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ESTUDO DA EXPRESSÃO E CARACTERÍSTICAS BIOQUÍMICAS DAS ECTONUCLEOTIDASES EM CULTURA DE CÉLULAS ACINARES DE GLÂNDULAS SALIVARES

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À minha familia, por entender a minha ausência em muitos momentos, pela ajuda, pelo suporte, amor, carinho...

Resplandecente é a sabedoria, e sua beleza é inalterável :

os que a amam, descobrem-na facilmente.

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RESUMO

Os nucleotídeos da adenina são moléculas sinalizadoras no meio extracelular e estão envolvidas em importantes condições fisiológicas e patológicas. O papel do ATP como um mediador extracelular e neurotransmissor têm sido descrito em vários sistemas, inclusive na glândula salivar. Nas glândulas salivares, o ATP é liberado juntamente com acetilcolina e noradrenalina a partir do sistema de inervação da glândula. Esse ATP atua sobre os receptores $P2X_4$, $P2X_7$, $P2Y_1$ e $P2Y_2$, regulando importantes processos secretórios. Os eventos induzidos pelos nucleotídeos extracelulares são controlados pelas ectonucleotidases, que incluem membros da família das ecto-nucleosídeo trifosfato difosfoidrolases (E-NTPDases), ecto-nucleotídeo pirofosfatase/fosfodiesterases (E-NPPs) e ecto-5'-nucleotidase (ecto-5'N). Assim, no presente estudo nós investigamos a presença dessas ectonucleotidases nas células acinares da glândula submandibular. A presença de uma E-NTPDase e uma ecto-5'-nucleotidase foi identificada com as seguintes propriedades bioquímicas para a hidrólise de ATP, ADP e AMP: pH ótimo entre 6,5-8,0, requerimento de cátions divalentes (Ca^{2+} e Mg^{2+}) e valores de Km_{app} na faixa de micromolar. As células acinares das glândulas salivares também foram capazes de hidrolisar o p-Nph-5´-TMP, sugerindo a presença de uma E-NPP. A enzima descrita nesse estudo é dependente de cátions metálicos divalentes (Ca^{+2} e Mg^{+2}), sendo que a hidrólise do *p*-Nph-5´-TMP foi máxima na presença de níveis basais de cátions e em pH entre 8,5 e 9,0. Também se analisou o efeito de vários compostos sobre a hidrólise do ATP, ADP e *p*-Nph-5´-TMP e verificou-se que a

azida sódica, inibidor de várias NTPDases, inibiu a hidrólise do ATP e do ADP, e não teve efeito sobre o *p*-Nph-5´-TMP.A ouabaína e o NEM, inibidores clássicos de ATPases, não afetaram significativamente a hidrólise de ATP, ADP e *p*-Nph-5´-TMP, já o AMP, inibiu a hidrólise do *p*-Nph-5´-TMP. Esses resultados sugerem a co-existência de diferentes ectonucleotidases nas células acinares, que estão relacionadas com a hidrólise dos nucleotídeos e que podem atuar em diferentes situações. Também avaliamos o efeito de fármacos antidepressivos na atividade e expressão das enzimas envolvidas na hidrólise dos nucleotídeos. Em relação à atividade enzimática, observamos que a fluoxetina induziu um aumento na hidrólise de ATP e ADP e o moclobemide e a imipramina induziram um aumento significativo na hidrólise de todos os nucleotídeos (ATP, ADP e AMP). A expressão relativa da NTPDase1-3 aumentou e da ecto-5´-nucleotidase diminuiu com a imipramina. A fluoxetina aumentou a expressão relativa da NTPDase3 e ecto-5´-nucleotidase e inibiu a expressão relativa da NTPDase2. O moclobemide aumentou a expressão relativa de todas as enzimas. Em relação à atividade enzimática da NPP, os tratamentos com imipramina, fluoxetina e moclobemide levaram a um aumento significativo dessa atividade, enquanto as expressões relativas das NPP-1, NPP-2 e NPP-3 não apresentaram alteração. A presença de ectonucleotidases modulando os níveis dos nucleotídeos nas células acinares é importante tendo em vista o papel do ATP nos mecanismos de secreção salivar.

ABSTRACT

Adenine nucleotides are extracellular signaling molecules and are involved in important physiological and pathological conditions. The role of adenosine triphosphate (ATP) as an extracellular mediator and neurotransmitter has been described in various systems, including salivary glands. In salivary glands, extracellular ATP is co-released with acetylcholine and noradrenaline from glands nerve system. ATP acts on $P2X_4$, $P2X_7$, $P2Y_1$ e $P2Y_2$ receptors and may regulate important secretory processes in salivary gland cells. The events induced by extracellular nucleotides are controlled by ectonucleotidases, that include family members of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase), ecto-pyrophosphatases/phosphodiesterases (E-NPP) and ecto-5'-nucleotidase. The presence of an E-NTPDase and ecto-5'-nucleotidase was identified with the following biochemical characteristics for ATP, ADP, and AMP hydrolysis: optimum pH between 6.5-8.0, dependence of divalent cations (Ca^{2+} e Mg^{2+}) and Km_{app} values in micromolar range. Acinar cells from salivary glands were capable to hydrolyze *p*-Nph-5´-TMP, suggesting the presence of an E-NPP. The enzyme described in this study is divalent cations-dependent (Ca^{+2} e Mg^{+2}), and the *p*-Nph-5´-TMP hydrolysis was maximal with basal levels of cations in pH between 8,5 and 9,0. The effect of different compounds in ATP, ADP e *p*-Nph-5´-TMP hydrolysis was evaluated and sodium azide, an inhibitor of several NTPDases, inhibited ATP and ADP hydrolysis, but have no effect in *p*-Nph-5´-TMP hydrolysis. Ouabain, NEM, classical ATPases inhibitors did not affect ATP, ADP e *p*-Nph-5´-TMP

hydrolysis; however, AMP inhibited the *p*-Nph-5´-TMP hydrolysis. These results suggest the co-existence of different ectonucleotidases in acinar cells that are related with nucleotide hydrolysis and can act under different situations. The effect of antidepressants in activity and expression of enzymes involved in nucleotide hydrolysis was also evaluated. In relation to enzymatic activity, fluoxetine induced an increase only in ATP and ADP hydrolysis, whereas moclobemide and imipramine induced a significant increase in hydrolysis of all nucleotides (ATP, ADP and AMP). The relative expression of NTPDase1-3 was enhanced and for ecto-5´-nucleotidase was diminished with imipramine. Fluoxetine enhanced relative expression of NTPDase3 and ecto-5´-nucleotidase and inhibited the relative expression of NTPDase2. Moclobemide enhanced the relative expression of all enzymes. In relation to E-NPPs activities, imipramine, fluoxetine e moclobemide treatmens lead to a significant increase in this activity whereas the relative expression of NPP-1, NPP-2, and NPP-3 did not show modifications. The presence of ectonucleotidases modulating the levels of nucleotides in acinar cells is important considering the ATP role in the mechanisms of salivary secretion.

APRESENTAÇÃO

Os resultados que fazem parte desta Tese estão apresentados sob a forma de artigos publicados e artigos submetidos à publicação, os quais encontram-se organizados em capítulos, no item 3 – ARTIGOS CIENTÍFICOS. Material e Métodos, Resultados, Discussão dos Resultados, Figuras, Tabelas e Referências Bibliográficas, encontram-se nos próprios artigos e representam a íntegra deste estudo.

O item 4 - DISCUSSÃO, encontrado no final desta Tese, apresenta interpretações e comentários gerais sobre todos os artigos científicos e manuscritos aqui representados.

No item 5, são apresentadas as conclusões referentes a este trabalho de pesquisa, organizadas de acordo com a ordem de citação dos artigos científicos.

O item 7 - REFERÊNCIAS BIBLIOGRÁFICAS refere-se somente às citações que aparecem na introdução e discussão desta Tese.

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ACR – regiões conservadas da apirase

ADP - adenosina 5'-difosfato

AMP - adenosina 5'-monofosfato

ATP - adenosina 5'-trifosfato

CD39 - antígeno de ativação celular linfóide/NTPDase 1

CD73 - ecto-5'-nucleotidase

D - ducto

Ecto-ATPDase - ecto-ATP difosfoidrolase

E-NPP family - familia ectonucleotídeo pirofosfatase/fosfodiesterase

E-NTPDase family – familia ectonucleosídeo trifosfato difosfoidrolase

GPI - glicosil-fosfatidilinositol

GMP - guanosina 5'- monofosfato

IMAOs - inibidores da monoamino-oxidase

MA - ácinos mucosos

NTPDase - nucleosídeo trifosfato difosfoidrolase

Pi- fosfato inorgânico

P2X - receptor purinérgico ionotrópico

P2Y - receptor purinérgico metabotrópico

p-Nph-5'-TMP - p-nitrofenil-5'-timidina-monofosfato

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

AS - ácinos serosos

SD - ducto estriado

SNC – sistema nervoso central

SSRIs - inibidores seletivos da recaptação de serotonina

TCAs - antidepressivos tricíclicos

UDP - uracil 5’-difosfato

UTP- uracil 5’-trifosfato

UMP - uracil 5’-monofosfato

5’NT – ecto- 5’- nucleotidase

1.INTRODUÇÃO

1.1.SALIVA

A saliva é um fluido complexo produzido pelas glândulas salivares. Embora conhecido como saliva, o termo mais apropriado seria fluido bucal, pois a saliva também é constituída pelo fluido crevicular que contém leucócitos e por células epiteliais descamadas. Mais de 99% da saliva é constituída por água e o total produzido em um dia varia de 600 a 1200 mililitros. A saliva encontrada na boca é do tipo mista, e sua constituição final também é influenciada pela microbiota e pelos alimentos. A saliva tem duas funções básicas: digestiva e protetora. A função protetora é expressa de várias maneiras. Como lubrificante, através de seu conteúdo glicoprotéico, forma uma barreira na mucosa protegendo-a de estímulos nocivos, pequenos traumas e toxinas bacterianas. Proporciona ações mecânicas de limpeza, removendo bactérias não aderentes e restos acelulares da cavidade bucal. Possui capacidade tampão, que atua impedindo que bactérias potencialmente patogênicas colonizem a cavidade bucal, e também neutraliza ácidos produzidos por bactérias a partir de açúcares da dieta. Esse tamponamento ocorre principalmente através dos íons bicarbonato e fosfato. Além disso, a saliva desempenha papel na gustação e tem grande influência ecológica sobre os microorganismos que tentam colonizar os tecidos bucais (Suddick e Dowd, 1984; Klein,1994; Tenovuo e Lagerlöf, 1995).

1.2. XEROSTOMIA

A saliva produzida pelas glândulas salivares pode ser modificada por influência de fatores intrínsecos fisiológicos, como regulação neurológica, emocional e hormonal, e podem influir também fatores extrínsecos como medicamentos e radiações ionizantes (Boraks, 1996; Scully, 2003). O fluxo é o parâmetro clínico mais importante da saliva, afetando a suscetibilidade a lesões de cárie (Tenovuo e Lagerlöf, 1995). A hipossalivação pode provocar xerostomia. A xerostomia é uma situação clínica subjetiva onde há menos saliva do que o normal. A boca seca pode ter várias causas, como, cigarro, álcool, bebidas com cafeína e medicamentos. A xerostomia pode ser induzida por vários tipos de fármacos, tais como os antidepressivos, antipsicóticos, anti-histamínicos, anti-hipertensivos e sedativos (Streckfus, 1995; Scully, 2003). Diversos tipos de receptores estão presentes na superfície das células da glândula salivar. Esses receptores medeiam ações exercidas por vários neurotransmissores que podem ser alteradas pela utilização de medicamentos como antidepressivos. Existem diferentes tipos de antidepressivos, como os IMAOs (inibidores da monoamino-oxidase), os TCAs (antidepressivos tricíclicos), e os SSRIs (inibidores seletivos da recaptação de serotonina). Estes fármacos produzem efeitos diversos sobre o fluxo salivar (Rafaelsen *et al.*, 1981; Hunter e Wilson, 1995; Mavissakalian, Perel e Guo, 2002). Entretanto, os mecanismos envolvidos na diminuição do fluxo salivar ainda foram pouco elucidados.

1.3. GLÂNDULAS SALIVARES

As glândulas salivares são glândulas exócrinas, ou seja, possuem um sistema de ductos que transporta e modifica a secreção primária. As glândulas salivares são divididas em dois grupos: as glândulas salivares maiores e as glândulas salivares menores. Estas, em grande número, encontram-se distribuídas na cavidade bucal e produzem cerca de 10% do volume total de saliva. Possuem uma secreção do tipo mucoso/misto e são denominadas de acordo com a sua localização em: labial, palatina, bucal, lingual, glossopalatina e glândulas sublinguais menores (Boraks, 1996; Tenovuo e Lagerlöf, 1995).

No homem, há três pares de glândulas salivares maiores, a parótida, a submandibular e a sublingual, que estão localizadas fora da cavidade bucal, e que possuem um extenso sistema de ductos que modificam e descarregam as suas secreções. As glândulas também podem ser classificadas de acordo com o tipo de secreção. A glândula parótida é tipicamente serosa (produz uma secreção aquosa e rica em enzimas). As glândulas submandibular e sublingual são mistas, sendo que a submandibular é principalmente serosa enquanto a sublingual é predominantemente mucosa (Boraks, 1996; Tenovuo e Lagerlöf, 1995).

1.4. ESTRUTURA DAS GLÂNDULAS SALIVARES

1.4.1. Porção Secretora Ou Ácino

O ácino é uma esfera ou um túbulo composto por uma única camada de células epiteliais que se assentam numa membrana basal e cercam a luz do ácino. O ácino é a porção mais distal do parênquima da glândula salivar em relação à cavidade bucal e é denominado unidade secretora terminal. Podem ser encontrados três tipos de células na unidade secretora terminal: células serosas, células mucosas e células mioepiteliais (Figura 1). O número e a distribuição de cada tipo de célula variam de glândula para glândula e de porção terminal para porção terminal (Dale, 2001). As células acinares normalmente têm forma piramidal, possuem um pólo basal que está voltado para o parênquima e um pólo apical voltado para a luz do ácino, onde são liberados os produtos secretórios. Ao redor dos ácinos, temos as células mioepiteliais (Wilborn e Shackleford, 1984; Klein,1994).

1.4.2. Células Mioepiteliais

As células mioepiteliais são encontradas junto às estruturas secretoras terminais (ácinos) e também junto aos ductos. São elementos com capacidade de contração com longos processos digitiformes. Pequenas terminações nervosas (junções neuroglandulares) podem ser observadas em íntima proximidade às células mioepiteliais. Essas pequenas terminações possuem vesículas sinápticas e pequenas mitocôndrias, com função desconhecida. (Suddick e Dowd, 1984; Klein, 1994).

1.4.3. Sistema de Ductos

Compreende uma rede bastante variada caracterizada por elementos progressivamente menores, constituído por três classes de ductos: os intercalares, os estriados e os excretores. Esse sistema participa ativamente na produção e modulação da saliva (Cook *et al.*, 1994; Cook *et al.*, 1998).

1.4.4.Ductos Intercalares

O ducto intercalar liga-se diretamente ao ácino. Esse ducto é ramificado e tem o menor diâmetro do sistema ductal. Suas paredes são revestidas por células cúbicas baixas e sua atividade funcional ainda não é bem conhecida. (Cook *et al.*, 1994; Cook *et al.*, 1998).

1.4.5. Ductos Estriados

Os ductos intercalares desembocam abruptamente nos estriados. São revestidos por células colunares e possuem uma estriação evidente na porção basal a qual lhes dá o nome. Desempenham papel na regulação da concentração eletrolítica da saliva. Nos ductos estriados, o sódio é reabsorvido e o potássio é transportado no sentido inverso, tornando a saliva hipotônica, com baixas concentrações de sódio e cloreto. Os íons bicarbonato também podem ser secretados ativamente (Cook *et al.*, 1994; Cook *et al.*, 1998).

1.4.6.Ductos Excretores Terminais

Após passar pelos ductos estriados, o fluido salivar é secretado para a cavidade bucal através dos ductos excretores terminais. Próximo ao ducto estriado as células epiteliais são simples e cúbicas, e à medida que se aproximam da cavidade bucal as células epiteliais se transformam em estratificadas cubóides ou colunares, unindo-se com o epitélio da boca junto ao orifício do ducto. O ducto excretor modifica a saliva final alterando a concentração de eletrólitos e acrescentando um componente mucoso (Cook *et al.*, 1994; Cook *et al.*, 1998).

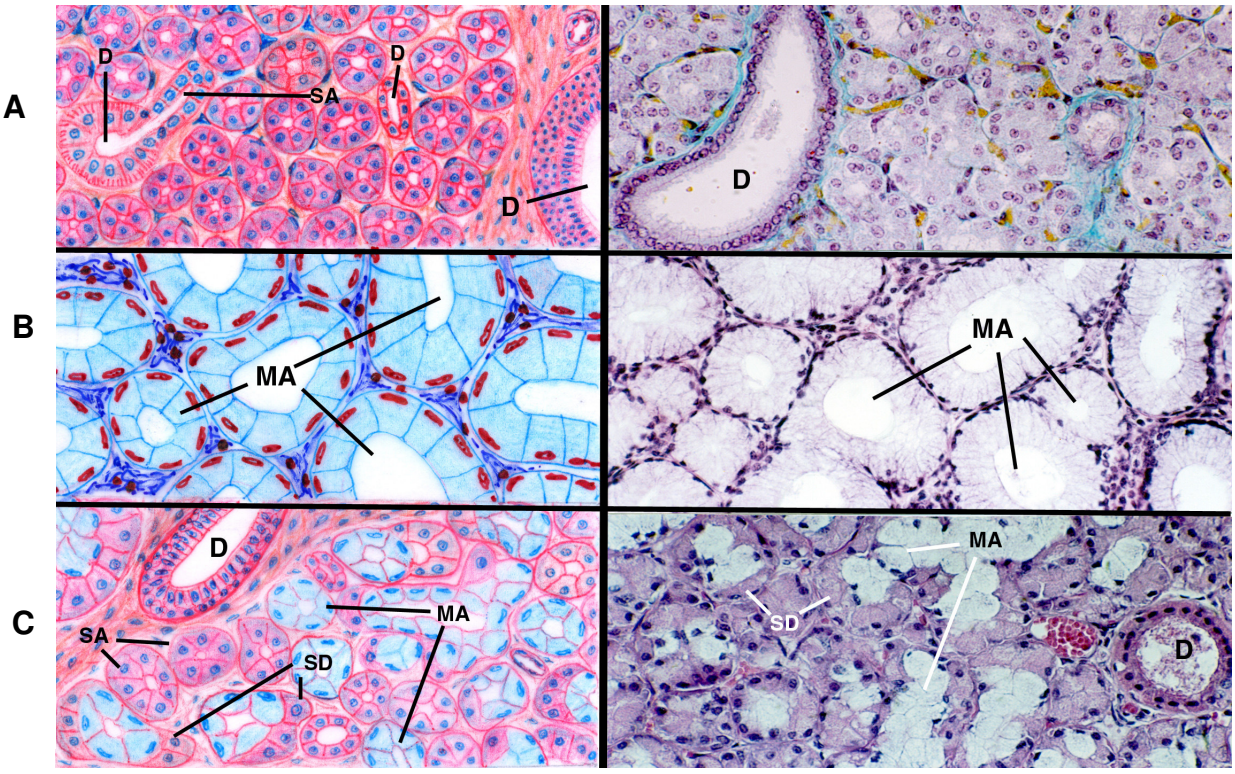

Figura 1: Essa imagem composta mostra os principais arranjos encontrados nas glândulas salivares da maioria dos animais. As imagens da esquerda são desenhos e as da direita são microfotografias de espécies reais, mostrando os diferentes arranjos de células encontrados nas glândulas. Em A: glândula serosa; B: glândula mucosa; C: glândula mista (serosa e mucosa); tipos de células: SA (ácinos serosos); MA (ácinos mucosos); SD (ducto estriado); D (ducto). education.vetmed.vt.edu/Curriculum/VM8054/Lab...

1.4.7. Suprimento Nervoso

As glândulas recebem nervos motores pós-ganglionares simpáticos e parassimpáticos. Os nervos penetram na glândula acompanhando os vasos sanguíneos e se dividem em ramos progressivamente menores até formarem

um plexo terminal junto ao parênquima. Nos plexos, os nervos consistem de axônios amielínicos envoltos pelo citoplasma da célula de Schwann e são distribuídos aos músculos das arteríolas, aos ácinos, às células mioepiteliais, aos ductos intercalares e estriados. Duas situações podem ocorrer: na primeira descrita como epilemal, os axônios permanecem no tecido conjuntivo e são separados das células secretoras pela membrana basal. Nos locais em que os axônios se aproximam das células secretoras, eles perdem sua cobertura da célula de Schwann. Neste caso, o local neuroefetor seriam as varicosidades do axônio que contém pequenas vesículas. As vesículas contêm os neurotransmissores, como noradrenalina e acetilcolina. Acredita-se que os neurotransmissores sejam liberados difundindo-se por 100 a 200 nm através da membrana basal antes de estimular a célula secretora. No segundo tipo, hipolemal, os axônios penetram na membrana basal. Nesse processo, perdem sua cobertura de células de Schwann e penetram por entre as células secretoras, ficando separados delas por um espaço de 10 a 20 nm. Pequenos gotejamentos ocorrem ao longo desses axônios amielínicos nos locais onde pequenas vesículas e muitas mitocôndrias se acumulam; considera-se que estes sejam os locais de liberação dos neurotransmissores. Com esse tipo de inervação um neurônio pode estimular rapidamente várias células secretoras, ou várias vezes as pequenas células (Dale, 2001).

1.4.8. Regulação da Secreção

A secreção salivar é regulada através de várias substâncias. Neurotransmissores como acetilcolina, norepinefrina, neuropeptídeos como a substância P ou peptídeo intestinal vasoativo estimulam receptores de membrana específicos acoplados a proteínas, que atuam sobre enzimas como a fosfolipase C e adenilato ciclase. Foi descrito que o ATP extracelular poderia aumentar o fluxo de ^{86}Rb e a secreção de amilase nos ácinos da parótida de ratos (Gallacher, 1982). Os receptores do ATP envolvidos ainda não foram identificados.

1.5. SISTEMA PURINÉRGICO

1.5.1 Nucleotídeos

Os nucleotídeos extracelulares purínicos (ATP, ADP e o nucleosídeo adenosina) e pirimidínicos (UTP e UDP) são moléculas sinalizadoras importantes, as quais induzem diversos efeitos biológicos via receptores celulares, denominados purinoreceptores. Os efeitos promovidos por essas moléculas incluem contração do músculo liso, neurotransmissão no sistema nervoso central (SNC) e periférico, secreção exócrina e endócrina, resposta imune, inflamação, agregação plaquetária, dor, modulação da função cardíaca, proliferação, diferenciação, apoptose, entre outras (Ralevic e Burnstock, 1998; Burnstock, 2006).

1.5.2. ATP

O papel do nucleotídeo ATP como fonte de energia química para várias funções celulares é bem conhecido (Vassort, 2001). Evidências de que o ATP poderia atuar como um neurotransmissor surgiram a partir de um trabalho que demonstrou a liberação de ATP em nervos sensoriais (Holton e Holton, 1954). Posteriormente, Burnstock (1972) demonstrou que existe uma transmissão purinérgica em sistema nervoso autônomo, além da transmissão colinérgica e noradrenérgica, onde o ATP atua como o principal neurotransmissor.

O ATP é liberado de fontes pré-sinápticas para o meio extracelular juntamente com outros neurotransmissores, como a acetilcolina (Vizi *et al*., 1997), a noradrenalina (Kennedy, 1996), a serotonina (Potter e White, 1980) e o glutamato (Inoue, 1998). A liberação de ATP ocorre através de exocitose em um processso dependente de cálcio (Phillis e Wu, 1981). Após ser liberado, o ATP pode interagir com receptores presentes na membrana (Burnstock e Willians, 2000), ser degradado por enzimas como as ectonucleotidases ou ser utilizado como substrato por ecto-kinases (Vizi e Sperlagh, 1999).

