UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA

MODULAÇÃO DO SISTEMA GLUTAMATÉRGICO: ESTUDO DOS EFEITOS DO ÁCIDO QUINOLÍNICO E DOS DERIVADOS DA GUANINA.

Rejane Giacomelli Tavares

Orientador: Prof. Dr. Diogo O. Souza
Co-orientadora: Prof[a]. Dr[a]. Carla Inês Tasca

Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas- Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul (UFRGS) como requisito parcial para obtenção do título de Doutor em Bioquímica.

Fevereiro, 2005

À Deus, fonte inesgotável de força.

AGRADECIMENTOS

Ao Diogo Souza, meu orientador, pela oportunidade, incentivo e confiança em mim depositada ao longo deste trabalho.

Á Carla Tasca, minha co-orientadora, por ter me apoiado durante todo o tempo, não só com sugestões e auxílio prático, mas principalmente com amizade, muito obrigada!

Ao André Schmidt, muito mais que um grande amigo, uma pessoa essencial para que este trabalho fosse realizado, pela paciência, atenção e constante disponibilidade, mesmo quando distante!

À Candice e Jamile, bolsistas de iniciação científica, pelo auxílio na execução da parte prática deste trabalho.

À Lúcia Martini, pela amizade, ajuda e incentivo.

A todo pessoal dos laboratórios 26 e 28, pela amizade e disponibilidade em ajudar.

Ao Departamento de Bioquímica- UFRGS, pela oportunidade.

Às fontes financiadoras de pesquisa: CNPq, FAPERGS, PROPESQ/ UFRGS e PRONEX.

Às minhas irmãs, pelo constante apoio.

Ao Eduardo, meu chefe, pela imensa compreensão, carinho, e por me possibilitar oportunidades de tempo, principalmente na fase final do trabalho. Muito obrigada!

SUMÁRIO

LISTA DE FIGURAS

LISTA DE SÍMBOLOS E ABREVIATURAS

$\Delta\Psi$	Potencial elétrico
ΔpH	Potencial de prótons
AG	Glutaminase
AMPA	Ácido α-amino-3-hidróxi-5-metil-4-isoxazol propiônico
AQ	Ácido quinolínico
ATP	Adenosina-5'-trifosfato
EAAC1	Transportador de aminoácido excitatório em ratos
EAAT1	Transportador de glutamato e aspartato em humanos
EAAT2	Transportador de glutamato em humanos
EAAT3	Transportador de aminoácido excitatório em humanos
EAAT4	Transportador de aminoácido excitatório em humanos
EAAT5	Transportador de aminoácido excitatório em humanos
GABA	Ácido γ-aminobutírico
GAD	Glutamato descarboxilase
GDP	Guanosina-5'-difosfato
GDPβ-S	Guanosina-5'-O-2-tiodifosfato
GFAP	Proteína glial fibrilar ácida
GLAST	Transportador de glutamato e aspartato em ratos
GLT-1	Transportador de glutamato em ratos
Gln	Glutamina
Glu	Glutamato
GMP	Guanosina-5'-monofosfato
GppNHp	5'-guanosina-5'-(imido)-trifosfato
GTP	Guanosina-5'-trifosfato
GUO	Guanosina
iGluR	Receptores glutamatérgicos ionotrópicos
i.c.v	Intra-cérebro-ventricular
i.p.	Intra-peritonial

KA	Ácido caínico
LTD	Depressão de longa duração
LTP	Potenciação de longa duração
mGluR	Receptores glutamatérgicos metabotrópicos
MK-801	(+)-5-metil-10,11-diidro-5-H-dibenzo[a,d]ciclohepten-5,10-imina ou dizocilpina
NAD^+	Nicotinamida adenina dinucleotídeo
NMDA	N-metil-D-aspartato
PTZ	Pentilenotetrazol
SNC	Sistema Nervoso Central
VGLUT1-3	Transportador de glutamato em vesículas sinápticas

Outras abreviaturas encontram-se detalhadas no texto.

Apresentação

A presente tese encontra-se organizada como a seguir descrito: No item **I. Introdução** encontram-se informações gerais, relativas aos assuntos foco deste trabalho. Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo publicado, artigos submetidos à publicação e artigo em fase final de revisão pelos autores, para ser submetido à publicação, os quais encontram-se organizados em Capítulos, no item **II.Resultados**. Material e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e representam a íntegra deste estudo.

O item **III. Considerações Finais**, encontrado no final desta tese, apresenta interpretações e comentários gerais sobre todos os artigos científicos e manuscritos aqui apresentados.

O item **IV. Conclusões** apresenta conclusões pontuais, relacionadas aos objetivos desta tese.

O item **V. Referências Bibliográficas** refere-se somente às citações que aparecem na introdução e considerações finais desta tese.

RESUMO

O aminoácido glutamato é o principal neurotransmissor excitatório do SNC de mamíferos e participa de funções importantes como cognição, memória, aprendizagem e plasticidade neuronal. Porém, excessiva estimulação dos receptores glutamatérgicos pode resultar em morte celular, processo este denominado excitotoxicidade e que está associado à processos neurodegenerativos. A remoção do glutamato da fenda sináptica, que ocorre através de transportadores dependentes de sódio de alta afinidade, localizados principalmente nos astrócitos, é o principal mecanismo modulatório das ações glutamatérgicas e responsável pela manutenção de concentrações extracelulares abaixo dos níveis neurotóxicos. O Ácido quinolínico (AQ), um agonista NMDA, é uma potente neurotoxina endógena, cujo acúmulo no cérebro parece estar envolvido na etiopatologia das convulsões. Entretanto, apesar do seu envolvimento em muitas doenças, os mecanismos moleculares e danos cerebrais ainda não são perfeitamente elucidados. Os derivados da guanina com funções extracelulares, sejam os nucleotídeos GTP, GDP ou GMP, e o nucleosídeo guanosina mostraram exercer ações tróficas em células neurais, bem como modular o sistema glutamatérgico. Os resultados demonstram que vários sistemas de transporte de glutamato são afetados pela ação do ácido quinolínico, e que os nucleotídeos da guanina podem exercer ação modulatória destas respostas. Nos estudos *in vitro*, o AQ estimulou a liberação de glutamato em sinaptossomas e inibiu a captação de glutamato por astrócitos. Observou-se ainda que o AQ inibiu a captação vesicular de glutamato, porém os nucleotídeos da guanina foram capazes de prevenir esta inibição, indicando uma possível modulação neste tranportador. Nos estudos *in vivo*, usando um modelo experimental de indução de convulsão por AQ, observou-se que a liberação sinaptossomal glutamatérgica também está estimulada, porém este efeito foi completamente abolido pela guanosina, quando este nucleosídeo foi capaz de prevenir a convulsão. AQ também estimula a captação vesicular de glutamato e inibe a captação vesicular de GABA; da mesma forma, os nucleotídeos da guanina exercem seus efeitos modulatórios, já que tanto a inibição quanto o aumento de captação retornaram aos níveis do controle quando houve prevenção da convulsão. Adicionalmente, estas alterações na captação vesicular de glutamato parecem ser relacionadas ao AQ, já que em outros modelos (picrotoxina, cainato, cafeína, PTZ ou eletrochoque transcorneal) não foram observadas alterações. Nossos resultados sugerem que os nucleotídeos da guanina exercem importante função como neuromoduladores e ainda neuroprotetores.

Abstract

Glutamate is the main excitatory neurotransmitter in mammalian CNS, involved in processes such as plasticity, learning and memory, and neural development. However, an excessive glutamate receptors activation can induce intracellular events which lead to the neural death through excitotoxic events, wich are associated to the etiology of neuodegeneratives disorders. The removal of glutamate from the synaptic cleft, which occurs by high affinity of sodium-dependent transporters, located mainly in astrocyte membranes, is the major mechanism for modulating of glutamate actions, responsible for maintaining its extracellular concentrations below neurotoxic levels. Quinolinic acid (QA), an NMDA agonist, is a potent endogenous neurotoxin. Accumulation of QA in the brain seems to be involved in the ethiopatogeny of convulsions. However, in spite of its involvement in many diseases, the molecular mechanisms linking QA and brain damage are far from understood. Extracellular guanine-based purines (GBPs), namely the nucleotides GTP, GDP, GMP and the nucleoside guanosine have been shown to exert trophic effects on neural cells, as well as to modulate of the glutamatergic system. The results demonstrate that various systems of glutamate transport are affected by action of QA, and guanine nucleotides exert modulatory effect. In *in vitro* studies, QA stimulates glutamate release in synaptosomes and inhibits the glutamato uptake by astrocytes. We observed that QA inhibits glutamate vesicular uptake, however guanine nucleotides prevent this inhibition, indicating a possible modulation of this transporter. In *in vivo* studies, using a experimental model of QA-induced seizures, we observed that synaptosomal glutamate release is stimulated, and this effect was completely abolished by guanosina. QA stimulates the glutamate uptake and inhibits the GABA uptake, and guanine nucleotides exert modulatory effect, because both effects are abolished when animals not displaying seizures. Additionally, this alterations in vesicular glutamate uptake appears are related with QA, because in other models of seizure (picrotoxin, kainate, caffeine, PTZ or maximal transcorneal electroshock) we do not observed any alterations. Our results suggest that guanine nucleotides exert an important role as neuromodulators and neuroprotectors.

I. INTRODUÇÃO

I.1- GLUTAMATO E TRANSMISSÃO SINÁPTICA

A transmissão sináptica, processo pelo qual os neurônios se comunicam com as células alvo, é essencial para todos os processos neuronais, desde um simples reflexo até o complexo processamento de informações nas regiões cerebrais especializadas. Esta transmissão sináptica é comumente mediada pela liberação de mensageiros químicos (neurotransmissores) do neurônio pré-sináptico e subsequente reconhecimento destas moléculas por receptores específicos nas células alvo pós-sinápticas apropriadas (Nicholls, 1993).

Estudos em junção neuromuscular de rã demonstraram que os neurotransmissores são liberados do terminal pré-sináptico em 'quanta', por um mecanismo dependente do influxo de cálcio no terminal nervoso (Katz, 1967). A microscopia eletrônica de tecido nervoso central e periférico revelou a presença de pequenas organelas, de tamanho uniforme e tendo aproximadamente 50 nm de diâmetro, localizadas nos terminais nervosos, denominadas vesículas sinápticas (De Robertis, 1964). Estas estruturas acumulam ativamente neurotransmissores, por um processo dependente de ATP.

Quando os terminais pré-sinápticos recebem um potencial de ação, ocorre a despolarização da membrana sináptica, com consequente influxo de íons cálcio através dos canais de cálcio sensíveis à voltagem (Nicholls, 1993). A seguir, as vesículas se fundem à membrana plasmática neuronal e ocorre a liberação do glutamato para a fenda sináptica, por exocitose (Bennet & Scheller,1994) (Figura 1). Uma vez liberado, este pode exercer suas ações através da ligação aos receptores ionotrópicos e/ou metabotrópicos,

principalmente pós-sinápticos, promovendo influxo de íons ou desencadeando a produção de segundos-mensageiros.

.

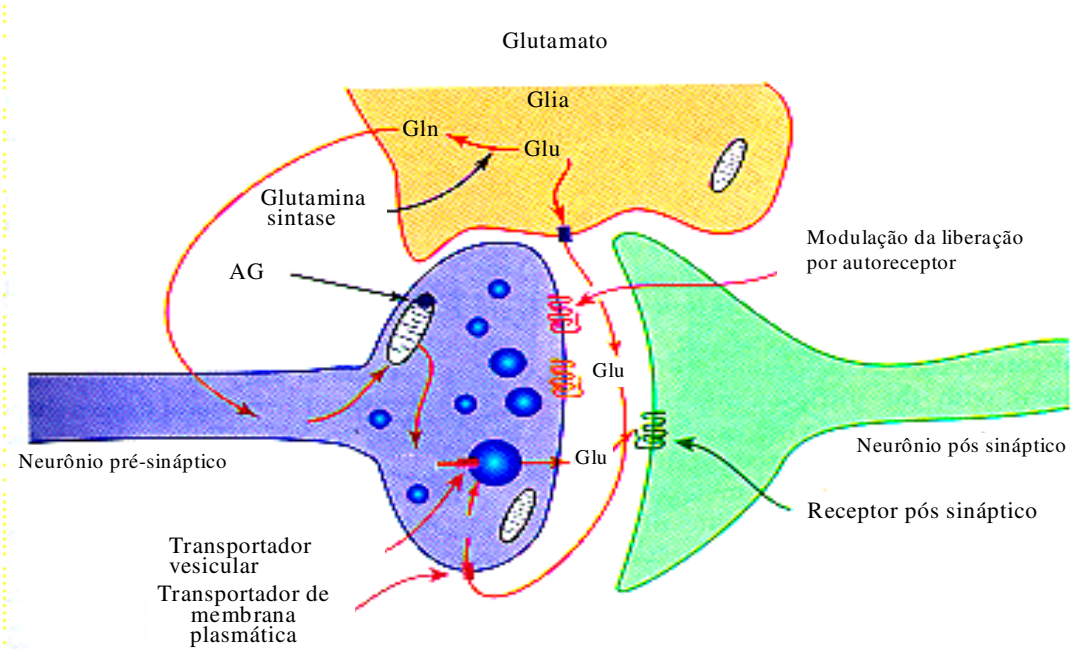


Fig. 1 - Neurotransmissão glutamatérgica. Abreviaturas: Glu, glutamato; Gln, glutamina; AG, glutaminase

O glutamato é o aminoácido livre mais abundante no sistema nervoso central (SNC). A maior parte do glutamato presente no tecido nervoso (70 %) apresenta funções metabólicas (preponderantemente biossíntese de proteínas, entre outras) idênticas às exercidas por este aminoácido nos outros tecidos (Dingledine & McBain, 1994). Além dessas funções, o glutamato é o principal neurotransmissor excitatório de mamíferos, participando em diversas funções cerebrais, como cognição, aprendizado e memória e

plasticidade neural (Collingridge & Lester, 1989; Izquierdo, 1994; Ozawa *et al*., 1998, Danbolt, 2001).

Além disso, o glutamato também promove alterações de longa duração na excitabilidade neuronal, como a indução da potenciação de longa duração (LTP), na transmissão sináptica em neurônios do hipocampo e do córtex visual, e a depressão de longa duração (LTD) no cerebelo e córtex visual (Artola & Singer, 1987; Ito, 1989). Por outro lado, é amplamente conhecido que a excitação excessiva dos receptores glutamatérgicos pode provocar dano ou morte neuronal, processo denominado de excitotoxicidade (Olney *et al*.,1978; Olney *et al*., 1980). Este mecanismo tem sido frequentemente relacionado com dano neural isquêmico (Choi & Rothman, 1990; Olney, 1990), a doenças neurodegenerativas agudas, como a epilepsia (Dingledine *et al*., 1991; Price, 1999), hipóxia, anóxia e traumatismo craniano; e doenças neurodegenerativas crônicas, como a doença de Alzheimer, a síndrome de Huntington, processos neurológicos relacionados à infecção pelo vírus HIV, encefalopatia hepática (Beal *et al*., 1986; Lipton & Rosenberg, 1994; Price, 1999), além de doenças genéticas, como a homocisteinúria, a hiperprolinemia e a hiperamonemia (Dingledine *et al*.,1991; Meldrum & Garthwate, 1991; Lipton & Rosenberg, 1994; Price, 1999; Obrenovitch *et al*., 2000; Maragakis & Rothstein, 2004).

O glutamato pode ser sintetizado a partir de numerosos precursores, tais como: i) outros aminoácidos, por transaminação, ii) oxaloacetato ou 2-oxoglutarato produzidos pela oxidação da glicose no ciclo de Krebs, iii) glutamina, pela ação da glutaminase, iv) outras vias menos importantes, a partir da ornitina e prolina (Fonnum, 1984). No entanto, ainda não se pode afirmar que alguma destas vias seja específica para o "pool" neurotransmissor deste aminoácido. A compartimentalização entre "pools" metabólico e neurotransmissor é

realizada través da captação e armazenamento do glutamato neurotransmissor em vesículas sinápticas, as quais podem acumular concentrações dez vezes maiores (100 mM) que as normalmente encontradas no citosol dos terminais pré-sinápticos (10 mM) (Nicholls & Atwell, 1990) .

Este fato é de crucial importância por permitir que as vesículas sinápticas sejam rapidamente preenchidas durante períodos de alta atividade, sem depleção do "pool" deste neurotransmissor nos terminais nervosos (Maycox *et al*., 1990). O cérebro possui uma grande quantidade de glutamato (aproximadamente 5-15 mmol/kg de peso, dependendo da região), mas somente uma pequena fração deste total é mantido extracelularmente. A concentração nos fluídos extracelulares e no líquor está normalmente em torno de 3-4 μM e 10 μM, respectivamente. Consequentemente, o gradiente de concentração de glutamato através da membrana plasmática é muito grande. A maior concentração deste neurotransmissor é encontrada dentro dos terminais nervosos (Danbolt, 2001). Assim, a distribuição do glutamato neste equilíbrio dinâmico (liberação e captação) é fundamental para a ação deste neurotransmissor, já que não existem enzimas extracelulares que metabolizem glutamato com alguma eficiência (Johnston, 1981).

A diversidade funcional do glutamato como neurotransmissor é devida à grande variedade de receptores existentes, que podem ser classificados de acordo com suas propriedades farmacológicas e fisiológicas (Ozawa *et al*, 1998). De uma forma geral, estes são divididos em duas classes: receptores ionotrópicos e receptores metabotrópicos (Figura 2).

Os *receptores ionotrópicos (iGluR)* são canais iônicos que permeiam a passagem de cátions específicos, e sua ativação promove a despolarização da membrana sináptica,

desencadeando uma resposta excitatória. Estes se subdividem, de acordo com a sua sensibilidade a agonistas, propriedadaes farmacológicas e fisiológicas, em receptores N-metil-D-aspartato (NMDA), ácido α-amino-3-hidróxi-5-metil-4-isoxazol propiônico (AMPA) e ácido caínico (KA). Todos os subtipos de receptores são ativados por glutamato, porém cada um deles é ativado seletivamente por um agonista diferente. Embora os recetores ionotrópicos glutamatérgicos sejam divididos nas três subclasses já referidas, estudos de clonagem molecular e de expressão gênica têm revelado uma diversidade muito maior (Ozawa *et al*.,1998). Os receptores AMPA e KA são entidades distintas, no entanto, como eles não podem ser claramente distinguidos pelo uso de agonistas ou antagonistas, geralmente recebem a denominação comum de receptores não-NMDA. Os receptores AMPA são amplamente distribuídos no SNC, com predomínio nas regiões do hipocampo, regiões CA1 e CA3 do giro denteado e na camada molecular do cerebelo (Cotman *et al*., 1995; Ozawa *et al*.,1998). Estes receptores medeiam a neurotransmissão excitatória rápida e são canais com grande permeabilidade a Na^+ e K^+ e, principalmente, com baixa permeabilidade a Ca^{+2}. Os receptores KA ainda não tem sua função bem definida, porém sabe-se que, ao contrário dos receptores AMPA, são potencialmente permeáveis a íons Ca^{+2} (Ozawa *et al*.,1998).

Os receptores NMDA estão amplamente distribuídos em todo o cérebro e também medeiam a neurotransmissão excitatória no SNC, porém por vias diferentes dos receptores AMPA. São caracterizados por serem altamente permeáveis aos íons Ca^{+2}, possuírem cinética de abertura lenta e dependente de voltagem, ou seja, a ativação do receptor NMDA e o influxo de íons através deste, só ocorrem se a membrana neuronal for previamente despolarizada, por exemplo, através da ativação de receptores do tipo AMPA, permitindo a

saída de íons Mg^{+2}, que bloqueiam este canal quando a membrana neuronal encontra-se em estado de repouso (Edmonds *et al*., 1995).

Embora a ativação dos receptores NMDA ocorra mais lentamente do que a ativação dos receptores AMPA, esta se prolonga por um período maior após a liberação de glutamato na fenda sináptica. Além do sítio de ligação para glutamato/NMDA, possuem também sítio para ligação do co-agonista endógeno glicina, sítio para união de bloqueadores (por exemplo, MK-801) e sítios regulatórios múltiplos (redox, zinco, poliaminas) (Martin *et al*., 1995; Sucher *et al*., 1996; Ozawa *et al*., 1998). Como a neurotoxicidade glutamatérgica é mediada, principalmente, por um grande influxo de íons Ca^{+2}, a ativação excessiva destes receptores têm sido altamente relacionada à excitotoxicidade e danos subsequentes, como a morte neural (Ozawa *et al*., 1998).

