressaltar que existe

uma grande dificuldade na obtenção de alguns anticorpos, pois existe uma reduzida disponibilidade comercial e os disponíveis muitas vezes possuem pouca especificidade, pois reconhecem apenas as regiões conservadas não sendo possível diferenciar os membros da família. Além disso, os anticorpos produzidos na sua grande maioria são policlonais, o que também dificulta na diferenciação das isoformas de E-NTPDases.

1.3 Análise cinética e bioquímica das E-NPPs em fração solúvel e microssomal

Em 2001, Vassort publicou uma revisão onde ele cita a falta de estudos sobre outras enzimas, além das ATPases, mas que sejam capazes de hidrolisar o ATP em tecido cardíaco, dentre elas as E-NPPs (Vassort, 2001). As E-NPPs influenciam muitos processos fisiológicos e alterações na sua atividade podem estar relacionadas com a fisiopatologia de várias doenças (Goding *et al.*, 2003). Alguns trabalhos mostram a presença destas enzimas em coração (Stefan *et al.*, 1999; Bollen *et al.*, 2000), no entanto, pouco se sabe sobre a sua importância com relação à hidrólise de nucleotídeos. Assim, no terceiro capítulo desta tese apresentamos investigações sobre as propriedades bioquímicas das E-NPPs em fração solúvel e microssomal de coração de ratos, utilizando o substrato artificial *p*-nitrofenil-5'-timidina-monofosfato, marcador para a atividade dessas enzimas.

A atividade enzimática encontrada em ambas frações nas condições avaliadas mostrou propriedades bioquímicas já descritas para E-NPPs, assim como pH alcalino e dependência por cátions divalentes (Kelly *et al.*, 1975; Sakura *et al.*, 1998; Hosoda *et al.*, 1999; Bollen *et al.*, 2000; Grobben *et al.*,

2000; Vollmayer *et al.*, 2003; Fürstenau *et al.*, 2006). As análises cinéticas também mostraram valores de K_M e V_{max} já relatados para as E-NPPs (Kelly *et al.*, 1975; Hosoda *et al.*, 1999; Vollmayer *et al.*, 2003).

A possível participação de outras enzimas na hidrólise do substrato artificial foi descartada pelo teste com alguns inibidores, além disso, a inibição observada por suramin sugere que, nas condições testadas, existe um predomínio de atividade NPP, uma vez que este composto é descrito como inibidor dessa atividade (Grobben *et al.*, 2000; Fürstenau *et al.*, 2006).

As E-NPPs1-3, sozinhas ou em combinação, tem se mostrado expressas em todos os tipos celulares em que se investigou a sua presença (Yano *et al.*, 1985; Jin-Hua *et al.*, 1997). No recente trabalho de Rücker e colaboradores (2007), a expressão do mRNA das NPP2 e NPP3 foi demonstrada em ventrículo esquerdo cardíaco de ratos através da técnica de RT-PCR. A presença de NPP1 já foi descrita em tecido de coração total (Stefan *et al.*, 1999; Goding *et al.*, 2003) e a ausência de expressão em ventrículo esquerdo sugere, provavelmente, a sua expressão em compartimentos específicos de acordo com suas funções fisiológicas.

A exata contribuição das E-NPPs em sistema cardíaco ainda permanece pouco ou nada estudada. A presença destas enzimas pode fazer parte da terminação da sinalização purinérgica e/ou geração de outras moléculas mensageiras como ADP, adenosina e pirofosfato (Stefan *et al.*, 2006). Uma vez que a presença de E-NTPDases também tenha sido demonstrada na fração solúvel e microssomal do coração, torna-se interessante entender o porquê de estas células estarem expressando tantas enzimas com funções semelhantes. A co-expressão de distintas ectonucleotidases com características semelhantes

em múltiplos sistemas tem sido sugerida por diversos trabalhos (Heine *et al.*, 1999; Kukulski & Komozynski, 2003; Oses *et al.*, 2004; Fürstenau *et al.*, 2006; Rücker *et al.*, 2007; Cognato *et al.*, 2008). Uma vez que essas enzimas apresentam diferentes propriedades cinéticas, elas podem atuar em condições fisiológicas distintas, além de poderem ser diferentemente reguladas. Dessa maneira, a possível co-localização de E-NTPDases e E-NPPs juntamente com ecto-5'-nucleotidases poderia constituir um complexo multi-enzimático importante para a modulação da sinalização purinérgica em diversos sistemas, incluindo as frações de coração estudadas.