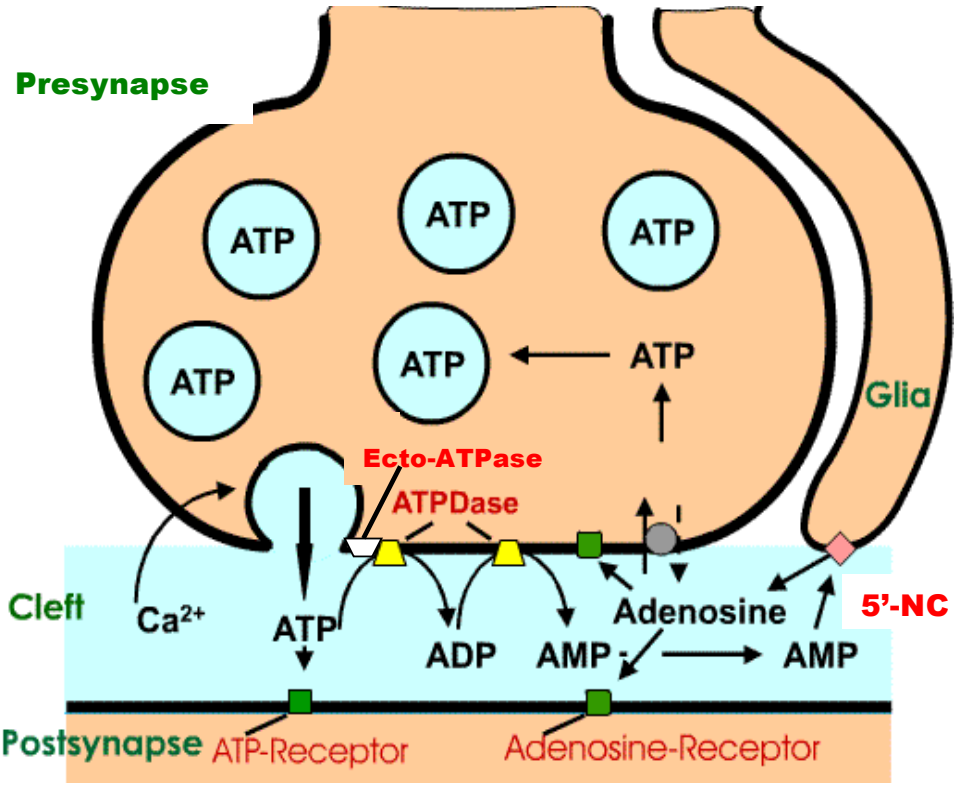

Figura 2. Liberação e degradação dos nucleotídeos da adenina no espaço extracelular durante o processo de neurotransmissão. (Adaptado de www.biozentrum.uni-frankfurt.de/prof/zimmermann).

1.5.3. Receptores Purinérgicos

Os efeitos dos nucleotídeos extracelulares são mediados por receptores presentes na superfície das membranas celulares. Esses receptores foram identificados como P1 e P2. Os receptores P1 reconhecem preferencialmente a adenosina, são acoplados a proteína G e subdivididos em quatro subtipos: A_1, A_{2A}, A_{2B} e A_3 (Fredholm *et al.*, 1997). 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 e Burnstock, 1998; Czajkowski e Baranska, 2002). Adicionalmente, os receptores

A_{2B} também podem ativar a fosfolipase C através da interação com a proteína G_q (Fredholm *et al.*, 2001).

Os receptores P2 são divididos em duas familias: P2X e P2Y. Os receptores P2 estão envolvidos na modulação da maioria dos eventos induzidos pelo ATP, incluindo os efeitos sobre a transmissão sináptica (Abrachio e Burnstock, 1998; Burnstock & Knight, 2004). Os receptores ionotrópicos P_2X, são ligados a canais iônicos ativados pelo ATP e são divididos em sete subtipos $P2X_{1-7}$ (Ralevic e Burnstock, 1998).

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 e Burnstock, 1998; Communi *et al.*, 2001; von Kugelgen e Wetter, 2000).

O ATP induz várias respostas através da estimulação dos receptores P2, em vários sistemas como SNC (Inoue *et al.*, 1998), e sistema vascular (Ralevic e Burnstock, 2003).

Na glândula salivar, tem sido descrito que o ATP é capaz de mobilizar estoques intracelulares de cálcio em células acinares da parótida de ratos, sugerindo que receptores P2Y estão envolvidos (Soltoff *et al.*, 1990, 1992). Posteriormente, foi observado que o principal efeito do ATP extracelular foi ativar receptores P2X e aumentar a captação de cálcio através de um canal de cátion não especifico (Soltoff *et al.*, 1990, 1992). Os receptores $P2X_4$ e $P2X_7$ foram identificados nos ácinos da glândula submandibular de ratos (Yu e Turner,1991; Gibb *et al.*, 1994; Buell *et al.*, 1996). O UTP aumentou o cálcio nas células

ductais da glândula submandibular. Essa resposta foi secundária a ativação da fosfolipase C e envolve o receptor $P2Y_2$ (Yu e Turner, 1991).O ATP parece ser um importante regulador da secreção salivar. O ADP tem mostrado ser menos potente do que o ATP, ao passo que o AMP é menos efetivo e a adenosina parece não ter efeito (Gallacher, 1982).

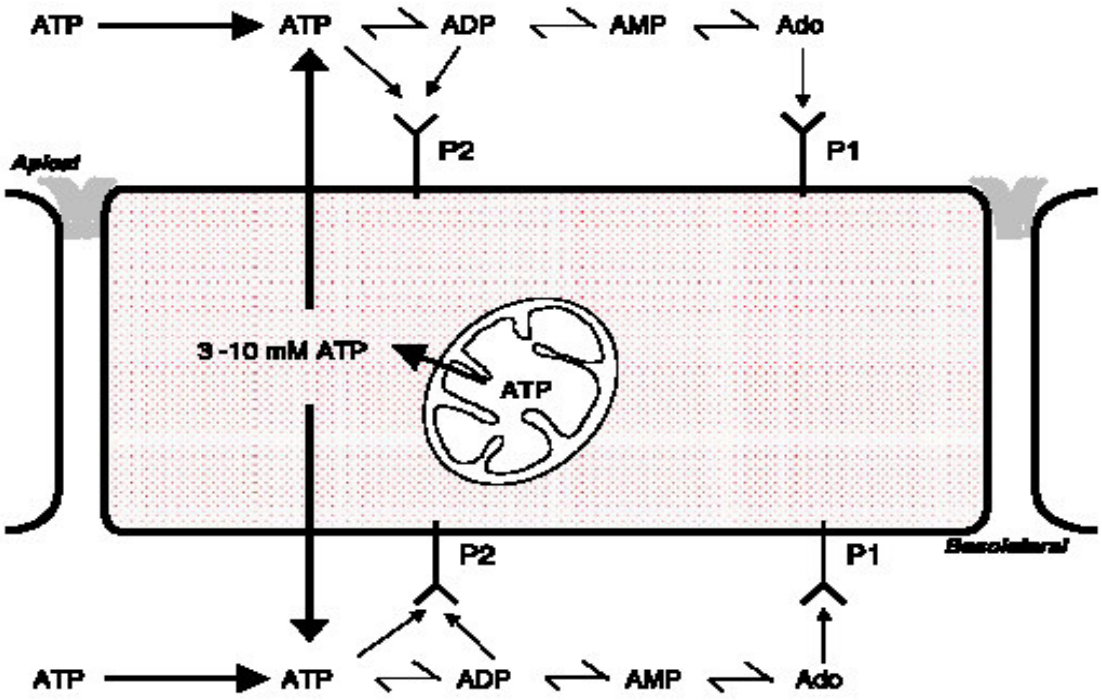

Figura 3. Princípios da sinalização extracelular do ATP em um modelo de célula epitelial nos espaços basolateral e apical. ATP gerado pela mitocôndria cria um enorme gradiente para o efluxo de ATP, secreção ou liberação. Somente 1% ou menos do pool de ATP citosólico é requerido para ativar os receptores P2 e estimular a transdução de sinal na mesma célula ou em células vizinhas. (Schwiebert and Zsembery, 2003).

1.5.4. Ectonucleotidases

Após a liberação no meio extracelular, os nucleotídeos da adenina exercem seus efeitos através da interação com receptores específicos localizados na membrana celular e posteriormente são metabolizados através da ação de ecto-enzimas. As ectonucleotidases constituem um eficiente mecanismo de controle dos níveis de nucleotídeos e nucleosídeos no espaço extracelular (Zimmermann, 1996; Zimmermann, 2001). Várias famílias de ectonucleotidases podem degradar os nucleotídeos extracelulares, dentre as quais podemos citar os membros da família das E-NTPDases (Ecto-nucleosídeo trifosfato difosfoidrolases), E-NPPs (Ecto-nucleotídeo pirofosfatase/ fosfodiesterases, PDEase, EC 3.1.4.1) e as fosfatases alcalinas, para a hidrólise de nucleotídeos di- e trifosfatados. Os nucleotídeos monofosfatados podem ser hidrolisados pela ecto-5'-nucleotidase e pelas fosfatases alcalinas (Zimmermann, 2001).

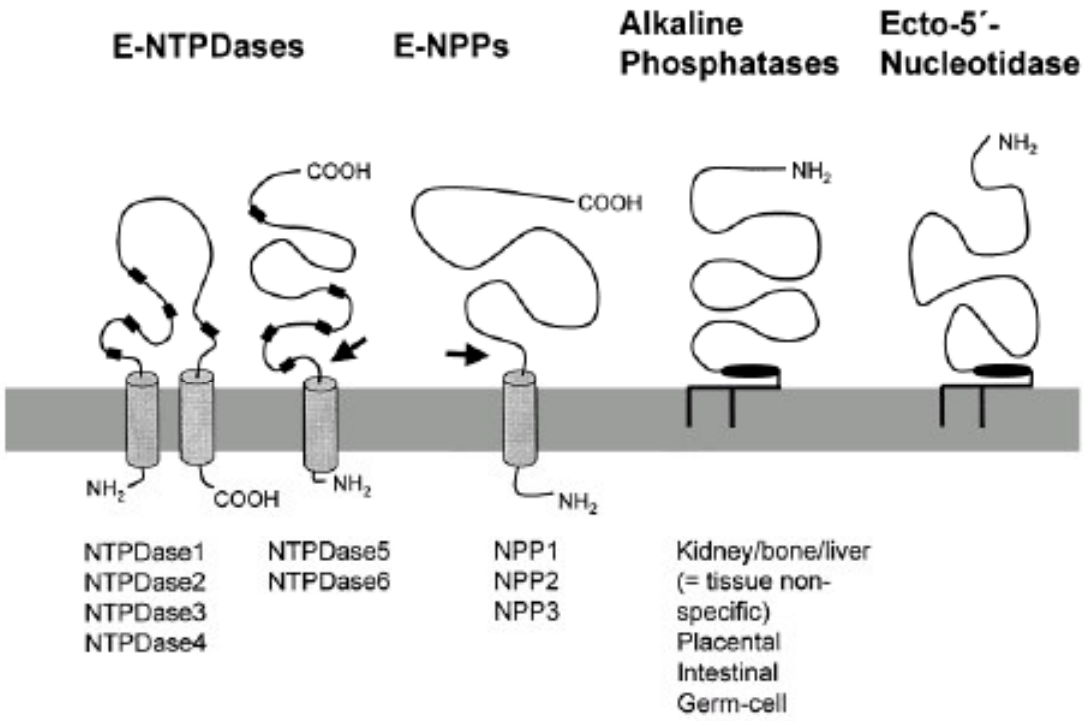

Figura 4. Topografia de membrana sugerida para os membros da família das ectonucleotidases (Zimmermann, 2001).

1.5.4.1. Ecto-nucleosídeo trifofato difosfoidrolases (E-NTPDases)

Os membros da família E-NTPDase previamente eram classificados como E-ATPases . Essa denominação foi adotada com o objetivo de prover uma ordem sistemática que indique a ordem de clonagem e a caracterização funcional das diferentes nucleotidases. As E-NTPDases constituem uma classe de ecto-enzimas ancoradas à membrana plasmática via domínios hidrofóbicos, com o sítio ativo voltado para o meio extracelular. Essas enzimas são caracterizadas pela sua capacidade em hidrolisar nucleotídeos tri- e difosfatados, dependência de cátions divalentes para exercer atividade catalítica,

insensibilidade a inibidores clássicos de P-, F- e V-ATPases e presença de cinco regiões altamente conservadas, denominadas de regiões conservadas da apirase (ACR) (Plesner, 1995). Em mamíferos, 8 membros dessa familia (nomeados NTPDase1-8) já foram clonados e caracterizados, apresentando diversidade de preferência por substrato e distribuição tecidual (Zimmermann, 2001; Bigonesse *et al.*, 2004).

As NTPDases de 1 a 3 compartilham semelhanças estruturais como o ancoramento na membrana através dos domínios NH_2 e COOH terminais, e um extensa porção extracelular onde estão as ACRs, diferindo em relação à razão de hidrólise dos nucleotídeos.

A NTPDase 1 (CD 39) hidrolisa ATP e ADP igualmente bem, sendo a proporção de hidrólise de 1:1 (Wang e Guidotti,1996; Heine *et al.*, 1999). A NTPDase 2 (CD39L1) hidrolisa o ATP numa proporção 30 vezes maior do que o ADP (Kirley, 1997). As NTPDases 3 e 8 hidrolisam o ATP preferencialmente ao ADP, numa proporção de cerca de 3:1 e 2:1, respectivamente (Zimmermann, 2001; Bigonesse *et al.*, 2004).

As NTPDases de 1 a 3 e 8 são ligadas à membrana plasmática por dois domínios transmembrana, N e C-terminal. As NTPDases de 4 a 7 são localizadas intracelularmente. A NTPDase 4 está ancorada ao aparelho de Golgi e prefere UDP como sustrato. A NTPDase 5 está ligada ao retículo endoplasmático e a NTPDase 6 está ancorada ao aparelho de Golgi. Ambas têm preferência por nucleosídeos difosfatados, não possuem o domínio transmembrana C-terminal e podem ser clivadas próximo ao domínio N-terminal

para formar uma proteína solúvel liberada. Já a NTPDase 7 se localiza em vesículas intracelulares e prefere nucleosídeos trifosfatados (Zimmermann, 2001; Robson *et al.*, 2006). Em glândulas salivares a presença uma ecto-ATPase/Cell-Cam 105 (Murphy *et al.*, 1994) foi descrita. Uma ecto-ATPase que atuaria sobre o ATP extracelular foi descrita (Dowd *et al.*, 1996, 1999), assim como a presença de uma fosfolipase C nas células ductais da glândula salivar (Pochet *et al.*, 2003). Além disso, um estudo de imunohistoquímica sugeriu a presença de uma NTPDase 1 e NTPDase 2 na glândula salivar (Kittel *et al.*, 2004).

1.5.4.2. Ecto-nucleotídeo pirofosfatase/ fosfodiesterase (E-NPP)

A família das ecto-nucleotídeo pirofosfatase/fosfodiesterases (E-NPP, PDEase, EC 3.1.4.1) é constituída por sete membros numerados de acordo com sua ordem de clonagem (NPP1-7) (Stefan *et al.*, 2005). Os membros da família E-NPP 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. 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). Somente as NPPs 1-3 são capazes de hidrolisar nucleotídeos extracelulares (Stefan *et al.*, 2006). Em células polarizadas, sua presença está restrita a áreas especializadas (Bollen *et al.*, 2000).

Essas enzimas apresentam atividade de fosfodiesterase alcalina bem como atividade nucleotídeo pirofosfatase, hidrolisando uma grande variedade de substratos, entre eles: 3´,5´-AMPc a AMP; ATP a AMP e PP_i; ADP a AMP e P_i, NAD^+ a AMP e nicotinamida mononucleotídeo; e AP_nA a AP_{n-1} e AMP (Zimmermann, 2001). O p-nitrofenil-5'-timidina-monofosfato (*p*-nitrophenyl-TMP) tem sido usado como um substrato artificial, específico para as E-NPPs (Sakura *et al.*, 1998).

As NPPs atuam em vários processos fisiológicos, mais notadamente proliferação e motilidade celular, angiogênese, mineralização óssea e digestão. Também parecem estar envolvidas na patofisiologia do câncer, resistência à insulina e mineralização ectópica (Stefan *et al.*, 2005; Stefan *et al.*, 2006).

Fosfodiesterases cíclicas (PDEs) têm sido detectadas na glândula submandibular de ratos, e parecem estar envolvidas na regulação de segundos mensageiros intracelulares relacionados com a secreção salivar (Komine & Shinomura, 2002; Imai *et al.*, 1999). A presença de uma fosfolipase C também foi demonstrada em células ductais (Pochet *et al.*, 2003).

1.5.4.3. Ecto-5´-nucleotidase

A ecto-5'-nucleotidase (CD73, EC 3.1.3.5) é um homodímero ligado a membrana plasmática através de uma âncora lipídica de GPI, tem o sítio catalítico voltado para o meio extracelular, e sua atividade hidrolítica é potencializada por cátions divalentes e inibida por ADP, ATP e 5'-α,β- metileno-difosfato. A ecto-5´-nucleotidase pode exercer uma ampla variedade de funções

dependendo de sua expressão tecidual e celular. Está presente na maioria dos tecidos e sua principal função é a hidrólise de nucleotídeos monofosfatados extracelulares, tais como AMP, GMP ou UMP, a seus respectivos nucleosídeos (Sträter, 2006), sendo que o AMP é o nucleotídeo hidrolisado com maior eficiência (Zimmermann, 1996).

A ecto-5´-nucleotidase atua em conjunto com as NTPDases e NPPs, hidrolisando o AMP produzido por essas enzimas até adenosina (Zimmermann, 1992). A adenosina, produto final da hidrólise do ATP, é um importante neuromodulador e neurotransmissor, desencadeando efeitos opostos aos do ATP (James and Richardson, 1993). Existem poucos estudos sobre o efeito da adenosina nas glândulas salivares, e alguns resultados sugerem que a adenosina tem pouca influência na glândula salivar (Gallacher, 1982).

2. OBJETIVOS

Sabendo-se que o ATP é co-liberado com neurotransmissores como a acetilcolina e a noradrenalina e que esses neurotransmissores estão envolvidos no processo de secreção salivar, consideramos importante verificar a potencial presença de ectoenzimas que possam estar envolvidas na hidrólise do ATP nas células das glândulas salivares. Além disso, medicamentos como os antidepressivos podem causar xerostomia. Portanto, após a identificação dessas ectoenzimas, procuramos avaliar possíveis papéis patofisiológicos para as mesmas nas células da glândula salivar. Portanto, os objetivos desta tese foram:

1- Caracterizar e estudar as propriedades bioquímicas da E-NTPDase e ecto-5´-nucleotidase em células acinares;
2- Caracterizar e estudar as propriedades bioquímicas da E-NPP em células acinares;
3- Investigar a influência de fármacos antidepressivos sobre as enzimas responsáveis pela hidrólise de ATP, ADP e AMP até adenosina nas células de glândulas salivares de ratos adultos e avaliar a expressão dos membros da família das E-NTPDases e ecto-5'-nucleotidase.
4- Investigar a influência de fármacos antidepressivos sobre a atividade e expressão das E-NPPS nas células de glândulas salivares de ratos adultos.

3. ARTIGOS

Capítulo 1- Kinetic characterization of ATP diphosphohydrolase and 5'-nucleotidase activities in cells cultured from submandibular salivary glands of rats

Sandra Liana Henz, Cristiane Guimarães Ribeiro, Aline Rosa, Rafael Augusto Chiarelli, Emerson André Casali, João José Freitas Sarkis*

Cell Biology International 30: 214-220, 2006.

Capítulo 2- Kinetic and biochemical characterization of an ectonucleotide pyrophosphatase/phosphodiesterase (EC 3.1.4.1) in cells cultured from submandibular salivary glands of rats.

Sandra Liana Henz, Cristina Ribas Fürstenau, Rafael Augusto Chiarelli, João José Freitas Sarkis *

Archives of Oral Biology 52: 916-923, 2007.

Capítulo 3- Antidepressants effects on ecto-nucleotidase activities from salivary glands of rats.

Sandra Liana Henz[1], Giana de Paula Cognato[1], Carla Denise Bonan[2], Maurício Reis Bogo[3], João José Freitas Sarkis[1,*]

Submetido a "*Life Sciences"*

Capítulo 4- Influence of antidepressant drugs on E-NPPs from salivary glands of rats

Sandra Liana Henz[1,4], Giana de Paula Cognato[1], Fernanda Cenci Vuaden[1], Carla Denise Bonan[2], Maurício Reis Bogo[3], João José Freitas Sarkis[1]

Submetido a "*European Journal of Pharmacology*"

ARTIGOS CIENTÍFICOS

3.1- Capítulo 1- Kinetic characterization of ATP diphosphohydrolase and 5'-nucleotidase activities in cells cultured from submandibular salivary glands of rats

Sandra Liana Henz, Cristiane Guimarães Ribeiro, Aline Rosa, Rafael Augusto Chiarelli, Emerson André Casali, João José Freitas Sarkis*

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Kinetic characterization of ATP diphosphohydrolase and 5′-nucleotidase activities in cells cultured from submandibular salivary glands of rats

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Abstract

The participation of ecto-ATP diphosphohydrolase (CD39; ecto-NTPDase) and ecto-5′-nucleotidase (CD73) activities in the nucleotide hydrolysis by salivary gland cells from rats was evaluated. We investigated the biochemical characteristics of these ectoenzymes in cells cultured from submandibular salivary glands of rats. The V_{max} for the hydrolysis of ATP, ADP and AMP were 2275 ± 153 (mean ± SEM, $n = 4$), 941 ± 96 (mean ± SEM, $n = 5$) and 175 ± 5 (mean ± SEM, $n = 5$) nmol Pi liberated per min per mg of protein, respectively. The K_m values for ATP, ADP and AMP were 224 ± 8 μM (mean ± SEM, $n = 4$), 163 ± 15 μM (mean ± SEM, $n = 5$) and 117 ± 5 μM (mean ± SEM, $n = 5$), respectively. The competition plot showed that ATP and ADP were hydrolyzed at the same active site on the enzyme. It may be postulated that the physiological role for this ecto-enzyme cascade is to terminate the action of the co-transmitter ATP, generating adenosine.

Keywords: Ectonucleotidases; Rat submandibular gland; Nucleotide hydrolysis

1. Introduction

In many tissues, extracellular ATP exerts a broad range of physiological responses, including neurotransmission (Edwards et al., 2002; Zimmermann, 2001), modulation of vascular tone, platelet aggregation, neutrophil aggregation, superoxide release, production of pro-inflammatory cytokines (Ford-Hutchinson, 1982; Kuroki and Minakami, 1989) and transepithelial chloride secretion by activation of plasma membrane receptors (Harden et al., 1997; Boarder and Hourani, 1998; King et al., 1998; Kunapuli and Daniel, 1998). These receptors can be ligand-gated ion channels (P2X-type) or coupled to heterotrimeric G proteins (P2Y-type). Many subtypes of both kinds of receptors have been described. Seven P2Y and P2X receptors have been currently identified (Buell et al., 1996; Ralevic and Burnstock, 1998). In salivary glands, four distinct subtypes of P2 ATP receptors have been identified in the ductal and acinar cells: P2Y1, P2Y2, P2X4 and P2X7 (Turner and Camden, 1990). At the end of its action, the ATP signaling molecule must be recaptured by the cell or hydrolyzed by an enzymatic system. We, and others (Sarkis and Saltó, 1991; Cunha et al., 1992), have described the presence of an enzymatic chain formed by an ecto-ATP diphosphohydrolase plus a 5′-nucleotidase, able to promote the hydrolysis of the neurotransmitter ATP to adenosine.