Os *receptores metabotrópicos (mGluR)* estão acoplados às proteínas ligantes de nucleotídeos da guanina (proteínas G) e modulam a atividade de efetores intracelulares, como a adenilato ciclase e fosfolipase C, responsáveis pela produção de segundos-mensageiros (AMP cíclico, diacilglicerol e inositol-3-fosfato), os quais ativam e/ou inibem diversos eventos de sinalização celular (Ozawa *et al*., 1998; Waxham, 1999, Bear *et al*, 2001). Até o momento, oito receptores metabotrópicos foram clonados (mGluR1 a mGluR2) (Conn & Pin, 1997). Devido à falta de ligantes específicos, sua classificação farmacológica foi dificultada. Assim, estes receptores estão subdivididos em três grupos, de acordo com a identidade de sua sequência de aminoácidos, afinidade por ligantes e segundos-mensageiros aos quais estão acoplados (Conn & Pin, 1997; Kommers *et al*., 1999). Em relação a sua localização, os receptores metabotrópicos estão presentes em ambos os terminais pré- e pós-sinápticos, bem como nas células gliais, e sua ativação pode promover efeitos inibitórios ou excitatórios (Ozawa *et al*., 1998).

Para o término do processo de neurotransmissão vários eventos, incluindo a retirada do neurotransmissor da fenda sináptica ou sua degradação metabólica estão envolvidos. Em relação ao glutamato, os eventos de retirada do neurotransmissor da fenda sináptica são de maior relevância devido ao fato de não haver enzimas extracelulares que o catabolizem. Portanto, o processo principal de término da transmissão sináptica é a recaptação do neurotransmissor pelas células neurais vizinhas, pela própria célula liberadora e por células gliais (Rudnick, 1998). Este processo é realizado por carreadores de alta afinidade, dependentes de sódio, localizados na região perisináptica e principalmente na glia (Nicholls & Atwell, 1990; Robinson & Dowd, 1997, Andreson & Swanson, 2000, Maragakis & Rothstein, 2004).

O glutamato captado pela glia pode ser metabolizado por duas diferentes rotas: i) transformado em glutamina, pela ação da glutamina sintetase, ou ii) convertido em α-cetoglutarato, por desaminação pela glutamato desidrogenase ou transaminação por uma transaminase, que será metabolizado através do ciclo do ácido tricarboxílico, produzindo em última instância lactato (Danbolt, 2001). Ambos, lactato e glutamina são liberados no fluído extracelular e podem entrar nos neurônios. Seguindo o clássico ciclo glutamina/glutamato, após a glutamina ser transportada para os neurônios, esta é novamente convertida em glutamato pela enzima mitocondrial glutaminase. O glutamato resultante poderá ser novamente captado pelas vesículas sinápticas, passando a fazer parte do "pool" neurotransmissor (Danbolt, 2001).

Esta captação vesicular é realizada através de carreadores de baixa afinidade, independentes de sódio, porém dependentes de um gradiente transmembrana próton-eletroquímico gerado por uma ATPase (V-ATPase) (Fig. 1). Este gradiente é constituído

por dois componentes: um potencial de prótons (ΔpH) e um potencial elétrico (ΔΨ). A captação de glutamato é dirigida principalmente pelo ΔΨ, enquanto a captação de GABA e glicina é dirigida por ambos os constituintes: ΔpH e ΔΨ (Fykse & Fonnum, 1996; Liu & Edwards,1997; Rudnick, 1998). A presença de cloreto interfere na captação de glutamato pelas vesículas sinápticas. Neste caso, existe um efeito bifásico em relação à concentração de cloreto: ativação do transporte de glutamato na presença de baixas concentrações (2-8 mM) e inibição com altas concentrações (>20 mM) (Tabb *et al*., 1992; Fykse & Fonnum, 1996; Wolosker *et al*., 1996). O funcionamento harmônico dos mecanismos acima descritos é fundamental para que os neurotransmissores sejam removidos do meio extracelular e armazenados de forma adequada, estando disponíveis para novamente serem liberados na fenda sináptica, após despolarização da membrana.

Atualmente, usando técnicas de clonagem molecular, já foram identificados cinco subtipos de transportadores de glutamato: GLT-1, GLAST, EAAC1, EAAT4 e EAAT5 (Tanaka, 2000; Maragakis & Rothstein, 2004). Em relação às suas localizações, GLAST (ou EAAT1, em humanos) é expresso em células gliais e em neurônios, e é o principal transportador de glutamato presente durante o desenvolvimento do SNC. GLT-1 (ou EAAT2, em humanos) é exclusivamente localizado em astrócitos, e é responsável por aproximadamente 90% de todo o transporte glutamatérgico no tecido adulto. Outro transportador importante para a recaptação do glutamato é EAAC1 (ou EAAT3, em humanos), de expressão exclusivamente neuronal, muito presente nas membranas pós-sinápticas. EAAT4 restringe-se às células de Purkinje do cerebelo e EAAT5 é encontrado em fotoreceptores e células bipolares da retina. Recentemente, identificou-se uma classe de

transportadores glutamatérgicos responsável pelo acúmulo de glutamato em vesículas pré-sinápticas: VGLUT1-3 (Maragakis & Rothstein, 2004).

Estudos com animais *knockout* para estes transportadores corroboram a fundamental importância da recaptação astroglial do glutamato. A perda de função ou a expressão reduzida de GLAST ou GLT-1 produziram elevadas concentrações extracelulares de glutamato, neurodegeneração característica de excitotoxicidade e paralisia progressiva. A expressão reduzida de EAAC1 não resultou em concentração extracelular de glutamato elevada, porém observaram-se eventos epilépticos (Rothstein *et al*., 1996; Maragakis & Rothstein, 2004).

Considerando-se o envolvimento destes neurotransmissores em várias patologias, vemos que anormalidades pré- ou pós-sinápticas, sozinhas ou em associação, podem ter efeitos lesivos ao sistema nervoso central, o que justifica a busca de um melhor entendimento destes sistemas transportadores.

I.2- MODULAÇÃO DO SISTEMA GLUTAMATÉRGICO PELOS DERIVADOS DA GUANINA.

Os derivados da guanina, incluindo os nucleotídeos (GTP, GDP e GMP) e o nucleosídeo (guanosina) são purinas que exercem inúmeras funções intracelulares, como fonte de energia para síntese proteica nos ribossomas, controle do movimento vesicular nas células, e são responsáveis pelo ciclo ativação/inativação das proteínas G (Bourne *et al*., 1990; Hepler & Gilman, 1992; Morris & Malbon, 1999).

Evidências atuais indicam que além das funções acima citadas, os derivados da guanina também modulam o sistema glutamatérgico agindo diretamente em sítios receptores na membrana plasmática, sem o envolvimento de proteínas G (Tasca *et al*.,

1995, Tasca *et al*., 1998, Tasca *et al.,* 1999). Além disto, tem sido demonstrado que os nucleotídeos e nucleosídeo da guanina exercem efeitos tróficos em células neurais (Rathbone *et al.,* 1999; Francisco et al., 2004).

Em estudos i*n vitro* observou-se que os nucleotídeos, inclusive seus análogos rígidos, inibem a ligação de glutamato e análogos (Baron *et al.,* 1989; Paz *et al*., 1994; Burgos *et al.,* 1998), previnem o dano isquêmico (Frizzo *et al*., 2002; Oliveira et al., 2002) e estimulam a captação de glutamato por astrócitos e fatias (Frizzo *et al*., 2001; Frizzo *et al*., 2002; Frizzo *et al*., 2003), num processo claramente envolvido em neuroproteção. Além disso, foi demonstrado que derivados da guanina (GUO, GMP, GTP, GDPβ-S e GppNHp) são capazes de modular a captação vesicular glutamatérgica, de maneira tempo- e concentração- dependente. GUO, GMP, GDPβ-S e GppNHp 1 mM inibiram a captação vesicular nos tempos iniciais de incubação (1,5 e 3 min); porém usando-se 10 min de incubação (*steady-state,* onde a velocidade de influxo e efluxo de glutamato são equivalentes), somente GTP e GppNHp 5 mM foram efetivos (Tasca *et. al*., 2004).

Ações neuroprotetoras destes nucleotídeos também foram observadas *in vivo*. Usando modelos de epilepsia induzida por agentes glutamatérgicos (agonistas NMDA) em ratos e camundongos, foi demonstrado que GMP e guanosina foram capazes de reduzir o aparecimento das convulsões observadas (Baron *et al*, 1989; Schmidt *et al*, 2000; Lara *et al*, 2001; Soares *et al*, 2004; Vinadé *et al*, 2003; Vinadé *et al*, 2005). Adicionalmente, recentes estudos em nosso grupo evidenciam que os efeitos anticonvulsivantes atribuídos ao GMP são, na verdade, resultantes da conversão do nucleotídeo monofosfatado (GMP) ao nucleosídeo (guanosina), por ação da enzima ecto-5'-nucleotidase (Soares *et al*., 2004).

GMP também mostrou-se neuroprotetor contra a morte celular mediada via receptor NMDA em *striatum* de ratos (Malcon *et al*., 2000), reduziu o dano neuronal em fatias de hipocampo submetidas à hipóxia (Regner *et al*., 1998) e hipoglicemia (Molz et al., 2005), além de proteger contra a toxicidade induzida por kainato em preparações de retina de aves (Burgos *et al*., 1998). Efeitos ansiolíticos e amnésicos foram observados em animais submetidos à tratamento crônico com guanosina (Vinadé *et al*., 2003, Vinadé *et al*., 2004; Vinadé *et al*., 2005).

Apesar do avanço no entendimento da interação dos nucleotídeos da guanina com o sistema glutamatérgico, o exato mecanismo desta modulação ainda não é perfeitamente elucidado. A demonstração de que os nucleotídeos da guanina agem como antagonistas glutamatérgicos, através da inibição de eventos fisiológicos estimulados por glutamato, pode levar à hipótese de que eles sejam neuroprotetores endógenos, devido à inibição de eventos neurotóxicos induzidos por glutamato, ou neuromoduladores, por influenciarem nas respostas de neurotransmissores.

I.3- ÁCIDO QUINOLÍNICO (AQ)

O ácido quinolínico (AQ), um metabólito endógeno do triptofano, é encontrado na natureza em muitos organismos, desde bactérias até humanos (Gholson, 1966; Isquith & Moat, 1966). Quimicamente, este composto é conhecido como ácido piridina-2,3-dicarboxílico. O anel aromático piridínico é praticamente inerte; assim, o AQ é muito estável nos tecidos e fluidos biológicos. Além disso, este composto possui duas carboxilas, o que permite a formação de complexos com íons divalentes (Sharma,89). Devido a esta afinidade, é possível que certas ações do AQ sejam atribuídas ao complexo quinolinato-metal.

A rota das quinureninas é a principal rota periférica de metabolismo do triptofano em mamíferos, e ocorre principalmente no fígado. Sua principal função é a biossíntese de nicotinamida adenina dinucleotídeo (NAD^+). Já em 1963, estudos mostraram que o AQ participa na biossíntese do cofator NAD^+ no fígado (Nishiuka & Hayaishi, 1963; Gholson, 1964). Além disso, sabe-se existir também uma rota extra-hepática, cuja função até o momento não é conhecida.

Até 1981 acreditava-se que o AQ era um metabólito fisiologicamente inativo do triptofano. Seu potencial fisiológico e significância farmacológica só foram reconhecidos com a descoberta de sua habilidade em ativar seletivamente os receptores glutamatérgicos NMDA (Stone, 2000). A importância do AQ como uma neurotoxina foi primeiramente evidenciada por Lapin (1978), onde demonstrou-se que a administração de AQ em camundongos causava convulsões. Entretanto, a presença de convulsões *per se* não era suficiente para classificar o AQ como uma neurotoxina, já que várias patologias, inclusive aquelas não originadas no SNC, apresentam este sintoma. Estudos eletrofisiológicos revelaram que o AQ se une a sítios de união dos aminoácidos excitatórios, normalmente ativados por glutamato e aspartato, sendo um agonista excitatório (Stone & Perkins, 1981). Mais tarde, Schwarcz e colaboradores (1983) administraram AQ diretamente no estriado de ratos e observaram um padrão anatômico e bioquímico de neurotoxicidade semelhante àquele encontrado na doença de Huntington. A partir desses achados, esta neurotoxina tem sido usada para estudos *in vivo*, simulando esta doença.

Estudos usando o modelo de injeção estriatal indicam que a toxicidade de AQ é mediada por receptores glutamatérgicos do tipo N-metil-D-aspartato (NMDA), já que a administração periférica de MK-801, um antagonista não-competitivo de receptores

NMDA, foi capaz de proteger contra a neurotoxicidade intraestriatal do AQ (Beal *et al*., 1988; Foster *et al*., 1988).

A neurotoxicidade do AQ tem sido demostrada *in vivo* e *in vitro*; entretanto, sua origem celular e sua concentração tecidual não estão bem definidas. Analiticamente, este tem sido determinado radioenzimaticamente (Foster *et al*., 1986) e por cromatografia gasosa com espectrometria de massa (Wolfensberger *et al.*, 1983; Moroni *et al*., 1984a; Heyes & Markey, 1988). Evidências indicam que o AQ não é sintetizado por todo o tecido cerebral normal, mas sim por uma população de macrófagos residentes, que pode constituir 0,1 % do cérebro (Heyes *et al*., 1992a; Reinhard *et al*., 1993; Saito *et al*., 1993b). Estima-se que o cérebro, em condições normais, possua concentrações na faixa de 50-100 pmol/g de AQ (Wolfensberger *et al*., 1983; Heyes & Markey, 1988). Níveis aumentados de AQ no cérebro e líquor, da ordem de micromolar, já foram encontrados em humanos após traumatismo crânio-encefálico (Bell *et al*., 1999), doenças inflamatórias do SNC (Heyes, 1996) e pacientes aidéticos (Heyes *et al*., 1989, 1991ab; Stone, 2001), principalmente naqueles pacientes com infecções oportunistas.

O aumento de AQ também está relacionado com vários estados infecciosos, tanto bacterianos como virais. Se microorganismos estimulam a síntese de AQ por liberação de citocinas, ativadoras indolamina 2,3-dioxigenase, doenças autoimunes, como a esclerose múltipla, também podem ser associadas com níveis aumentados desta neurotoxina (Heyes *et al*., 1992b, Stone, 2001). A secreção de AQ por células ativadas do sistema imune é importante, na medida em que existem evidências de ativação de receptores glutamatérgicos por citocinas (Stone, 2001). Outro achado importante é que o AQ induz ao aumento da peroxidação lipídica (Rios & Santamaria, 1991), e este fato pode ser responsável pela desmielinização observada na esclerose múltipla, neuropatia mielo-óptica

subaguda e mielopatia associada ao HTLV. Doenças metabólicas também podem ser associadas com níveis elevados de AQ. Heyes sugeriu que as lesões neurológicas observadas na acidemia glutárica tipo I podem ser, pelo menos em parte, relacionadas com aumento desta substância (Heyes, 1987).

Outra patologia na qual provavelmente existe envolvimento do AQ é o coma hepático, situação resultante de falência hepática. A análise dos níveis de AQ no líquor e no córtex frontal revelaram níveis de AQ 7 vezes maiores no líquor e 3 vezes maiores no córtex dos pacientes afetados do que no dos controles (Moroni *et al*., 1986). O mecanismo deste aumento não está elucidado, mas níveis plasmáticos elevados de 3-hidroxiquinurenina, também encontrados no coma hepático (Pearson & Reynolds, 1991), sugerem um possível bloqueio da rota hepática, talvez envolvendo a enzima quinureninase. Este derivado hepático, a 3-hidroxiquinurenina, pode atravessar a barreira hematoencefálica e ser um substrato para o aumento de AQ (Erickson *et al*., 1992).

Dados obtidos a partir de estudos em ratos sugerem haver um aumento dos níveis cerebrais de AQ dependente da idade (Moroni *et al*., 1984b). Ratificando esta hipótese, medidas de AQ no líquor de humanos apresentaram uma correlação positiva com a idade (Heyes *et al*., 1992b). Entretanto, o AQ parece não estar elevado no cérebro ou líquor de pacientes com doença de Alzheimer (Sofic *et al*., 1989; Heyes *et al*., 1992b).

Além de efeitos tóxicos diretos sobre os neurônios, o AQ pode induzir disfunção mitocondrial, o que pode ser um fator que contribui para a neurodegeneração (Bordelon *et al*., 1997). A neurotoxicidade produzida pelo AQ pode ser devida também, ao menos em parte, pela formação de espécies reativas de oxigênio, já que estudos demonstraram que antioxidantes como a melatonina foram efetivos no bloqueio da atividade neurotóxica do AQ (Stone, 2001).

Assim, neste trabalho buscou-se elucidar os mecanismos envolvidos na ação neurotóxica do AQ, bem como comprovar o a ação neuromoduladora e/ou neuroprotetora dos derivados da guanina sobre estes eventos.

I.4- OBJETIVOS

Sabendo-se que, (i) o aminoácido glutamato é o principal neurotransmissor excitatório no Sistema Nervoso Central, sendo relacionado não somente a eventos fisiológicos, mas também com eventos patológicos quando de sua ação sobre seus receptores; (ii) que o ácido quinolínico , até muito pouco tempo atrás considerado apenas um metabólito inativo do triptofano, tem se mostrado uma potente neurotoxina envolvida em muitas patologias e suas consequências neurológicas ainda permanece com seu papel fisiológico e mecanismo de ação não perfeitamente elucidado; (iii) e que muitas evidências sugerem que os derivados da guanina estejam envolvidos na modulação de respostas glutamatérgicas, foram estabelecidos os seguintes objetivos:

1- Verificar a ação do ácido quinolínico nos processos de captação e liberação de glutamato em sinaptossomas, bem como sobre a captação astrocitária de glutamato.

2- Verificar qual o mecanismo envolvido na inibição da captação vesicular de glutamato por ácido quinolínico, bem como verificar se os derivados da guanina exercem algum efeito modulatório no sistema de transporte vesicular.

3- Verificar se o ácido quinolínico e outros agentes usados para induzir convulsões em modelos experimentais alteram o transporte vesicular de glutamato.

4- Verificar se a ação vesicular do ácido quinolínico *in vitro* pode ser observada também quando este é usado experimentalmente *in vivo*, e se os nucleotídeos da

guanina possuem alguma ação modulatória sobre este sistema, seja *in vitro* ou *in vivo.*

5- Verificar se o ácido quinolínico, *in vivo*, exerce alguma interferência nos processos de captação e liberação de glutamato em sinaptossomas.

II. RESULTADOS

Capítulo 1

Quinolinic acid stimulates synaptosomal glutamate release and inhibits glutamate uptake into astrocytes.

Neurochem. Int, 40: 621-627, 2002.

ELSEVIER

Neurochemistry International 40 (2002) 621–627

NEUROCHEMISTRY International

www.elsevier.com/locate/neuint

Quinolinic acid stimulates synaptosomal glutamate release and inhibits glutamate uptake into astrocytes

Rejane G. Tavares [a], Carla I. Tasca [b], Candice E.S. Santos [a], Leticia B. Alves [a], Lisiane O. Porciúncula [a], Tatiana Emanuelli [c], Diogo O. Souza [a,*]

[a] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 (Anexo), 90035-003 Porto Alegre, RS, Brazil*

[b] *Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil*

[c] *Departamento de Tecnologia e Ciência dos Alimentos, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil*

Received 24 May 2001; received in revised form 20 June 2001; accepted 26 October 2001

Abstract

Quinolinic acid (QA) is an endogenous neurotoxin involved in various neurological diseases, whose action seems to be exerted via glutamatergic receptors. However, the exact mechanism responsible for the neurotoxicity of QA is far from being understood. We have previously reported that QA inhibits vesicular glutamate uptake. In this work, investigating the effects of QA on the glutamatergic system from rat brain, we have demonstrated that QA (from 0.1 to 10 mM) had no effect on synaptosomal L-[^{3}H]glutamate uptake. The effect of QA on glutamate release in basal (physiological K^+ concentration) or depolarized (40 mM KCl) conditions was evaluated. QA did not alter K^+-stimulated glutamate release, but 5 and 10 mM QA significantly increased basal glutamate release. The effect of dizolcipine (MK-801), a noncompetitive antagonist of *N*-methyl-D-aspartate (NMDA) receptor on glutamate release was investigated. MK-801 (5 μM) did not alter glutamate release per se, but completely abolished the QA-induced glutamate release. NMDA (50 μM) also stimulated glutamate release, without altering QA-induced glutamate release, suggesting that QA effects were exerted via NMDA receptors. QA (5 and 10 mM) decreased glutamate uptake into astrocyte cell cultures. Enhanced synaptosomal glutamate release, associated with inhibition of glutamate uptake into astrocytes induced by QA could contribute to increase extracellular glutamate concentrations which ultimately lead to overstimulation of the glutamatergic system. These data provide additional evidence that neurotoxicity of QA may be also related to disturbances on the glutamatergic transport system, which could result in the neurological manifestations observed when this organic acid accumulates in the brain.