1.4 Atividade das NTPDases, ecto-5'-nucleotidase e NPP em coração de ratos tratados com cafeína

Conforme observado no Anexo desta tese, os resultados obtidos com a administração aguda e crônica de cafeína demonstram alterações significativas e com diferentes perfis para cada substrato em cada fração cardíaca estudada.

Os estudos sobre as diferenças da exposição aguda e crônica aos ligantes de adenosina mostram que os efeitos da administração aguda de um ligante em particular podem ser opostos aos seus efeitos crônicos. Isso pode acontecer devido a diferenças entre os períodos de tratamento, as doses estudadas, as vias de administração, ao gênero e à idade (Jacobsen *et al.*, 1996; Fredholm *et al.*, 1999; Léon *et al.*, 2002). No nosso trabalho, tanto o tratamento agudo quanto o crônico foram administrados por via oral na tentativa de minimizar possíveis diferenças que possam ser relacionadas à via de administração. As doses de cafeína administradas, tanto no tratamento

agudo quanto no crônico, foram escolhidas de acordo com a literatura a respeito dos efeitos dessa molécula sobre o sistema cardiovascular.

Quando nós testamos os efeitos agudos da cafeína sobre a atividade das ectonucleotidases, observamos que as doses de 5 e 45 mg/Kg foram capazes de aumentar significativamente a hidrólise de ATP na fração solúvel (Anexo – Figura 1A). A hidrólise dos outros nucleotídeos não foi significativamente alterada na mesma fração. Do mesmo modo, nenhuma alteração significativa foi encontrada na hidrólise dos substratos testados na fração microssomal (Anexo – Figura 1B). Embora outros parâmetros precisem ser avaliados na tentativa de relacionar as atividades alteradas das ectonucleotidases com os efeitos da cafeína no coração, podemos sugerir que a administração aguda de cafeína pode desenvolver um papel modulatório na via das ectonucleotidases modulando, principalmente, a disponibilidade de ATP no meio extracelular. Uma vez que a hidrólise de ATP foi encontrada como sendo preferencial na atividade descrita para as E-NTPDases, a alteração somente na atividade desse substrato não parece surpreendente, e poderia sugerir uma modulação sensível da atividade dessa enzima. Considerando os efeitos da cafeína descritos sobre o coração (principalmente a arritmia), o aumento da hidrólise do nucleotídeo ATP na fração solúvel poderia sugerir um mecanismo compensatório para a diminuição da disponibilidade desse nucleotídeo extracelular, uma vez que se sabe da liberação dessas moléculas em processos como este e também da sua capacidade de induzir arritmias no músculo cardíaco.

Os resultados observados sobre o efeito do tratamento crônico com cafeína na hidrólise dos nucleotídeos da adenina em fração solúvel,

demonstram que houve alteração significativa da hidrólise de ATP e ADP somente na concentração de 1 g/L. Como pode ser observado na Figura 2A do Anexo, houve um aumento na hidrólise de ATP e uma diminuição na hidrólise de ADP. Esse efeito contraditório encontrado, pode ser resultado da ação de diferentes E-NTPDases. Embora nossos resultados preliminares sobre a identificação das isoformas dessas enzimas tenham indicado apenas a presença da NTPDase5 nessa fração (Capítulo II – Figura 4), mais estudos são necessários para enfatizar esses dados.

Os dados sobre os efeitos do tratamento crônico com cafeína na fração microssomal demonstram uma diminuição da hidrólise dos nucleotídeos ATP, ADP e AMP, porém com significância somente para as hidrólises de ATP e AMP (Anexo – Figura 2B). Esse resultado nos parece interessante e poderia ser relacionado com os efeitos da cafeína descritos no coração. Os nucleotídeos da adenina e a adenosina são capazes de afetar de maneira direta o fluxo de Ca^{2+} no retículo sarcoplasmático modulando o processo de excitação-contração do coração, sendo que o ATP parece causar a liberação desse cátion para o citoplasma ativando o processo, e a adenosina, apresenta um efeito contrário (Meissner, 1984; Meissner & Henderson, 1987; Duke & Steele, 1998). Além disso, algumas evidências sugerem que a saída anormal de Ca^{2+} por estes canais pode estar associada com certas patologias, incluindo arritmias e falência cardíaca (Györke et al., 2002; Hove-Madsen et al., 2006), a diminuição observada na hidrólise de ATP nos microssomas, poderia resultar em concentrações elevadas dessa molécula no micro-ambiente do retículo sarcoplasmático ativando esses processos. De maneira contrária, a diminuição

da hidrólise do AMP diminuiria a disponibilidade da adenosina nesse meio, acentuando a possibilidade do desenvolvimento dos processos relacionados.