The extracellular hydrolysis of ATP to adenosine by ectonucleotidases has been reported for several cell types (Ralevic and Burnstock, 1998; Battastini et al., 1995). These enzymatic activities can regulate the extracellular concentration of adenine nucleotides and nucleosides, modulating their local effects. Degradation of ATP and other nucleotides occurs through a cascade of cell-surface-bound enzymes such as ecto-ATPase (EC

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3.6.1.3), ecto-apyrase/ATP diphosphohydrolase/NTPDase (EC 3.6.1.5) and ecto-5′-nucleotidase (EC 3.1.3.5), resulting in the formation of ADP, AMP and adenosine (Zimmermann, 2001). The presence of ATP diphosphohydrolase activity has been demonstrated in: (a) plant tissue; (b) insects; (c) parasites; (d) peripheral nervous system from synaptosomes of electric ray and in a number of mammalian sources (Kettlun et al., 1992; Ribeiro et al., 1984; Sarkis and Saltó, 1991; Frasseto et al., 1993; Battastini et al., 1995; Mans et al., 1998; Heine et al., 1999; Zimmermann, 2001). Apyrase is the enzyme that hydrolyzes ATP and ADP (and other tri- and diphosphates nucleosides) to the monophosphated esters plus inorganic phosphate (Pi), releasing 2 mol Pi/mol ATP and 1 mol Pi/ mol ADP.

Many papers have discussed the role of ectonucleotidases not only in the nervous system (Battastini et al., 1995; Fontella et al., 2004), but also in other systems, including salivary glands (Murphy et al., 1994; Dowd et al., 1996).

In the salivary glands of blood-feeding arthropods, similar enzymes have been studied, where a role to prevent blood coagulation has been described (Ribeiro et al., 1984; Valenzuela et al., 2001). Others findings, in the parotid gland, point to a possible role for these enzymes in reducing luminal ATP concentrations, while at the same time suggesting a lesser role for the enzyme in regulating ATP on the basolateral border of the gland (Murphy et al., 1994; Dowd et al., 1996). In the parotid gland ATP probably has a neurotransmitter/neuromodulator function (Dowd et al., 1999).

A possible ecto-ATPase activity in rat salivary gland cells was identified and characterized (Dowd et al., 1996, 1999). In the present study, we demonstrate that salivary gland cells in culture were able to promote the hydrolysis of ATP, ADP and AMP and we present evidence for the first time that the enzymes responsible for nucleotide hydrolysis were a true ecto-apyrase (ATP and ADP hydrolysis) and an ecto-5′-nucleotidase (AMP hydrolysis). The kinetic parameters for nucleotide hydrolysis and the effect of divalent cations, calcium and magnesium on enzymatic activities were determined. Our study may provide new information regarding the role of enzymes in the regulation of nucleotide signaling pathways in the salivary glands. This enzymatic chain could be involved in the degradation of the co-transmitter ATP to adenosine in the vicinity of the salivary glands.

2. Materials and methods

2.1. Materials

Culture medium, Dulbecco's modified Eagle's medium (DMEM), was purchased from Grand Island Biological Company (Grand Island, NY, USA). The lactate dehydrogenase (LDH) kit was obtained from Labtest Diagnóstica, MG-Brazil, collagenase I-S, nucleotides and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Cultilab Ltda (São Paulo, SP, Brazil). All the other chemical reagents were of the highest available quality.

2.2. Cell isolation and culture

Salivary gland cell (SGC) clusters were obtained from 35-day-old male Wistar rats. The animals were sacrificed and the submandibular glands were removed quickly, trimmed of fat and fascia tissues and minced in a small volume of Hank's buffer saline solution (HBSS), pH 7.4. The minced salivary glands were dispersed in HBSS supplemented with collagenase I-S (1 mg/ml). The gland cells were dissociated by pipetting 10 times every 20 min with a Pasteur pipette during 2 h. The collagenase was removed by centrifugation at 700 × *g* (5 min) and the cell clusters were then washed with HBSS and centrifuged twice at 40 × *g* (5 min) to remove the lysed and contaminants (red and endothelial cells).

Clusters with 4–5 cells (as observed by phase-contrast microscopy) were maintained in a water-saturated atmosphere with 95% air and 5% CO_2 in DMEM with 5% fetal bovine serum (FBS), pH 7.4 for 24–48 h (modified from Murphy et al., 1994; Dowd et al., 1996).

2.3. Enzymatic assays

After 24 or 48 h in culture the SGC were washed and centrifuged three times at 1000 × *g* for 3 min with the reaction medium containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose and 10 mM Hepes (pH 7.4). The cell clusters were filtered through a nylon filter (100 mesh) to homogenize the cluster size. All samples were pre-incubated for 1 min. The reaction medium, containing ATP, ADP or AMP as substrate (at concentrations indicated), was incubated with aliquots of 20 μl of cell suspension (approximately 15–20 μg of protein) at 37 °C for 5 min in a final volume of 200 μl. The reaction was stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 5% (v/v) and the samples were chilled on ice. Incubation times and protein concentration were chosen to ensure the linearity of the reaction. In order to correct non-enzymatic hydrolysis, we performed controls by adding the SGC after the reaction was stopped with TCA. All assays were carried out in triplicate. Samples were centrifuged at 14 000 × *g* for 5 min at 4 °C to precipitate protein and the supernatant was used to measure the amount of inorganic phosphate (Pi) liberated using the colorimetric method described by Chan et al. (1986). Enzyme activities were expressed as nmoles of Pi released per minute per milligram of protein.

2.4. Protein determination

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

2.5. Cellular integrity

Determination of cellular integrity was performed by measuring the activity of the cytosolic enzyme lactate dehydrogenase (LDH) present in the supernatant obtained after incubation at 37 °C and comparing it to total enzyme activity in cells lysed with 1% Triton X-100. The LDH activity in the supernatant of the incubation medium in relation to the LDH activity in the Triton X-100 disrupted fraction (100%) was indicative of cellular integrity.

2.6. Statistical analysis

The mean ± SEM data for groups of three or four experiments were analyzed by ANOVA and, when necessary, the post hoc Student–Newman–Keuls test using the statistical program SPSS 6.0 for Windows. Values of $P < 0.05$ were considered significant. The computer program Graph Pad Prism was used for kinetic analysis of linear regression.

3. Results

3.1. ATP, ADP and AMP hydrolysis

To ensure the linearity as a function of time of the enzymatic reaction, the cellular suspension was incubated as described in Section 2 with 2.0 mM of ATP, ADP or AMP for up to 20 min. Fig. 1 shows that culture of salivary gland cells promoted ATP,

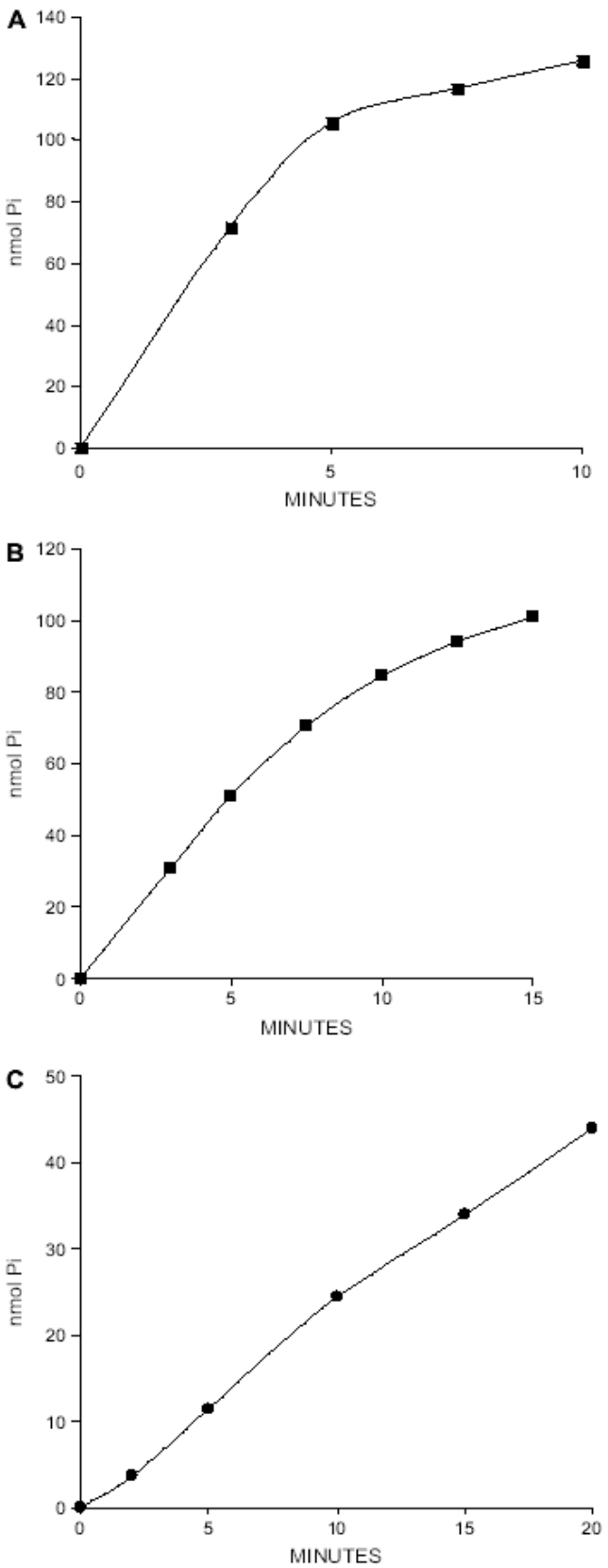

Fig. 1. Time-course of extracellular hydrolysis of ATP, ADP and AMP. The acinar cells (15–20 μg) were incubated with ATP (A), ADP (B) or AMP (C), 2.0 mM plus 2.0 mM Ca^{2+}, as described in Section 2. Plots are representative of three isolated experiments for each nucleotide.

ADP and AMP hydrolysis that was linear up to 2.5 min for ATP, up to 7.5 min for ADP and up to 20 min for AMP (Fig. 1). Conversely, the protein curve (Fig. 2) demonstrated that the incubation with 2.0 mM ATP, ADP or AMP for 5 min was linear when up to 20 μg of protein was used as the amount of protein in the assay.

In order to investigate the possibility of cation dependency for the salivary gland enzymes, we tested the hydrolysis rate for the nucleotides in the presence or absence of divalent cations or EDTA as indicated in Section 2. In the presence of 3.0 mM EDTA, ATP, ADP and AMP hydrolysis was practically negligible when compared with controls (without the addition of divalent cations) (Fig. 3). The hydrolysis of all nucleotides was significantly increased by addition of Ca^{2+} or Mg^{2+} in relation to basal level (Fig. 3). Significant differences were observed in enzymatic activation by different cations at different concentrations tested for ATP and ADP hydrolysis when compared with the basal group, however AMP hydrolysis was not increased when compared with the basal group. These results indicate that the AMP hydrolysis is maximal in the presence of basal levels of divalent cations secreted by the cells or present in the biological fraction. Thus, we can conclude that the enzymes responsible for the hydrolysis of ATP, ADP and AMP in salivary gland cells are cation dependent. The maximal enzyme activation for ATP and ADP is possible with 2.0 mM or less of both cations tested (Fig. 3). No additive effects were observed when the two divalent cations tested were added to the reaction medium at the same time, suggesting that both Ca^{2+} and Mg^{2+} are competing for the same activation site. In addition the activation of the enzyme at low concentrations of ions (0.1 mM of each) and both at the same time in the incubation was not additive (results not shown). Based on these results, we established

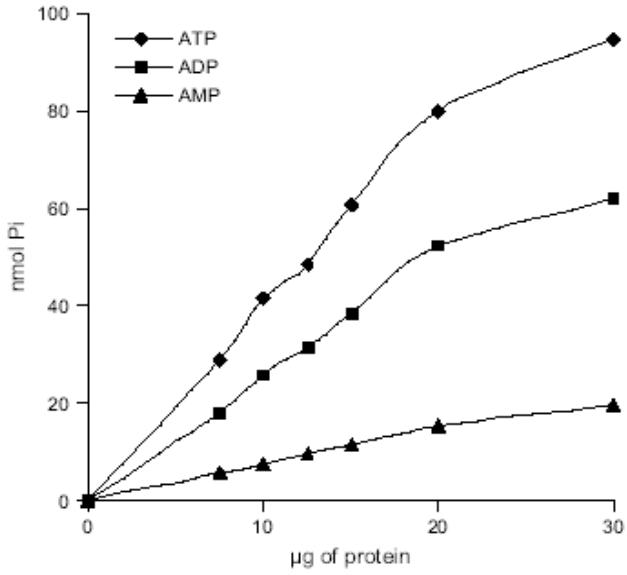

Fig. 2. Protein concentration curve for extracellular hydrolysis of ATP, ADP and AMP. The different quantities of acinar cells (0–30 μg) were incubated with ATP (A), ADP (B) or AMP (C) 2.0 mM plus 2.0 mM Ca^{2+}, as described in Section 2. The plot is representative of three independent experiments for each nucleotide.

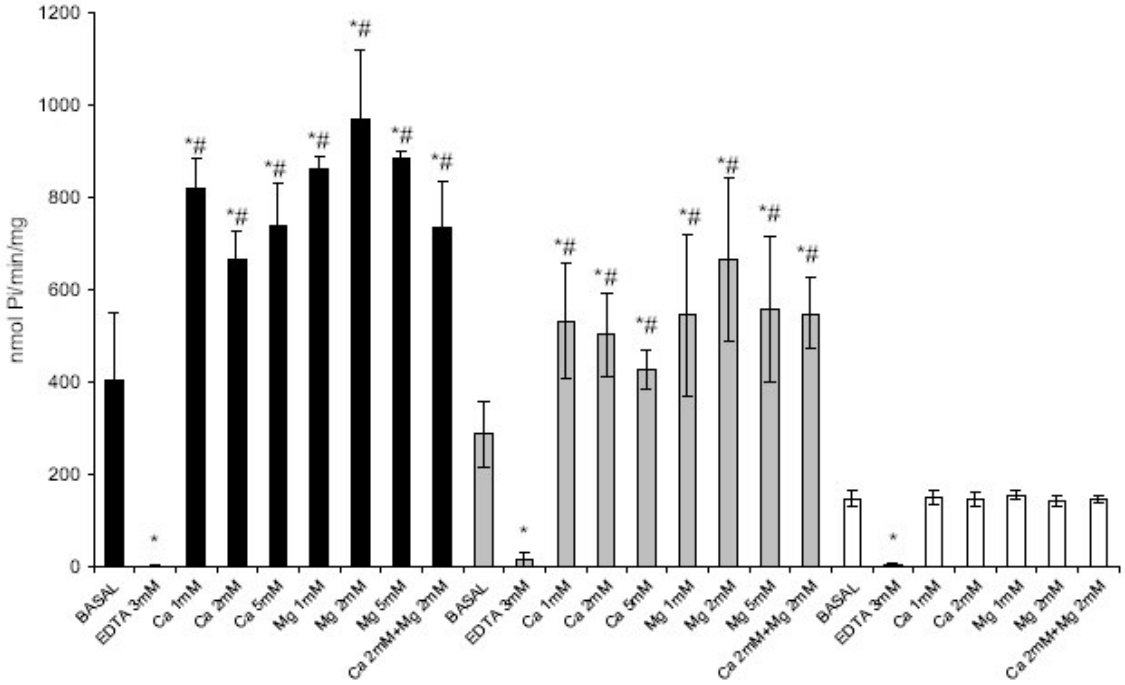

Fig. 3. Cation dependence for extracellular hydrolysis of ATP, ADP and AMP. The acinar cells (15–20 μg) were incubated with 2.0 mM ATP (black bars), ADP (gray bars) or AMP (white bars) with or without addition of cations or EDTA, as indicated. The data (mean ± SEM) are representative of three different experiments. $^{*}P < 0.05$ compared to basal group, $^{\#}P < 0.05$ compared to the 3.0 mM EDTA group (Student–Newman–Keuls test).

the ratio of 2.0 mM/2.0 mM for nucleotides/divalent cations as optimal conditions for measuring the ectonucleotidase activity. In this manner, the ectonucleotidase activities were measured in a physiological range of divalent cations and nucleotides.

3.2. Kinetic parameters

Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis were determined at ATP and ADP concentrations ranging from 0.075 to 1.2 mM and 0.075–1.0 mM, respectively. Mg^{2+}-AMP hydrolysis was determined at AMP concentrations ranging from 0.05 to 1.0 mM. The results (Fig. 4, insets) indicated that all enzymatic activities increased with increasing nucleotide concentration until saturation with less than 1.0 mM. The Eadie–Hofstee plot for the hydrolysis of ATP, ADP and AMP is shown in Fig. 4. The apparent Michaelis–Menten constants ($K_{m,app}$) calculated by linear regression were 224 ± 8 μM (mean ±SEM, $n = 4$) for ATP and 163 ± 15 μM (mean ± SEM, $n = 5$) for ADP. The calculated maximum velocities ($V_{max,\ app}$) for ATP hydrolysis were 2275 ± 153 (mean ± SEM, $n = 4$) and for ADP 941 ± 96 (mean ± SEM, $n = 5$) nmol Pi/min per mg. The values of K_m were not statistically different. The kinetic parameters for AMP hydrolysis were 117 ± 5 μM (mean SEM, $n = 5$) for K_m and 175 ± 5 (mean ± SEM, $n = 5$) nmol Pi/min per mg for V_{max}.

3.3. A single active site

ATP and ADP hydrolysis could be catalyzed by an ATP diphosphohydrolase or by combinations of ecto-enzymes that are able to mimic apyrase activity. To show that ATP and ADP hydrolysis occurs due to an ATP diphosphohydrolase and that one active site is able to hydrolyze the two substrates, we used the Chevillard competition plot enzyme (Chevillard et al., 1993). To assay the combination of substrate concentrations in a Chevillard competition plot we chose concentrations at which the rate of hydrolysis was the same when either ATP or ADP (from Fig. 4, insets) was used as substrate. The P values ranged from 1 to 0. The horizontal straight line obtained in the competition plot (Fig. 5) indicates a constant hydrolysis rate at all substrate combinations tested and the interpretation is that the hydrolysis of both substrates, ATP and ADP, occurs at the same active site of a single enzyme.

3.4. Cellular integrity

The protocol was carried out according to the manufacturer's instructions. The measurement of LDH activity showed that the majority of salivary gland cells remained intact during the incubation in the reaction medium with or without ATP, ADP or AMP. The results (not shown) indicated fewer than 20% disrupted cells. Thus, the participation of cytosolic enzymes in extracellular nucleotide hydrolysis by salivary glands cells was excluded.

4. Discussion

In the present study, we demonstrate that the salivary gland was able to hydrolyze nucleotides extracellularly. Our results strongly suggest that the enzyme responsible for ATP and ADP hydrolysis in the salivary glands is an ATP

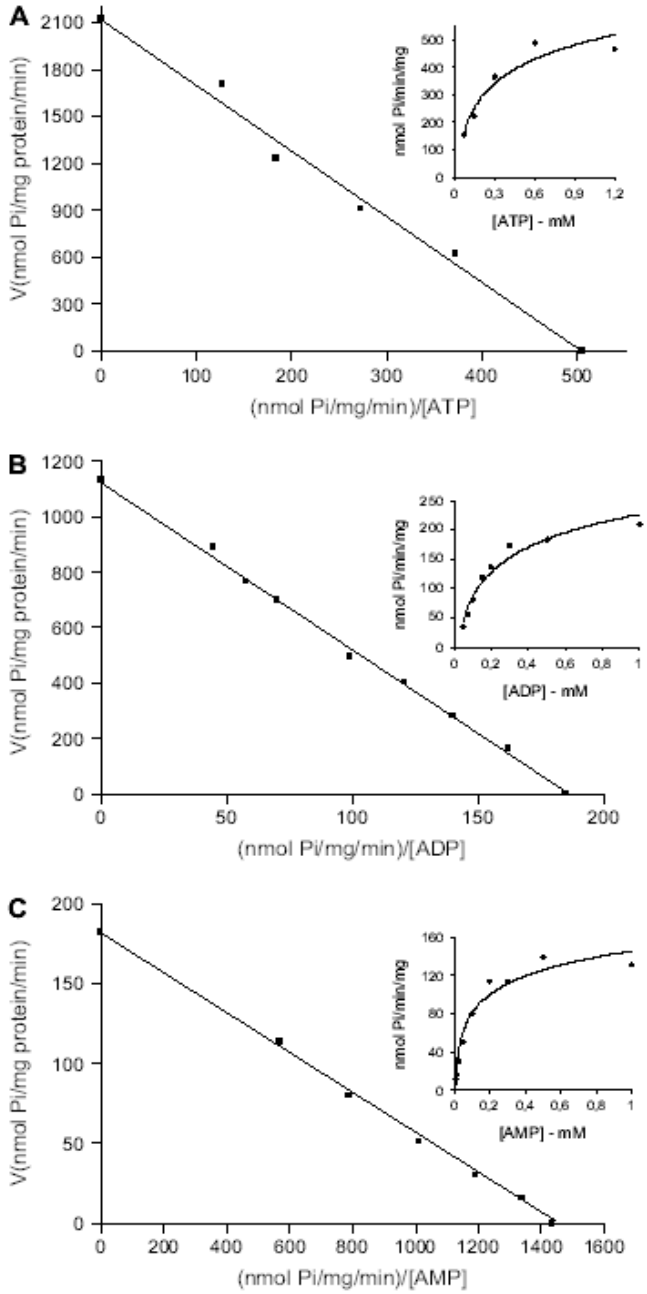

Fig. 4. Eadie–Hofstee plots for extracellular hydrolysis of ATP (A), ADP (B) and AMP (C). Reaction rate was measured by released Pi, as described in Section 2. Results were obtained with a nucleotide concentration ranging from 0.075 to 1.2 mM for ATP, from 0.075 to 1.0 mM for ADP and from 0.05 to 1.0 mM for AMP, plus 2.0 mM Ca^{2+}, as described in Section 2. The insets show activation of the enzyme for all substrates. Best-fit analysis indicated a linear relationship. Plots are representative of at least four independent experiments for each nucleotide.

diphosphohydrolase, probably an NTPDase 3, on the basis of the ratio ATPase/ADPase (Zimmermann, 2001). This enzyme is a functional intermediate that hydrolyzes ATP approximately three times better than ADP (Smith and Kirley, 1998). Thus, the enzyme described by us has a hydrolysis ratio for ATPase/ADPase of approximately 2.42. This ratio indicates the relative preference of this enzyme to hydrolyze ATP more

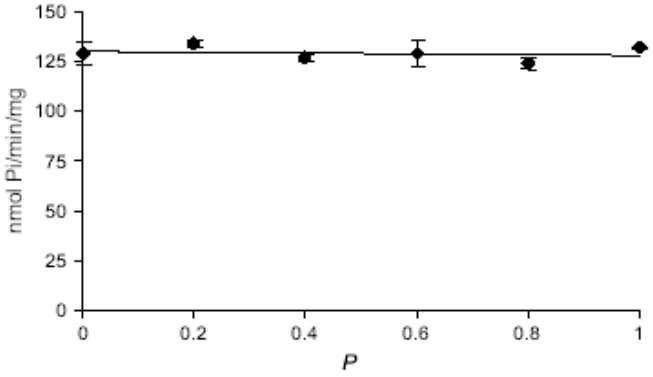

Fig. 5. Competition plot for extracellular hydrolysis of ATP and ADP. The concentration at which the velocities were the same for ATP and ADP was chosen for the Chevillard plot. Assay conditions are described in Section 2. The incubation time was 5 min, the concentration of substrate A (ADP) at $P = 0$ was 0.2 mM and substrate B (ATP) at $P = 1$ was 0.075 mM. Plot represents an experiment carried out in triplicate. No significant difference was found between different points.

effectively with a delayed hydrolysis of ADP. The similar cation dependence for ATP and ADP hydrolysis is in accordance with the idea that the enzyme responsible is an ATP diphosphohydrolase. We used the Chevillard competition plot (Fig. 5), which is a decisive method to determine if two substrates are hydrolyzed at the same active site of an enzyme (Chevillard et al., 1993). The constant velocity presented by the Chevillard plot indicates that ATP and ADP are hydrolyzed at only one active site. Notably, this same protocol was used to demonstrate the presence of an NTPDase enzyme in human platelets (Pilla et al., 1996) and in slices obtained from the central nervous system (Bruno et al., 2002). Furthermore, for both substrates Ca^{2+}-ATP and Ca^{2+}-ADP the values of $K_{m,app}$ and V_{max} (Fig. 4) reinforce the possibility for the presence of an ecto-ATP diphosphohydrolase in the salivary gland.