Keywords: Quinolinic acid; Glutamate release and uptake; Synaptosomal preparations; Astrocyte cell cultures; Rat brain

1. Introduction

Quinolinic acid (QA), an endogenous metabolite of tryptophan, is a potent endogenous neurotoxin, first demonstrated by Lapin (1978). QA is a neurotoxic metabolite that is normally present in low nanomolar concentrations in human brain and cerebrospinal fluid. However, substantial increasing in QA levels to micromolar concentrations are found in the brain and CSF of patients with infectious and inflammatory neurological diseases (Heyes et al., 1996; Stone, 2001). Accumulation of QA in the brain seems to be involved in the ethiopathology of convulsions and occurs in patients with hepatic encephalopathy, acquired immune deficiency syndrome (AIDS)-related neurological disorders, Huntington's disease, and glutaric aciduria type I (Stone, 2001). Although the amount of quinolinic acid in the brain rarely exceed 1 μM, it has been shown that in AIDS-related dementia this concentration may attain low milimolar levels (Connick and Stone, 1988, 1989; Stone, 2001). In other disorders, quinolinic acid concentration was not exactly defined, but increased QA production is shown to be related with observed neurotoxicity (Stone, 2001) in spite of its involvement in many diseases, the exact molecular mechanism linking QA and brain damage are far from being understood. However, some studies demonstrated that QA exerts its toxicity in the central nervous system (CNS) via glutamatergic receptors (Stone, 2001).

Glutamate, the main excitatory neurotransmitter in the mammalian brain, has important roles in several physiological and pathological events (Ozawa et al., 1998).

* Corresponding author. Tel.: +55-51-316-5557; fax: +55-51-316-5540/5535.
E-mail address: diogo@ufrgs.br (D.O. Souza).

PII: S0197-0186(01)00133-4

Glutamatergic neurotransmission is achieved through ionotropic (ligand-gated ion channels) and metabotropic (G protein-coupled) receptors. Specifically, the *N*-methyl-D-aspartate (NMDA) ionotropic glutamate receptor subtype seems to be crucial in plasticity processes associated with normal brain function (Izquierdo and Medina, 1997; Ozawa et al., 1998). However, overstimulation of the glutamatergic system, as observed when glutamate concentration in the synaptic cleft increases, may be neurotoxic. Glutamate neurotoxicity is implicated in acute neurological disorders as well as in neurodegenerative diseases (Lipton and Rosenberg, 1994; Ozawa et al., 1998; Price, 1999).

In order to avoid the excessive increases of extracellular glutamate concentration and consequently, its excitotoxicity, glutamate must be taken up from synaptic cleft to the cytosol of glial and neuronal cells and stored into synaptic vesicles on neuronal terminals. Glutamate uptake processes involve two transport systems located at distinct cellular levels: high affinity Na^+-dependent carriers located at cell membranes of astrocytic and neuronal cells (Robinson and Dowd, 1997; Anderson and Swanson, 2000), and a low affinity Na^+-independent carrier located at the membrane of synaptic vesicles (Fykse and Fonnum, 1996; Wolosker et al., 1996). The Na^+-dependent glutamate uptake system in astrocytes is the most significant by maintaining the glutamate levels below neurotoxic concentrations, as clearly demonstrated by studies using knock-out of astrocytic glutamate transporters (Rothstein et al., 1996). The coordinated actions of vesicular and cell membrane systems effectively maintain glutamate concentrations in the synaptic cleft at low micromolar levels. Glutamate stored inside synaptic vesicles is available for release when presynaptic membranes are further depolarized. Among the various factors involved in the modulation of glutamate release, activation of presynaptic NMDA receptors may stimulate glutamate release (Bradford et al., 1998).

Based upon studies aimed at investigating the involvement of the glutamatergic system in QA toxicity, we previously reported that QA inhibits glutamate uptake into synaptic vesicles obtained from rat brain (Tavares et al., 2000). Considering this finding and the involvement of QA and glutamatergic system in brain dysfunction, the purpose of the present study was to evaluate the effects of this neurotoxin on glutamate uptake into astrocytes and synaptosomes as well as the release of this neurotransmitter by presynaptic terminals (synaptosomal preparations).

2. Experimental procedures

2.1. Animals

Wistar rats from our local breeding colony were used. The animals were kept on a 12 h light/12 h dark cycle, at a room temperature of 22 °C, with free access to food and water. The NIH guide for the care and use of laboratory animals (NIH publications no. 80-23, revised 1978) was followed in all experiments.

2.2. Synaptosomal preparations

Animals were decapitated and the forebrain was used to prepare synaptosomes on a discontinuous Percoll gradient according to Dunkley et al. (1988). Synaptosomes were all used in the same day of preparation. Protein concentration was measured according to the method of Lowry et al. (1951).

2.3. L-[^{3}H]Glutamate uptake by synaptosomal preparations

Synaptosomal preparations were washed twice by resuspending in three volumes of 0.3 M sucrose with 15 mM Tris/acetate buffer (pH 7.4) and centrifuging at 13,000 × *g* for 15 min. The final pellet was resuspended in 0.3 M sucrose with 15 mM Tris/acetate buffer (pH 7.4), and incubated in HBSS, pH 7.4 (Hepes/Cl buffered salt solution, composition in mM: HEPES 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 12, $CaCl_2$ 1.0), in the presence of L-[^{3}H]glutamate (Amersham International, UK, specific activity 53 Ci/mmol, final concentration 100 nM), with or without QA (0.1–10 mM), for 1 min at 37 °C. The reaction was stopped by filtration through GF/B filters. The filters were washed three times with 3 ml of ice-cold 15 mM Tris/acetate buffer (pH 7.4) in 155 mM ammonium acetate. The radioactivity retained on the filters was measured in a Wallac scintillation counter. Specific L-[^{3}H]glutamate uptake was calculated as the difference between uptake obtained in the incubation medium as described above, and uptake obtained with a similar incubation medium containing choline chloride instead of NaCl (nonspecific uptake).

2.4. L-[^{3}H]Glutamate release from synaptosomal preparations

L-[^{3}H]Glutamate release was measured according to Mignes et al. (1999), with minor modifications. Synaptosomal preparations were incubated in HBSS, pH 7.4, for 15 min at 37 °C in the presence of L-[^{3}H] glutamate (final concentration, 500 nM). Aliquots of labeled synaptosomes (1.4 mg protein) were centrifuged at 13,000 × *g* for 1 min. Supernatants were discarded, and the pellets were washed 4 times in HBSS by centrifugation at 13,000 × *g* for 1 min at 4 °C. In order to measure the basal release of L-[^{3}H]glutamate, the final pellet was resuspended in HBSS and incubated for 60 s at 37 °C in the absence (control) or presence of QA (0.1–10 mM). Incubation was terminated by immediate centrifugation (16,000 × *g* for 1 min at 4 °C). Radioactivity present in supernatants and pellets was separately determined in a Wallac scintillation counter. The released L-[^{3}H]glutamate was calculated as a percentage of

the total amount of radiolabel in the synaptosomal preparation at the start of the incubation period. K^+-stimulated L-[^{3}H]glutamate release was assessed as described for basal release, except that the incubation medium contained 40 mM KCl (NaCl decreased accordingly) in order to induce synaptosomal depolarization. The effect of QA (0.1–10 mM) on L-[^{3}H]glutamate release from synaptosomal preparations in the absence of Ca^{2+} was performed in a medium as described above, except that $CaCl_2$ was omitted while 2 mM EGTA was added.

2.5. Primary cortical astrocyte cultures

The primary cortical astrocyte cultures were prepared as described by Swanson et al. (1997), with minor modifications. The cortical tissue of newborn rats was dissociated mechanically. Dissociated cells were washed, suspended in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and plated in 24-well tissue culture plates at an approximate density of 5×10^4 cells/cm^2. Cultures were maintained in a humidified, 5% CO_2 incubator at 37 °C. The medium was exchanged weekly. At confluence (days 13–15), 10 μM cytosine arabinoside was added and after 48 h this medium was replaced with medium containing 3% FBS and 0.15 mM dibutyryl cyclic AMP (dBcAMP). Addition of dBcAMP to the media induced the expression of GLT-1 and increased the expression of GLAST (Swanson et al., 1997). Cultures were used at 25–30 days in vitro.

2.6. L-[^{3}H]Glutamate uptake by astrocyte cultures

Glutamate uptake was measured as described by Leal et al. (2001). Briefly, the culture media were replaced with a modified HBSS containing 2 mM glucose buffered to pH 7.2 with 5 mM PIPES, in the absence or presence of QA (0.5–10 mM). The uptake was started by adding 0.01 μCi/ml L-[^{3}H]glutamate plus 100 μM of unlabeled glutamate to each culture well. Uptake was terminated after 7 min of incubation (37 °C), by washing cell cultures twice with ice-cold HBSS, followed immediately by cell lysis in 0.5 N NaOH/0.05% lauryl sulfate. Nonspecific uptake was measured in HBSS containing choline chloride instead of NaCl. Aliquots were taken for scintillation counting and protein assays (Lowry et al., 1951).

2.7. Measurement of lactate dehydrogenase (LDH) activity

In order to evaluate the integrity of the synaptosomal preparations and astrocyte cell cultures after the incubation in the presence of QA, an aliquot of the supernatant was withdrawn and frozen for determination of LDH leakage. LDH activity was evaluated by using an assay kit (Doles Reagents, Brazil) which measured the amount of a colored complex derived from the NADH formed by the enzymatic reaction using a spectrophotometric method (510 nm).

2.8. Statistical analysis

Statistical significance was assessed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test when appropriate. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of QA on glutamate uptake into synaptosomal preparations

In order to evaluate whether QA interferes with the neuronal presynaptic glutamate uptake, we assayed L-[^{3}H]glutamate uptake into rat brain synaptosomal preparations. Fig. 1 shows that QA (in a concentration range from 0.1 to 10 mM) had no effect on glutamate uptake into synaptosomes when compared to control.

3.2. Glutamate release from synaptosomal preparations

The effect of QA on L-[^{3}H]glutamate release in basal (physiological extracellular K^+ concentration) or depolarized (40 mM KCl) conditions was evaluated. QA (0.1–10 mM) did not alter K^+-stimulated glutamate re-

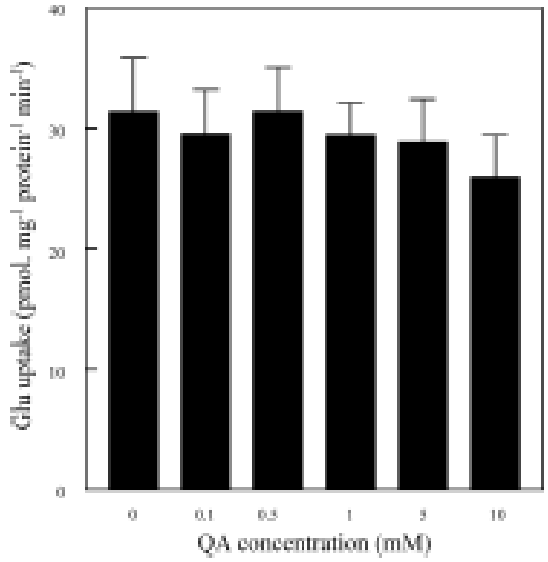


Fig. 1. Effect of QA on L-[^{3}H] glutamate uptake into brain synaptosomes of rats. QA was used in the concentration range from 0.1 to 10 mM. Glutamate uptake is expressed as pmol mg of protein^{-1} min^{-1}. Data are mean ± S.E.M. from six independent experiments performed in triplicates. No statistically significant differences were observed between control and groups in the presence of QA.

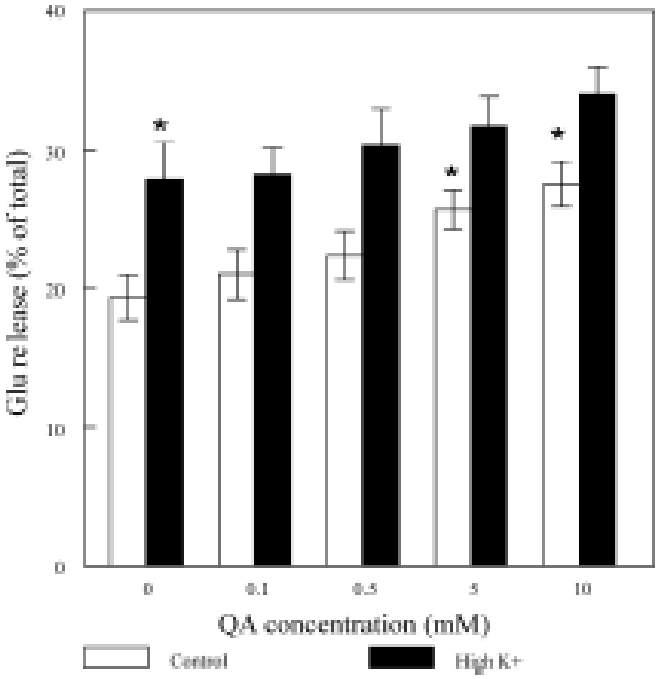


Fig. 2. Effect of QA on basal and K^+-stimulated L-[^{3}H]glutamate release from rat brain synaptosomes. QA was used in the concentration range from 0.1 to 10 mM. K^+-stimulated release was obtained by using 40 mM KCl in the incubation media. Glutamate release is expressed as percentage of total radioactivity content. Data are mean ± S.E.M. from six independent experiments performed in triplicate. The symbol (*) represents values significantly different from the control group (basal release in the absence of QA); $P < 0.05$, by ANOVA followed by Duncan's multiple range test.

lease. However, 5 and 10 mM QA increased basal glutamate release significantly from 19–26 to 28%, respectively (Fig. 2).

In order to determine Ca^{2+}-dependence of QA effect on L-[^{3}H]glutamate release, synaptosomal preparations were incubated in the absence of Ca^{2+} (in the presence of 2 mM EGTA) (Fig. 3). Control release was not affected by the absence of Ca^{2+}. However, both QA-induced and K^+-stimulated L-[^{3}H]glutamate release were abolished in the absence of Ca^{2+}.

3.3. The involvement of NMDA receptors

We also investigated the effect of dizolcipine (MK-801), a noncompetitive antagonist of NMDA receptor, on basal L-[^{3}H]glutamate release (Fig. 4). MK-801 (5 μM) did not modify glutamate release per se, but completely abolished the QA-induced (10 mM) glutamate release, suggesting that the effect of QA was exerted via NMDA receptors. The effect of NMDA (50 μM) on basal glutamate release was also investigated (Fig. 5). NMDA also stimulated glutamate release from 12 to 17%, without affecting QA-induced glutamate release, indicating that both compounds probably exert their actions through the same receptor.

3.4. Effect of QA on glutamate uptake into astrocytes

We also investigated the effect of QA on glutamate uptake into primary astrocyte cell cultures. Fig. 6 shows that QA decreased the glutamate uptake into astrocytes (maximum inhibition of 60%) at the same concentrations which it increased glutamate release from synaptosomal preparations (from 5 mM QA) (Fig. 2).

3.5. Measurement of LDH leakage

Synaptosomal preparations and astrocyte cell cultures did not show any significant leakage of the cytosolic marker LDH after incubations with 5 and 10 mM QA (data not shown), indicating that this compound did not disrupt plasma membranes in our assay conditions.

4. Discussion

Evidence suggests that QA is associated with neuronal damage through the overactivation of the postsynaptic NMDA subtype of glutamate receptors (for a review, see Stone, 2001). Striatal administration of QA is associated

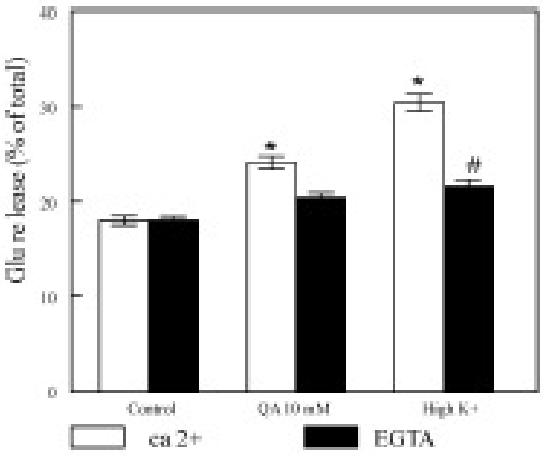


Fig. 3. Effect of the absence of calcium on the release of L-[^{3}H]glutamate from rat brain synaptosomes induced by QA or stimulated by high K^+ concentration. 10 mM QA was used. Depolarizing conditions were obtained by increasing KCl concentration to 40 mM. In calcium-free medium the release of glutamate was performed without addition of $CaCl_2$ and in the presence of EGTA (2 mM). Glutamate release is expressed as percentage of total radioactivity content. Data are mean ± S.E.M. from 7–10 independent experiments performed in triplicates. The symbol (*) represents values significantly different from the control group (basal release in the absence of QA); $P < 0.05$. The symbol (#) represents value significantly different from the group: High K^+ in the presence of Ca^{2+}, $P < 0.05$, by ANOVA followed by Duncan's multiple range test.

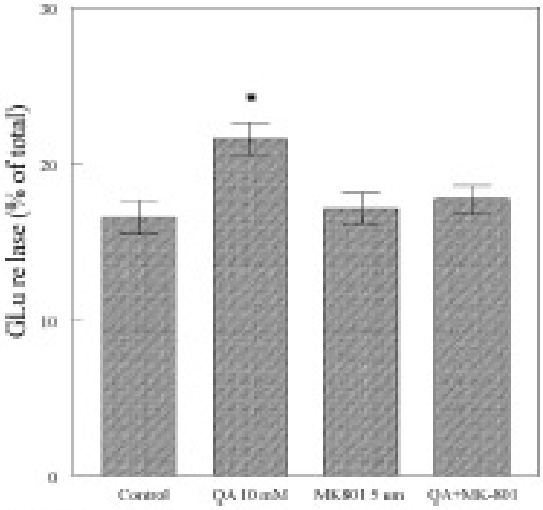


Fig. 4. Effect of MK-801 on L-[^{3}H] glutamate release induced by QA from rat brain synaptosomes. MK-801 (5 μM) was assayed in the absence or presence of 10 mM QA. Glutamate released is expressed as percentage of total radioactivity content. Data are mean ± S.E.M. from six independent experiments performed in triplicates. The symbol (*) represents values significantly different from the control group (basal release in the absence of QA), $P < 0.05$, by ANOVA followed by Duncan's multiple range test.

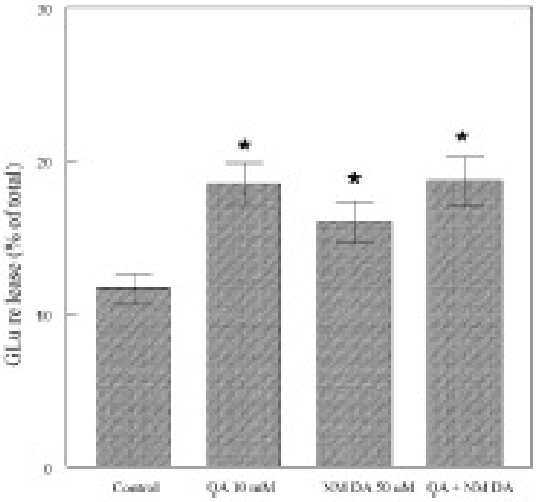


Fig. 5. Effect of NMDA on L-[^{3}H] glutamate release induced by QA from rat brain synaptosomes. NMDA (50 μM) was assayed in the absence or presence of 10 mM QA. Glutamate released is expressed of total radioactivity content. Data are mean ± S.E.M. from six independent experiments performed in triplicates. The symbol (*) represents values significantly different from the control group (basal release in the absence of QA), $P < 0.05$, by ANOVA followed by Duncan's multiple range test.

with neurotoxicity and development of neurological damage which resembles that observed in Huntington's disease and probably results from excessive NMDA receptor activation (Beal et al., 1991; Malcon et al., 1997). Moreover, intracerebroventricular injection of QA induces seizures (Lapin, 1978; Schmidt et al., 2000; Lara et al., 2001). In this context, increasing QA levels in cerebrospinal fluid to micromolar concentrations may be of neurological significance (Beal et al., 1999). In experiments aimed to investigate dose-response effects, we started with micromolar QA concentrations. The observed effects were obtained from 5 mM, which is higher than physiopathological concentrations. However, in some experiments we used only 10 mM QA, because this concentration provided a means to investigate the mechanism for QA toxicity, based on an approach previously reported (Reinhard et al., 1994).