Nossos resultados mostram que a cafeína administrada tanto de forma aguda, quanto crônica é capaz de alterar a hidrólise dos nucleotídeos da adenina nas preparações de tecido cardíaco estudadas. Todavia, mais dados são necessários para tentar relacionar os resultados encontrados e os conhecidos efeitos da cafeína no coração.

1.5 Ectonucleotidases em frações subcelulares – possível papel das ectonucleotidases solúveis e de retículo sarcoplasmático

A variedade de respostas, mediadas pelos nucleotídeos/nucleosídeo da adenina não depende somente do tipo de receptor ativado, mas também da célula com a qual esta molécula interagiu; além disso, deve-se levar em consideração a modulação da concentração extracelular destas purinas pela complexa cascata de ecto-enzimas e enzimas solúveis, que são capazes de hidrolisar completamente o ATP até adenosina (Zimmermann, 2001; Robson *et al.*, 2006). Não menos importante, pode-se ainda citar as nucleotidases intracelulares, que também podem participar do controle da concentração extracelular destas moléculas, uma vez que elas têm a capacidade de ser transportadas através da membrana plasmática.

No nosso estudo, as enzimas caracterizadas na fração solúvel podem ser provenientes do rompimento da membrana celular durante o processo de homogeneização do tecido cardíaco. Apesar disso, não podemos descartar a presença de verdadeiras nucleotidases solúveis na fração estudada. E-NTPDases solúveis têm sido detectadas em soro ou plasma de humanos e

outras espécies (Ireland & Mills, 1966; Coade & Pearson, 1989; Birk *et al.*, 2002; Yegutkin *et al.*, 2003); todavia, a origem dessas enzimas é pouco entendida e geralmente é atribuída à sua liberação após diferentes estímulos celulares (Todorov *et al.*, 1997; Yegutkin *et al.*, 2000; Sorensen *et al.*, 2003; Yegutkin *et al.*, 2006). Até momento, o papel fisiológico de E-NTPDases solúveis ainda não é claro, mas estudos recentes tem considerado a administração dessas enzimas para terapias antiplaquetárias, sob condições experimentais pró-trombóticas (Pinsky *et al.*, 2002; Robson *et al.*, 2005; Kohler *et al.*, 2007). Assim como em outros tecidos, E-NTPDases solúveis poderiam representar um importante sistema efetor auxiliar para a inativação local de níveis elevados agudos de nucleotídeos, especialmente nos sítios de injúria e inflamação no coração. Contudo, mais estudos são necessários para entender a importância dessas atividades.

As características das 5'-nucleotidases solúveis, derivadas de vários tecidos, tem sido demonstradas em um grande número de estudos, principalmente com a enzima purificada (Naito & Lowenstein, 1981; Truong *et al.*, 1988; Zimmermann, 1992; Bianchi & Spychala, 2003). A expressão dessas enzimas na forma solúvel no coração tem uma importante função fisiológica pela geração de adenosina durante o processo de isquemia e na regulação dos "pools" de nucleotídeos (Bianchi & Spychala, 2003).

A presença de atividade correspondendo a uma E-NTPDase, hidrolisando os nucleotídeos da adenina, tem sido descrita em microssomas de vários tecidos (Miura *et al.*, 1987; Valenzuela *et al.*, 1989; Alleva *et al.*, 2002). Essa atividade, juntamente com a da ecto-5'-nucleotidase também descrita em microssomas cardíacos nessa tese, pode apresentar outros importantes papéis

além daqueles bem estabelecidos. A fração microssomal é enriquecida por vesículas derivadas do retículo sarcoplasmático, a maior organela intracelular que seqüestra e libera Ca^{2+} do citoplasma, regulando o desenvolvimento de tensão e relaxamento no miocárdio (Meissner, 1984; Meissner & Henderson, 1987). Altas concentrações intracelulares de Ca^{2+} ativam um rápido bombeamento do íon para dentro do retículo por ação de uma Ca^{2+}-ATPase, por outro lado, a despolarização da membrana plasmática abre canais de Ca^{2+} do retículo, liberando Ca^{2+} para o citosol. Algumas evidências sugerem que a saída anormal de Ca^{2+} por estes canais pode estar associada com certas patologias, incluindo arritmias e falência cardíaca (Györke *et al.*, 2002; Hove-Madsen *et al.*, 2006). Estudos prévios têm demonstrado que os nucleotídeos da adenina, a adenosina e a cafeína são capazes de afetar diferentemente a liberação de Ca^{2+} do retículo sarcoplasmático tanto em músculo esquelético quanto em fibras cardíacas (Meissner, 1984; Smith *et al.*, 1986; Meissner & Henderson, 1987; Rousseau *et al.*, 1988; Duke & Steele, 1998; Butanda-Ochoa *et al.*, 2003; Hleihel *et al.*, 2006). A modulação destes efeitos pode ocorrer indiretamente através da sinalização via purinoceptores de membrana celular (Butanda-Ochoa *et al.*, 2003; Hove-Madsen *et al.*, 2006), todavia, ativação direta do canal de Ca^{2+} tem sido observada em estudos com canais isolados ou vesículas de retículo sarcoplasmático (microssomas) (Meissner, 1984; Meissner & Henderson, 1987). Evidência adicional da influência da sinalização purinérgica sobre a liberação de Ca^{2+} do retículo sarcoplasmático pode ser visualizado em um estudo recente, no qual a adenosina foi descrita por modular a liberação de Ca^{2+} através da ligação aos receptores A_{2A} presentes no retículo (Hleihel *et al.*, 2006). Sustentados pelos resultados discutidos