AMP, the final product of ATP and ADP hydrolysis by an ecto-ATP diphosphohydrolase/NTPDase is the substrate in salivary glands for an ecto-5′-nucleotidase activity. This last enzyme was demonstrated by cation dependence and kinetic parameters (Figs. 3 and 4). The ecto-5′-nucleotidase is usually considered to be only Mg^{2+} dependent (Zimmermann, 1992), however, our results (in accordance with other previous studies) indicate that Ca^{2+} could also activate this enzyme (Casali et al., 2001). The co-existence of these two enzymes is very important for their participation in an "enzyme chain" for the complete hydrolysis of ATP to adenosine and for the control of the nucleotide/nucleoside ratio in the vicinity of the salivary gland.

ATP can activate plasma membrane receptors, such as P2X or P2Y (Gibbons et al., 2001). The effects of ATP on salivary gland cells have been investigated by many authors (Tenneti et al., 1998; Pochet et al., 2003) and the control of concentrations of agonists of P2-receptors by NTPDases is an important factor in nucleotide signaling (Turner et al., 1999; Liu et al., 2000; Kittel et al., 2004). The parotid gland is essentially serous and the submandibular gland is a mixed gland. Parotid gland acinar cells can co-express P2X4 and P2X7 receptors (Gibbons et al., 2001) and the rat submandibular gland ductal

cells express P2X7 receptors (Pochet et al., 2003). This indicates that salivary gland acinar cells have P2X7 receptors with properties similar to those in other tissues. Receptors coupled to a G-protein, P2Y1 and P2Y2, also have been described in salivary glands (Buell et al., 1996; Gibbons et al., 2001; Yu and Turner, 1991). Some authors have shown that P2Y2 receptors have a role in the response to damage and disruption of normal salivary gland structure and function (Ahn et al., 2000). Furthermore, with regard to the nucleotide hydrolysis enzymes, a number of factors should be taken into consideration: (a) strong immunoreactivity and enzyme activity were described in the membranes of myoepithelial cells and their caveolae and (b) acini attached to myoepithelial cells showed weak immunoreactivity for NTPDase 1, while NTPDase 2 activity (ecto-ATPase) was strong in the plasma membranes of nerve terminals and membranes of nerve fibers (Kittel et al., 2004). Phospholipase D activity in rat submandibular ductal cells has been shown to be stimulated by ATP in a concentration dependent manner (Pochet et al., 2003).

The ATP molecule may also participate in other functions in salivary glands. The ability of ATP to activate Cl^- currents in submandibular gland acinar and duct cells has been demonstrated (Lee et al., 1997; Zeng et al., 1997), however, the levels and source of endogenous ATP remain enigmatic. Data from other tissues suggest that P2X7 receptors may have a role in mediating inflammation or cell death in salivary glands (Gibbons et al., 2001).

According to Ralevic and Burnstock (1998), when fluids flow in tubes such as salivary ducts, they cause a shear stress and distension that provokes the release of ATP through the apical membrane. Since the P2X7 receptors are located on this membrane, ATP might have an autocrine effect (Zeng et al., 1997).

Our study shows that extracellular hydrolysis of ATP and ADP in salivary gland acinar cells is probably performed by an ATP diphosphohydrolase and that the AMP produced could be hydrolyzed by an ecto-5′-nucleotidase activity. We cannot disregard the possible presence of two enzymes to hydrolyze ATP, an ecto-ATP diphosphohydrolase and an ecto-ATPase (Dowd et al., 1996), which may be co-expressed, as occurs in many other rat tissues (Kegel et al., 1997; Heine et al., 1999; Vlajkovic et al., 2002; Kukulski and Komoszynsky, 2003). For the first time, in submandibular salivary glands, we present some evidence to support the presence of an ecto-ATP diphosphohydrolase, an enzyme able to promote the hydrolysis of ATP and ADP at the same active site. Some aspects of this mechanism should be considered when extrapolating these observations to in vivo conditions. ATP release, especially from neuronal sources, occurs in close proximity to the acinar cells, which would result in concentrations of ATP at neuroeffector junctions that are difficult to determine (Dowd et al., 1996).

In summary, it may be postulated that this enzyme cascade (ecto-ATP diphosphohydrolase plus ecto-5′-nucleotidase) may participate in the hydrolysis of ATP released as a co-transmitter from nerve terminals at the basal border of cells in the salivary glands. This cascade, now described in salivary glands, has been previously described in the central and peripheral nervous system (Sarkis and Saltó, 1991; Battastini et al., 1995) and is attributed to the same function. The ectonucleotidases described herein could contribute to reducing the cytotoxic effect of extracellular ATP on the cell. More studies are necessary to demonstrate the physiological and pathological role of these enzymes in the salivary glands. However our results reinforce that salivary gland ecto-ATP diphosphohydrolase is involved in important physiological functions and suggests that this activity may be regulatory of ratio ATP/adenosine in the vicinity of the nerve terminal.

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3.2- Capítulo 2- Kinetic and biochemical characterization of an ectonucleotide pyrophosphatase/phosphodiesterase (EC 3.1.4.1) in cells cultured from submandibular salivary glands of rats.

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Kinetic and biochemical characterization of an ecto-nucleotide pyrophosphatase/phosphodiesterase (EC 3.1.4.1) in cells cultured from submandibular salivary glands of rats

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ABSTRACT

The participation of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity in the nucleotide hydrolysis by salivary gland cells of rats was evaluated using *p*-nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP) as a substrate for this enzyme. We investigated the biochemical characteristics of this ectoenzyme in cells cultured from submandibular salivary glands of rats. Primary cell cultures 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, dependence of pH alkaline and inactivation by a metal ion chelator. The K_m value for the hydrolysis of *p*-Nph-5′-TMP was 280.7 ± 34.2 μM (mean ± S.D., $n = 4$) and V_{max} was 721.31 ± 225 nmol *p*-nitrophenol/min/mg (mean ± S.D., $n = 4$). We suggest that E-NPP is co-localized with an ecto-ATP diphosphohydrolase/ecto-NTPDase and an ecto-5′-nucleotidase, since these enzymes probably act under different conditions. It may be postulated that the physiological role for these ecto-enzymes is to terminate the action of the co-transmitter ATP, generating adenosine.

1. Introduction

Many publications have discussed the role of adenosine triphosphate (ATP) as an extracellular mediator and neurotransmitter in various systems, including salivary glands.[1,2] Extracellular ATP exerts a broad range of physiological responses, including neurotransmission,[1] modulation of vascular tone, platelet aggregation, neutrophil aggregation, superoxide release, production of pro-inflamatory cytokines[3,4] and transepithelial chloride secretion by activation of plasma membrane receptors.[5] ATP and other extracellular nucleotides influence epithelial cell functions via a variety of P2 receptors.[6] These receptors can be ligand-gated ion channels (P2X-type) or coupled to heterotrimeric G proteins (P2Y-type). In salivary glands, four distinct P2 subtypes of ATP receptors have been identified in the ductal and acinar cells: $P2Y_1$, $P2Y_2$, $P2X_4$ and $P2X_7$.[7] At the end of its action, the ATP signaling molecule must be recaptured by the cell or hydrolyzed by enzymatic system(s).

The extracellular hydrolysis of ATP to adenosine by ectonucleotidases has been reported in several cell types.[6,8–10] These enzymatic activities can regulate the extracellular

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concentration of adenine nucleotides and nucleosides, thus modulating their local effects. Degradation of ATP and other nucleotides can occur through a cascade of cell-surface-bound enzymes such as ecto-ATPase (EC 3.6.1.3), ecto-apyrase/ATP diphosphohydrolase/NTPDase (EC 3.6.1.5), ecto-pyrophosphatase/phosphodiesterase/NPP (EC 3.1.4.1) and ecto-5′-nucleotidase (EC 3.1.3.5).[1] These enzymes seem to have multiple roles in extracellular nucleotide metabolism and in the regulation of intercellular signalling.[11] E-NTPDase describes a family of mammalian enzymes that catalyze the hydrolysis of γ- and β-phosphate residues of nucleosides 5′-tri and 5′-diphosphates, and these enzymes require the presence of divalent cations for their catalytic activity.[1] We, recently, described the presence of an NTPDase as an ecto-enzyme in cell cultures obtained from submandibular glands of adult rats.[12]

The family of E-NPPs is known to hydrolyze 5′-phosphodiester bonds in nucleotides and their derivatives, resulting in the release of 5′-monophosphates.[11,13] E-NPPs hydrolyze a broad range of substrates such as UDP-galactose, NAD^+, cAMP, ATP and ADP. On the other hand, AMP is not a substrate for NPPs, as expected from the absence of phosphodiester or pyrophosphate bonds.[13] The *p*-nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP) has been used as an artificial substrate marker for E-NPPs characterization, generating *p*-nitrophenol as a final product. Current evidence suggests that E-NPPs have multiple and largely related physiological roles, including nucleotide recycling, modulation of purinergic receptor signaling, regulation of extracellular pyrophosphate levels, stimulation of cell motility, activity of ecto-kinases and, probably, regulation of the insulin receptor.[11] These families of enzymes demonstrate not only overlapping tissue distributions, but also overlapping substrate specificities and may also present overlapping function.[1] Nucleotides can exert different responses in diverse tissues, for this reason cells can co-exhibit two or more different families of enzymes, probably with slightly different nucleotide hydrolysis properties. NPP4-5 have a predicted type I membrane orientation, but have not yet been functionally characterized.[13]

Dowd et al.[14,15] identified and characterized a possible ecto-ATPase activity in rat salivary gland cells. In the submandibular gland cyclic nucleotide phosphodiesterase, PDE1 and PDE5[16,17] were detected in the rat. NPP1 is present on the distal convoluted tubules of the kidney, epithelium of salivary glands ducts, brain capillary endothelium and epididymis.[11]

In a previous study, we described the presence of an E-NTPDase and 5′-nucleotidase in salivary gland cells.[12] In the present study, we demonstrate that salivary gland cells in culture, in addition to these enzymes, were able to promote the extracellular hydrolysis of *p*-Nph-5′-TMP (a substrate marker for the ecto-pyrophosphatase/phosphodiesterase). The kinetic parameters for substrate hydrolysis and the effects of divalent cations, calcium and magnesium on enzymatic activities were determined. We postulated the co-existence of a multiple enzymatic system in the salivary glands for extracellular nucleotide hydrolysis. Our study may provide new information regarding the presence of different families of enzymes with similar functions in the salivary gland cells, which probably act under distinct physiological situations in the regulation of nucleotide signaling pathways.

2. Materials and methods

2.1. Materials

Dulbecco's modified eagle medium (DMEM) was purchased from Grand Island Biological Company (Grand Island, NY, USA). Collagenase I-S, nucleotides, Hepes, *p*-Nitrophenyl thymidine 5′-monophosphate, EDTA, Trizma Base, ouabain, lanthanum chloride, levamisole, NEM (*N*-ethylmaleimide) sodium azide and phenylalanine were obtained from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Cultilab Ltda (São Paulo, SP, Brazil). All the other chemical reagents were of the highest available quality.

2.2. Cell isolation and culture

Salivary gland cell (SGC) clusters were obtained from 35-day-old male Wistar rats. The animals were sacrificed with ether overdose and the submandibular glands were removed quickly, trimmed of fat and fascia tissues and minced in a small volume of Hank's buffer saline solution (HBSS), pH 7.4. The minced salivary glands were dispersed in HBSS supplemented with collagenase I-S (1 mg/ml). The gland cells were dissociated by pipetting 10 times every 20 minutes with a Pasteur pipette for 2 h. The collagenase was removed by centrifugation at $700 \times g$ (5 min) and the cell clusters were then washed with HBSS and centrifuged twice at $40 \times g$ (5 min) to remove the lysed cells and contaminants (red and endothelial cells).

Clusters with 4–5 cells (Fig. 1) (as observed by phase-contrast microscopy) were maintained in a water-saturated atmosphere with 95% air and 5% CO_2 in Dulbecco's modified eagle medium (DMEM) with 5% fetal bovine serum (FBS), pH 7.4 for 24–48 h [modified from[14,18]].

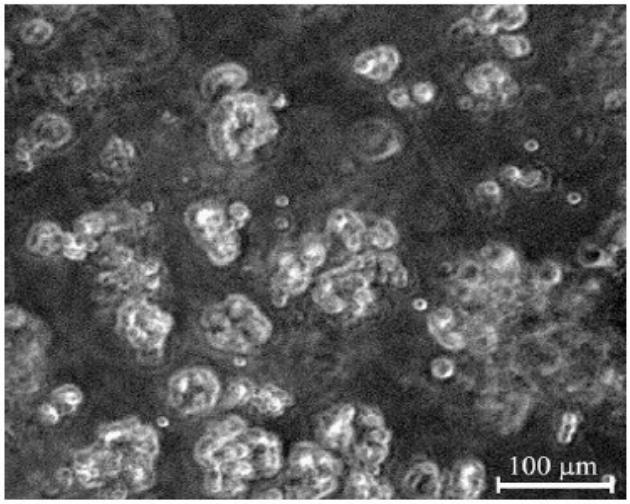

Fig. 1 – Representative picture of submandibular gland cells cultured to 24 h showing cell clusters. The cell culture was performed as described in material and methods of the manuscript. Cells were viewed with a Nikon inverted microscope and images transferred to computer with a digital camera (Sound Vision Inc. Wayland, MA).

2.3. Enzymatic assays

2.3.1. Assay of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) activity

After 24 or 48 h in culture, the SGC were washed and centrifuged three times at 1000 × *g* for 3 min with a medium containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose and 10 mM Hepes (pH 7.4). The cell clusters were filtered through a nylon filter (100 mesh) to homogenize the cluster size. The reaction medium, containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose, 2 mM $CaCl_2$ and 10 mM Hepes (pH 7.4) was pre incubated with aliquots of 20 μl of cell suspension (approximately 15–20 μg of protein per tube) at 37 °C for 1 min in a final volume of 200 μl. The reaction was started with the addition of ATP or ADP as substrate at a final concentration of 2.0 mM. The incubation time for ATP was 4 min and the incubation time for ADP was 5 min. The reaction was stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 5% (v/v) and the samples were chilled on ice. Incubation times and protein concentration were chosen to ensure the linearity of the reaction. In order to correct non-enzymatic hydrolysis, we performed controls by adding the SGC after the reaction was stopped with TCA. All assays were carried out in triplicate. Samples were centrifuged at 14,000 × *g* for 5 min at 4 °C to precipitate protein and the supernatant was used to measure the amount of inorganic phosphate (Pi) liberated using the colorimetric method described by Chan.[19] Enzyme activities were expressed as nmoles of Pi released per minute per milligram of protein.

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

After 24 or 48 h in culture, the SGC were washed and centrifuged three times at 1000 × *g* for 3 min with a medium containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose and 10 mM Hepes (pH 7.4). The cell clusters were filtered through a nylon filter (100 mesh) to homogenize the cluster size. The artificial substrate for E-NPPs, *p*-nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP), was used as a substrate marker to evaluate the enzymatic activity, generating *p*-nitrophenol.[20] The reaction medium, containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose and 50 mM Tris–HCl buffer (pH 8.9), was pre-incubated with aliquots of 20 μl of cell suspension (approximately 10–15 μg of protein per tube) at 37 °C for 10 min 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 6 min of incubation, the reaction was stopped by the addition of 200 μl 0.2N NaOH and the samples were chilled on ice. Incubation times and protein concentration were chosen to ensure the linearity of the reaction. In order to correct non-enzymatic hydrolysis, we performed controls by adding the SGC after the reaction was stopped with 0.2N NaOH. All assays were carried out in triplicate. 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. Enzyme activities were generally expressed as nmol *p*-nitrophenol released per minute per milligram of protein.

2.4. Protein determination

Protein was measured by the Comassie Blue method, according to Bradford,[21] using bovine serum albumin as standard.

2.5. Cellular integrity

Determination of cellular integrity was performed by measuring the activity of the cytosolic enzyme, lactate dehydrogenase (LDH). In a previous study,[12] we demonstrated that the majority of salivary gland cells remained intact (fewer than 20% of the cells were disrupted).

2.6. Statistical analysis

The mean ± S.D. data for groups of three or more experiments were analyzed by ANOVA and, when necessary, the post hoc Student–Newman–Keuls test using the statistical program SPSS 6.0 for Windows. Values of $P < 0.05$ were considered significant. The Graph Pad Prism program was used for kinetic analysis of linear regression.

3. Results

3.1. Hydrolysis of *p*-Nph-5′-TMP

To ensure linearity, as a function of enzymatic reaction time, the cellular suspension was incubated as described in Section 2 with 0.5 mM of *p*-Nph-5′-TMP and the amount of *p*-nitrophenol released was measured. Fig. 2(A) shows that the culture of salivary gland cells promoted *p*-Nph-5′-TMP hydrolysis that was linear up to 20 min. The hydrolysis of *p*-Nph-5′-TMP was linear up to 15 μg of protein per tube (Fig. 2B), as such this amount of protein was used in the assays. Fig. 2A and B are representative of three independent experiments.

3.2. Cation dependence

In order to investigate the possibility of cation dependence for the salivary gland enzyme, we tested the hydrolysis rate for the *p*-Nph-5′-TMP in the presence or absence of divalent cations or EDTA (cation chelator), as indicated in Section 2. The hydrolysis of *p*-Nph-5′-TMP was not significantly increased by addition of 0.5 and 1.0 mM Ca^{2+} or Mg^{2+} in relation to basal preparation levels of cations (Fig. 3). In the presence of 1.5 mM EDTA, hydrolysis of *p*-Nph-5′-TMP was practically negligible when compared with control (without the addition of any divalent cations) (Fig. 3). When we added 1.5 mM EDTA plus Ca^{2+} or Mg^{2+} (0.5 and 1.0 mM), the hydrolysis of *p*-Nph-5′-TMP was lower when compared with the same concentrations of cations without EDTA and the basal levels. At high concentrations of Ca^{2+} or Mg^{2+} (2.0 and 3.0 mM) plus 1.5 mM EDTA, the activities were higher when compared with lower concentrations of both cations under the same conditions, however the addition of cation did not revert the activities to basal levels (Fig. 3). These results indicate that the *p*-Nph-5′-TMP hydrolysis is maximal in the presence of basal levels of divalent cations present in the biological fraction. Thus, we may conclude that the enzyme responsible

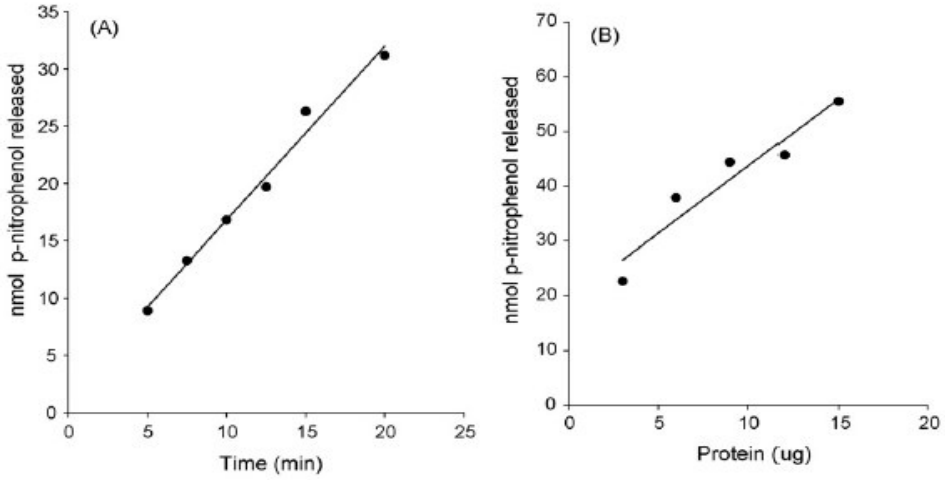

Fig. 2 – (A) Time course of extracellular hydrolysis of *p*-Nph-5′-TMP. The glandular cells (10 μg) were incubated with 0.5 mM *p*-Nph-5′-TMP, as described in Section 2; (B) protein concentration curve for extracellular hydrolysis of *p*-Nph-5′-TMP. The different quantities of glandular cells (0–20 μg) were incubated with 0.5 mM *p*-Nph-5′-TMP, as described in Section 2. Plots are representative of three isolated experiments.

for the hydrolysis of *p*-Nph-5′-TMP in salivary gland cells is cation dependent, but that no additive effects are observed when the two divalent cations tested are added to the reaction medium compared to the basal level. On basis of these results, cations were not added to the reaction medium during further analysis of ectonucleotidase activity. Thus, the ectonucleotidase activities were measured at basal levels of divalent cations.

3.3. *Kinetic parameters*

The hydrolysis of *p*-Nph-5′-TMP was determined at concentrations ranging from 0.075 to 1.0 mM. The results (Fig. 4, inset) indicated that enzymatic activities increased with increasing *p*-Nph-5′-TMP concentration, with a tendency towards saturation at less than 1.0 mM. The Eadie–Hofstee plot for the hydrolysis of *p*-Nph-5′-TMP is shown in Fig. 4. The apparent Michaelis–Menten constant (K_m, app), calculated by linear regression for *p*-Nph-5′-TMP was 280.7 ± 34.2 μM (mean ± S.D., $n = 4$). The calculated maximum velocity (V_{max}, app) for *p*-Nph-5′-TMP hydrolysis was 721.31 ± 225 nmol *p*-nitrophenol/min/mg of protein (mean ± S.D., $n = 4$).

3.4. *Effects of some compounds on ATP, ADP and p-Nph-5′-TMP hydrolysis*

In order to clarify possible differences between the previously described NTPDase[12] and the enzyme described (E-NPP) in the present study in salivary glands, we tested different compounds that act in a distinct manner on these enzymes. Several studies[5,22–25] have shown that these compounds affect ATP and ADP hydrolysis, as well as E-NPP activity. ATP, ADP (2.0 mM) and *p*-Nph-5′-TMP (0.5 mM) were chosen as substrates for the enzymes (Table 1). The E-NPP assays were performed at pH 8.9, while E-NTPDase activity determinations were carried out at pH 7.4. Ouabain, NEM, Levamisole and gadolinium chloride (final concentrations 0.05 and 0.1 mM) did not affect significantly the hydrolysis of any of the three

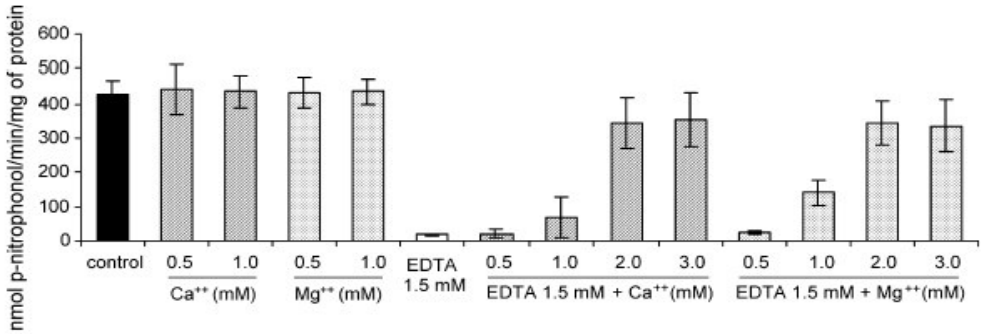

Fig. 3 – Cation dependence for extracellular hydrolysis of *p*-Nph-5′-TMP. The glandular cells (10 μg) were incubated with 0.5 mM *p*-Nph-5′-TMP without cations in the control group (black bar), with Ca^{2+} or Mg^{2+} (hachured bars), only with 1.5 mM EDTA (white bar) and with or without addition of cations (Ca^{2+} or Mg^{2+}) and 1.5 mM EDTA, as indicated. The data are representative of four different experiments.