Our results show that in synaptosomal preparations, QA acts at presynaptic membranes by stimulating glutamate release without affecting glutamate uptake. The observation that QA did not alter glutamate uptake in synaptosomal preparations, contrasts with the previously demonstrated inhibitory effect of QA on glutamate uptake into synaptic vesicles (Tavares et al., 2000). This discrepancy may be attributed to specific characteristics and functional differences between the presynaptic and vesicular glutamate carriers, as those related to dependence on energy, glutamate affinity and modulatory effect of ions (Robinson and Dowd, 1997; Dunbolt, 2001).

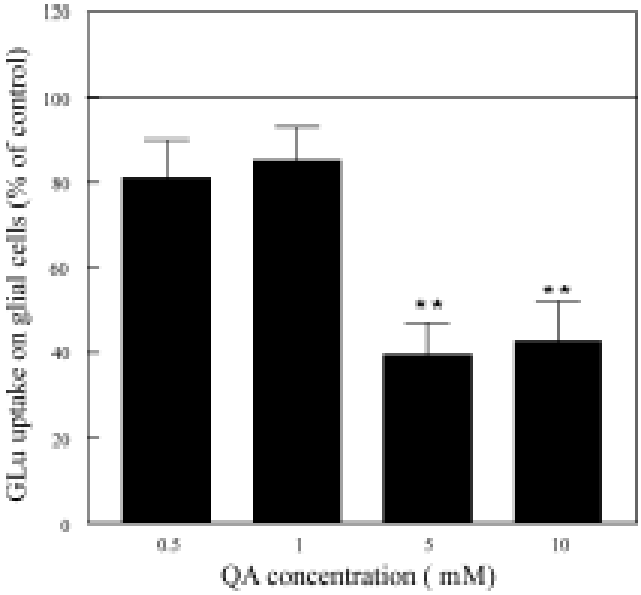


Fig. 6. Effect of QA on L-[^{3}H] glutamate uptake by astrocyte cell cultures. QA was used in the concentration range from 0.5 to 10 mM. Glutamate uptake is expressed as percentage of control (100%). Data are mean ± S.E.M. from six independent experiments performed in triplicates. The symbol (**) represents values significantly different from the control group (in the absence of QA), $P < 0.01$, by ANOVA followed by Duncan's multiple range test.

QA stimulated synaptosomal glutamate release in basal conditions, but had no effect in the presence of depolarizing K^+ concentrations. Importantly, removing Ca^{2+} ions from the medium equally abolished both K^+-stimulated and QA-induced glutamate release. These data could indicate that QA induces synaptosomal depolarization through mechanism(s) dependent on exogenous Ca^{2+}. This Ca^{2+} dependency could reflect an effect of this neurotoxin on mobilization of the stored vesicular glutamate, instead of releasing glutamate from a cytosolic pool, although we cannot totally exclude the role of glutamate cytosolic contribution to this QA stimulatory effect. In addition, our results indicate that this effect occurred through the activation of presynaptic NMDA receptors, because MK-801 completely abolished QA-induced glutamate release and NMDA presented similar stimulatory effect, which was no additive with QA. A recent study demonstrated that activation of presynaptic NMDA autoreceptors may provide a positive feedback on presynaptic glutamate release in synaptosomal preparations (Brustkel et al., 1998). Additionally, Connick and Stone have demonstrated that in vivo, QA, as other glutamate ligands, is able to stimulate the efflux of endogenous glutamate and aspartate; this effect was also abolished by NMDA antagonists (Connick and Stone, 1988).

Additionally, our results also indicate that QA strongly inhibited glutamate uptake by astrocytes, which is considered the most important process to maintain the extracellular glutamate below toxic concentrations. Considerable attention has been focused on the mechanisms that regulate neural cell membrane glutamate transporters, since numerous data have been published indicating that inhibition, impairment or reversal of this transport system may contribute to increasing the level of extracellular glutamate. Moreover, is well documented that impairment of glutamate transport, specially by astrocytes, is involved in the development of acute or progressive neurodegenerative processes (Rothstein et al., 1996; Anderson and Swanson, 2000; Maragakis and Rothstein, 2001).

The absence of significant LDH leakage after incubation of synaptosomes and astrocytes with QA suggests that increased glutamate release and inhibition of glutamate uptake are not due to impairment of plasma membrane integrity.

The enhanced synaptosomal glutamate release associated with inhibition of glutamate uptake into astrocytes induced by QA could contribute to increase extracellular glutamate concentrations, which ultimately leads to overstimulation of the glutamatergic system. These effects may be related to the neurotoxicity observed when this organic acid accumulates in the brain. Therefore, the neurotoxic effects of QA could be attributed to these alternative or additional sites of action on the glutamate transport systems.

Acknowledgements

This study was supported by grants from the Brazilian National Research Council (CNPq), CAPES and Pronex (No. 41960904-366/96 to D.O. Souza).

References

Anderson, C.M., Swanson, R.A., 2000. Astrocyte glutamate transport: review of properties, regulation and physiological functions. Glia 32, 1–14.

Beal, M.F., Ferrante, R.J., Swartz, K.J., Kowall, N.W., 1991. Chronic quinolinic acid lesions in rat closely resemble Huntington's disease. J. Neurosci. 11, 1649–1659.

Ball, M.J., Kocharnick, P.M., Hayes, M.P., Wisniewski, S.R., Sinz, E.H., Clark, R.S.B., Blight, A.R., Marion, D.W., Adelson, P.D., 1999. Quinolinic acid in the cerebrospinal fluid of children after traumatic injury. Crit. Care Med. 27, 493–497.

Braidol, A.I.M., Beaudoin, E., Lopes da Silva, F.H., Chijsen, W.E.J.M., 1998. A presynaptic *N*-methyl-D-aspartate autoreceptor in rat hippocampus modulating amino acid release from a cytoplasmic pool. Eur. J. Neurosc. 10, 106–114.

Connick, J.H., Stone, T.W., 1988. Quinolinic acid effects on amino acid release from the rat cerebral cortex in vitro and in vivo. Br. J. Pharmacol. 93, 868–876.

Connick, J.H., Stone, T.W., 1989. Quinolinic acid neurotoxicity: protection by intracerebral physostigmine and prostanoid (PIA) and potentiation by hypotension. Neurosci. Lett. 101, 191–196.

Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65, 1–105.

Dunkley, P.R., Heath, J.W., Harrison, S.M., Jarvie, P.E., Glenfield, P.J., Rostas, J.A., 1988. A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: homogeneity and morphology of subcellular fractions. Brain Res. 441, 59–71.

Fykse, E.M., Fonnum, F., 1996. Amino acid neurotransmission: dynamics of vesicular uptake. Neurochem. Res. 21, 1053–1060.

Heyes, M.P., Achim, C.L., Wiley, C.A., Major, E.O., Saito, K., Markey, S.P., 1996. Human microglia convert L-tryptophan into the neurotoxin quinolinic acid. Biochem. J. 320, 595–597.

Izquierdo, I., Medina, J.H., 1997. Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiol. Learn. Memory 68, 285–316.

Lapin, I.P., 1978. Stimulant and convulsant effects of kynurenines injected into brain ventricles in mice. J. Neural. Trans. 42, 37–43.

Lara, D.R., Schmidt, A.P., Prizzo, M.E.S., Burgos, J.S., Ramirez, G., Souza, D.O., 2001. Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. Brain Res. 912, 176–180.

Leal, M.B., Ermanoski, T., Perciavalle, L.O., Souza, D.O., Elisabetsky, E., 2001. Ibogaine alters synaptosomal and glial glutamate release and uptake. Neuro. Report. 12, 1–5.

Lipton, S.A., Rosenberg, P.A., 1994. Mechanisms of disease: excitatory amino acids as a final common pathway for neurological disorders. New Eng. J. Med. 330, 613–622.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Malcon, C., Achaval, M., Komlos, F., Portela, W., Sarmento, M., Ramirez, G., Souza, D.O., 1997. GMP protects against quinolinic acid-induced loss of NADPH-diaphorase-positive cells in the rat striatum. Neurosci. Lett. 225, 145–148.

Maragakis, N.J., Rothstein, J.D., 2001. Glutamate transporters in neurologic disease. Arch. Neurol. 58, 365–370.

Miguez, P.V., Leal, R.B., Martinovic, M., Nicolau, M., Oshiro, N.H., 1999. Synaptosomal glutamate release induced by the fraction Bo2 from the venom of the sea anemone *Bunodosoma caissarum*. Neuro. Rep. 10, 67–70.

Ozawa, S., Kamiya, H., Tsuzuki, K., 1998. Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol. 54, 581–618.

Price, D.L., 1988. New order from neurological disorders. Nature 398, A3–A5.

Reinhard, J.F., Erickson, J.B., Flanagan, E.M., 1994. Quinolinic acid in neurological disease: opportunities for novel drug discovery. Adv. Pharmacol. 30, 85–127.

Robinson, M.B., Dowd, L.A., 1997. Heterogeneity and functional subtypes of sodium-dependent glutamate transporters in the mammalian central nervous system. Adv. Pharmacol. 37, 69–115.

Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M.A., Wang, Y., Schielke, J.P., Welty, D.F., 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16, 675–686.

Schmidt, A.P., Lara, D.R., Maraschin, J.F., Perla, A.S., Souza, D.O., 2000. Guanosine and GMP prevent seizures induced by quinolinic acid in mice. Brain Res. 884, 40–43.

Stone, T.W., 2001. Kynurenines in the CNS: from endogenous obscurity to therapeutic importance. Prog. Neurobiol. 64, 185–218.

Swanson, R.A., Liu, J., Miller, J.W., Rothstein, J.D., Farrell, K., Stein, B.A., Longuemare, M.C., 1997. Neuronal regulation of glutamate transporter subtype expression in astrocytes. J. Neurosci. 17, 932–940.

Tavares, R.G., Tasca, C.I., Santos, C.E.S., Wajner, M., Souza, D.O., Dutra-Filho, C.S., 2000. Quinolinic acid inhibits glutamate uptake into synaptic vesicles from rat brain. Neuroreport 27, 249–253.

Wolosker, H., Souza, D.O., de Meis, L., 1996. Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient. J. Biol. Chem. 271, 11726–11731.

Capítulo 2

Guanine Based Purines Abolish The Inhibitory Effect of Quinolinic Acid on Uptake of Glutamate by Synaptic Vesicules From Rat Brain.

Artigo em revisão para submissão.

Guanine Based Purines Abolish The Inhibitory Effect of Quinolinic Acid on Uptake of Glutamate by Synaptic Vesicules From Rat Brain.

Rejane G. Tavares[1], Carla I. Tasca[2], Candice E. S. Santos[1], Carlos S. Dutra-Filho[1] and Diogo O. Souza[1,CA].

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 anexo, 90035-003 Porto Alegre, RS, Brasil.

[2]Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Trindade, 88040-900 Florianópolis, SC, Brasil.

[CA] Corresponding Author
Address correspondence to:
Diogo Onofre Souza
Departamento de Bioquímica
Instituto de Ciências Básicas da Saúde
Universidade Federal do Rio Grande do Sul
Rua Ramiro Barcelos, 2600 (Anexo)
90035-003, Porto Alegre, RS, Brasil.
Telephone number: +55-51 316-5558
FAX number: +55-51 316-5535
E-mail: diogo@ufrgs.br

Abstract

Quinolinic acid (QA) is an endogenous and potent neurotoxin associated with a number of important neurological diseases. The uptake of neurotransmitters to synaptic vesicles is an important event involved in the storage and release of neurotransmitters. In this work we investigated the mechanisms of QA action in vesicular glutamate uptake and a possible interaction of Guanine Based Purines (GBPs) in this effect. Results show that QA inhibits the vesicular glutamate uptake in a time- and concentration-dependent manner. Moreover, we investigated the action of QA in a vacuolar H^+-ATPase, responsible for provision of the energy to generation of a proton eletrochemical gradient that drives a glutamate uptake. QA not affect the ATP hydrolysis by V-ATPase, neither proton electrochemical gradient. formation. The maximum velocity of the initial rate of glutamate uptake was decreased by QA. GBPs [guanine, guanosine, GMP, guanosine-5'-*O*-2-thiodiphosphate (GDPβ-S) or 5'-guanylylimidodiphosphate (GppNHp)] prevent specifically this inhibition induced by QA in the vesicular uptake, while adenosine-monophosphate (AMP) or inosine-monophosphate (IMP) were ineffective. This findings might be related to the neurotoxic effects of QA and GBPs might be a neuroprotective function.

Key Words: Quinolinic acid; Glutamate; Guanine Based Purines; Synaptic vesicles; Rat brain.

Introduction

Glutamate is the main excitatory neurotransmitter in mammalian CNS, involved in processes such as plasticity, learning and memory, and neural development (Ozawa, 1998; Danbolt, 2001). Additionally, when glutamate concentrations increase in the synaptic cleft, it may be neurotoxic, as observed in acute (hypoxia, ischemia, brain traumatism) or chronic (neurodegenerative disorders) pathologic processes (Lipton e Rosenberg, 1994; Ozawa, 1998; Maragakis e Rothstein, 2001).

The synaptic actions of glutamate are terminated by its uptake from the synaptic cleft, through two transport systems: (i) a high affinity Na^+-dependent carriers, located in the cell membranes of neural and glial cells, and (ii) a low affinity Na^+-independent carriers, located in the membrane of synaptic vesicles, that is driven by a vacuolar H^+-ATPase (V-ATPase), located in the vesicles membrane (Naito e Ueda, 1985; Wolosker et al, 1996) . These both carriers, simultaneously store glutamate in synaptic vesicles and decrease enormously its concentration in synaptic cleft (Fykse and Fonnum, 1996; Danbolt, 2001). Under normal physiological conditions, glutamate stored inside the synaptic vesicles is released from synaptic vesicles in the nerve terminal by a Ca^{+2}-signaled exocytotic mechanism, when the pre-synaptic membranes are further depolarized. Hence, the modulation of glutamate uptake from the synaptic cleft by glial cells has been shown to be essential for modulation of the physiological and pathological effects of glutamate.

The intracellular roles of guanine based purines (GBPs) in events such as transmembrane signal transduction, protein synthesis, protein translocation into the endoplasmic reticulum, cell diferentiation and proliferation, and guidance of vesicular traffic within cells, have been already described (Bourne et al, 1990; Hepler at al, 1992). GTP/GDP modulate transmembrane signal transduction by binding to G-proteins, coupling neurotransmitters receptors to cell effectors (Morris and Malbon, 1999). Extracellular guanine-based purines (GBPs), namely the nucleotides GTP, GDP, GMP and the nucleoside guanosine have been shown to exert trophic effects on neural cells (Rathbone et al, 1999) as well to modulate of the glutamatergic system. Concerning their effects on the glutamatergic activity i*n vitro,* GBPs inhibit the binding of glutamate and analogs (Baron et al, 1989; Burgos et al, 1998; Paz et al, 1994), prevent cell responses to excitatory amino acids (Baron et al, 1989; Paz et al, 1994) and increase the glutamate uptake by astrocytes, a process involved in

neuroprotection (Frizzo et al, 2001; Frizzo et al, 2002; Frizzo et al, 2003). *In vivo*, GBPs protected against seizures induced by glutamatergic agents in rats and mice (Baron et al, 1989; Lara et al, 2001, Schmidt et al, 2000, Soares et al, 2004; Vinadé et al, 2003; Vinadé et al, 2005). Additionally, GBPs present amnesic and anxiolytic effects in mice (Vinadé et al, 2003; Vinadé et al, 2004; Vinadé et al, 2005). These behavioral effects seem to be related, at least partially, to antagonism of the glutamatergic system. GBPs also inhibit cell responses evoked by glutamate or its analogues by acting on the outside of cell membranes without the involvement of G-proteins (Tasca et al., 1995; Regner et al., 1998; Tasca et al., 1998; 1999a, 1999b).

Quinolinic acid (QA), an endogenous metabolite of tryptophan, is a potent endogenous neurotoxin. The neurotoxic role of QA, first demonstrated by Lapin (1978), who showed the convulsive effect of QA administration to mice (Lapin, 1978), is exerted via glutamatergic receptors in CNS. Accumulation of QA in the brain seems to be involved in the ethiopatogeny of convulsions and occurs in patients with hepatic encephalopathy, acquired immune deficiency syndrome (AIDS)-related neurological disorders, Huntington's disease and in glutaric aciduria type I (Reinhardt et al, 1994; Stone, 2001). However, in spite of its involvement in many diseases, the molecular mechanisms linking QA and brain damage are far from understood.

Considering, i) the role of synaptic vesicles in the clearance of glutamate, modulating its physiological and neurotoxic roles; ii) the attributable neuroprotective effect of GBPs against glutamate toxicity; and iii) the neuropathological effects caused by accumulation of QA, in this study we investigated the effect of GBPs on the inhibitory effect of QA on glutamate uptake into rat brain synaptic vesicles, aiming to clarify the underlying mechanisms of neurotoxicity/neuroprotective actions of these endogenous compounds

MATERIAL AND METHODS

Animals

Male Wistar rats (3 months, 200-250 g) maintained on a 12 hour light-12 hour dark schedule at 25^{o}C, with food and water *ad libitum*, were obtained from our local breeding

colony. The "Principles of laboratory animal care" (NIH publication n^{o} 85-23, revised 1985) were followed for all the experiments.

Materials

Quinolinic acid, AMP, IMP, ATP, L-glutamate, guanine, guanosine (GUO), GMP, guanosine-5'-O-2-thiodiphosphate (GDPβ-S), 5'-guanylylimidodiphosphate (GppNHp), and Bafilomycin A_1 were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[G-^{3}H]Glutamate (57.0 Ci/mmol) was obtained from Amersham International (Buckinghamshire, U.K.). Acridine orange, oxonol V and carbonylcyanide-*p*-(trifluoromethoxy)phenylhydrazone (FCCP) were kindly provided by Dr. L. de Meis. All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

Synaptic vesicles preparation

Synaptic vesicles were isolated from rat brains as described by Fykse and Fonnum (1988). Homogenates (10% wt/vol) from two whole rat brains were made in 0.32 M sucrose, 10 mM Mops-Tris (pH 7.4), and 1 mM EGTA and centrifuged for 10 min at 800 *g* (Sorvall RC-5C). The pellet was suspended, homogenized and spun again, and both supernatants were pooled and centrifuged for 30 min at 20.000 *g* to obtain crude synaptosomal fractions (P_2). The crude synaptosomal fractions were osmotically shocked by resuspension in a small volume (approximately 0.8 ml/g of fresh tissue) of 10 mM Mops-Tris (pH 7.4) containing 0.1 mM EGTA and centrifuged at 17.000 *g* for 30 min. The remaining supernatant containing synaptic vesicles were subjected to a discontinuous (0.4 M and 0.6 M) sucrose density gradient centrifugation at 65.000 *g* (Optima L Preparative Ultracentrifuge - SW28 rotor) for 2 h. Synaptic vesicle fractions were isolated from the bottom of the 0.4 M sucrose band and stored at -70^{o} C. In order to evaluate a possible contamination by plasme membrane vesicles, we performed control incubations with the addition of high sodium concentrations, since glutamate uptake by plasme membranes is Na^{+}-dependent. In none of the synaptic vesicle preparations the uptake was increased by sodium ions (data not shown). Additionally, we performed the electronic microscopy of our preparation, were we observed the purity of synaptic vesicles preparation. Protein

concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Glutamate Vesicular Uptake

Uptake was performed as described by Wolosker et al. (1996) and detailed by Tavares et al. (2000; 2001). Briefly, standard uptake medium were composed by 10 mM Mops-Tris (pH 7.4), 4 mM KCl, 140 mM potassium gluconate, 0.12 M sucrose, 2 mM $MgCl_2$, 2 mM ATP, 50 μM L-glutamate, 3 μCi/ml L-[^{3}H]glutamate.. In experiments performed to study the QA effect, 0.01 to 10 mM QA was added in the uptake medium. In order to evaluate the action of GBPs, 100 nM to 1 mM of guanine, guanosine, GMP, GDPβ-S or GppNHp were added in the uptake medium. AMP or IMP were used in concentration of 1 mM. Uptake was started by the addition of synaptic vesicles (40-50 μg of protein/tube), carried out for 1.5 to 10 min at 35^o C, and the reaction was stopped by rapid filtration of the assay medium through 0.45 μm Millipore filters. The filters were quickly flushed with three times 4 ml of 10mM Mops-Tris (pH 7.4) at room temperature. Glutamate uptake was corrected for nonspecific binding, which was measured in the absence of ATP. Radioactivity was counted in a liquid scintillation counter.