acima, nós somos capazes de sugerir que a atividade E-NTPDase e ecto-5'-nucleotidase encontradas na fração microssomal poderiam modular a liberação de Ca^{2+} do retículo sarcoplasmático, modulando, consequentemente, o processo de excitação-contração do coração.

Como descrito anteriormente, por serem capazes de hidrolisar os mesmos substratos que as E-NTPDases, porém com características cinéticas diferenciadas, as E-NPPS podem estar relacionadas a funções semelhantes mas em condições fisiológicas distintas.

De uma maneira geral, a presença de E-NTPDases, E-NPPs e ecto-5'-nucleotidase na fração solúvel pode contribuir para o melhor entendimento da modulação da sinalização purinérgica no coração. Em adição, a caracterização dessas enzimas nas vesículas derivadas de retículo sarcoplasmático pode adicionar novas informações sobre os seus papéis no meio intracelular.

Os diferentes efeitos observados da administração de cafeína sobre a atividade das nucleotidases identificadas em fração solúvel e microssomal de tecido cardíaco de ratos acrescentam evidências sobre os possíveis papéis dessas enzimas na modulação da sinalização purinérgica no coração.

2. CONCLUSÕES GERAIS

Nossos resultados sugerem que tanto a preparação solúvel quanto a microssomal cardíaca foram capazes de hidrolisar os nucleotídeos ATP, ADP e AMP com características bioquímicas e cinéticas que sugerem a atividade de enzimas da família das E-NTPDases (para a hidrólise de ATP e ADP) e ecto-5'-nucleotidase (para a hidrólise de AMP).

As análises preliminares de Western blot revelaram a presença da NTPDase5 na fração solúvel e da NTPDase2 na fração microssomal. Em adição, a enzima ecto-5'-nucleotidase foi identificada em ambas frações, mas com mais intensidade na fração microssomal. Uma atividade relacionada à E-NPP foi caracterizada nas duas preparações cardíacas estudadas.

A possível co-localização de E-NTPDases e E-NPPs juntamente com ecto-5'-nucleotidases poderia constituir um complexo multi-enzimático importante para a modulação da sinalização purinérgica em diversos sistemas, incluindo as frações de coração estudadas.

Foi demonstrado que a administração crônica e aguda de cafeína apresentou diferentes efeitos sobre a hidrólise de nucleotídeos da adenina na preparação solúvel e microssomal cardíacas. Esses resultados, ainda preliminares, necessitam ser melhor investigados na tentativa de relacionar essas alterações com os conhecidos efeitos da cafeína sobre o coração.

Finalmente, a partir de nossos resultados, consideramos que existe um complexo sistema de hidrólise de nucleotídeos que pode representar uma nova abordagem de sinalização purinérgica cardíaca.

3. PERSPECTIVAS

1) Verificar a possível presença dos subtipos de receptores P1 e P2 na fração microssomal cardíaca de ratos pelo método de Western blot;

2) Investigar o efeito direto (*in vitro*) da cafeína sobre as atividades das ectonucleotidases estudadas;

3) Analisar através de Western blot possíveis alterações no imunoconteúdo das ectonucleotidases estudadas frente aos tratamentos agudo e crônico com cafeína;

4) Verificar o perfil dos nucleotídeos da adenina e seus metabólitos, pelo método de HPLC, nas frações estudadas de ratos tratados com cafeína;

5) Investigar alguns parâmetros hemodinâmicos, através do método de coração isolado, em ratos tratados com cafeína;

6) Solubilizar, purificar, caracterizar e identificar a enzima de microssoma.

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