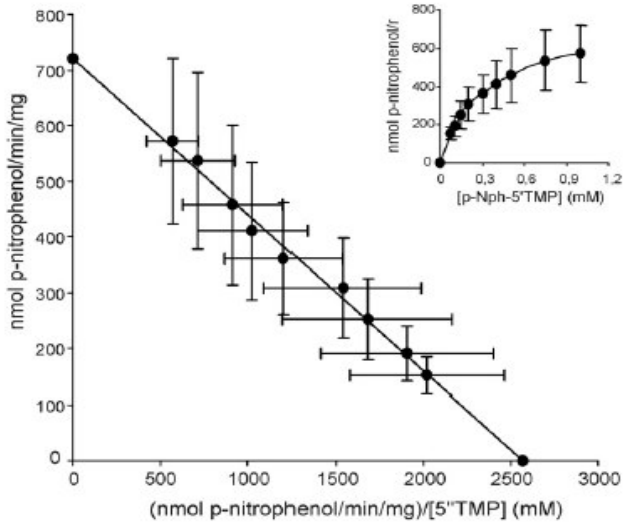

Fig. 4 – Eadie–Hofstee plot for extracellular hydrolysis of p-Nph-5′-TMP. Reaction rate was measured by release of p-nitrophenol, as described in Section 2. Results were obtained with a nucleotide concentration ranging from 0.075 to 1.0 mM p-Nph-5′-TMP without cation addition, as described in Section 2. The insets show activation of the enzyme with the substrate. Best-fit analysis indicated a linear relationship. The Eadie–Hofstee plot was obtained with mean ± S.D. for four independent experiments.

substrates tested. Phenylalanine (0.5 mM) had no effect on ATP and p-Nph-5′-TMP hydrolysis, however, we observed a slight increase in ADP hydrolysis. Suramin (0.25 mM) significantly inhibited the hydrolysis of p-Nph-5′-TMP and ATP by approximately 21.7 and 26.4%, respectively, however, ADP hydrolysis was slightly affected. Sodium azide promoted significant inhibition when tested at concentrations of 10, 20 and 30 mM in ATP (13.7, 12.6 and 15.0%, respectively) and ADP (29.1, 35.9 and 41.8%, respectively) hydrolysis. Conversely, no inhibition was obtained with 1.0 and 5.0 mM sodium azide; this inhibitor did not affect NPP activity. Orthovanadate (0.1 mM) tested at low concentration did not affect ATP and ADP hydrolysis, however, the hydrolysis of p-Nph-5′-TMP was significantly inhibited (27%). Gadolinium chloride, inhibited ATP, ADP and p-Nph-5′-TMP hydrolysis when tested at a final concentration of 0.5 mM; this compound decreased hydrolysis by approximately 30% for ATP and ADP and 20% for p-Nph-5′-TMP. The hydrolysis of p-Nph-5′-TMP was inhibited by AMP (0.5 mM) by approximately 30%, while ADP hydrolysis was significantly increased by 104.2%. Hydrolysis of ATP, ADP and p-Nph-5′-TMP were apparently diminished in the presence of cAMP.

4. Discussion

The ecto-pyrophosphatase/phosphodiesterase (E-NPP) multigene family contains five members. NPP1-3 is a type II transmembrane metalloenzyme with a modular structure composed of a short intracellular domain, a single transmembrane domain and an extracellular domain containing a

Table 1 – Effects of different compounds on p-Nph-5′-TMP, ATP and ADP hydrolysis

Compound	Concentration (mM)	n	% Control enzyme activity		
			ATPase	ADPase	PDEase
Ouabain	10	3	98.39 ± 13.74	104.11 ± 13.72	112.01 ± 6.56
NEM	1.0	5	90.42 ± 8.0	111.31 ± 20.0	100.32 ± 11.0
Levamisole	10	3	92.16 ± 10.8	101.8 ± 5.51	92.98 ± 4.95
Phenylalanine	5	3	99.34 ± 7.07	108.55 ± 2.12*	87.21 ± 11.20
Suramim	0.25	3	73.60 ± 13.02*	90.28 ± 5.6	78.32 ± 8.83*
Sodium azide	1	3	99.60 ± 7.43	104.43 ± 13.8	101.66 ± 32.57
	5	3	98.64 ± 5.73	95.22 ± 17.10	111.94 ± 5.33
	10	4	87.31 ± 2.18*	70. 9 7 ± 15.24*	101.61 ± 5.72
	20	4	87.40 ± 1.79*	64.13 ± 18.22*	101.33 ± 1.11
	30	4	85.08 ± 12.27*	58.21 ± 13.72*	102.61 ± 8.22
Orthovanadate	0.1	5	96.13 ± 10.0	112.30 ± 11.0	73.79 ± 7.0*
Gadolinium Chloride	0.05	3	91.48 ± 11.0	97.13 ± 7.0	93.02 ± 8.0
	0.1	3	80.87 ± 3.0*	88.45 ± 11.0*	93.31 ± 13.0
	0.5	6	62.82 ± 7.0*	61.52 ± 23.0*	81.41 ± 15.0*
AMP	0.5	3	121.92 ± 9.25	204.21 ± 18.37*	69.65 ± 8.92*
camp	3	2	69.47 ± 8.5	72.12 ± 9.89	85.8 ± 4.72

Differential effects of distinct compounds on p-Nph-5′-TMP, ATP and ADP hydrolysis. Results are expressed as mean ± S.D. of at least three experiments for each substrate p-Nph-5′-TMP hydrolysis (pH 8.9) and three experiments for ATP and ADP hydrolysis (pH 7.4). Data were analyzed by one-way ANOVA, followed by Student–Neumann–Keuls post hoc test. Asterisk (*) represents difference from control enzyme activity (100%) ($P < 0.005$). Data are expressed as percentage of control enzyme activity. The 100% values correspond to 487.5 ± 64.5 nmol p-nitrophenol/min/mg of protein for p-Nph-5′TMP hydrolysis and 824.2 ± 183.5 and 480.6 ± 82.3 nmol Pi/min/mg for ATP and ADP hydrolysis, respectively.

conserved catalytic site. NPP4-5 has a predicted type I membrane orientation, but has not yet been functionally characterized.[13] In vitro, these enzymes can hydrolyze both pyrophosphate and phosphodiester bonds and, thereby, produce nucleoside 5′-monophosphates.[13,29] NPP1-5 show a distinct but partially overlapping tissue distribution with other ecto-enzymes. Thus, different isozymes of E-NPPPs can be present in the same tissue.[13,23] Current evidence suggests that E-NPPs have multiple and largely related physiological roles, including nucleotide recycling, modulation of purinergic receptor signaling, regulation of extracellular pyrophosphate levels, stimulation of cell motility and, probably, regulation of the insulin receptor and the activity of ecto-kinases.[11]

In the present study, we showed that the salivary gland cells were able to extracellularly hydrolyze nucleotides. The *p*-Nph-5′-TMP hydrolysis observed, resulting in *p*-nitrophenol formation in vitro, suggests the existence of E-NPP activity in salivary gland cells. In a previous study, we showed that E-NPP has an optimal activity in a pH ranging from 8.5 to 9.0.[13,30] In our study, we tested a Tris–HCl buffering system and the highest enzyme activity was observed at between pH 8.5 and 8.9 (data not shown). These pH values are in accordance with those previously described for E-NPPs.[13,30] We chose a pH of 8.9 to perform the enzyme assays since, at this pH, the specific activity was optimal. Another enzyme that has an optimal activity in alkaline pH is a phosphatase alkaline that hydrolyses ATP and ADP. We discarded the participation of this enzyme using levamisole, a classical inhibitor, which did not affect either of the nucleotides hydrolysis (Table 1). In order to discard the possible participation of classical ATPases in the hydrolysis of the substrates, ATP and ADP, we used ouabain a Na K – ATPase inhibitor, which did not significantly affect the hydrolysis of ATP, ADP and *p*-Nph-5′-TMP. Similar results were observed for NEM, a Ca^{2+}, Mg^{2+}, ATPase inhibitor.

It has been demonstrated that E-NPPs are metalloenzymes, and that their catalytic activity depends on divalent cations.[1,13,11] The enzyme described in this study is dependent on divalent metal cations (Mg^{2+} and Ca^{2+}) since, following the addition of 1.5 mM EDTA to the reaction medium the E-NPP activity was almost completely abolished. The activity was fully restored in the presence of cations (Mg^{2+} and Ca^{2+}), but no additive effects were observed when compared to the control group (basal) without cation addition (Fig. 2). These results indicate that the *p*-Nph-5′-TMP hydrolysis is maximal in the presence of basal levels of divalent cations secreted by the cells or present in the biological fraction. Based on these results, further analysis was carried out without the addition of cations (see Section 2).

Our results demonstrated that salivary gland cells hydrolyze extracellular adenine nucleotides ATP, ADP and *p*-Nph-5′-TMP, suggesting the presence of an E-NTPDase and an E-NPP. In order to prove our hypothesis, we tested the effects of different compounds on ATP, ADP and *p*-Nph-5′-TMP hydrolysis. We observed that some compounds presented different effects with respect to ATP/ADP hydrolysis and *p*-Nph-5′-TMP hydrolysis. Phenylalanine affected only ADP hydrolysis (Table 1). Suramin (0.25 mM), an inhibitor of E-NTPDase and E-NPP,[25,30] significantly inhibited the hydrolysis of ATP and *p*-Nph-5′-TMP, while ADP hydrolysis was slightly affected. Sodium azide, an inhibitor of various ATP diphosphohydrolases,[22,26,27] significantly inhibited ATP and ADP hydrolysis, when tested at high concentrations of 10, 20 and 30 mM (13.7, 12.6 and 15.0%, respectively, and 29.1, 35.9 and 41.8%, respectively) (Table 1). *p*-Nph-5′-TMP hydrolysis was not affected by sodium azide at any concentration, suggesting a co-existence of two enzyme activities responsible for di- and tri-phosphate nucleotide hydrolysis in salivary gland cells.

Orthovanadate (0.1 mM) is known to inhibit transport ATPases and has also been described as an inhibitor of E-NTPDases of salivary gland, mammary gland and rat uterus.[32] Surprisingly, orthovanadate did not affect ATP/ADP hydrolysis, but inhibited *p*-Nph-5′-TMP hydrolysis. Vanadate can mimic transition states for enzymes involving monoester substrates (which are generally presumed to follow a dissociative mechanism) as well as diester substrates (presumed to have a more associative mechanism).[33] As would be expected for such a transition state mimic, vanadate is a potent inhibitor of many phosphoryl transfer enzymes.[34,35] In our study, the E-NPP inhibition probably occurred since vanadate can mimic the transition state for cleavage of a phosphodiester substrate, with histidine, tyrosine and ribose as ligands,[33] and compete with the artificial substrate *p*-Nph-5′-TMP. Furthermore, AMP (0.5 mM) significantly decreased *p*-Nph-5′-TMP hydrolysis; previous studies report that AMP competitively inhibits the E-NPP reaction,[31] probably because it occupies the catalytic site as a product of the E-NPP reaction.[23] On the other hand, AMP significantly enhanced (104.21%) ADP hydrolysis, probably due to an overlapping hydrolysis of ADP by an E-NTPDase and AMP hydrolysis by an ecto-5′-nucleotidase with production of large amounts of Pi. These results are important to support our hypothesis of a co-existence of an E-NPP and an E-NTPDase in salivary gland cells.

Gadolinium has been described as the most potent inhibitor for both membrane-bound and soluble E-NTPDases.[28] In this study, 0.5 mM gadolinium chloride significantly inhibited ATP and ADP hydrolysis and, unexpectedly, we also observed an inhibitory effect on the hydrolysis of *p*-Nph-5′-TMP.

In this study, we evaluated the possible co-localization of an E-NTPDase, 5′-nucleotidase and an E-NPP. We observed that AMP, suramin, orthovanadate, sodium azide (high concentrations) and phenylalanine had different effects on ATP/ADP hydrolysis and on *p*-Nph-5′-TMP, suggesting the presence of two distinct enzyme activities on the ecto-surface of salivary gland cells.

In a previous study, we showed that extracellular hydrolysis of ATP and ADP in salivary gland cells is probably performed by an ATP diphosphohydrolase and that the AMP produced could be hydrolyzed by an ecto-5′-nucleotidase activity.[12] We cannot disregard the possible presence of two or more enzymes that hydrolyze ATP, an ecto-ATP diphosphohydrolase, an ecto-ATPase[14] and a cyclic nucleotide-NPP,[17] which may be co-expressed, as occurs in many other rat tissues.[36,37] E-NPP comprises a versatile group of enzymes with broad substrate specificity. For the first time, in submandibular salivary glands, we present evidence to support the co-localization of an ecto-ATP diphosphohydrolase and an E-NPP. Some aspects of this mechanism should be considered when extrapolating these observations to *in vivo*

conditions. ATP release, especially from neuronal sources, occurs in close proximity to the glandular cells, which may result in the concentration of ATP at neuroeffector junctions that are difficult to determine.[14] In summary, it may be postulated that this enzyme cascade (ecto-ATP diphosphohydrolase plus ecto-5′-nucleotidase), and E-NPP, may participate in the hydrolysis of ATP released as a co-transmitter from nerve terminals at the basal border of cells in the salivary glands. This co-existence, now described in salivary glands, has been previously described in the blood serum and platelets.[25,38] According to Zimmermann,[29] the E-NPP might act as a protection mechanism to prevent subversion of the cell by destroying incoming DNA or RNA. The ectonucleotidases described herein could contribute to reducing the cytotoxic effect of extracellular ATP on the cell. The co-existence of these two enzymes is very important because they can act under different conditions and in a complementary manner for the complete hydrolysis of ATP to adenosine. Further studies are necessary to understand how these enzymes control the nucleotide/nucleoside ratio in different physiological and pathological conditions in the salivary glands.

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3.3.- Capítulo 3- Antidepressants effects on ecto-nucleotidase activities from salivary glands of rats.

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Antidepressant effects on ecto-nucleotidase activities from salivary glands of rats

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Abstract

Adenosine is the product of extracellular hydrolysis of adenine nucleotides in submandibular gland cells (SGC), which occurs by the action of an ecto-nucleotidase cascade constituted by E-NTPDases and ecto-5´-nucleotidase. Antidepressant drugs commonly cause xerostomy and ATP can influence the saliva production. In this study, we have evaluated the effect of three different antidepressant drugs on the activities and expression of ecto-nucleotidases in cell culture from salivary glands. Rats received imipramine (IMI; 10 mg/mL), fluoxetine (FLU; 20 mg/mL) or moclobemide (MOC; 30 mg/mL) by gavage. The drugs were administered once a day for 14 days and then rats were euthanized for cell culture and gene expression analysis. There was a significant increase in ATP (IMI 30.9%; FLU 32.5%; MOC 34.5%) and ADP hydrolysis (IMI 34.3%; FLU 44.5%; MOC 54.1%) in all treatments. AMP hydrolysis was increased after IMI (64.1%) and MOC (65.3%) treatments and was not influenced by FLU. Thus, an increase in the relative expression of NTPDase1 (IMI 96%; MOC 288%), NTPDase2 (IMI 15%; MOC 124%), NTPDase3 (IMI 200%; FLU 91%; MOC 40%), and ecto-5´-nucleotidase (FLU 19%; MOC 74%) from salivary glands of rats submitted to antidepressant therapy was observed. Downregulation of enzyme expression was also observed through PCR analysis for NTPDase2 (FLU 22%) and 5'-nucleotidase (IMI 42%). Relative expression of NTPDase1 was not affected by FLU therapy. These findings indicate that the antidepressant drugs modulate the activities and expression of ecto-nucleotidase and this effect could be involved in the xerostomy mechanism.

Keywords: E-NTPDases; ecto-5´-nucleotidase; antidepressants; xerostomy; salivary glands.

Introduction

Xerostomy has several possible causes and the complaint of dry mouth comes usually from patients treated for hypertensive, psychiatric or urinary problems (Scully, 2003). Pharmacological therapy is the most common cause of reduced salivation and the drugs implicated in dry mouth are antipsychotics, atropine, beta-blockers, antihistamines, and tricyclic antidepressants, which are the most often prescribed psychoactive substances (Streckfus, 1995).

The therapeutic effects of antidepressants are related to effects on neurotransmitters, particularly by inhibiting the monoamine transporter proteins for serotonin and norepinephrine. Selective serotonin re-uptake inhibitors (SSRIs) specifically prevent the re-uptake of serotonin (thereby increasing the level of serotonin in synapses of the brain) whereas earlier monoamine oxidase inhibitors (MAOIs) blocked the degradation of neurotransmitters by enzymes which normally break them down. Tricyclic antidepressants (TCAs) prevent the re-uptake of various neurotransmitters, including serotonin, norepinephrine, and dopamine (Scully, 2003). The SSRIs, like fluoxetine, tend to have fewer side effects than other antidepressants, which include dry mouth, nausea, nervousness, insomnia, sexual problems and headache. Imipramine, a tricyclic antidepressant, has dry mouth as a common side effect and moclobemide (MAOI

antidepressant) has dry mouth as a mild side effect (Rafaelsen et al., 1981; Nelson et al., 1984).

In recent years, a marked increase in knowledge about the mechanisms of action and the effects of extracellular nucleotides in a wide variety of systems occurred. In the salivary glands, Gallacher (1982) presented the first evidence that P2 receptor are localized in parotid salivary gland cells of mouse and extracellular nucleotides, mainly ATP, may regulate important secretory processes in these cells. ATP and other extracellular nucleotides influence epithelial cell functions via P2 receptors (Ralevic and Burnstock, 1998). P2 receptors can be ligand-gated ion channels (P2X) or coupled to heterotrimeric G proteins (P2Y). In salivary glands, four distinct P2 subtypes of ATP receptors have been identified in the ductal and acinar cells: $P2Y_1$, $P2Y_2$, $P2X_4$ and $P2X_7$ (Turner et al., 1999).
At the end of its action, ATP may be recaptured by the cell or hydrolyzed by a cascade of cell-surface-bound enzymes, such as E-NTPDases (nucleoside triphosphate diphosphohydrolases), E-NPPs (ecto-pyrophosphatase/ phosphodiesterases) and ecto-5´-nucleotidase (Zimmermann, 2001). Members of the E-NTPDases family are dominant ecto-nucleotidases (Zimmermann, 2001). Four of the eight members of this family NTPDase1, NTPDase2, NTPDase3, NTPDase8 appear relevant to the control of P2 receptor signaling since they are located at the surface of plasma membrane and hydrolyze nucleotides in the range of concentrations that activates P2 receptors (Picher et al., 1996; Smith and Kirley, 1999; Mateo et al., 1999; Lavoie et al., 2004; Bigonesse et al., 2004). Several types of enzymes mediate the saliva production. In previous studies we

have described the presence of an E-NTPDase and ecto-5´-nucleotidase in acinar cells (Henz et al., 2006, 2007). Other enzymes have been also described in salivary glands such a possible ecto-ATPase activity in rat salivary gland cells and a phospholipase D activity has been shown to be stimulated by ATP in ductal rat submandibular cells (Dowd et al., 1996, 1999; Pochet et al., 2003). Kittel et al., (2004) showed weak immunoreactivity for NTPDase1 whereas NTPDase2 activity (ecto-ATPase) was strong in the plasma membranes of nerve terminals and membranes of nerve fibers in salivary glands.

Studies have shown a relationship between antidepressants and reduction of salivary flow, but the mechanisms involved in this process remain unclear (Koller et al., 2000; Scully, 2003; Kopittke et al., 2006; Choi et al., 2006). Since in previous studies we have described the presence of an E-NTPDase and ecto-ecto-5´-nucleotidase in salivary glands, here we have evaluated the activities and expression of ecto-nucleotidases from salivary glands of rats submitted to antidepressant therapy.

Materials and Methods

Materials

Dulbecco´s Modified Eagle Medium (DMEM) was purchased from Grand Island Biological Company (Grand Island, NY, USA). Collagenase I-S, nucleotides, Hepes and EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal

bovine serum (FBS) was purchased from Cultilab Ltda (São Paulo, SP, Brazil). All the other chemical reagents were also of the analytical grade.

Animals

Male Wistar rats (30 days-old) were maintained in groups of four animals per cage. The animals were kept at room temperature of 22 ± 2°C on a light cycle from 7 a.m. to 7 p.m., receiving rat chow (Nutrilab1, Brazil) and water ad libitum. 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).

Antidepressant Therapy

The animals were divided in four treatment groups: saline, imipramine (IMI; Tofranil1, Biogalênica) 10 mg/mL, fluoxetine (FLU; Prozac, Eli Lilly) 20 mg/mL or moclobemide (MOC; Aurorix, Roche) 30 mg/mL, suspended in saline. All solutions were administered by oral gavage, 1 mL/kg. Antidepressant doses were chosen according to Kopittke et al., (2005). Animals received the antidepressant treatment once a day for 14 days and, subsequently (15 days after the beginning of therapy) they were euthanized for culture procedures.

Isolation and Culture of Submandibular Gland Cells

The animals were sacrificed and the submandibular glands were removed quickly, trimmed of fat and fascia tissues and minced in a small volume of Hank's Buffer Saline Solution (HBSS), pH 7.4. The minced salivary glands were

dispersed in HBSS supplemented with collagenase I-S (1mg/mL). The gland cell clusters were dissociated by pipetting 10 times every 20 minutes with a Pasteur pipette for 2 hours. The collagenase was removed by centrifugation at 700g (5 min) and the cell clusters were then washed with HBSS and centrifuged twice at 40g (5 min) to remove the lysed cells and contaminants (red and endothelial cells). Clusters with 4-5 cells (as observed by phase-contrast microscopy) were maintained in a water-saturated atmosphere with 95% O_2 and 5% CO_2 in Dulbecco´s Modified Eagle Medium (DMEM) with 5% fetal bovine serum (FBS), pH 7.4 for 24 hours (modified from Murphy et al., 1994 and Dowd et al., 1996).

E-NTPDase and ecto-5´-nucleotidase activities

Enzyme assays were performed according to Henz et al., (2006). Briefly, after 24 hours in culture, the SGC were washed and centrifuged three times at 1000g for 3 min with a medium containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose and 10 mM Hepes (pH 7.4). The cell clusters were filtered through a nylon filter (100 mesh) to homogenize the cluster size. The reaction medium, containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose, 2 mM $CaCl_2$ and 10 mM Hepes (pH 7.4) was preincubated with aliquots of 20µL of cell suspension (approximately 15-20 µg of protein per tube) at 37ºC for 1 min in a final volume of 200 µL. The reaction was started by the addition of ATP, ADP or AMP as substrates at a final concentration of 2.0 mM. Incubation times for ATP, ADP and AMP hydrolysis were 4, 5, and 6 min, respectively. The reaction was stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 5% (v/v) and the samples were chilled on ice.