Measurement of ATPase activity

ATP hydrolysis was assayed in standard uptake medium, as described above, in the absence of radioactive glutamate. Vesicles were pre-incubated for 10 minutes in the presence of 1 μM Bafilomycin A_1 (Bafilomycin-sensitive ATPase) or not for evaluation of total Mg^{+2}-ATPase. Synaptic vesicle preparations were added to reaction medium containing 50 μM glutamate and QA (0.5 to 10 mM), and incubated for 10 min at 35^o C. Reaction was stopped by the addition of trichloroacetic acid 10 % (vol/vol). Samples were chilled on ice for 10 min and 100 μl aliquots were withdrawn for assaying the released inorganic phosphate (Pi) through the method of Chan et al. (1986), using malachite green as the color reagent. Controls with addition of the synaptic vesicle preparation after trichloroacetic acid were used for correcting non-enzymatic Pi releasing.

Electrochemical proton gradient

Fluorescence measurements were made at 25^{o} C in a F-3010 Hitachi fluorescence spectrophotometer using pH and potential-sensitive probes as described previously (Maycox et al, 1988; Cidon and Sihra, 1989). The proton gradient (ΔpH) was determined by measuring the fluorescence quenching of acridine orange (2 μM) at 492 (excitation) and 537 nm (emission). The transmembrane potential (ΔΨ) was determined by measuring the fluorescence quenching of oxonol V (1.5 μM) at 617 (excitation) and 643 nm (emission). The reaction was started by the addition of 2 mM ATP to a 1.5 ml cuvette containing 100 μg synaptic vesicle protein/ml, 10 mM Mops-Tris (pH 7.0), 140 mM KCl (for ΔpH measurement) or 140 mM potassium gluconate (for ΔΨ measurement). The reaction was stopped by the addition of the protonophore FCCP to a final concentration of 10 μM.

Statistical analysis

Results were analyzed by one-way ANOVA followed by Duncan's multiple-range test when appropriate.

Results

In a previous study we demonstrated that Quinolinic Acid (QA) inhibited vesicular uptake of glutamate when incubated for 10 min (steady-state). In order to clarify the mechanism of this inhibition, we performed a time course analyses (ranging from 0.5 to 10 min) by using 1 mM QA (Fig. 1). In the presence of 50 μM glutamate, QA displaying an inhibitory effect from 1.5 min. The effects of increasing concentrations (0.1 to 10 mM) of QA on the initial rate (1.5 min) of glutamate uptake was studied (Fig. 2). QA inhibited the glutamate uptake significantly from 0.5 mM.

The kinetic parameters were determinated, using Linewever-Burk analysis. When assaying glutamate uptake for 10 min incubations, the K_M values observed were not different in the absence or presence of 1 mM QA: 0.73 ± 0.18 mM and 0.69 ± 0.24 mM, respectively. However, V_{max} values for vesicular glutamate uptake were significantly decreased from 4.31 ± 0.79 nmol/mg protein.min^{-1} to 2.62 ± 0.52 nmol/mg protein.min^{-1} in

the presence of 1 mM QA (Fig 3). Thus, QA appears to act as noncompetitive inhibitor with respect to glutamate. In order to verify whether the inhibitory effect of QA could be due to an effect on the V-ATPase, we measured the activity of the vesicular Bafilomycin-sensitive ATPase, enzyme involved in the uptake by generation of energy. Activity of total vesicular Mg^{+2}-ATPase also was measurement for functional control of the synaptic vesicles preparation. We observed that QA from 0.5 to 10 mM did not affect the ATP hydrolysis by Bafilomycin-sensitive V-ATPase in the vesicle preparations, indicating that inhibitory effect of QA was occurring probably through action on glutamate carrier (Fig. 4). The formation of a proton gradient (ΔpH) and transmembrane potential (Δψ) are necessary to drive glutamate uptake. The evaluation of this electrochemical proton gradient showed that 1 mM QA did not alter the proton gradient or transmembrane potential (data not shown).

Previous studies from our group demonstrated importance of guanine based purines (GBP) in the modulation of the glutamatergic responses. Fig 6 shows the interaction between 0.5 mM QA and GBP (100 nM to 1 mM) on vesicular glutamate uptake. The inhibitory effect caused by 0.5 mM QA was reversed from of all GBP used (GUA, GUO, GMP, GDPβ-S or Gpp(NH)p), in concentration from 10 μM to 1 mM. We have previously demonstrated that GBP caused a delay in vesicular glutamate uptake, however at 10 min. incubations, no GBP alone affected the glutamate uptake (data not shown). Adenine or inosine derivatives (AMP or IMP) did not revert the inhibition induced by 0.5 mM QA on the vesicular glutamate uptake (Fig. 7), indicating a specific modulatory effect of guanine derivatives on QA-induced vesicular glutamate uptake inhibition.

Discussion

Brain synaptic vesicles actively accumulate glutamate from the cytosol by a process driven by a transmembrane electrochemical proton gradient generated by a Mg^{2+}-ATPase. This uptake is mainly driven by the membrane potential (Rudnick, 1998). Glutamate uptake in synaptic vesicles were shown to occur in many animal species, as fish, avians (Roseth and Fonnum, 1995) and rats (Roseth et al, 1995). This system can be modulated by endogenous or exogenous compounds; the inhibition of glutamate uptake into synaptic

vesicles by an endogenous cytosolic factor (Lobur et al, 1990) or nitric oxide (Wolosker et al, 1996) and by exogenous factors, as mitochondrial uncouplers and ionophores, some dyes (Wang e Floor, 1998; Ogita et al, 2001), or some pesticides (Vaccari et al, 1999) and also by glutamate analogs, including naturally occurring amino acids (Fykse et al, 1989), have been demonstrated. The impairment of this uptake lead to increased concentrations of extracellular glutamate, which can evoke excitotoxic actions. Therefore, the clearance of extracellular glutamate must be precisely modulated (Fykse et al, 1989; Rudnick, 1998).

The intracellular roles of guanine based purines (GBP) in many events have been already described (for a review, see Bourne et al, 1990). GTP modulates transmembrane signal transduction by binding to G-proteins, leading them to the active state; an intrinsic GTPase activity of G-proteins produces GDP, which remains bound to G-proteins in their inactive state (Hepler and Gilman, 1992). Moreover, recents studies suggested that neuroprotective GBPs effects against toxic overstimulation of the glutamatergic system were exerted specifically by guanosine or through the conversion of guanine nucleotides to guanosine (Soares et al, 2004).

Several reports have suggested that QA is associated with neuronal damage, through the overactivation of NMDA subtype of glutamate receptors. Striatal administration of QA may be associated with neurotoxicity and development of neurological damage resembling consequences observed in Huntington's disease (Beagles et al, 1998). Recently we demonstrated that QA inhibits the vesicular glutamate uptake, without interfering on GABA or glycine uptake (Tavares et al, 2000), stimulates the glutamate synaptosomal release and inhibits the uptake in astrocytes (Tavares et al, 2002).

The inhibition time and concentration-dependent of vesicular glutamate uptake produced by QA may be related to the mechanisms involved in neurotoxicity induced by this compound, besides NMDA receptor stimulation. The absence of some inhibitory effect on vesicular ATPase activity indicates that QA is acting directly on the vesicular carrier, were normally glutamate binds. By inhibiting vesicular glutamate uptake, QA might alter the synaptic turnover of glutamate.

QA inhibitory effect on vesicular glutamte uptake was reversed by GBP. Considering that QA is an endogenous neurotoxin, these results point to an additional site of GBP action as neuroprotective compound. This is a important evidence that GBP may be

neuroprotectors by acting in other sites than glutamatergic receptors in neural cell membranes. The ineffectivity of AMP or IMP on QA inhibition could indicate some specificity of guanine purines as compared with other purines.

ACKNOWLEDGMENTS:

This study was supported by grants from the Brazilian National Research Council (CNPq), the Financing Agency for Studies and Projects (FINEP), and the Pronex (# 41960904).

References

DANBOLT, N.C. Glutamate uptake. *Progress in Neurobiology*. 65: 1-105, 2001.

OZAWA, S.; KAMIYA, H.; TSUZUKI, K. Glutamate receptors in the mammalian central nervous system. *Progress in Neurobiology* 54: 581-618, 1998.

Lipton, A. S., and Rosenberg, P. A. 1994. Excitatory amino acids as a final common pathway for neurological disorders. New England J. Med. 330:613-622.

MARAGAKIS, N.J. & ROTHSTEIN, J.D. Glutamate transporters: animal models to neurologic disease. *Neurobiology of Disease*. 15:461-473, 2004.

NAITO, S., AND T. UEDA. 1985. Characterization of glutamate uptake into synaptic vesicles. *J. Neurochem*. **44**: 99-109.

WOLOSKER, H.; SOUZA, D. O.; de MEIS, L. Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient. *J. Biol. Chem*. 271: 11726-11731, 1996.

Bourne, H. R., Sanders, D. A., and McCormick, F. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348:125-131.

Hepler, J. R., and Gilman, A. G. 1992. G Proteins. Trends Biochem. Sci. 17:383-387.

17.

MORRIS, J.A. & MALBON, C.C. Physiological regulation of G protein-linked signaling. *Physiol. Rev*. 79: 1373-1430, 1999.

RATHBONE, M.P., MIDDLEMISS, P.J., GYSBERGS, J.W., ANDREW, C., HERMA, M.A.R., REE, J.K., CICCARELLI, R., DI IORIO, P., CACIAGLI, F. Trophic effects of purines in neurons and glial cells. *Prog. Neurobiol*. 59: 663-690, 1999.

B.M. Baron, M.W. Dudley, D.R. McCarty, F.P. Miller, I.J. Reynolds, C.J. Schmidt, Guanine nucleotides are competitive inhibitors of N-Methyl-D-Aspartate at its receptor site both *in vitro* and *in vivo*, J. Pharmacol. Exp. Ther. 250 (1989) 162-169.

J.S. Burgos, A. Barat, D.O. Souza, G. Ramírez, Guanine nucleotides protect against kainate toxicity in an ex vivo chick retinal preparation, FEBS Lett. 430 (1998) 176-180.

PAZ, M.M., RAMOS, M., RAMIREZ, G., SOUZA, D.O. Differential effects of guanine nucleotides on kainic acid binding and on adenylate cyclase activity in chick optic tectum *FEBS Lett*. 355: 205-208, 1994.

FRIZZO, M.E.S.; LARA, D.R.; DAHM, K.C.S.; PROKOPIUK, A.S.; SWANSON, R.; SOUZA, D.O. Activation of glutamate uptake by guanosine in primary astrocyte cultures. *NeuroReport* 12: 879-881, 2001.

FRIZZO, M.E.S., LARA, D.R., PROKOPIUK, A.S., VARGAS, C.R., SALBEGO, C.G., WAJNER, M., SOUZA, D.O. Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. *Cell. Mol. Neurobiol*. 22: 353-63, 2002.

FRIZZO, M.E.S., SOARES, F.A., DALL'ONDER, L.P., LARA, D.R., SWANSON, R.A., SOUZA, D.O. Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. *Brain Res*. 972: 84-89, 2003.

FYKSE, E. M.; & FONNUM, F. Amino acid neurotransmission: dynamics of vesicular uptake. *Neurochem. Res*. 21: 1053-1060, 1996.

Fykse EM, Christensen H, Fonnum F. Comparison of the properties of gamma-aminobutyric acid and L-glutamate uptake into synaptic vesicles isolated from rat brain. J Neurochem 1989;52:946-51.

LAPIN, I. P. Stimulant and convulsant effects of kynurenines injected into brain ventricles in mice. *J. Neural Trans*. 42: 37-43, 1978.

LARA, D.R., SCHMIDT, A.P., FRIZZO, M.E.S., BURGOS, J.S., RAMIREZ, G., SOUZA, D.O.. Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. *Brain Res*. 912: 176-180, 2001.

FYKSE, E. M., AND F. FONNUM. 1988. Uptake of gamma-aminobutyric acid by a synaptic vesicle fraction isolated from rat brain. *J. Neurochem*. **50**: 1237-1242.

SCHMIDT, A.P., LARA, D.R., MARASCHIN, J.F., PERLA, A.S., SOUZA, D.O. Guanosine and GMP prevent seizures induced by quinolinic acid in mice. *Brain Res*. 864: 40-43, 2000.

SOARES, F.A., SCHMIDT, A.P., FARINA, M., FRIZZO, M.E., TAVARES, R.G., PORTELA, L.V., LARA, D.R., SOUZA, D.O. Anticonvulsant effect of GMP depends on its conversion to guanosine. *Brain Res*. 1005: 182-186, 2004

VINADÉ, E.R., SCHMIDT, A.P., FRIZZO, M.E.S., IZQUIERDO, I., ELIZABETSKY, E., SOUZA, D.O. Chronically administered guanosine is anticonvulsant, amnesic and anxiolytic in mice. *Brain Res*. 977: 97-102, 2003.

VINADE, E.R., IZQUIERDO, I., LARA, D.R., SCHMIDT, A.P., SOUZA, D.O. Oral administration of guanosine impairs inhibitory avoidance performance in rats and mice. *Neurobiol. Learn. Mem*. 81: 137-143, 2004.

VINADÉ, E.R., SCHMIDT, A.P., FRIZZO, M.E.S., PORTELA, L.V., SOARES, F.A., SCHWALM, F.D., ELISABETSKY, E., IZQUIERDO, I., SOUZA, D.O. Effects of Chronic Administered Guanosine on Behavioral Parameters and Brain Glutamate Uptake in Rats. *Journal of Neurosci. Res*. 79: 248-253, 2005.

TASCA, C.I.; WOFCHUK, S.T.; SOUZA, D.O.; RAMÍREZ, G.; RODNIGHT, R. Guanine nucleotides inhibit the stimulation of GFAP phosphorylation by glutamate. *NeuroReport,* 6: 249-252, 1995.

REGNER, A.; RAMÍREZ, G.; BELLÓ-KLEIN, A.; SOUZA, D.O. Effects of guanine nucleotides on glutamate-induced chemiluminescence in rat hippocampal slices submitted to hypoxia. *Neurochem, Res*. 23: 523-528, 1998.

TASCA, C.I.; CARDOSO, L.F.; MARTINI, L.H.; RAMÍREZ, G.; SOUZA, D.O. Guanine nucleotides inhibit cAMP accumulation induced by metabotropic glutamate receptors activation. *Neurochem. Res.* 23: 183-188, 1998.

TASCA, C.I.; CARDOSO, L.F.; SOUZA, D.O. Effects of guanine nucleotides on adenosine and glutamate modulation of cAMP levels in optic tectum slices from chicks. *Neurochem. Int*. 34: 213-220, 1999a.

TASCA, C.I.; BURGOS, J.S.; BARAT, A.; SOUZA, D.O.; RAMIREZ, G. Chick Kainate binding protein lacks GTPase activity. NeuroReport 10:1981-1983, 1999b

STONE, T.W. Kynurenines in the CNS: from endogenous obscurity to therapeutic importance. *Progress in Neurobiology*. 64: 185-218, 2001.

REINHARD, J.F., ERICKSON, J.B., FLANAGAN, E.M., 1994. Quinolinic acid in neurological disease: opportunities for novel drug discovery. Adv. Pharmacol. 30, 85-127.

LOWRY, O. H., R. L. ROSENROUGH, R. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193**: 265-275.

TAVARES, R.G.; TASCA, C.I.; SANTOS, C.E.S.; WAJNER, M.; SOUZA, D.O.; DUTRA-FILHO, C.S. Quinolinic acid inhibits glutamate uptake into synpatic vesicles from rat brain. *NeuroReport* 11: 249-253, 2000.

TAVARES, R.G.; SANTOS, C.E.S.; TASCA, C.I.; WAJNER, M.; SOUZA, D.O.; DUTRA-FILHO, C.S. Inhibition of glutamate uptake into synaptic vesicles from rat brain by 3-nitropropionic acid in vitro. Exp. Neurol. 172:250-254, 2001

CHAN, K., D. DELFERT AND K. D. JUNGER. 1986. A direct colorimetric assay for Ca^{+2}-ATPase activity. *Anal. Biochem*. **157**: 375-380.

CIDON, S., AND T. S. SIHRA. 1989. Characterization of a H^{+}-ATPase in rat brain synaptic vesicles. *J. Biol. Chem*. **264**: 8281-8288.

MAYCOX, P.R.; DECKWERTH, T.; HELL, J.W., JAHN, R. 1988. Glutamate Uptake by brain synaptic vesicles. J. Biol. Chem. 263, 15423-15428.

ROSETH, S., AND F. FONNUM. 1995. A study of the uptake of glutamate, γ-aminobutyric acid (GABA), glycine and β-alanine in synaptic brain vesicles from fish and avians. *Neurosci. Lett*. **183**: 62-66.

RUDNICK, G. 1998. Bioenergetics of neurotransmitter transport. *J. Bioenerg. Biomemb*. **30**: 73-185.

ROSETH S, FYKSE EM, FONNUM F. Uptake of L-glutamate into rat brain synaptic vesicles: effect of inhibitors that bind specifically to the glutamate transporter. J Neurochem 1995;65:96-103.

LOBUR AT, KISH PE, UEDA T. Synaptic vesicular glutamate uptake: modulation by a synaptosomal cytosolic factor. J Neurochem 1990;54:1614-8.

VACCARI A, SABA P, MOCCI I, RUIU S. Dithiocarbamate pesticides affect glutamate transport in brain synaptic vesicles. J Pharmacol Exp Ther 1999;288:1-5.

OGITA, K.; HIRATA, K.; BOLE, D.G.; YOSHIDA, S.; TAMURA, Y.; LECKENBY, A.M.; UEDA, T. 2001 Inhibition of vesicular glutamate storage and exocytotic release by Rose Bengal. J. Neurochem. 77, 34-42.

R.G. TAVARES, C.I. TASCA, C.E. SANTOS, L.B. ALVES, L.O. PORCIÚNCULA, T. EMANUELLI, D.O. SOUZA, Quinolinic acid stimulates synaptosomal glutamate release and inhibits glutamate uptake into astrocytes, Neurochem. Int. 40 (2002) 621-627.

Legends

Figure 1: Effect of QA on time-course of glutamate uptake into rat brain synaptic vesicles. QA 1 mM was incubated in the presence of 50 μM L-[^{3}H]glutamate, for 0.5 to 10 min at 35° C. Results are expressed in mean ± S.E.M. (nmol.mg protein^{-1}.min^{-1}) and represent 6 separate experiments from different synaptic vesicles preparations. Incubations were performed in triplicates. *Indicates means significantly different from the control group, $p<0.05$.

Figure 2: Effect of increasing QA on glutamate uptake into synaptic vesicles. QA from 0.01 to 10 mM was incubated for 1,5 min at 35° C in the presence of 50 μM L-[^{3}H]glutamate. Results were expressed in mean ± S.E.M. (nmol.mg protein^{-1}.min^{-1}) and represent 6 separate experiments from different synaptic vesicles preparations. Incubations were performed in triplicates. *Indicates means significantly different from the control group, $p<0.05$.

Figure 3: Effect of increasing glutamate concentration on glutamate uptake into rat brain synaptic vesicles in the absence or presence of 1 mM QA. Lineweaver-Burk analysis in 10 min incubations displaying the K_M values in the absence or presence of 1 mM QA: 0.73 ± 0.18 mM and 0.69 ± 0.24 mM, respectively. V_{max} values were 4.31 ± 0.79 nmol/mg protein.min^{-1} and 2.62 ± 0.52 nmol/mg protein.min^{-1} ($p<0.05$). Data represent means of 12 separate experiments carried out in triplicate. Standard deviations did not reach 20% of the values of means.

Figure 4: Effect of increasing QA concentrations on Bafilomycin-sensitive V-ATPase activity in the rat brain synaptic vesicles. QA (0.5 to 10 mM) was incubated for 10 min at 35° C in the presence of 2 mM ATP and standard incubation medium. Data represent means ± S.E.M. of 4 separate experiments carried out in triplicate. *Indicates means significantly different from the Total V-ATPase activity, $p<0.05$.

Figure 5: Effect of GBP (guanine, guanosine, GMP, GDPβ-S or Gpp(NH)p) (100 nM to 1 mM) on glutamate uptake into rat brain synaptic vesicles. The incubation lasts for 10 min at 35° C in the presence of 50 µM glutamate plus 0.5 mM QA. Data represent means of 6 experiments carried out in triplicate. Standard errors did not reach 20% of the values of means. *indicates means significantly different from the control group, $p<0.05$.