Incubation times and protein concentration were chosen to ensure the linearity of the reaction. In order to correct for non-enzymatic hydrolysis, we performed controls by adding the SGC after the reactions had been stopped with TCA. All assays were carried out in triplicate. Samples were centrifuged at 14 000g for 5 min at 4ºC to precipitate protein and the supernatant was used to measure the amount of inorganic phosphate (Pi) released using the colorimetric method described by Chan et al., (1986). Enzyme activities were expressed as nmoles of Pi released per minute per milligram of protein.

Protein determination

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

Statistical Analysis

The data obtained are expressed as means ± S.D. values of at least eight different experiments and were analyzed by Student's t test. Values of $P \leq 0.05$ were considered statistically significant.

Analysis of Gene Expression by Semi-quantitative RT-PCR

The expression analysis of the E-NTPDases 1-3 and 8, and 5'-nucleotidase was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. In the fifteenth day after treatment with different antidepressants, the salivary glands of rats were isolated and total RNA

extraction, free of protein and DNA contamination, was obtained using TRIzol® reagent (Invitrogen) in accordance with the manufacturer's instructions. The cDNA species were synthesized with SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen, USA) from 3 μg of RNA following the suppliers. RT-PCR reactions were performed for 50 min at 42°C. cDNA (0.5 μL) was used as a template for PCR reactions using specific primers. Rat DNA sequences encoding to NTPDase1 (NM_022587), NTPDase2 (O35795), NTPDase3 (NM_178106), and NTPDase8 (AY536920) were retrieved from GenBank database and aligned using ClustalX program. Regions with low scores of similarity among sequences were used to construct specific primers using Oligos 9.6 program. Each primer was blasted against rat genome in order to confirm its specificity. The strategy adopted to design the primers does not allow cross-amplification. Specific primers were also constructed to 5'-nucleotidase (NM_021576) and β-actin (NM_031144) (Table 1). RT-PCR conditions were optimized in order to determine the number of cycles that would allow product detection within the linear phase of mRNA transcripts amplification. PCR reactions had a volume of 25 μL using a concentration of 0.4 μM of each primer, 200 μM of dNTP, $MgCl_2$ 2mM and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. The following conditions were used for PCR reactions: Initial 1 min denaturation step at 94°C, 1 min annealing step (Table 1), 1 min extension step at 72°C for 35 cycles and a final 10 min extension at 72°C. The fragments length of PCR reactions was confirmed with Low DNA Mass Ladder (Invitrogen, USA) and β-actin was carried out as an internal standard. The relative abundance of each mRNA versus β-

actin was determined by densitometry using the freeware ImageJ 1.37 for Windows.

Results

In order to verify the effects of antidepressant therapy on ecto-nucleotidase activities we have studied the influence of this treatment on ATP, ADP and AMP hydrolysis from submandibular glands cells. ATP hydrolysis (Fig. 1A) was significantly enhanced for all treatments ($P<0.05$): IMI 30.9%, FLU 32.5% and MOC 34.5% when compared to the saline. Fig. 1B shows ADP hydrolysis, which was significantly increased ($P<0.05$) by IMI 34.3%, FLU 44.5% and MOC 54.1% in rats submitted to antidepressants therapy when compared to saline group (216.1 ± 57.6 nmol Pi. min^{-1}. mg^{-1}).

The AMP hydrolysis (Fig. 1C) was also significantly increased ($P<0.05$) for IMI 64.1%) and for MOC 65.3% when compared with the saline. In contrast, FLU did not alter AMP hydrolysis.

The relative expression of enzymes was also analyzed by semi-quantitative RT-PCR. NTPDase8 was not expressed in salivary gland of rats (Fig. 3). Animals submitted to IMI treatment presented an increase in the relative expression of NTPDase1 (96%), NTPDase2 (15%), and NTPDase3 (200%). Meanwhile, the relative expression of ecto-5'-nucleotidase from salivary glands was decreased in 42% by IMI treatment (Fig. 2A). MOC therapy was able to enhance the relative expression of NTPDase1 (288%), NTPDase2 (124%), NTPDase3 (40%) and ecto-5'-nucleotidase (74%) (Fig.2C). Salivary glands of FLU-treated rats have

also shown an increase in relative expression of NTPDase3 (91%) and ecto-5'-nucleotidase (19%). However, relative expression of NTPDase2 was diminished by FLU administration in 22% and NTPDase1 expression was not affected by this therapy (Fig. 2B).

Discussion

The extracellular hydrolysis of ATP to adenosine by ecto-nucleotidases has been reported in several cell types (Casali et al., 2001; Robson et al., 2006; Buffon et al., 2006), including salivary glands (Henz et al., 2006, 2007). These enzymatic activities can regulate the extracellular concentration of adenine nucleotides and nucleoside, modulating their local effects. Degradation of ATP and other nucleotides occurs through a cascade of cell-surface-bound enzymes, including E-NTPDases, E-NPPs and ecto-5´-nucleotidase (EC 3.1.3.5), resulting in the formation of ADP, AMP and adenosine (Zimmermann, 2001). In this study, we demonstrated that FLU, IMI, and MOC are able to alter both activities and expression of E-NTPDases and ecto-5´-nucleotidase in submandibular gland cells.

Tricyclic antidepressants have a wide pharmacological spectrum. Unwanted side effects are common (Feighner, 1999) and become more frequent with age (Koller et al., 2000). Tricyclic antidepressants may alter indirectly (central nervous system), and/or directly (salivary glands) salivary gland functions as a result of their action on modulating α-β-adrenergic and muscarinic neural transmission. In our study, we observed an increase in enzyme activities and expression of E-

NTPDases when ATP and ADP were used as substrates in rats submitted to IMI treatment. Scarpace et al., (1992, 1993) observed that the tricyclic antidepressant desipramine altered signal transduction in rat parotid and submandibular glands. It has been observed that salivary flow was significantly decreased when rats were submitted to IMI treatment (Kopittke et al., 2005). Therefore, considering that ATP and adenosine are important signaling molecules, it is possible to suggest that modulation in nucleotide levels by ecto-nucleotidases during antidepressant therapy can be involved in the regulation of salivary flow. One possible explanation could involve the activation of ecto-nucleotidases, leading to an enhanced degradation of extracellular ATP and its metabolites ADP and AMP. For this reason, the stimulus for Ca^{2+} entrance in the acinar cells is diminished and could reduce the salivary flow. In addition, it has been demonstrated that IMI alters cortical membrane fluidity for in vitro and in vivo studies, and that chronic IMI treatment affects brain membrane architecture and enzyme activities, such as Na^+,K^+-ATPase (Zanatta et al., 2001).

Moreover, our findings have shown that FLU treatment induced an increase on ATP and ADP hydrolysis in submandibular gland cells. Similar results were observed in another study evaluating the effect of FLU on Na^+,K^+-ATPase activity (Zanatta et al., 2001). However, we did not observe activation in ecto-5´-nucleotidase activity and, in a pre-clinical study, fluoxetine showed a tendency to decrease salivation (Kopittke et al., 2005).

Moclobemide induced a significant increase in the hydrolysis of all nucleotides tested. It has been suggested that activation of purinergic receptors by ATP is a

more effective stimulus than activation of muscarinic receptors in terms of elevating $[Ca^{+2}]_i$. Thus, ATP can effectively regulate secretory processes in salivary glands (Soltoff et al., 1990; Dehaye et al., 1999) through the control of its levels by ecto-nucleotidase activities in rats treated with moclobemide.

The alterations observed in ecto-nucleotidase activities could be a consequence of transcriptional control and/or post-translational mechanisms. The antidepressant drugs have been studied in the central nervous system and it is known that chronic treatment by various classes of these drugs may result in a common final pathway of changes in gene expression in the brain (Wong et al., 2004). We have studied the relative expression of E-NTPDases1-3 and ecto-5'-nucleotidase from salivary glands of antidepressant-treated rats since this pharmacological affects the kinetic of ecto-nucleotidases. We have observed a general increase in relative expression of NTPDase1-3 in submandibular salivary glands of rats treated with IMI for 14 days. However, relative expression of ecto-5'-nucleotidase was decreased by this treatment. We also have evaluated the effect of MOC chronic therapy in the expression of ecto-nucleotidases. All enzyme expression tested here was strongly up-regulated by this drug. FLU chronic therapy was also able to up-regulate the relative expression of NTPDase3 and ecto-5'-nucleotidase. However, expression of NTPDase2 was inhibited by FLU and NTPDase1 was not modified by this therapy. Some evidence shows that these antidepressants were able to modulate expression of several different proteins: IMI can decrease the expression of Ndrg2, a member of the N-Myc downstream-regulated genes (Takahashi et al., 2005). Moreover,

MOC, IMI and FLU can enhance the mRNA expression of PRAX-1, a protein involved in the action mechanism of several antidepressants (Chardenot et al., 2002). In addition, FLU can activate the expression of Bcl-2 and Bcl-Xl, proteins involved in the cellular survival (Chiou et al., 2006). On the other hand, chronic treatment with FLU can up or down-regulate several kinds of clock genes, depending on the cerebral region (Uz et al., 2005). These evidences support the modulatory effect of antidepressant drugs on protein expression. The regulation in relative expression of ecto-nucleotidases from salivary gland of rats treated with antidepressants could contribute in part with the increase in enzyme kinetics. The other plausible explanation for the changes in the enzyme activities after antidepressant treatment may involve post-translational events. According to analysis performed in NetPhosk, a kinase-specific prediction of protein phosphorylation sites tool (http://www.cbs.dtu.dk/dk/), E-NTPDases1-3 and ecto-5'-nucleotidase sequences present possible PKC phosphorylation sites. In fact, accumulating evidence suggested that signal transduction cascade including protein phosphorylation is implicated in the neurochemical action of antidepressant agents (Racagni et al., 1992; Hyman and Nestler, 1996). In addiction, chronic antidepressant therapy has been shown to induce changes in the function of protein kinase C, cyclic AMP-dependent protein kinase, and calcium/calmodulin-dependent protein kinase in the brain (Popoli et al., 2000). In addition, there is some evidence that the expression of protein kinase C (PKC) is up-regulated by fluoxetine (Rausch et al., 2002). The activity of PKA can be altered in rat cerebral cortex following two or three weeks of treatment with

different antidepressant agents such as tricyclics, monoamino oxidase inhibitors and selective serotonin re-uptake inhibitors (Nestler et al., 1989; Perez et al., 1989, 1991). Despite all data cited above are related to the rat brain, it could lead us to the hypothesis that phosphorylation may exert a modulation on these enzyme activities in the salivary glands of antidepressant-treated rats. Based on the different expression profiles promoted by different antidepressant therapies, it is possible to suggest that the regulation of relative expression of NTPDase1-3 and ecto-5'-nucleotidase may act in synergy with post-translational events, such as phosphorylation, in salivary glands of rats.

Knowing that ATP can improve the Ca^{2+} influx and this fact contributes to the production of saliva, we can hypothesize that ATP hydrolysis to adenosine by E-NTPDases, E-NPPs and 5'-nucleotidases can be involved with xerostomy. The results reported here showed that the ecto-nucleotidases could be implicated in the most common side effect caused by antidepressant therapy. Further studies are necessary to understand the different extracellular signaling pathways and their influence in the salivary glands and, consequently, in salivary flow or composition.

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Legends to Figures

Figure 1: Effect of antidepressants, imipramine, fluoxetine and moclobemide on the hydrolysis of ATP (A), ADP (B), and AMP (C) by submandibular gland cells from *Wistar* rats. Results are presented as means ± S.D. for at least 8 independent experiments. The symbol * represents the statistical difference in comparison to the saline group. All data were analyzed using Student´s t test. Statistical significance was set at $P \leq 0.05$.

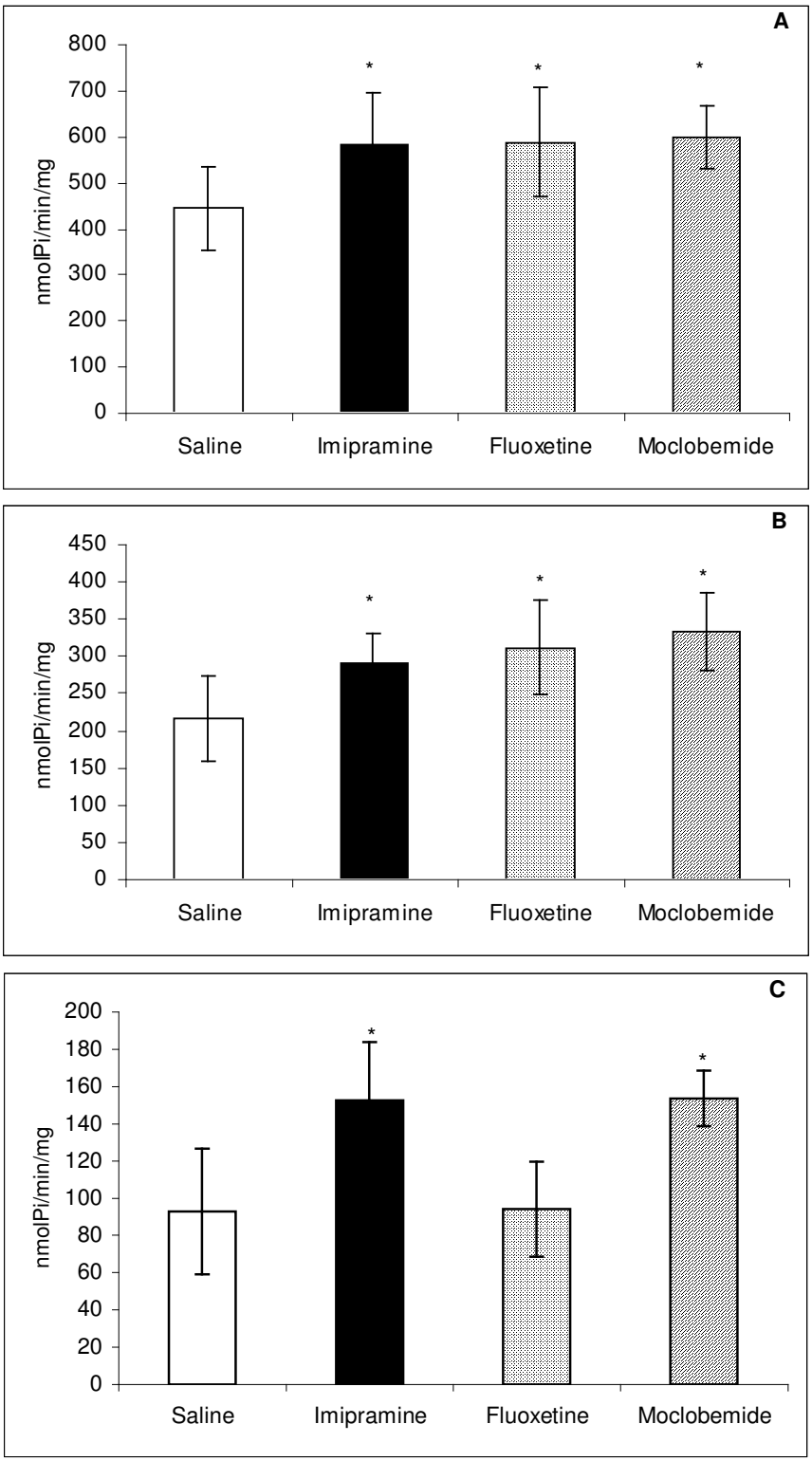
Figure 2: Representative semi-quantitative RT-PCR mRNA for NTPDase1, 2, 3 and ecto-5´-nucleotidase from salivary glands of rats submitted to saline, imipramine, fluoxetine, and moclobemide therapy. The expression was evaluated by ecto-nucleotidases to β-actin mRNA ratio. Three independent experiments were performed with entirely consistent results.

Figure 3: Representative semi-quantitative RT-PCR mRNA for NTPDase8 from liver and salivary glands of näive rats. Three independent experiments were performed with entirely consistent results.

Table 1: Primer sequences and PCR amplification conditions

Gene	1.1.1 GenBank accession number	Primers (5'–3')	T_m (ºC)	PCR product
NTPDase1	NM_022587	F - GATCATCACTGGGCAGGAGGAAGG R - AAGACACCGTTGAAGGCACACTGG	65	543 bp
NTPDase2	O35795	F - GCTGGGTGGGCCGGTGGATACG R - ATTGAAGGCCCGGGGACGCTGAC	66	331 bp
NTPDase3	NM_178106	F - CGGGATCCTTGCTGTGCGTGGCATTTCTT R - TCTAGAGGTGCTCTGGCAGGAATCAGT	65	267 bp
NTPDase8	AY536920	F - CCACACTGTCACTGGCTTCCTTG R - ACGAGGATGTATAGGCCTGAGG	65	394 bp
5'-nucleotidase	NM_021576	F - CCCGGGGGCCACTAGCACCTCA R - GCCTGGACCACGGGAACCTT	65	403 bp
β-actin	NM_031144	F - TATGCCAACACAGTGCTGTCTGG R - TACTCCTGCTTCCTGATCCACAT	58.5	210 bp

Figure 1


A
800
700
600
500
400
300
200
100
0
nmolPi/min/mg
*
*
*
Saline
Imipramine
Fluoxetine
Moclobemide
B
450
400
350
300
250
200
150
100
50
0
nmolPi/min/mg
*
*
*
Saline
Imipramine
Fluoxetine
Moclobemide
C
200
180
160
140
120
100
80
60
40
20
0
nmolPi/min/mg
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Saline
Imipramine
Fluoxetine
Moclobemide

Figure 2

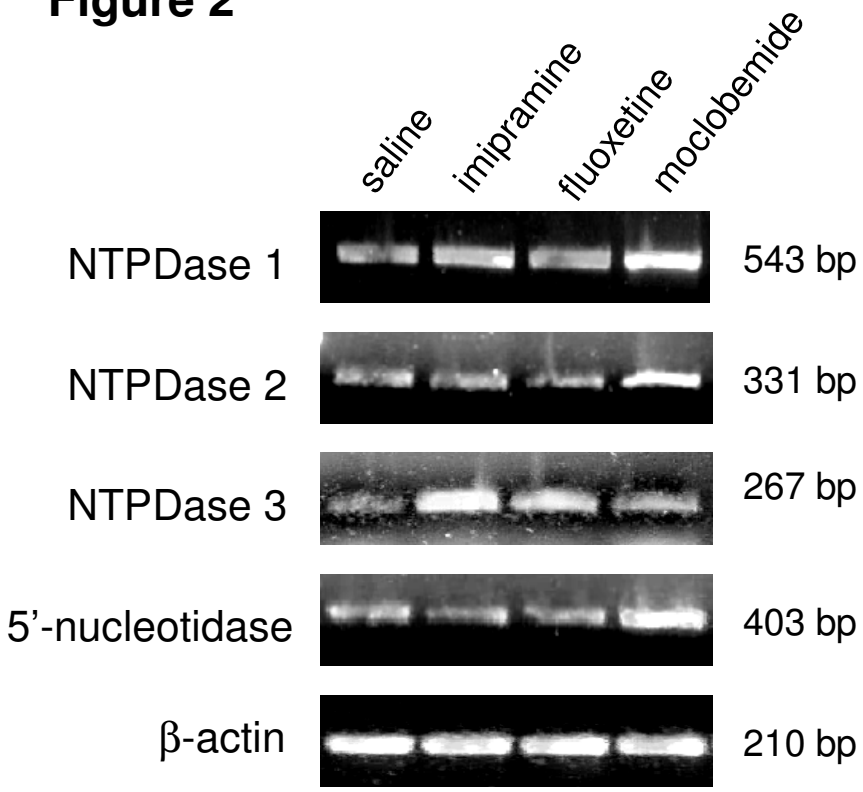
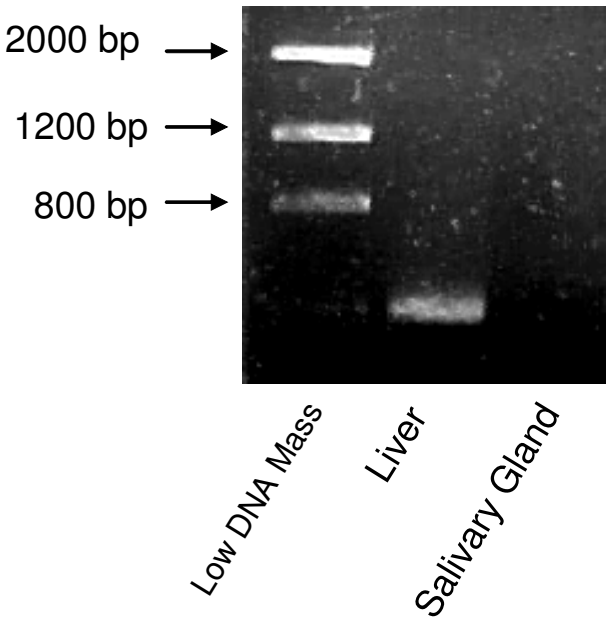

saline
imipramine
fluoxetine
moclobemide
NTPDase 1
543 bp
NTPDase 2
331 bp
NTPDase 3
267 bp
5'-nucleotidase
403 bp
β-actin
210 bp

Figure 3



3.4- Capítulo 4- Influence of antidepressant drugs on E-NPPs from salivary glands of rats

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Influence of antidepressant drugs on E-NPPs from salivary glands of rats

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Abstract

Xerostomy is commonly caused by antidepressant drugs and ATP can influence the saliva production. Adenosine is the product of extracellular hydrolysis of adenine nucleotides in submandibular gland cells, which occurs by the action of ecto-nucleotidases. In this study, we have evaluated the effect of three different antidepressants in the activities of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP1-3) in cells cultured from salivary glands. Rats received imipramine (10 mg/ml), fluoxetine (20 mg/ml) or moclobemide (30 mg/ml) by oral gavage. The drugs were administered once a day for 14 days. Our results showed that the hydrolysis of *p*-nitrophenyl-5'-thymidine monophosphate increased in all treatments. These effects were not consequence of transcriptional control of E-NPP1-3 genes, but it was probably due to changes in phosphorylation state. The results reported here can highlight the importance of ecto-nucleotidases in the most common side effect caused by antidepressant therapy.

Keywords: antidepressants, E-NPPs, salivary glands, xerostomy.

Introduction

Several studies have discussed the role of adenosine triphosphate (ATP) as an extracellular mediator and neurotransmitter in various systems, including salivary glands (Zimmermann, 2001; Kittel et al., 2004). ATP and other extracellular nucleotides influence epithelial cell functions via P2 receptors (Ralevic and Burnstock, 1998). In the salivary glands, ATP can regulate important secretory processes by two types of receptors (Gallacher, 1982; Dehaye et al., 1999). Four ATP receptors have been identified in the ductal and acinar cells: $P2Y_1$, $P2Y_2$, $P2X_4$ and $P2X_7$ (Turner and Camden, 1990; Turner et al., 1999). The inactivation of ATP signaling is promoted by a cascade of cell-surface-bound enzymes constituted by nucleoside triphosphate diphosphohydrolases (NTPDases), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), alkaline phosphatases and ecto-5´-nucleotidase (EC 3.1.3.5) (Zimmermann, 2001).

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). Only NPP1-3, which have a common ancestor, have been implicated in the hydrolysis of nucleotides (Zimmermann, 2000; Bollen et al., 2000; Volmayer et al., 2003; Stefan et al., 2006) whereas NPP6-7 are only known to hydrolyze phosphodiester bonds in lysophospholipids or other choline phosphodiesters (Stefan et al., 2006).

Current evidence suggests that E-NPPs have multiple and largely related physiological roles, including nucleotide recycling, modulation of purinergic receptor signaling, regulation of extracellular pyrophosphate levels, stimulation of cell motility,

activity of ecto-protein kinases and probably regulation of insulin receptor (Goding et al., 2003). These families of enzymes reveal not only overlapping tissue distributions, but also overlapping substrate specificities and functions (Zimmermann, 2001).