Figure 6: Effect of AMP or IMP (1 mM) on glutamate uptake into rat brain synaptic vesicles. The incubation lasts for 10 min at 35° C in the presence of 50 µM glutamate plus 0.5 mM QA. Results were expressed in mean ± S.E.M. (nmol.mg protein^{-1}.min^{-1}) and represent 6 separate experiments from different synaptic vesicles preparations. Incubations were performed in triplicates. *indicates means significantly different from the control group, $p<0.05$.

Figura 1

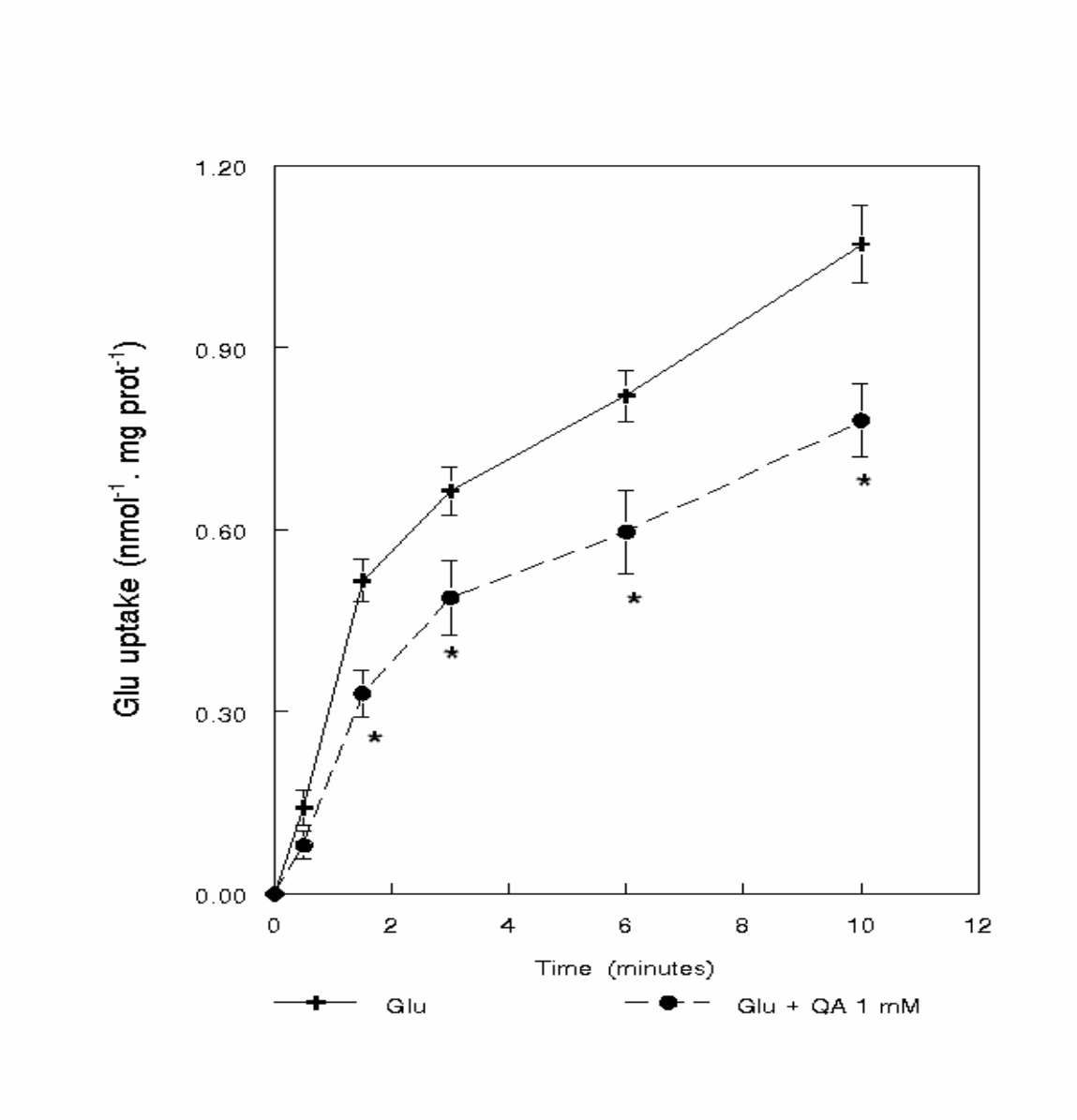

1.20
0.90
0.60
0.30
0.00
Glu uptake (nmol-1. mg prot-1)
0
2
4
6
8
10
12
Time (minutes)
Glu
Glu + QA 1 mM

Figura 2

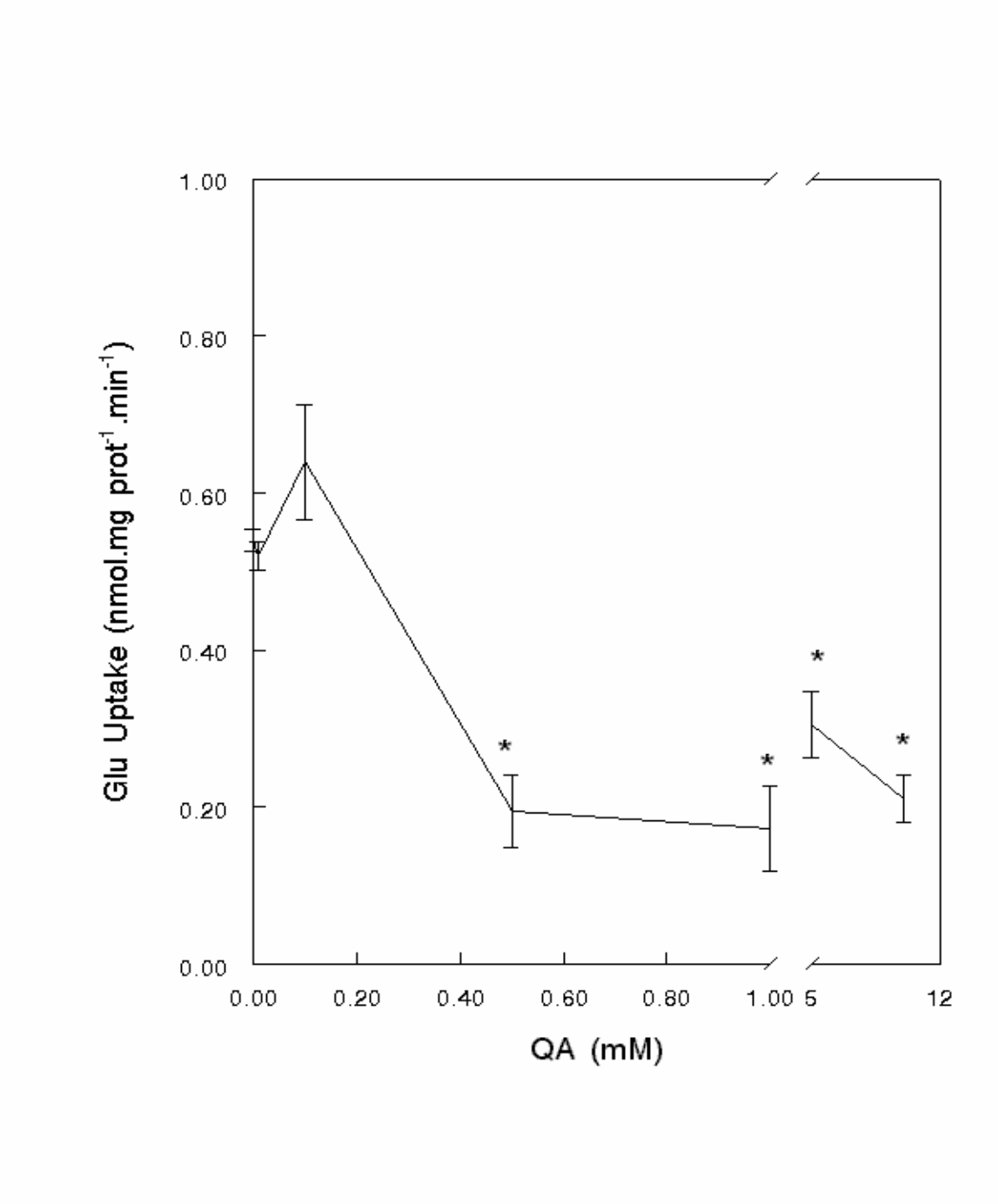

1.00
0.80
0.60
0.40
0.20
0.00
Glu Uptake (nmol.mg prot-1.min-1)
0.00
0.20
0.40
0.60
0.80
1.00
5
12
QA (mM)
*
*
*
*

Figura 3

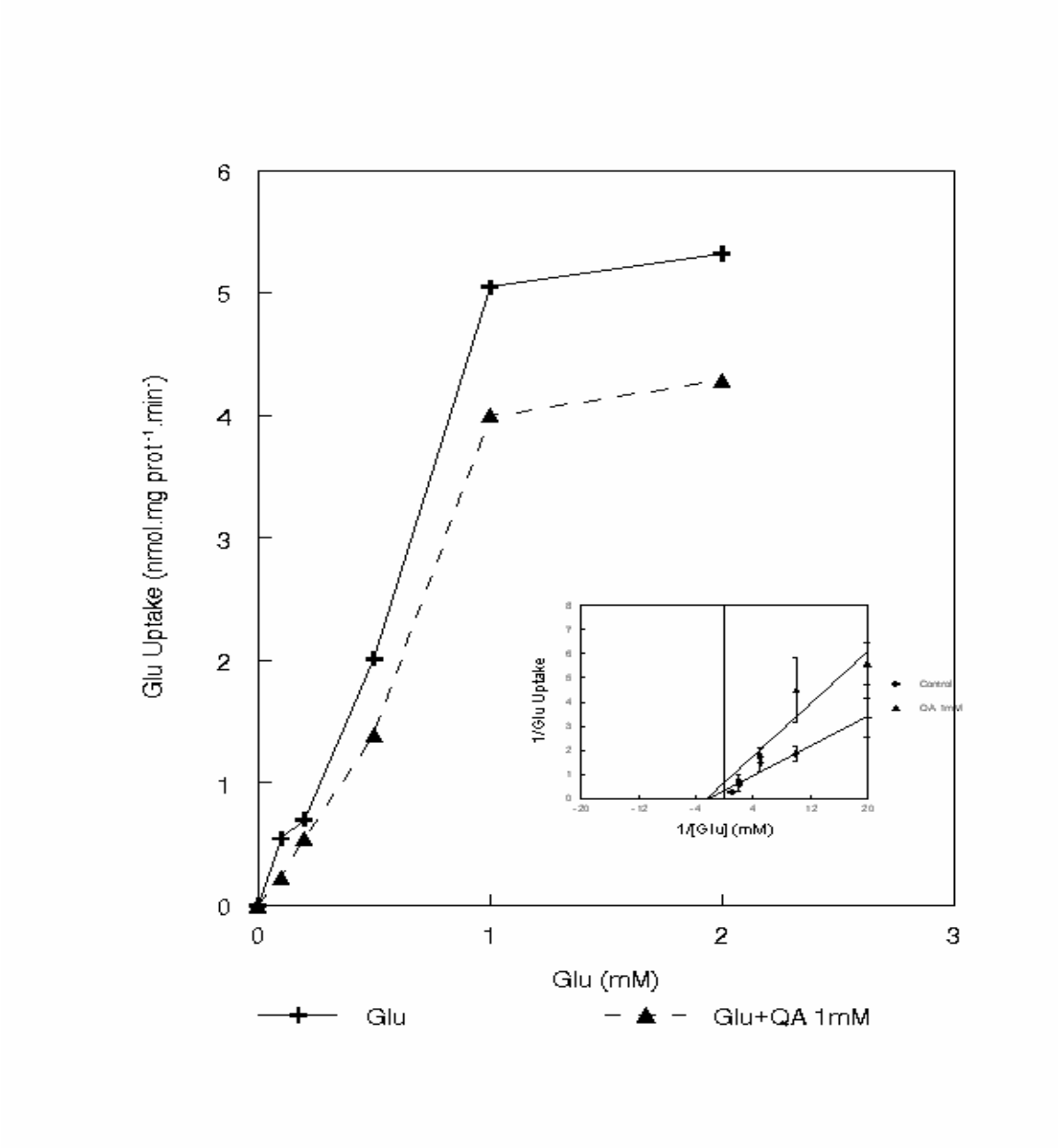

Glu Uptake (nmol.mg prot-1.min-1)
Glu (mM)
Glu
Glu+QA 1mM
1/Glu Uptake
1/[Glu] (mM)
Control
QA 1mM

Figura 4

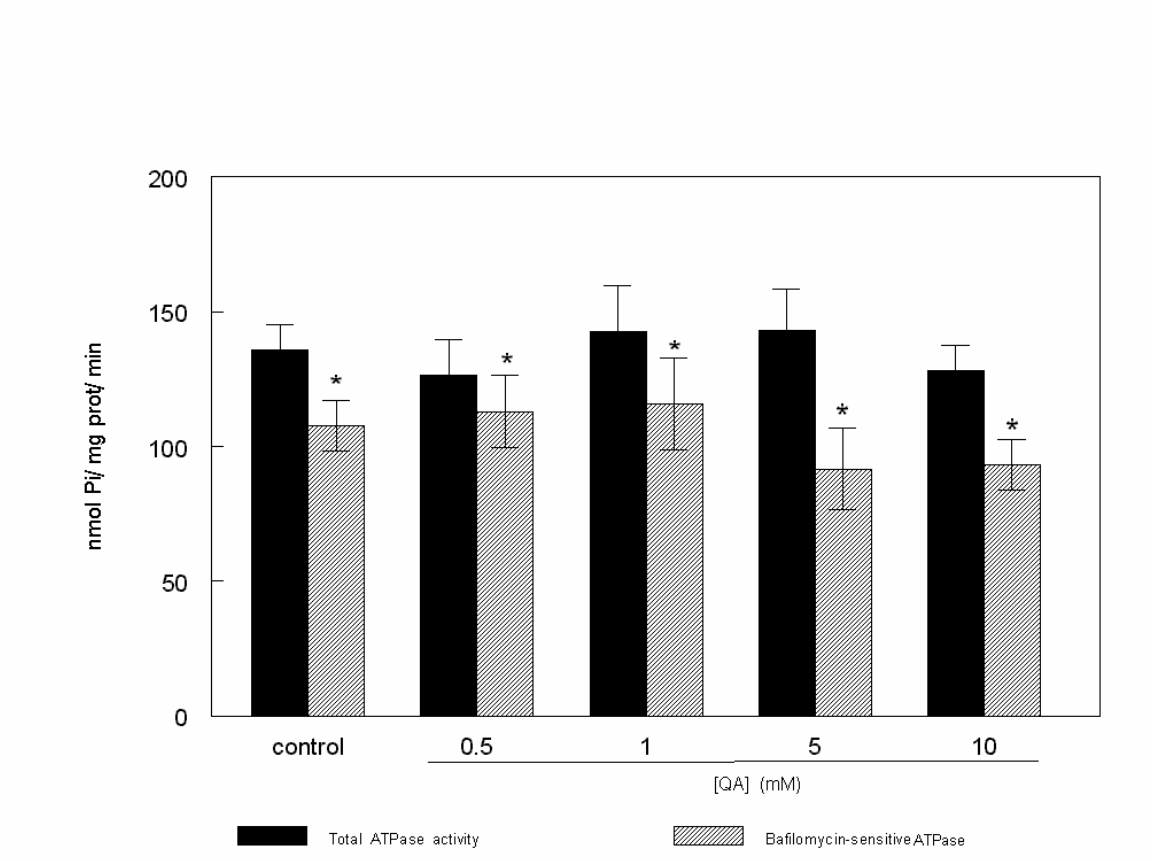

200
150
100
50
0
nmol Pi/ mg prot/ min
*
*
*
*
*
control
0.5
1
5
10
[QA] (mM)
Total ATPase activity
Bafilomycin-sensitive ATPase

Figura 5

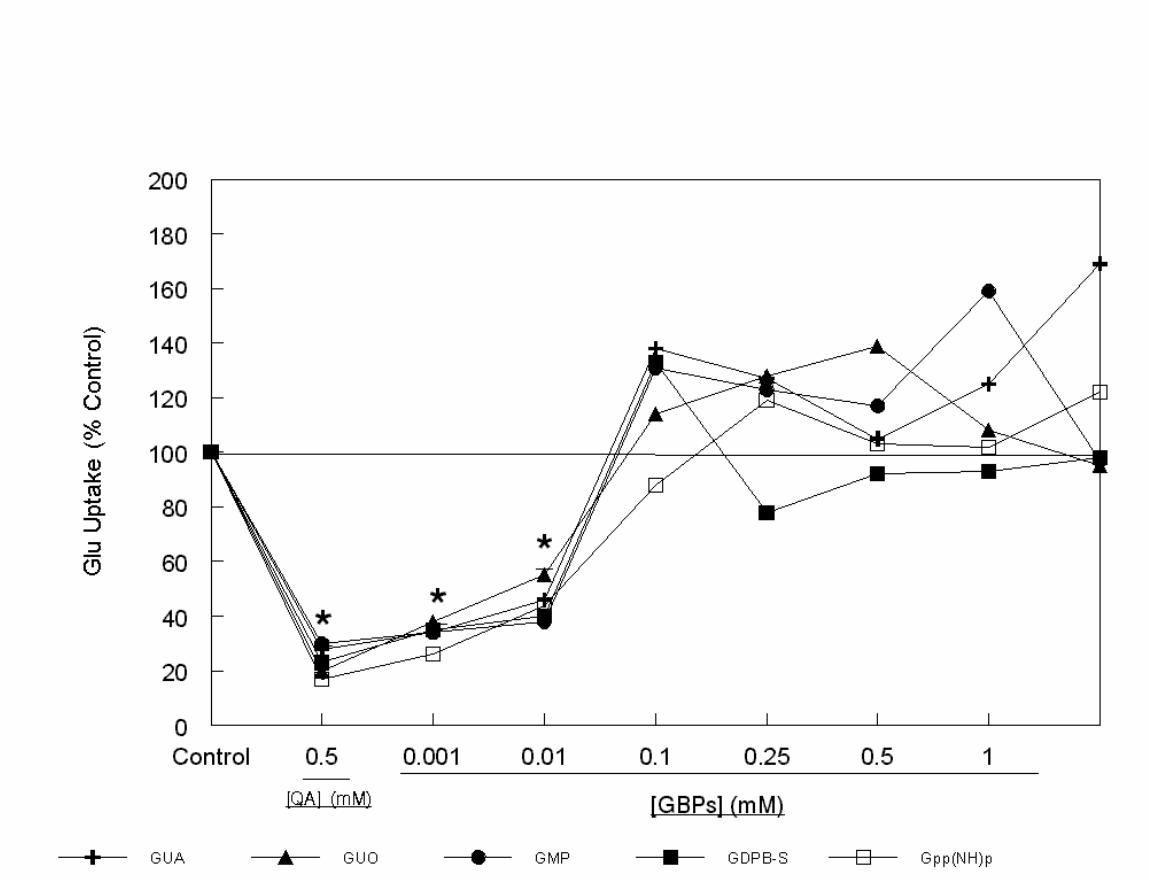
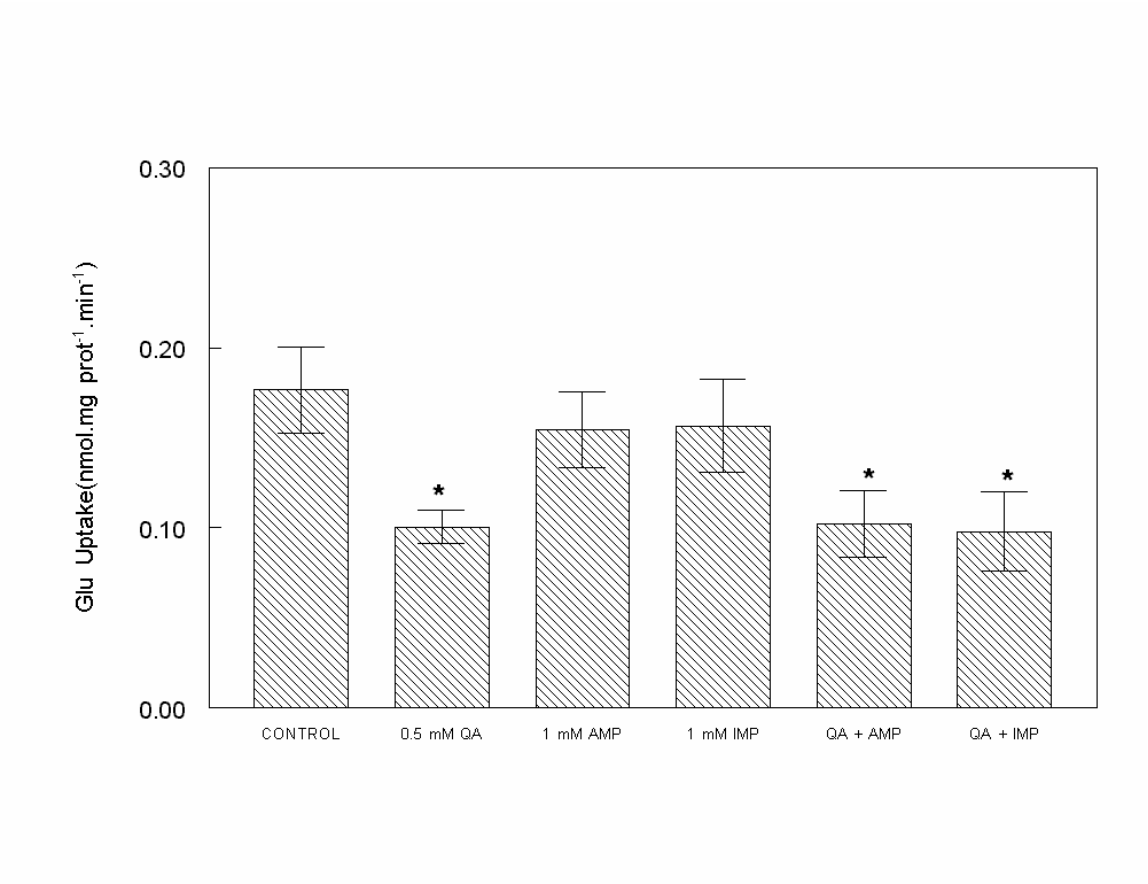

Glu Uptake (% Control)
200
180
160
140
120
100
80
60
40
20
0
Control
0.5
0.001
0.01
0.1
0.25
0.5
1
[QA] (mM)
[GBPs] (mM)
GUA
GUO
GMP
GDPB-S
Gpp(NH)p

Figura 6


0.30
0.20
0.10
0.00
Glu Uptake(nmol.mg prot-1.min-1)
*
*
*
CONTROL
0.5 mM QA
1 mM AMP
1 mM IMP
QA + AMP
QA + IMP

Capítulo 3

Synaptic vesicle glutamate uptake is increased after seizures induced by quinolinic acid in rats

Artigo submetido ao *NeuroReport*

Synaptic vesicle glutamate uptake is increased after seizures induced by quinolinic acid in rats

Rejane G. Tavares[1], André P. Schmidt[1], Carla I. Tasca[2] and Diogo O. Souza[1] (✉)

[1]Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil

[2]Department of Biochemistry, CCB, Federal University of Santa Catarina, Florianópolis, SC, Brazil

(✉) Diogo O. Souza

Avenida Ramiro Barcelos, 2600-Anexo

CEP 90035-003

Porto Alegre - RS - Brazil

Phone: (55-51) 33165557 / 33165558

Fax: (55-51) 33165540 / 33165535

E-mail: diogo@ufrgs.br

Running Head**: Quinolinic acid stimulates glutamate uptake in seizures.**

Abstract

Glutamate is the main neurotransmitters, mediating excitatory synaptic events. Giving the relevance of glutamate in epileptogenesis and the role of animal models in understanding epilepsy pathogenesis, the aim of this study was to investigate the effects of *in vivo* administration of several convulsant agents on glutamate uptake by synaptic vesicles in rats. Animals were treated with vehicle (saline 0.9%), QA (quinolinic acid) 156.8 nmoles, kainate 30 mg/kg, picrotoxin 6 mg/kg, PTZ (pentylenetetrazole) 60 mg/kg, caffeine 150 mg/kg or MES (maximal transcorneal electroshock) 80 mA. All convulsants induced seizures up to 100 % of animals but only QA stimulated glutamate uptake by synaptic vesicle. This study provided new evidence on the role of QA on glutamate synaptic vesicle uptake in rats.

Keywords: Epilepsy, seizures, quinolinic acid, glutamate uptake, synaptic vesicles.

1 - Introduction:

Glutamate, by acting via ionotropic and metabotropic receptors, is the main excitatory neutransmitter in mammalian central nervous system (CNS), being essential for its normal function [1]. However, overstimulation of the glutamatergic system such as increasing concentrations of glutamate in the synaptic cleft has been implicated in the pathogenesis of several acute and chronic brain diseases such as epilepsy [1-4]. An altered glutamate neurotransmission will lead to an excess of excitation, which may play a pivotal role in the initiation and/or spread of abnormal discharges that can result in seizures [5].

Glutamate is taken up and stored for neurotransmission in synaptic vesicles by a $Mg(^{2+})$-ATP dependent process. The main driving force for vesicular glutamate uptake is the transmembrane potential [6]. Upon substantial increase in the intracellular free calcium concentration, synaptic vesicles fuse with the plasma membrane and release their neurotransmitter contents, initiating synaptic neurotransmission [6,7]. Altered transport properties (i.e. increased glutamate uptake activity) could be related to an enhanced excitatory neurotransmission, which can lead to seizure behavior [8].

A great variety of animal models have been used in epilepsy research in the last 40 years [9]. These range from electrical induced seizures to genetic models of epilepsy in rats and mice [8,9]. These models have proved to be useful in understanding of the pathogenesis of epilepsy and testing the anticonvulsant properties of agents given systemically [9]. Quinolinic acid (QA), an agonist of N-methyl-D-aspartate (NMDA) receptors and glutamate releaser, induces seizures, has been proposed to be involved in the etiology of epilepsy [10,11] and has proven to be a useful animal model of seizures involving the glutamatergic system. Other toxic agents such as kainate, picrotoxin, pentylenetetrazole (PTZ), caffeine and maximal transcorneal electroshock (MES) promote seizures, provide adequate animal models to study epilepsy and may modulate, at least partially, the glutamatergic system [9].

Giving the relevance of glutamate in pathological processes such as epilepsy, the potential modulation of glutamatergic neurotransmission by synaptic vesicles and the pivotal role of animal models in understanding the epilepsy pathogenesis, the aim of this study was to investigate and compare the effects of *in vivo* administration of several electral

and chemical convulsant agents on glutamate uptake by synaptic vesicles from rat brain.

2 - Material and methods:

2.1. Drugs

QA, kainate, picrotoxin, PTZ and caffeine were obtained from Sigma Chemicals (St Louis, MO, USA). L-[^{3}H]Glutamate (57.0 Ci/mmol) was purchased from Amersham International (UK). The anesthetic sodium thiopental was obtained from Cristália (SP, Brazil). All solutions were dissolved in saline 0,9% and buffered with 0,1 N NaOH or 0,1 N HCl to pH 7,4 when necessary.

2.2. Animals

Male adult Wistar rats (250-350 g) were kept on a 12 hour light/dark cycle (light on at 7:00 am) at a constant temperature of 22 ± 1ºC. They were housed in plastic cages (five per cage) with commercial food and tap water *ad libitum.* Our institutional protocols for experiments with animals, designed to minimize suffering and limit the number of animals sacrificed, were followed throughout.

2.3. Animal models of seizures

2.3.1 Quinolinic acid-induced seizures

Animals were anesthetized with sodium thiopental (40 mg/kg, 1 ml/kg, i.p.). In an stereotaxic apparatus, the skin of the skull was removed and a 27 gauge 9 mm guide cannula was placed at 0.9 mm posterior to bregma, 1.5 mm right from de midline and 1.0 mm above the lateral brain ventricle. Through a 2 mm hole made at the cranial bone, the cannula was implanted 2.6 mm ventral to the superior surface of the skull, and fixed with jeweler acrylic cement. Experiments were performed 72 hours after surgery. A 30 gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a microsyringe. The tip of the infusion cannula protruded 1,0 mm beyond the guide cannula aiming the lateral brain ventricle. Animals were treated with an i.c.v. infusion of either 4 µL of vehicle (saline 0.9%) or QA (39,2 mM, the lowest dose causing seizures in all control animals). Rats were observed in plexiglas chambers for the occurrence of tonic-clonic seizures lasting more than 5 seconds [12]. Ten minutes after first seizure, rats were sacrified by decaptation and brain used for synaptic vesicle preparation. Animals not displaying

tonic-clonic seizures during the first 10 minutes of behavior observation were discarded from synaptic vesicle preparation.

2.3.2. *Kainate-induced seizures*

The kainate model has been described previously [13]. In summary, animals received a single i.p. dose of vehicle (saline 0.9%) or kainate (30 mg/kg) and observed for 60 minutes for the ocurrence of seizures. Ten minutes after the first seizure, rats were sacrified by decaptation and brain used for sinaptic vesicle preparation. Only animals that reached generalized motor seizures were used for the synaptic vesicle preparation.

2.3.3. *Picrotoxin-induced seizures*

Picrotoxin seizure model has been described in detail previously [14]. Rats were treated with a subcutaneous (s.c.) infusion of vehicle (saline 0.9%) or picrotoxin (6 mg/kg) and observed for 60 minutes for the presence of clonic seizures. Ten minutes after the first seizure, rats were sacrified by decaptation and brain used for sinaptic vesicle preparation. Animals not displaying clonic seizures during these 60 minutes were discarded from synaptic vesicle preparation..

2.3.4. *PTZ-induced seizures*

A single i.p. injection of PTZ induces seizures in rats as described previously [15]. Rats were treated with an i.p. infusion of vehicle (saline 0.9%) or PTZ (60 mg/kg) and observed for 60 minutes for the presence of tonic-clonic seizures. Ten minutes after the first seizure, rats were sacrified by decaptation and brain used for sinaptic vesicle preparation. Animals not displaying tonic-clonic seizures were discarded from synaptic vesicle preparation.

2.3.5. *Caffeine-induced seizures*

As previously described [18], i.p. administration of caffeine can induce seizures in rodents. In this study, rats recieved an i.p. injection of vehicle (saline 0.9%) or caffeine (150 mg/kg) and observed for 60 minutes for the presence of clonic seizures. Ten minutes after the first seizure, rats were sacrified by decaptation and brain used for sinaptic vesicle preparation. Animals not displaying clonic seizures were not included in the synaptic vesicle preparation.

2.3.6. *Maximal transcorneal electroshock (MES)-induced seizures*

Seizures induced by transcorneal electroshock has been described in detail previously [16,17]. Rats were challenged via saline-soaked copper electrodes to a bilateral transcorneal stimulation of a constant current of 80 mA. The tonic hind-limb extension was taken as the end-point for seizure activity. Ten minutes after the first seizure, rats were sacrified by decaptation and brain used for sinaptic vesicle preparation. Animals not displaying tonic seizures (hind-limb extension) were discarded from synaptic vesicle preparation.

2.4. Synaptic vesicles preparation

Synaptic vesicles were isolated from rat brains as described elsewhere [19]. Two whole brains were homogenized (10% wt/vol) in a buffer containing 0.32 M sucrose, 10 mM Mops/Tris, pH 7.4, and 1 mM EGTA and centrifuged twice for 10 min at 1000 x g. Both supernatants were pooled and centrifuged for 30 min at 20,000 x g to obtain the crude synaptosomal fraction (P2). This fraction was osmotically shocked by resuspension in 10 mM Mops/Tris, pH 7.4, containing 0.1 mM EGTA (~0.8 mL/g of fresh tissue) and centrifuged at 17,000 x g for 30 min. The supernatant containing synaptic vesicles was subjected to 0.4 M and 0.6 M sucrose density gradient centrifugation at 65,000 x g for 2 h. The synaptic vesicle fraction was isolated from the 0.4 M sucrose band and stored at –70 °C.

2.5. [^{3}H]Glutamate uptake by synaptic vesicles

Uptake experiments were performed in a standard medium (final volume of 200 μL) composed of 10 mM Mops/Tris, pH 7.4, 4 mM KCl, 140 mM potassium gluconate, 0.12 M sucrose, 2 mM $MgCl_2$, 2 mM ATP, with 50 μM [^{3}H]glutamate (3 μCi/mL), as previously described [20]. Incubation was carried out for 10 min at 35° C, and stopped by rapid filtration of the suspension through 0.45 μm Millipore filters. The filters were quickly flushed three times with 4 mL of 10 mM Mops/Tris, pH 7.4, at room temperature. Specific uptake was calculated by discounting the uptake measured in the absence of ATP. Radioactivity was measured with a Wallac scintillation counter.

2.6. Measurement of protein content

Protein content was determined according Lowry [21], using serum bovine albumin as standard.

2.7. Statistical analysis

For behavioral data, statistical analysis between groups was performed by the Fisher exact test for the occurrence of seizures. For synaptic vesicles preparation, statistical significance was assessed by ANOVA followed by Duncan's multiple range test when appropriate. All synaptic vesicle experiments were performed at least in triplicates and the mean was used for the calculations. All results with $p<0.05$ were considered significant.

3 – Results

An acute i.c.v. administration of QA (156,8 nmoles) induced seizures in 90% of animals. Kainate (30 mg/kg i.p.) and caffeine (150 mg/kg i.p.) caused seizures in 83% and 85% of rats, respectively. Picrotoxin (6 mg/kg s.c.), PTZ (60 mg/kg i.p.) or maximal electroshock (80 mA) produced seizures in all animals (Figure 1). Neither s.c., i.p. nor i.c.v. admistration of saline produced seizures in rats.

We also studied the effects of *in vivo* administration of the convulsant agents on the vesicular uptake of glutamate. I.c.v. administration of QA was able to stimulate the uptake of glutamate, an effect not reproduced by any other convulsant (Figure 2).

4- Discussion:

This study shows that an acute *in vivo* i.c.v. administration of QA induces seizures and increases glutamate uptake into synaptic vesicle from rat brain. This effect seems to be exclusively related to QA, since a similar acute administration of kainate, picrotoxin, PTZ, caffeine or MES did not affect glutamate uptake into synaptic vesicle.

Glutamate accumulation into synaptic vesicles is a vital step in glutamate synaptic transmission. The up-regulation of glutamate storage capacity could lead to an increase in quantal size and play a role in modulation of glutamate transmission efficiency [22].

As evidenced previously, prolonged depolarization of synaptosomes leads to an increase in vesicular glutamate content [22] and glutamate uptake by synaptic vesicles is increased in a brain region-specific manner in epileptic mice, probably in response to overstimulation of glutamatergic system [8]. Thus, disturbance in glutamate-accumulating vesicles may occur as a consequence of repeated seizure activity. Since glutamatergic

synapses and the vesicular glutamate uptake system are widely distributed in the CNS, it is reasonable that abnormal vesicular accumulation or transport function may have a pivotal role in seizures induced by glutamatergic agents such as QA [8].

Although QA is an endogenous NMDA agonist and presents effects impaired by NMDA antagonists [23,24], it has been proposed to stimulate the release of glutamate, an effect that could be related, at least partially, to its convulsant effect [25]. Guanine-based purines are compounds known to induce astrocytic glutamate uptake and protect against seizures induced by QA in rodents [23,24,26]. Thus, it is tempting to presume that glutamate release induced by QA could be directly related to an up-regulation of glutamate storage capacity in synaptic vesicle. However, remains uncertain if QA-induced glutamate uptake is the initial cause, an effect of seizure activity or represent the cause of continued seizures by augmenting glutamate synaptic strength [22], since *in vitro* administration of QA inhibits glutamate uptake into synaptic vesicles from rat brain [27].

In vitro administration of kainate was not uptaken by synaptic vesicle and was a non-competitive inhibitor of the glutamate uptake [28]. No increase in dialysate glutamate content was detected during picrotoxin-induced seizures, even when the K^+ concentration in the perfusion medium was raised to 50% above that measured previously during paroxysmal activity [29]. In mice, GABA antagonists such as bicuculline and picrotoxin were unable to change levels of aspartate, glutamate, GABA and taurine in nerve endings [30]. Also, administration of PTZ did not change forebrain concentrations of amino acids [31]. Systemic administration of caffeine increased locomotor activity but did not increase extracellular levels of glutamate [32]. Sodium-independent binding and sodium-dependent glutamate high affinity uptake were measured in hippocampal membranes of rats administered electroshock seizures. There were no differences in these glutamatergic synaptic markers among electroshocked or kindled animals [33].

Altogether, these findings indicate that seizure activity is not necessarily accompanied by an overall increase in extracellular glutamate concentration and QA effects on glutamate uptake may be related specifically to its mechanism of action in spite of other convulsants. Increased glutamatergic excitation in epilepsy may result from other abnormalities such as increased density of glutamate receptors, enhanced activation subsequent to reduced modulation, or sprouting of glutamatergic synapses [14,29].

5- Conclusion

In conclusion, this study provided new evidence on the role of QA-induced seizures on glutamate synaptic vesicle uptake in rats *in vivo*. This effect seems to be not mediated by other neurotransmitter systems since neither the non-NMDA agonist kainate, the $GABA_A$ antagonists picrotoxin or PTZ, the adenosine receptor antagonist caffeine nor the electrical stimulus affected the synaptic vesicle glutamate uptake.

Acknowledgments:

This research was supported by the Brazilian funding agencies FAPERGS, CAPES, CNPq and PRONEX (#41960904)

References

[1] Ozawa S, Kamiya H and Tsukuki K. Glutamate receptors in the mammalian central nervous system. *Prog. Neurobiol.* 1998; **54** : 581-618.

[2] Castellano C, Cestari V, Ciamei A. NMDA receptors and learning and memory processes. *Curr Drug Targets* 2001; **2**:273-283.

[3] Lipton SA, Rosemberg PA. Excitatory amino acids as a final common pathway for neurologic disorders. *New Engl J Med* 1994; **330**:613-622.

[4] Meldrum BS.The role of glutamate in epilepsy and other CNS disorders. *Neurology* 1994; **44**:S14-S23.

[5] Meldrum BS. Excitotoxicity and epileptic brain damage. *Epilepsy Res*. 1991; **10**: 55-61. Review.

[6] Fykse EM, Fonnum F. Amino acid neurotransmission: dynamics of vesicular uptake. *Neurochem Res*. 1996; **21**:1053-1060.

[7] Nicholls D, Attwell D. The release and uptake of excitatory amino acids. *Trends Pharmacol Sci*. 1990; **11**:462-468.

[8] Lewis SM, Lee FS, Todorova M, et al. Synaptic vesicle glutamate uptake in epileptic (EL) mice. *Neurochem Int* 1997; **31**:581-585

[9] Bradford HF. Glutamate, GABA and epilepsy. *Prog Neurobiol*. 1995; **47**: 477-511. Review.

[10] Heyes MP, Wyler AR, Devinsky O, Yergey JA, Markey SP, Nadi NS. Quinolinic acid concentrations in brain and cerebrospinal fluid of patients with intractable complex partial seizures. *Epilepsia* 1990; **31**:172-177.

[11] Nakano K, Takahashi S, Mizobuchi M, Kuroda T, Masuda K, Kitoh J. High levels of quinolinic acid in brain of epilepsy-prone E1 mice. *Brain Res* 1993; **619**:195-198.

[12] Lapin I.P. Stimulant and convulsant effects of kynurenines injected into brain ventricles in mice. *J. Neural Trans* 1978; **42**: 37-43.

[13] Bonan CD, Walz R, Pereira GS, Worm PV, Battastini AM, Cavalheiro EA, Izquierdo I, Sarkis JJ. Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy. *Epilepsy Res*. 2000; **39**:229-238.

[14] Millan MH, Obrenovitch TP, Sarna GS, Lok SY, Symon L, Meldrum BS. Changes in rat brain extracellular glutamate concentration during seizures induced by systemic picrotoxin or focal bicuculline injection: an in vivo dialysis study with on-line enzymatic detection. *Epilepsy Res*. 1991; **9** :86-91.

[15] Bruno AN, Oses JP, Bonan CD, Walz R, Battastini AM, Sarkis JJ. Increase of nucleotidase activities in rat blood serum after a single convulsive injection of pentylenetetrazol. *Neurosci Res*. 2002; **43**:283-288.

[16] Krupp E, Loscher W. Anticonvulsant drug effects in the direct cortical ramp-stimulation model in rats: comparison with conventional seizure models. *J Pharmacol Exp Ther.* 1998; **285**:1137-1149.

[17] Wlaz P, Potschka H, Loscher W. Frontal versus transcorneal stimulation to induce maximal electroshock seizures or kindling in mice and rats. *Epilepsy Res*. 1998; **30**:219-229.

[18] Koryntova H, Kubova H, Tutka P, Mares P. Changes of cortical epileptic afterdischarges under the influence of convulsant drugs. *Brain Res Bull*. 2002; **58**:49-54.

[19] Fykse EM, Fonnum F. Uptake of gamma-aminobutyric acid by a synaptic vesicle fraction isolated from rat brain. *J Neurochem* 1988; **50**: 1237-1242.

[20] Wolosker H, de Souza DO, de Meis L. Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient. *J Biol Chem*. 1996; **271**:11726-11731.