Various types of enzymes mediate the saliva production and we have described in a previous study the presence of an E-NTPDase, an ecto-5´-nucleotidase (Henz et al., 2006) and an E-NPP (Henz et al., 2007) in acinar cells. NTPDase1 showed weak immunoreactivity whereas NTPDase2 activity was slightly higher in plasma membranes of nerve terminals and membranes of nerve fibers in salivary glands (Kittel et al., 2004).

Pharmacological therapy is the most common cause of reduced salivation and the most frequently drugs implicated in dry mouth are the tricyclic antidepressants, antipsychotics, atropine, beta-blockers and antihistamines (Streckfus, 1995). Dry mouth is a common side effect caused by antidepressant therapy (Scully, 2003). The main function of antidepressants is to increase the extracellular neurotransmitter concentrations, inhibiting the metabolism and reuptake (BezchlibnyK-Butler and Virani, 2004). Antidepressants include monoamine oxidase inhibitors, tricyclic compounds, selective serotonin and norepinefrine reuptake inhibitors, as well as, some atypical drugs (Galeotti et al., 2002).

Selective serotonin reuptake inhibitors (SSRIs) specifically prevent the reuptake of serotonin (thereby increasing the level of serotonin in synapses of the brain) whereas earlier monoamine oxidase inhibitors (MAOIs) block the degradation of neurotransmitters by enzymes. Tricyclic antidepressants (TCAs) prevent the reuptake of various neurotransmitters, including serotonin, norepinephrine and dopamine (Scully, 2003). The SSRIs, such as fluoxetine, tend to have fewer side effects than other

antidepressants. Some of the side effects that can be caused by SSRIs include dry mouth, nausea, nervousness, insomnia, sexual problems and headache. Imipramine, a tricyclic antidepressant, and moclobemide have also dry mouth as a common side effect (Rafaelsen et al., 1981; Nelson et al., 1984). Several studies have shown a relationship between antidepressants and reduction of salivary flow, but the mechanisms involved in this process remain unclear (Koller et al., 2000; Scully, 2003; Choi et al., 2006; Kopittke et al., 2005). In this study we have evaluated the activities and expression of ecto-pyrophosphatase/phosphodiesterases from salivary glands of rats submitted to antidepressant therapy.

Materials and Methods

Materials

Dulbecco´s Modified Eagle Medium (DMEM) was purchased from Grand Island Biological Company (Grand Island, NY, USA). Collagenase I-S, nucleotides, Hepes and EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Cultilab Ltda (São Paulo, SP, Brazil). All the other chemical reagents were of the highest available quality.

Animals

Male Wistar rats (n=52 / 45-day-old rats) were maintained in groups of four animals per cage. The animals were kept at room temperature of 22 ± 2°C and light cycle from 7 a.m. to 7 p.m. receiving rat chow (Nutrilab1, Brazil) and water *ad libitum*.

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).

Antidepressant Therapy

The animals were divided in four groups: saline, imipramine (IMI; Tofranil, Biogalênica) 10 mg/ml, fluoxetine (FLU; Prozac, Eli Lilly) 20 mg/ml or moclobemide (MOC; Aurorix, Roche) 30 mg/ml, suspended in saline. All solutions were administered by oral gavage, 1 ml/kg. Antidepressant doses were chosen according to Kopittke et al. (2005). Animals received the antidepressant treatment once a day for 14 days and subsequently (15 days after the beginning of therapy) they were euthanized for culture procedure.

Cell Isolation and Culture

Submandibular gland cell (SGC) clusters were obtained from 45-day-old rats. The animals were euthanized and the submandibular glands were removed quickly, trimmed of fat and fascia tissues and minced in a small volume of Hank's Buffer Saline Solution (HBSS), pH 7.4. The minced salivary glands were dispersed in HBSS supplemented with collagenase I-S (1mg/ml). The gland cells were dissociated by pipetting 10 times every 20 minutes with a Pasteur pipette for 2 hours. The collagenase was removed by centrifugation at 700 x *g* (5min) and the cell clusters were then washed with HBSS and centrifuged twice at 40 x *g* (5 min) to remove the lysed cells and contaminants (red and endothelial cells).

Clusters with 4-5 cells (as observed by phase-contrast microscopy) were maintained in a water-saturated atmosphere with 95% air and 5% CO_2 in Dulbecco´s Modified Eagle Medium (DMEM) with 5% fetal bovine serum (FBS), pH 7.4 for 24 hours (modified from Murphy et al., 1994 and Dowd et al., 1996).

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

After 24 hours in culture, the SGC were washed and centrifuged three times at 1000 *g* for 3 min with a medium containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose and 10 mM Hepes (pH 7.4). The cell clusters were filtered through a nylon filter (100 mesh) to homogenize the cluster size. The artificial substrate for E-NPPs, *p*-nitrophenyl 5'-thymidine monophosphate (*p*-Nph-5'-TMP), was used as a substrate marker to evaluate the enzymatic activity, producing p-nitrophenol (Sakura et al., 1998). The reaction medium, containing 135 mM NaCl, 5.0 mM KCl, 10 mM glucose and 50 mM Tris-HCl buffer (pH 8.9), was preincubated with aliquots of 20µl of cell suspension (approximately 10-15µg of protein per tube) at 37ºC for 10 min in a final volume of 200µl. The enzyme reaction was started by the addition of *p*-Nph-5'-TMP to a final concentration of 0.5 mM. After 6 min of incubation, the reaction was stopped by the addition of 200µl 0.2N NaOH and the samples were chilled on ice. Incubation times and protein concentration were chosen to ensure the linearity of the reaction. In order to correct non-enzymatic hydrolysis, we performed controls by adding the SGC after the reaction was stopped with 0.2N NaOH. All assays were carried out in triplicate. 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. Enzyme activities were generally expressed as nmol *p*-nitrophenol released per minute per milligram of protein.

Protein determination

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

Statistical analysis

The data obtained are expressed as means ± S.D of at least five experiments. The results of antidepressant treatments were analyzed by Student's t test or one way ANOVA followed by Tukey test as post hoc. Values of $P<0.05$ were considered significant.

Analysis of gene expression by semi-quantitative RT-PCR

Rat DNA sequences encoding to E-NPP1 (**NM_022587.1**), E-NPP2 splice isoforms (**Q64610**), and E-NPP3 (**NM_178106**) was retrieved from GenBank database and aligned using ClustalX program. Regions with low scores of similarity among sequences were used for searching specific primers, which were designed using the program Oligos 9.6. NPP2 splice isoforms were identified using primers localized upstream and downstream of the splice junction resulting in different PCR products. NPP2 isoform 1 PCR product is 587 bp long (with the intron) whereas NPP2 isoform 2 PCR product is 512 bp long (without the intron) (Table 1). In order to confirm the primers specificity, each primer was blasted against rat genome and it was able to

recognize only its specific target sequence. Thus, the strategy adopted to construct the primers did not allow cross-amplification.

The analysis of the expression of E-NPP1, E-NPP2, and E-NPP3 was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Fourteen days after treatment with different antidepressants, the salivary glands of rats were dissected and immediately frozen with liquid nitrogen for storage in - 80°C freezer. The total RNA extraction, free of protein and DNA contamination, were obtained using Trizol® Reagent (Invitrogen) in accordance with the manufacture instructions. The cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT-PCR from 1 g of total RNA and oligo (dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 50 ºC. cDNA (1 µL) was used as a template for PCR with specific primers for E-NPP1, E-NPP2, and E-NPP3. β-actin PCR was performed as a control for cDNA synthesis. PCR reactions were performed (total volume of 25 µL) using a concentration of 0.4 µM of each primer indicated below and 200 µM and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. Conditions for all PCRs were as follow: Initial 1 min denaturation step at 94 ºC, 1 min at 94 ºC, 1 min annealing step (NPP1: 60 ºC; NPP2: 67 ºC; NPP3: 65 ºC; β -actin: 58.5 ºC), 1 min extension step at 72 ºC for 35 cycles and a final 10 min extension at 72 ºC.

PCR products were submitted to electrophoresis using a 1% agarose gel. The relative abundance of each mRNA versus β-actin was determined by densitometry using the freeware ImageJ 1.37 for Windows.

Each experiment was repeated three times using RNA isolated from independent extractions. The expression analysis was performed in replicate and representative

findings were shown. The normalized expression levels of E-NPP1-3 genes were expressed as mean ± S.E.M and statistically compared by Student´s t test. *P* values ≤ 0.05 were considered significant.

Results

In order to verify if male Wistar rats undergo biochemical changes induced by antidepressant therapy, we have studied the influence of this treatment on *p*-Nph-5'-TMP hydrolysis from cells of SGC. Figure 1 demonstrates the *p*-Nph-5'-TMP nucleotide hydrolysis in cells cultured of salivary submandibular glands for three different antidepressants. The *p*-Nph-5'-TMP hydrolysis (Fig. 1) was significantly enhanced for all treatments ($P<0.05$). The treatment of IMI, FLU and MOC induced an increase of 29% (290 ± 40 nmol Pi. min^{-1}. mg^{-1}), 35% (311 ± 64 nmol Pi. min^{-1}. mg^{-1}) and 34% (333 ± 53 nmol Pi. min^{-1}. mg^{-1}) when compared to the control (216 ± 57 nmol Pi. min^{-1}. mg^{-1}).

The upregulation of E-NPPs activities could be consequence of transcriptional control and/or posttranslational modifications. We evaluated E-NPP1-3 transcripts in SGC after antidepressant treatments. Although the mRNAs of both NPP2 splice isoforms could be easily identified in different brain structures with the strategy adopted (data not shown), it was not possible to detect NPP2 isoform 1 (587 bp) in SGC (Fig. 2).

Animals submitted to antidepressant treatments have not shown an increase in the relative expression for E-NPP1, E-NPP2 and E-NPP3 in SGC. Meanwhile, the relative expression of E-NPP from salivary glands was slightly decreased to E-NPP1 by imipramine and fluoxetine treatments (Fig 2A and B). These results suggest that the increased activity observed after antidepressant treatments could be consequence of

changes in phosphorylation state. In order to verify this hypothesis, the E-NPP1 (NP_445987), E-NPP2 (Q64610) and E-NPP3 (AAH97326) amino acid sequences were analyzed in NetPhosk, a kinase-specific prediction of protein phosphorylation sites tool (http://www.cbs.dtu.dk) (Blom et al., 2004). The results obtained from E-NPP1 sequence indicated the residues Ser271 and Thr713 as potential Protein Kinase C phosphorylation. The same approach with E-NPP2 sequence indicated the residues Ser176, Thr412, Thr493 and Thr866 for Protein Kinase C and Ser396 for Protein Kinase B phosphorylation sites. The residues Ser17, Thr647 and Ser786 as potential Protein Kinase C phosphorylation were recognized in E-NPP3 sequence. The residues were identified with high prediction scores in E-NPP1-3 sequences.

Discussion

The results of the present study demonstrated that IMI, FLU and MOC are able to alter the activities whereas the E-NPP1-3 expressions were not changed in salivary gland.

Antidepressants have been frequently studied in the central nervous system, but in other organs their effects remain unclear. A common side effect caused by antidepressant treatment is dry mouth (Scully, 2003). Systemic diseases, radiation therapy, prescription and non-prescription drugs are important causes of salivary gland disturbances (Mandel, 1980). Saliva components are critical in maintaining oral health and supporting other oral functions. It is well known that several neurotransmitters, such as acetylcholine, substance P, vasoactive intestinal polypeptide, and ATP are co-released in salivary glands (Looms et al., 1998; Harmer et al, 2001). Besides the ACh and ATP co-release following parasympathetic stimulation, extracellular ATP also functions as a regulator of salivary

gland function, probably by the action in four distinct subtypes of P2 receptors identified in the ductal and acinar cells: $P2Y_1$, $P2Y_2$, $P2X_4$ and $P2X_7$ (Turner and Camden, 1990).

Degradation of ATP and other nucleotides can occur through a cascade of cell-surface-bound enzymes named ecto-nucleotidases, including E-NTPDase family, E-NPP family and ecto-5´-nucleotidase (Ralevic and Burnstock, 1998). Extracellular hydrolysis of ATP to adenosine promoted by ecto-nucleotidases has been reported for several cell types (Casali et al., 2001; Robson et al., 2006; Buffon et al., 2006), including salivary glands (Henz et al., 2006; 2007).

Tricyclic antidepressants have shown common side effects, that can occur indirectly (central nervous system) and/or directly (salivary glands) (Feighner, 1999, Koller et al, 2000). In this study, we observed an increase in enzyme activity when *p*-Nph-5'-TMP was used as substrate in rats submitted to IMI treatment. However, there was no significant changes on the expression of E-NPPs. Scarpace et al., (1992, 1993) observed that the tricyclic antidepressant desipramine altered signal transduction in rat parotid and submandibular glands. It has been observed that salivary flow was significantly decreased when rats were submitted to IMI treatment (Kopittke et al., 2005). In addition, it has been demonstrated that IMI alters cortical membrane fluidity for *in vitro* and *in vivo* studies, and that chronic IMI treatment affects brain membrane architecture and enzyme activities, such as Na^+,K^+-ATPase (Zanatta et al, 2001). Our findings have also shown that fluoxetine and moclobemide treatment induced an increase on *p*-Nph-5'-TMP hydrolysis in acinar cells. Similar results were observed in evaluating the effect of fluoxetine on Na^+,K^+-ATPase activity (Zanatta et al., 2001). It has been suggested that activation of purinergic receptors by ATP is a more effective stimulus than

activation of muscarinic receptors in terms of elevating $[Ca^{+2}]_i$. Thus, ATP can effectively regulate secretory processes in salivary glands (Soltoff et al, 1990; Dehaye et al, 1999). Therefore, considering that ATP and adenosine are important signaling molecules, it is possible to suggest that the control of ATP levels by ecto-nucleotidases during antidepressant therapy can be involved in the regulation of salivary flow. One possible hypothesis could involve the activation of ecto-nucleotidases, leading to an enhanced degradation of extracellular ATP and its metabolites ADP and AMP. For this reason, the stimulus for Ca^{2+} entrance in the acinar cells is diminished, promoting a reduction in the salivary flow.

The alterations observed in ecto-nucleotidase could be a consequence of transcriptional control and/or post-translational mechanisms. The antidepressant drugs have been studied in the central nervous system and it is known that chronic treatment by various classes of these drugs may result in a common, final pathway of changes in gene expression in the brain (Wong et al., 2004). We have studied the relative expression of E-NPP 1, 2, 3 from salivary glands of antidepressant-treated rats, since this pharmacological therapy seems to affect the kinetic of ecto-nucleotidases. The results have shown that animals submitted to antidepressant treatments did not present significant changes in the relative expression for E-NPP1, E-NPP2 and E-NPP3 in SGC. However, a slight decrease of E-NPP1 expression was observed for the imipramine and fluoxetine treatments (Fig 2A and 2B).

The plausible explanation for the changes in the enzyme activities after antidepressant treatment may involve post-translational events. According to analysis performed in NetPhosk, E-NPP1-3 sequences showed high prediction scores for PKC phosphorylation sites. In fact, accumulating evidence suggested that signal transduction

cascade including protein phosphorylation is implicated in the neurochemical action of antidepressant agents (Racagni et al., 1992; Hyman & Nestler, 1996). In addiction, chronic antidepressant therapy has been shown to induce changes in the function of protein kinase C, cyclic AMP-dependent protein kinase, and calcium/calmodulin-dependent protein kinase in the brain (Popoli et al., 2000). In addition, there is some evidence that the expression of protein kinase C (PKC) is upregulated by fluoxetine (Rausch et al., 2002). The activity of PKA can be altered in rat cerebral cortex following two or three weeks of treatment with different antidepressant agents such as tricyclics, monoamino oxidase inhibitors and selective serotonin reuptake inhibitors (Nestler et al., 1989; Perez et al., 1989, 1991). Despite all data cited above are related to the rat brain, it could lead us to the hypothesis that phosphorylation may exert a modulation on these enzyme activities in the salivary glands of antidepressant treated rats.

The role of nucleotides in regulating saliva have not been reported, although the effects of nucleotides on second messenger levels, ion fluxes, and protein secretion in salivary cells suggest important roles for P2 receptors in modulating the production and composition of saliva (Turner et al., 1999). ATP and other nucleotides can promote an increase in membrane condutance, and this fact contributes to the production of saliva. In our study, nucleotide hydrolysis by E-NPPs was enhanced; this fact can modify the membrane conductance and may be influencing the common side effects of antidepressant therapy, including the xerostomy. Further studies are necessary to understand the different extracellular signaling pathways involved in salivary secretion.

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Legend to Figures

Figure 1: Effect of antidepressant drugs on E-NPP activity in submandibular gland cells. Bars represent mean± S.D for five different experiments. *Represents significant statistical difference by One-way ANOVA followed by Tukey test as post hoc.

Figure 2: Relative gene expression patterns after imipramine (IMI), fluoxetine (FLU), and moclobemide (MOC) chronic treatment for NPP1 (A and B), NPP2 (C and D), NPP3 (E and F), and β-actin in salivary glands of rats. Three independent experiments were performed, with entirely consistent results.

Table 1: PCR primer design

NPP	GenBank accession number	Primers sequences	PCR product
NPP1[#]	NP_445987	F 5'-GAATTCTTGAGTGGCTACAGCTTCCTA-3' R 5'-CTCTAGAAATGCTGGGTTTGGCTCCCGGCA-3'	410 bp
NPP2 (1)* NPP2 (2)	Q64610	F 5'-CCATGCCAGACGAAGTCAGCCGACC-3' R 5'-CCAAACACGTTTGAAGGCGGGGTAC-3'	587 bp 512 bp
NPP3	AAH97326	F 5'-GAGAAGACAAATTTGCCATTTGGGAGG-3' R 5'-TCTCATTATTTCCTTTGATTGCGGGAG-3'	301 bp
β-actin	NP_112406	F 5'-TATGCCAACACAGTGCTGTCTGG-3' R 5'-TACTCCTGCTTCCTGATCCACAT-3'	210 bp

(*) Represents the splice isoform of NPP2. (#) Primer sequences were obtained from Vollmayer et al., 2001.

Figure 1

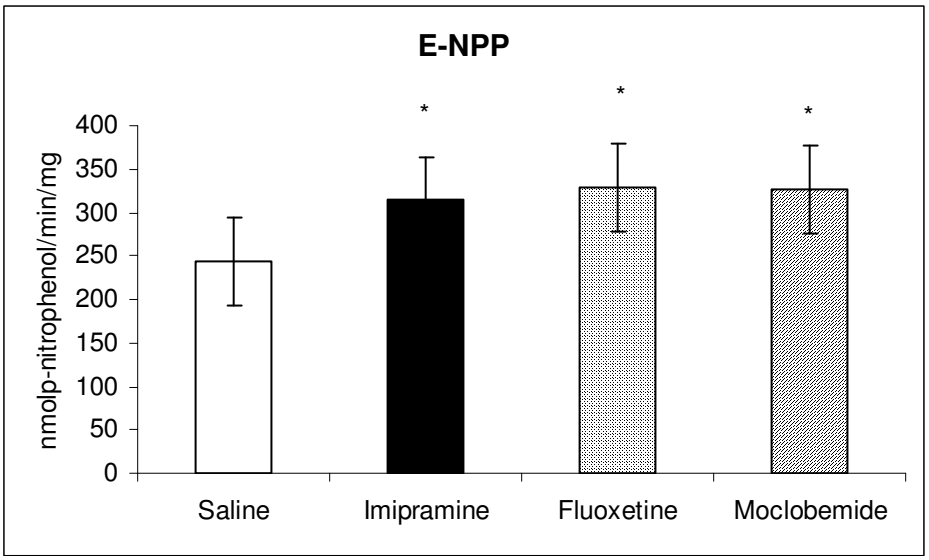
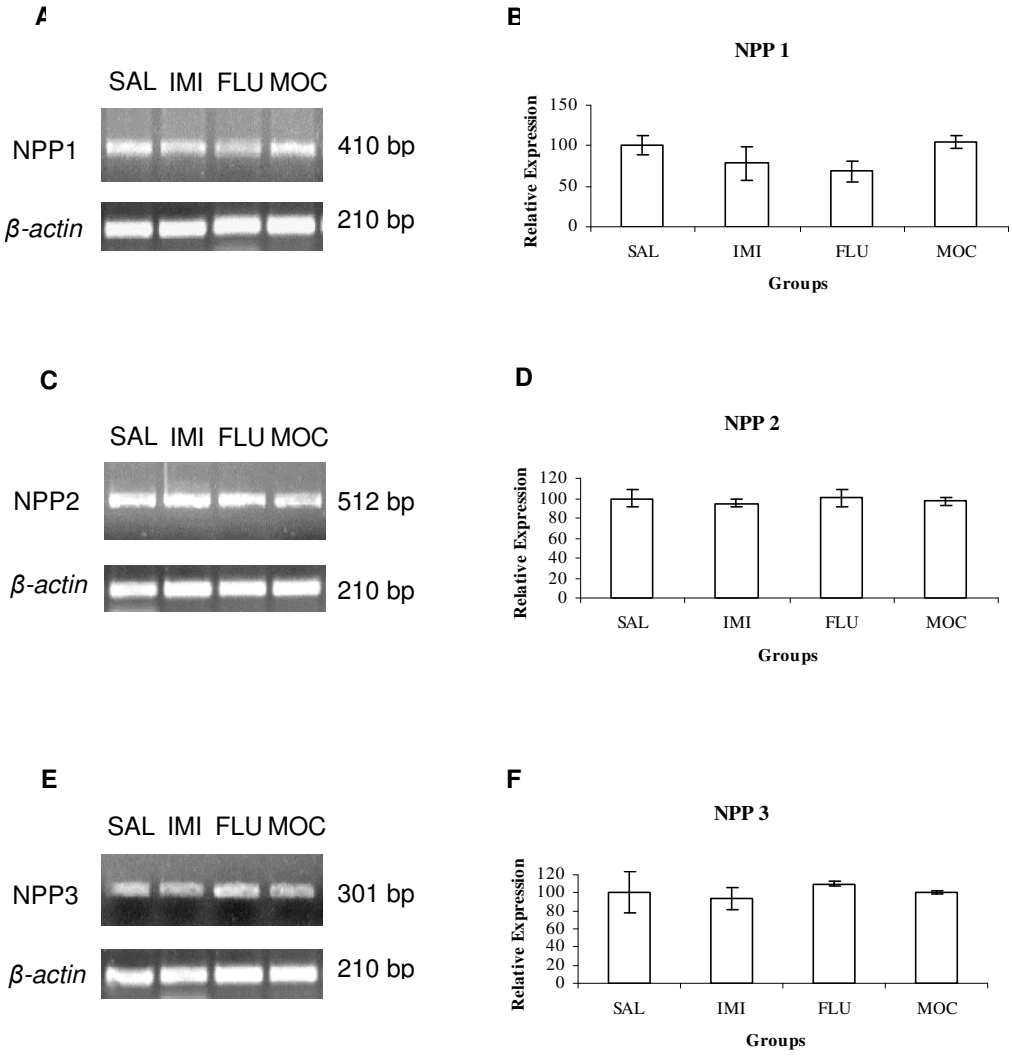

E-NPP
nmolp-nitrophenol/min/mg
400
350
300
250
200
150
100
50
0
*
*
*
Saline
Imipramine
Fluoxetine
Moclobemide

Figure 2


A
SAL IMI FLU MOC
NPP1
410 bp
β-actin
210 bp
B
NPP 1
Relative Expression
150
100
50
0
SAL
IMI
FLU
MOC
Groups
C
SAL IMI FLU MOC
NPP2
512 bp
β-actin
210 bp
D
NPP 2
Relative Expression
120
100
80
60
40
20
0
SAL
IMI
FLU
MOC
Groups
E
SAL IMI FLU MOC
NPP3
301 bp
β-actin
210 bp
F
NPP 3
Relative Expression
120
100
80
60
40
20
0
SAL
IMI
FLU
MOC
Groups

4. DISCUSSÃO

A saliva é produzida pelas glândulas salivares e desempenha um papel fundamental na manutenção da saúde bucal. Quando a produção de saliva é prejudicada por doenças ou pela utilização de medicamentos, a prevalência de cárie e doença periodontal aumenta. A secreção salivar é um processo constante que é estimulado pelo sistema nervoso autônomo. A função secretora das glândulas salivares é regulada pelo sistema simpático e parassimpático e por seus receptores (Baum, 1993; Looms *et al*., 1998). Neurotransmissores como a acetilcolina, noradrenalina, peptídeo vasointestinal ativo e substância P estimulam receptores presentes na membrana plasmática das células das glândulas salivares (Dehaye *et al*., 1999). Além disso, receptores e neurotransmissores não-adrenérgicos e não-colinérgicos podem desempenhar um importante papel na fisiologia salivar (Baum e Wellner, 1999).

Nucleotídeos extracelulares purínicos (ATP, ADP) e pirimidínicos (UTP e UDP) e o nucleosídeo adenosina são moléculas sinalizadoras importantes. Entre os efeitos promovidos por essas moléculas encontram-se as secreções exócrinas, tais como a saliva (Ralevic e Burnstock, 1998; Burnstock, 2006). Nucleotídeos e nucleosídeos derivados da adenina também podem ser liberados por diversos tipos celulares em condições patofisiológicas, modulando uma variedade de efeitos (Schwiebert e Zsembery *et al*, 2003) .O ATP é liberado de fontes pré-sinápticas para o meio extracelular juntamente com outros neurotransmissores, como a acetilcolina (Vizi *et al*., 1997), a noradrenalina

(Kennedy, 1996), a serotonina (Potter e White, 1980) e o glutamato (Inoue, 1998). A liberação de ATP ocorre através de exocitose em um processso dependente de cálcio (Phillis e Wu, 1981). Após ser liberado, o ATP pode interagir com receptores presentes na membrana (Burnstock e Willians, 2000), ser degradado por enzimas como as ectonucleotidases ou ser utilizado como substrato por ecto-kinases (Vizi e Sperlagh, 1999).

Tem sido demonstrado que o ATP extracelular é uma importante molécula sinalizadora em diferentes tecidos constituídos por células epiteliais, como é o caso da glândula salivar (Schwiebert e Zsembery, 2003). Estudos mostram que o ATP poderia mobilizar estoques intracelulares de cálcio nos ácinos da glândula parótida (Soltoff *et al*., 1990). Também foi demonstrado que o UTP aumenta o cálcio intracelular em uma linhagem de células ductais (Yu e Turner, 1991). Um aumento no cálcio intracelular nas células acinares desencadeia o movimento de íons e água, contribuindo efetivamente para a elaboração da saliva primária. O ATP também parece regular a secreção salivar e influenciar trocas iônicas, inclusive nos ductos (Novak, 2003). Entretanto, o ADP tem mostrado ser menos potente do que o ATP, ao passo que o AMP é menos efetivo e a adenosina parece não ter efeito (Gallacher, 1982). Tem sido observado que o ATP secretado para o lúmen dos ductos funciona como um sinalizador ductal (Turner *et al*., 1999). O ATP também pode ativar canais de Na^{+}/Ca^{+2} e transportadores que irão trocar Na^{+}/H^{+} e Cl^{-}/HCO_3^{+}(Novak, 2003). O ATP e seu produto final de reação, a adenosina, normalmente tem efeitos antagônicos, providenciando um elegante mecanismo homeostático de regulação (Fields e Burnstock, 2006).

Normalmente, a ativação dos receptores para adenosina leva de uma maneira geral a uma redução de trabalho e consumo de oxigênio por células e órgãos, ou seja, a adenosina tem o efeito de um “metabólito retaliatório” (Newby, 1984). Embora a adenosina seja um importante sinalizador em vários tipos celulares, sua ação nas glândulas salivares ainda não foi estudada, nem seus receptores descritos.

Efeitos dos nucleotídeos nos níveis de segundos mensageiros, fluxo de íons e secreção protéica, em glândulas salivares, fortemente sugerem importantes papéis para os receptores P2 na modulação da composição e produção da saliva (Turner *et al*., 1990). Nas glândulas salivares, quatro subtipos distintos de receptores P2 têm sido identificados nas células acinares e ductais: $P2Y_1$, $P2Y_2$, $P2X_4$ e $P2X_7$ (Turner e Camden, 1990). Os receptores $P2X_4$ e $P2X_7$ são canais de cátions não seletivos ativados pelo ATP com alta e baixa afinidade, respectivamente (Soltoff *et al*, 1992; 1993). Os receptores $P2X_7$ foram descritos em células acinares da glândula parótida, mas seu significado funcional permanece desconhecido (Gibbons *et al*., 2001). Efeitos citotóxicos do ATP em células da microglia, macrófagos e outras células imunes que expressam receptores $P2X_7$ foram relatados (Pizzo *et al*., 1992; Zambon *et al*., 1994). Entretanto, um papel similar nas glândulas salivares não foi descrito. Pochet *et al*. (2003) demonstraram que o ATP foi capaz de estimular a atividade da fosfolipase D em células ductais da glândula submandibular de uma maneira dose dependente. Os receptores $P2X_7$ podem ativar essa enzima em linfócitos humanos, macrófagos, placenta humana, astrócitos tipo 2 de ratos e células

acinares da glândula submandibular (Pochet *et al*., 2003). O receptor $P2X_4$ é ativado por concentrações em torno de 10 µM de ATP, tanto na glândula parótida quanto na submandibular (Turner *et al*., 1999). Ambos os receptores são expressos nos ácinos, mas sua localização ainda não foi determinada (Buell *et al*., 1996). O receptor $P2Y_1$ têm sido detectado por RT-PCR e a mobilização de cálcio em resposta ao ADP, ATP e análogos dos nucleotídeos da adenina, sugere que esse receptor é mais ativo em células isoladas a partir de animais imaturos (Park *et al*., 1997). Entretanto, a localização desse receptor ainda não foi definida (ácinos x ductos). Amsallem *et al*. (1996) apresentaram dados farmacológicos consistentes em que os receptores $P2Y_1$ funcionais foram identificados em células ductais de ratos adultos. O receptor $P2Y_2$ estaria relacionado com situações de dano tecidual nas glândulas salivares (Turner *et al*., 1997). A expressão do receptor $P2Y_2$ também estava aumentada na glândula submandibular em um modelo que simula a doença auto-imune da Síndrome de Sjögren em ratos (NOD.B10) (Ann *et al*., 2005). Como as células acinares expressam receptores $P2X_4$ e $P2X_7$ e também $P2Y_1$ e $P2Y_2$, a hidrólise de ATP é importante, uma vez que os receptores $P2X_7$ podem mediar inflamações e morte celular (Pizzo *et al*., 1992; Zambom *et al*., 1994).

A sinalização mediada pelo ATP pode ser inativada pela ação de um grupo de enzimas denominadas de ectonucleotidases. As ectonucleotidases constituem um eficiente mecanismo de controle dos níveis de nucleotídeos e nucleosídeos no espaço extracelular (Zimmermann, 1996; Zimmermann, 2001). Várias famílias de ectonucleotidases podem degradar os nucleotídeos extracelulares, dentre as quais

podemos citar os membros da família das E-NTPDases (Ecto-nucleosídeo trifosfato difosfoidrolases), os membros da familia das E-NPPs (Ecto-nucleotídeo pirofosfatase/fosfodiesterases, PDEase, EC 3.1.4.1) e as fosfatases alcalinas. Os nucleotídeos monofosfatados podem ser hidrolisados pela ecto-5'-nucleotidase e pelas fosfatases alcalinas (Zimmermann, 2001).

Nosso grupo de pesquisa tem estudado alterações nas atividades das ectonucleotidases em diferentes condições patológicas e fisiológicas nas mais diversas frações biológicas, como por exemplo: alteração na hidrólise de ATP, ADP e AMP em soro de ratas que tiveram privação de hormônios esteróides (Pochmann *et al*, 2004); diminuição na hidrólise de ATP e ADP em plaquetas de ratos com tumor tratados com aspirina (Buffon *et al*., 2004); ratos com alteração nos hormônios tireoidianos (hipertireoidismo ou hipotireoidismo) tiveram a hidrólise de ATP, ADP e AMP alterada em sinaptossomas de hipocampo e córtex cerebral (Bruno *et al*, 2005).

O efeito do ATP nas células da glândula salivar tem sido investigado (Murphy *et al*., 1994; Tenneti *et al*., 1998; Pochet *et al*., 2003) e o controle da concentração de agonistas dos receptores P2 por ectonucleotidases é um fator importante na sinalização mediada pelos nucleotídeos (Turner *et al*., 1999; Dowd e Zeng, 1999; Kittel *et al*., 2004).Entretanto, o papel das ectonucleotidases nas células das glândulas salivares ainda não foi elucidado. Dessa forma, considerando o papel dos nucleotídeos extracelulares, a ampla distribuição dos purinoreceptores, a sua presença nas células das glândulas salivares e a importância das ectonucleotidases no controle dos efeitos dos nucleotídeos,

consideramos importante avaliar a presença e o comportamento das ectonucleotidases nas células das glândulas salivares.

No primeiro capítulo, descrevemos uma atividade enzimática em células acinares que compartilha características bioquímicas já descritas para a familia das E-NTPDases: 1) pH ótimo em torno de 7,4; 2) dependência de cátions divalentes tanto para a hidrólise do ATP quanto do ADP; 3) valores calculados para a hidrólise do ATP de K_{Mapp} 224 ± 8 µM e $V_{máx}$ 2275 ± 153 nmol Pi/mim/mg, e para o ADP de K_{Mapp} 163 ± 15 µM e $V_{máx}$ 941 ± 96 nmol Pi/mim/mg. A hidrólise do ATP pode ocorrer através de uma E-NTPDase ou por combinações de enzimas que mimetizam essa atividade enzimática. Para nos assegurarmos que a hidrólise dos nucleotídeos era realizada pela mesma enzima, utilizamos o plote de competição de Chevillard, e os resultados demonstraram que as hidrólises do ATP e ADP ocorrem no mesmo sítio ativo. A razão de hidrólise ATP/ADP foi de aproximadamente 2.42, sugerindo a presença de uma NTPDase 3, que hidrolisa ATP aproximadamente 3 vezes mais do que o ADP.

Os dados demonstraram que o AMP, produto final da hidrólise de ATP e ADP pelas E-NTPDases e substrato para a ecto-5'-nucleotidase, também foi hidrolisado pelas células acinares. As constantes cinéticas para o AMP foram: K_{Mapp} 117 ± 5 µM e $V_{máx}$ 175 ± 5 nmol Pi/mim/mg. Esta enzima também demonstrou dependência de cátions divalentes. Embora normalmente a ecto-5'-nucleotidase seja considerada Mg^{+2}–dependente, em nosso estudo a enzima foi ativada também pelo Ca^{+2}, estando de acordo com observações de outro estudo (Casali *et al*., 2001).

No capítulo 1, demonstramos que as células acinares foram capazes de hidrolisar o ATP, ADP e o AMP, e que essa hidrólise ocorreu provavelmente pela ação de uma E-NTPDase e uma ecto-5'- nucleotidase. A co-existência dessas duas enzimas é muito importante, pois sugere sua participação em uma cascata enzimática, que irá levar à completa hidrólise do ATP até adenosina, controlando dessa maneira a concentração de nucleotídeos/nucleosídeos nas células acinares.

O ATP também pode ser hidrolisado por ectonucleotidases da familia das E-NPPs. Por este motivo, no segundo capítulo caracterizamos essa familia de enzimas nas células acinares. O substrato marcador da atividade NPP, *p*-Nph-5'-TMP, foi testado e verificamos que as células acinares foram capazes de hidrolisar esse substrato artificial. Além disso, outras características bioquímicas já descritas para as E-NPPs também foram observadas, como atividade enzimática ótima em pH alcalino (entre 8.5 e 8.9), dependência de cátions divalentes, valores de K_{Mapp} de 280.7 ± 34.2 µM e $V_{máx}$ de 721.31 ± 225 nmol *p*-nitrophenol/mim/mg.

Para confirmar a presença deste sistema multi-enzimático nas células acinares, testamos *in vitro* o efeito de diferentes compostos sobre a hidrólise de ATP, ADP e *p*-Nph-5'-TMP. Com o objetivo de descartar a participação de ATPases clássicas na hidrólise do ATP foram testados a ouabaína, um inibidor clássico da Na^{+},K^{+}-ATPase, e o NEM, inibidor da Ca^{+2}, Mg^{+2}- ATPase, sendo que ambos não afetaram a hidrólise de nenhum dos substratos testados. Para descartar a participação da fosfatase alcalina, uma enzima que tem atividade

ótima em pH alcalino e que hidrolisa ATP e ADP, foi testado o levamisole, um inibidor clássico dessa enzima. Foi observado que a hidrólise do ATP e do ADP não foi afetada. Suramin, um inibidor de E-NTPDases e E-NPPs, inibiu significativamente a hidrólise de ATP e *p*-Nph-5'-TMP, enquanto que a hidrólise de ADP foi levemente afetada. A azida sódica, considerado um inibidor de ATP difosfoidrolases (NTPDase1) em altas concentrações (10, 20 e 30 mM), inibiu a hidrólise do ATP e do ADP, e não teve efeito sobre a hidrólise de *p*-Nph-5'-TMP. O AMP inibiu a hidrólise do *p*-Nph-5'-TMP, o que está de acordo com outros estudos que relatam uma competição do AMP pela NPP (Bollen *et al*., 2000).

Baseados nos resultados obtidos, sugerimos pela primeira vez a co-existência de uma E-NPP, uma E-NTPDase e uma 5´-nucleotidase na superfície extracelular das células acinares. A presença de diferentes ectonucleotidases parece ser importante na modulação do efeito do ATP liberado na proximidade das células acinares. A co-existência dessas enzimas já foi descrita em outros sistemas, como no soro e em plaquetas (Oses *et al*., 2004; Fürstenau *et al*., 2006), e parece estar relacionada com a diminuição da citoxicidade do ATP no meio extracelular. Além disso, estas enzimas podem atuar em diferentes situações, e de uma maneira complementar na hidrólise do ATP até adenosina.

A fim de investigarmos possíveis papéis para as enzimas caracterizadas nas células acinares, foi avaliado o efeito de fármacos antidepressivos nessas células. A xerostomia tem várias causas e uma das mais comuns é o uso de medicamentos como antipsicóticos, beta-bloqueadores, anti-histamínicos e antidepressivos tricíclicos, que são as substâncias psicoativas mais

freqüentemente prescritas (Streckfus, 1995). O efeito terapêutico dos antidepressivos está relacionado com o controle dos níveis de neurotransmissores, principalmente a norepinefrina e a serotonina. Os recaptadores seletivos de serotonina (SSRIs) previnem a recaptação de serotonina enquanto que os inibidores da monoamino oxidase (MAOIs) bloqueiam a destruição dos neurotransmissores. Os antidepressivos tricíclicos (TCAs) previnem a recaptação de vários neurotransmissores, como serotonina, norepinefrina e dopamina (Scully, 2003). Todos os fármacos antidepressivos citados possuem efeitos colaterais, entre eles a xerostomia em diferentes graus (Rafaelsen *et al*., 1981; Nelson *et al*., 1984). Uma vez que em estudos pré-clínicos foi demonstrado que os antidepressivos alteraram a composição e o fluxo salivar, consideramos importante avaliar o comportamento das ectonucleotidases em ratos submetidos à terapia com antidepressivos. Portanto, no terceiro capítulo investigamos o efeito de fármacos antidepressivos sobre a expressão e a atividade das E-NTPDases em células acinares e no quarto capítulo avaliamos a expressão e a atividade das E-NPPs após o tratamento com imipramina (IMI), fluoxetina (FLU) e moclobemide (MOC). Podemos observar que a hidrólise do ATP foi significativamente aumentada em aproximadamente 30% em todos os tratamentos. Em relação à hidrólise do ADP, também observamos um aumento promovido pela IMI (34.3%), FLU (44.5%) e MOC (51%). A hidrólise do AMP foi significativamente aumentada em torno de 60% para a IMI e MOC, enquanto para a FLU permaneceu inalterada.

A degradação mais rápida do ATP e seus metabólitos ADP e AMP poderia influenciar o fluxo salivar, uma vez que a entrada de Ca^{+2} nas células acinares é modulada pelo ATP. Além disso, foi demonstrado em estudos *in vivo* e *in vitro* que a fluidez da membrana cortical está alterada em tratamentos com a IMI, e que seu uso crônico afeta a arquitetura da membrana cerebral e atividades enzimáticas como a Na^{+},k^{+}-ATPase (Zanatta *et al*., 2001). O aumento na hidrólise de ATP e ADP pela FLU e de todos os nucleotídeos para a IMI e MOC poderia afetar o influxo de cálcio para o interior da célula, bem como diminuir o estímulo causado pelo ATP, levando dessa maneira a uma menor produção de saliva.

A análise da expressão relativa das enzimas foi realizada através de RT-PCR. Avaliamos a presença da NTPdase 1, 2, 3, e também da NTPdase 8, pois essa ecto-enzima foi recentemente caracterizada e seria um intermediário funcional em relação à hidrólise dos nucleotídeos (Bigonesse *et al*., 2004). Em nosso estudo não identificamos a presença da NTPDase 8 nas células acinares. O tratamento com IMI aumentou a expressão em 96% da NTPDase 1, em 15% da NTPDase 2, e em 200% da NTPDase 3, enquanto a expressão da ecto-5'-nucleotidase diminuiu. O MOC foi capaz de aumentar a expressão relativa da NTPDase 1 em 288%, da NTPDase 2 em 124%, da NTPDase 3 em 40%, e da ecto-5'-nucleotidase em 74%. A FLU aumentou a expressão da NTPDase 3 em 91% e da ecto-5'-nucleotidase em 19%. Os antidepressivos podem modular a expressão de diferentes tipos de proteínas. A IMI pode diminuir a expressão do Ndrg2, um membro do N-Myc gene "downstream" regulador (Takahashi *et al*.,

2005). Além disso, a IMI, FLU e o MOC podem aumentar a expressão de mRNA da PRAX-!, uma proteína envolvida no mecanismo de ação de vários antidepressivos (Chardenot *et al*., 2002). O tratamento com FLU também pode aumentar ou diminuir a regulação de “clock” genes e ativar a expressão de proteínas envolvidas na sobrevivência celular (Uz *et al*., 2005; Chiou *et al*., 2006).

No quarto capítulo avaliamos o comportamento das E-NPPs após o tratamento com os antidepressivos. Observamos que a hidrólise do *p*-Nph-5'-TMP estava significativamente aumentada para todos os tratamentos ($P<0.05$). Esse aumento foi de 29% para a IMI, 35% para a FLU e 34% para o MOC.

A expressão relativa da E-NPP1, E-NPP2 e E-NPP3 não mostrou alteração em nenhum dos tratamentos testados (IMI, FLU e MOC) nas células acinares. Por outro lado, a expressão relativa estava levemente diminuída para a E-NPP1 após o tratamento com IMI e FLU (Fig 2A and B).

Uma possibilidade para as alterações observadas nas atividades enzimáticas após o tratamento com os antidepressivos pode envolver eventos pós–translacionais. As análises realizadas no “NetPhosk” (http://www.cbs.dtu.dk/dk/), uma ferramenta de predição de sítios de fosforilação para quinases específicas, demonstraram que as ectonucleotidases avaliadas em nosso trabalho, E-NTPDases1,2,3, E-NPPs1-3 e ecto-5’-nucleotidase, apresentam possíveis sítios de fosforilação pela PKC. Evidências sugerem que a cascata de transdução de sinal envolvendo fosforilação de proteínas está implicada na ação neuroquímica dos antidepressivos (Racagni *et al*., 1992;

Hyman e Nestler, 1996). Tem sido demonstrado que a terapia com antidepressivos induz mudanças na função da proteína quinase dependente de AMP cíclico e na proteína quinase dependente de cálcio/calmodulina no cérebro (Popoli *et al*, 2000). A expressão da proteína quinase C parece ser aumentada pela FLU (Raush *et al*., 2002). Diferentes antidepressivos como os tricíclicos, inibidores da monoamino oxidase e recaptadores seletivos de serotonina têm alterado a atividade da PKA no córtex cerebral após 2-3 semanas de tratamento (Nestler *et al*., 1989; Perez *et al*., 1989,1991). Esses dados, embora sejam relacionados ao cérebro, nos permitem sugerir que a fosforilação poderia modular efeitos dos antidepressivos na glândula salivar. Além disso, alterações na fluidez da membrana poderiam modificar a atividade das ectonucleotidases nas células acinares. Como a liberação dos neurotransmissores atua diretamente na estimulação da produção da saliva, a modulação dos níveis de ATP poderia influenciar a diminuição do fluxo salivar observado em pacientes submetidos à terapia com antidepressivos. Levando-se em consideração que os neurotransmissores são fundamentais para a produção de saliva, e de que o ATP aumenta o influxo de Ca^{+2} para o interior da célula ativando o processo secretório, parece razoável sugerir que as ectonucleotidases, como as E-NTPDases, E-NPPs e ecto-5'-nucleotidase possam estar envolvidas nos processos que levam a xerostomia. Entretanto, mais estudos são necessários para elucidar os mecanismos envolvidos e procurar alternativas terapêuticas.

O principal achado dessa tese foi demonstrar a presença de um sistema multi-enzimático nas células acinares capaz de metabolizar as purinas do meio

extracelular. Nossos resultados abrem novas perspectivas para o estudo e o modo de atuação das ectonucleotidases nas glândulas salivares, uma vez que a cultura das células acinares foi padronizada em nosso laboratório. Investigações adicionais usando esse modelo podem ser úteis para elucidar os efeitos de medicamentos e de condições patológicas *in vivo*, que possam interferir na produção e na constituição da saliva.

5. CONCLUSÕES

Os resultados encontrados nesse trabalho mostraram que as células acinares foram capazes de hidrolisar ATP, ADP e AMP, sugerindo a presença de E-NTPDases e de uma ecto-5'-nucleotidase. Também verificamos que as células acinares das glândulas salivares foram capazes de hidrolisar *p*-Nph-5´-TMP, sugerindo a presença de uma E-NPP;

Os resultados encontrados nos permitem sugerir pela primeira vez a co-existência de um sistema multi-enzimático nas células acinares das glândulas salivares, ou seja, a presença de diferentes famílias na mesma célula, que poderiam atuar de uma maneira complementar em diferentes situações fisiológicas ou patológicas controlando os níveis extracelulares de nucleotídeos.

Verificamos que a atividade e a expressão das enzimas descritas nesse trabalho estavam alteradas em ratos submetidos à terapia com diferentes antidepressivos, mostrando que o controle dos níveis extracelulares de nucleotídeos pelas ectonucleotidases pode ser influenciado por situações sistêmicas, que por sua vez podem interferir na secreção salivar.

Um melhor entendimento da função das ectonucleotidases nas células acinares, modulando os níveis dos nucleotídeos da adenina é importante tendo em vista o papel do ATP nos mecanismos de secreção salivar.

6. PERSPECTIVAS

1) Estudar o efeito *in vivo* de outros medicamentos amplamente utilizados, como os anti-hipertensivos, no comportamento das enzimas descritas e correlacionar com o fluxo e a composição da saliva.
2) Analisar o efeito de doenças sistêmicas, como o diabetes, na atividade e expressão das enzimas descritas e sua correlação com o fluxo e composição da saliva.
3) Determinar a localização das enzimas nas células das glândulas salivares
4) Estudar a presença dos receptores de adenosina nas células das glândulas salivares e possíveis papéis para a adenosina nessas células.
5) Estudar possíveis drogas sialogogas que possam minimizar os efeitos da xerostomia.

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