[21] Lowry OH, Rosebrough NR, Farr AL and Randall RJ.Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. 1951; **193**: 265- 275.

[22] Bole DG, Hirata K and Ueda T. Prolonged depolarization of rat cerebral synaptosomes leads to an increase in vesicular glutamate content. *Neurosci. Lett*. 2002; **322**:17-20.

[23] Lara DR, Schmidt AP, Frizzo MES, Burgos JS, Ramirez G, Souza DO. Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. *Brain Res*. 2001; **912**: 176-180.

[24] Schmidt AP, Lara DR, Maraschin JF, Perla AS, Souza DO. Guanosine and GMP prevent seizures induced by quinolinic acid in mice. *Brain Res*. 2000; **864**: 40-43.

[25] Connick JH, Stone TW. Quinolinic acid effects on amino acid release from the rat cerebral cortex in vitro and in vivo. *Br J Pharmacol*. 1988; **93**: 868-876.

[26] Baron BM, Dudley MW, McCarty DR, Miller FP, Reynolds IJ, Schmidt CJ. Guanine nucleotides are competitive inhibitors of N-Methyl-D-Aspartate at its receptor site both *in vitro* and *in vivo*. *J Pharmacol Exp Ther*. 1989; **250**:162-169.

[27] Tavares RG, Tasca CI, Santos CE, Wajner M, Souza DO, Dutra-Filho CS. Quinolinic acid inhibits glutamate uptake into synaptic vesicles from rat brain. *NeuroReport*. 2000; **11**: 249-253.

[28] Fykse EM, Iversen EG, Fonnum F. Inhibition of L-glutamate uptake into synaptic vesicles. *Neurosci Lett*. 1992; **135**:125-128.

[29] Obrenovitch TP, Urenjak J, Zilkha E. Evidence disputing the link between seizure activity and high extracellular glutamate. *J Neurochem*. 1996; **66**: 2446-2454.

[30] Wood JD, Johns KL. Stability of the amino acid content of nerve endings after administration of gamma-aminobutyrate agonists and antagonists. *Neuropharmacology*. 1985; **24**: 685-687.

[31] Yudkoff M, Daikhin Y, Nissim I, Horyn O, Lazarow A, Nissim I. Metabolism of brain amino acids following pentylenetetrazole treatment. *Epilepsy Res*. 2003; **53**: 151-162.

[32] Dalia A, Uretsky NJ, Wallace LJ. Dopaminergic agonists administered into the nucleus accumbens: effects on extracellular glutamate and on locomotor activity. *Brain Res.* 1998; **788**:111-117.

[33] Slevin JT, Ferrara LP. Lack of effect of entorhinal kindling on L-[3H]glutamic acid presynaptic uptake and postsynaptic binding in hippocampus. *Exp Neurol*. 1985; **89**: 48-58.

Figure 1:

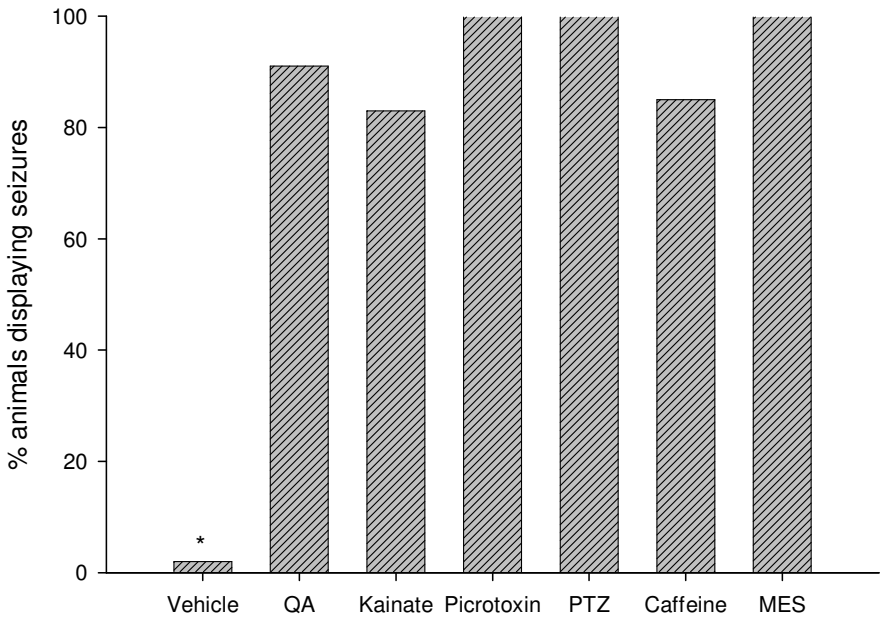
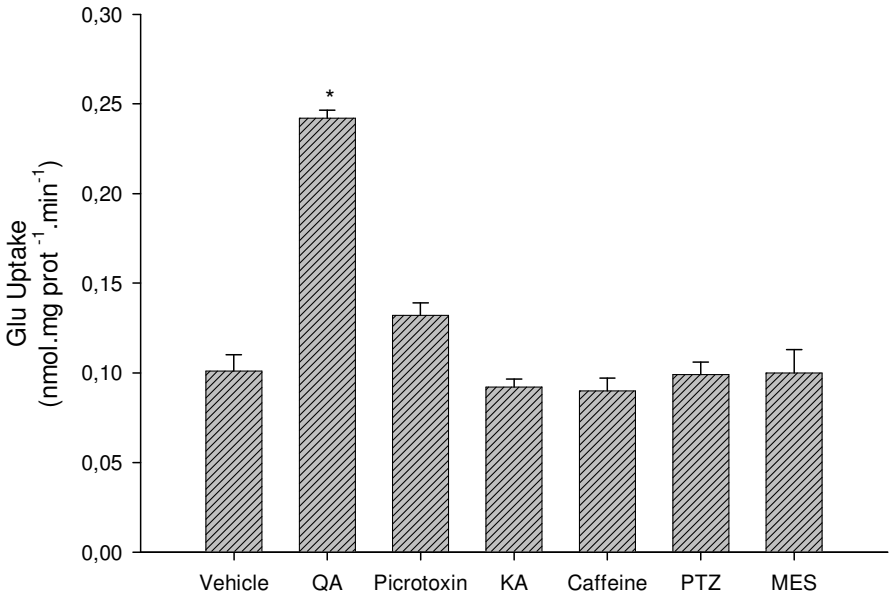

100
80
60
40
20
0
% animals displaying seizures
*
Vehicle
QA
Kainate
Picrotoxin
PTZ
Caffeine
MES

Figure 2:


0,30
0,25
0,20
0,15
0,10
0,05
0,00
Glu Uptake
(nmol.mg prot -1 .min-1)
*
Vehicle
QA
Picrotoxin
KA
Caffeine
PTZ
MES

Legends:

Figure 1 – Seizures induced by vehicle (saline 0.9%), QA (quinolinic acid – 156.8 nmoles), Kainate (30 mg/kg), picrotoxin (6 mg/kg), PTZ (pentylenetetrazole – 60 mg/kg), caffeine (150 mg/kg) or MES (maximal transcorneal electroshock – 80 mA). n = 6 – 10 animals per group. * = $p < 0.01$ (Fisher exact test), as compared with other groups.

Figure 2 – Effects of in vivo administration of vehicle (saline 0.9%), QA (quinolinic acid – 156.8 nmoles), Kainate (30 mg/kg), picrotoxin (6 mg/kg), PTZ (pentylenetetrazole – 60 mg/kg), caffeine (150 mg/kg) or MES (maximal transcorneal electroshock – 80 mA) on glutamate uptake into brain synaptic vesicles. Glutamate uptake is expressed as nmol.mg of protein^{-1}.min^{-1}. Data are mean ± SD. * = $p < 0.01$ (ANOVA followed by Duncan's multiple range test), as compared with vehicle group.

III. CONSIDERAÇÕES FINAIS

Há mais de 40 anos, Lucas demonstrou que glutamato, quando injetado sistemicamente, causava degeneração da retina (Lucas,1957). Em 1967, Olney mostrou que o glutamato extracelular pode causar lesões no sistema nervoso central (Olney, 1969); após publicou sua hipótese de 'excitotoxicidade' para a ação do glutamato (Olney, 1971). Atualmente, vários estudos sugerem que este mecanismo glutamatérgico de excitotoxicidade está envolvido na fisiopatologia de muitas alterações neurológicas, incluindo as doenças neurodegenerativas. Além disso, outras evidências têm sugerido a participação do dano oxidativo, de alterações na homeostase do cálcio e do comprometimento metabólico nos mecanismos patológicos das doenças neurodegenerativas (Ying, 1996). Todos estes comprometimentos no metabolismo celular podem ser provocados pelo excesso de ativação dos receptores glutamatérgicos (Pellegrini-Giampietro *et al*., 1990; Lafon-Cazal *et al*., 1995).

Até o momento, muitas excitotoxinas endógenas e exógenas têm sido estudadas, como o AQ e o próprio glutamato; porém, seus mecanismos de ação não estão completamente elucidados (Reynolds Jr. *et al.,* 1997). Baseado no conceito de excitotoxicidade, e em resultados obtidos em nosso laboratório que demonstraram ser o AQ um inibidor da captação de glutamato em vesículas sinápticas (Tavares *et al*, 2000), buscou-se investigar se este também apresentava efeitos sobre os sistemas transportadores presentes nas membranas pré- e pós-sinápticas, através da avaliação dos processos de captação e liberação de glutamato em sinaptossomas, já que estes transportadores Na^+-dependente representam um papel importante na manutenção do equilíbrio dinâmico da concentração glutamatérgica intra e extracelular, impedindo a ação tóxica deste neurotransmissor.

In vitro, observamos que somente o processo de liberação de glutamato é estimulado em presença do AQ. Buscando-se verificar se esta liberação aumentada de glutamato ocorria por despolarização via influxo de íons Ca^{+2}, estudou-se o efeito do AQ em sinaptossomas já despolarizados (em presença de alta concentração de íons K^{+}) e também em presença de EGTA, um agente conhecidamente quelante de íons Ca^{+2}. Nestas situações, AQ não induziu nenhum aumento adicional da liberação de glutamato, além de ter seu efeito estimulatório completamente abolido quando da ausência de íons Ca^{+2} exógenos. Além disso, utilizando-se um antagonista do receptor NMDA, o MK-801, o efeito do AQ também deixou de ser observado, o que ratifica que a ação do AQ ocorre via ativação de receptores glutamatérgicos do tipo NMDA (Stone, 2001).

A consequência primária de uma inibição na captação vesicular de neurotransmissores seria a depleção das suas reservas vesiculares, reduzindo a liberação deste neurotransmissor na fenda sináptica de maneira dependente de íons Ca^{+2} (induzida por despolarização). Além disso, caso as vias de metabolização intracelular do neurotransmissor já se encontrem saturadas, a inibição desta captação vesicular poderia causar ainda um aumento na sua concentração intracelular. No entanto, as consequências finais da inibição da captação vesicular parecem depender ainda do funcionamento dos transportadores presentes na membrana plasmática, onde poderíamos especular que um aumento na concentração intracelular poderia resultar em liberação espontânea de glutamato através da inversão do sentido de transporte deste neurotransmissor pelos transportadores de membrana plasmática (dependentes de íons Na^{+}). A proposição deste mecanismo nos parece razoável considerando as diversas evidências de que déficits na produção celular de energia favorecem a reversão do fluxo de neurotransmissor por este transportador (Sánchez-Prieto & González, 1988; Szatowski *et al*., 1990).

Atualmente sabe-se que a efetiva retirada do glutamato do meio extracelular é feita pelas células gliais (Anderson & Swanson, 2000). Interessantemente, investigando o efeito do AQ na captação de glutamato em astrócitos, encontramos uma diminuição significativa da mesma, ou seja, torna-se plausível a hipótese de que um acúmulo de glutamato da fenda sináptica, resultante da combinação de liberação sinaptossomal aumentada e de um retardo ou diminuição da eficiência de retirada do glutamato do meio extracelular via astrócitos seja um fator colaborador para os efeitos neurotóxicos do AQ. Evidências indicando serem os receptores NMDA a via preferencial de ação do AQ despertaram a curiosidade: é possível haver alguma forma de modulação desta ação?

Nosso grupo tem demonstrado que os derivados da guanina, incluindo os nucleotídeos e também o nucleosídeo guanosina, inibem repostas fisiológicas do glutamato, como a fosforilação da proteína marcadora de astrócitos, GFAP (Tasca *et al*., 1995) bem como respostas excitotóxicas, exercendo assim um papel neuroprotetor. Pode-se citar, entre os vários efeitos observados, a ação protetora contra a morte celular induzida via receptor NMDA em striatum (Malcon *et al*., 1997), a redução da ocorrência de convulsões induzidas via ativação de receptores NMDA em modelos experimentais (Schmidt *et al*., 2000 ; Lara *et al*., 2001, Vinadé *et al*., 2003), além de efeitos comportamentais amnésicos e ansiolíticos (Vinadé *et al*., 2003; Vinadé *et al*., 2005).

Evidências recentes sugerem que a ação anticonvulsivante observada é devida à ação do nucleosídeo guanosina, resultante da hidrólise enzimática do GMP (Soares *et al.,* 2004). Além disso, estes parecem exercer função neurorregulatória ou neuromoduladora em vesículas sinápticas (Tasca *et al*., 2004). Corroborando estes processos já descritos, em nosso estudo tanto os nucleotídeos da guanina como o nucleosídeo, preveniram o efeito inibitório sobre a captação vesicular de glutamato induzida por AQ. É importante ressaltar,

que ao contrário de outras respostas glutamatérgicas estudadas, no caso da captação vesicular, os derivados da guanina estão agindo em um sítio intracelular, compartimento onde estão normalmente disponíveis.

A utilização de AQ por administração i.c.v. em modelos experimentais para indução de convulsões, já está bem caracterizada (Lapin *et al*., 1978). Utilizando-se este modelo, estudos subsequentes foram realizados para um melhor entendimento tanto da ação do AQ quanto da ação dos derivados da guanina. Resumidamente, verificamos que vesículas sinápticas provenientes do cérebro de animais que convulsionaram apresentam uma capacidade de captação glutamatérgica aumentada e captação GABAérgica diminuída.

Em relação ao glutamato, vemos que este efeito é contrário àquele observado *in vitro*, porém recentes evidências demonstram que o volume de neurotransmissor estocado em vesículas apresenta-se aumentado em situações de despolarização sinaptossomal via influxo de íons Ca^{+2} (Bole *et al*., 2002), o que é perfeitamente compatível com o mecanismo de ação do AQ via receptores NMDA. Soma-se à isso o fato de que, nos estudos *in vivo*, temos ainda a participação de outros sistemas de transporte envolvidos, principalmente os transportadores astrocitários e sinaptossomais, e não somente o transportador vesicular, como nos estudos de captação vesicular *in vitro*.

Outro estudo usando animais geneticamente modificados, com predisposição ao aparecimento de convulsões, também demonstrou que a captação vesicular de glutamato está aumentada nos animais que apresentaram eventos convulsivos. Assim, estes achados nos permitem sugerir que o "quanta" vesicular de glutamato envolvido na neurotransmissão pode ser modulado, e que este fato não seja a causa inicial das convulsões, mas sim um efeito da mesma, ou ainda um fator contribuinte para a manutenção da atividade epiléptica (Lewis et al., 1997).

Também nos estudos *in vivo* o efeito modulatório dos derivados da guanina foi observado. Schmidt *et al*. (2000) já haviam demonstrado que administração i.p. de guanosina e GMP protegem os animais de convulsões induzidas por AQ em até 80 %. Adicionalmente, trabalhos recentes demonstraram que a convulsão induzida por AQ neste modelo pode também ser modulada por pré-condicionamento dos receptores NMDA, com uso de doses subtóxicas desta substâncias. Nesta situação, parece haver envolvimento não só dos receptores NMDA, mas também dos receptores adenosinérgicos do tipo A_1 (Boeck *et al*., 2004).

Nossos resultados mostraram que a ocorrência ou não de convulsões modifica a atividade de captação vesicular. Nos animais onde foi observada uma proteção contra a indução de convulsões, os níveis de captação vesicular, tanto glutamatérgica quanto GABAérgica, foram semelhantes aos encontrados nos animais controle. No entanto, nos animais onde os derivados da guanina não foram eficientes na proteção contra a indução de convulsões, os níveis de glutamato e GABA encontraram-se, respectivamente, aumentados e diminuídos, de acordo com os resultados previamente relatados. Pode-se sugerir, então, que o efeito protetor dos derivados da guanina seja devido, pelo menos em parte, à modulação e/ou regulação do transporte vesicular de neurotransmissores. Em relação à diminuição da captação vesicular de GABA observada neste estudo, pode-se relacionar a mesma com o mecanismo convulsivo, onde um desbalanço entre os neurotransmissores é observado (Allen *et al*., 2004; Bettler *et al*., 2004), ou ainda à ação direta do AQ, já que estudos demonstraram que lesões em consequência da administração de AQ no *striatum* resultaram em considerável depleção de GABA (Maeda et al., 1997).

Outro achado interessante é que a captação glutamatérgica vesicular só se encontra alterada no modelo de indução de convulsão usando o agonista NMDA AQ, pois em outros

modelos, usando ácido caínico, cafeína (modelo adenosinérgico), picrotoxina (modelo GABAérgico), PTZ ou eletrochoque transcorneal, nenhuma alteração foi verificada.

Utilizando o mesmo modelo de indução de convulsões por AQ, este possível efeito modulatório foi também avaliado nos terminais pré- e pós-sinápticos, usando-se preparações de sinaptossomas. Observou-se que a liberação basal de glutamato, estimulada na presença de AQ injetado i.c.v., retorna aos níveis do controle (salina 0,9 %) quando os derivados da guanina são eficientes na proteção contra a indução de convulsões. Novamente pode-se pensar que este aumento na liberação sinaptossomal de glutamato induzida por AQ, é causado por um influxo de Ca^{+2} e resultante despolarização.

Outros estudos sugerem que os mecanismos envolvidos na ação do AQ sejam bem mais complexos, incluindo déficit energético (diminuição de produção e/ou utilização do ATP) por disfunção mitocondrial (Bordelon *et al*., 1997), produção de radicais livres e peroxidação lipídica (Santamaria *et al*., 2001). Embora cada um destes processos seja distinto, efeitos sinérgicos parecem ocorrer.

Um mecanismo interessante de interligação entre os diversos efeitos das neurotoxinas tem sido proposto por Alexi *et al*. (1998): toxinas mitocondriais agem primariamente produzindo a depleção dos níveis de ATP. O resultado imediato do dano mitocondrial é o aumento da produção de radicais livres. O déficit energético resulta em alterações na bomba de íons (Na^+,K^+-ATPase) e nos canais iônicos como os receptores NMDA, promovendo influxo e conseqüente acúmulo intracelular de cálcio com todos os seus efeitos danosos inclusive a intensificação do processo de formação de radicais livres. Estes podem promover a morte celular por ação direta na membrana, destruindo proteínas e oxidando o DNA. Embora os detalhes da interação entre os três componentes deste

mecanismo não sejam completamente elucidados, esta interação nos parece plausível e explicaria muitos dos efeitos observados na ação do AQ.

A presente demonstração de que o AQ age alterando vários processos glutamatérgicos, propicia um melhor entendimento dos complexos mecanismos envolvidos na sua ação, bem como no desencadeamento e/ou manutenção das convulsões induzidas por esta neurotoxina. A demonstração de mais uma ação modulatória e/ou neurorreguladora dos nucleotídeos da guanina frente a transmissão glutamatérgica, também contribui para a melhor compreensão dos mecanismos envolvidos na excitotoxicidade. Entretanto, é fundamental que estudos adicionais sejam realizados para determinar com clareza quais são os sítios de interação dos nucleotídeos da guanina, seus possíveis receptores e um maior detalhamento de sua função sináptica, para que tenhamos uma melhor compreensão do papel destas substâncias em situações fisiológicas e patológicas no sistema nervoso.

IV. CONCLUSÕES

- O ácido quinolínico *in vitro* estimula a liberação sinaptossomal de glutamato, sem interferir na captação sinaptossomal do mesmo. Este efeito provavelmente seja devido à ação direta do ácido quinolínico nos receptores NMDA, já que antagonistas NMDA (MK-801) aboliram o efeito estimulatório.
- O ácido quinolínico inibe a captação vesicular de glutamato *in vitro*, de modo não-competitivo, dependente de tempo e concentração. Além disso, a atividade da V-ATPase e a formação do gradiente próton-eletroquímico não são alterados, o que reforça a provável ação do ácido quinolínico diretamente sobre o transportador vesicular.
- Os derivados da guanina exercem um efeito modulatório desta ação do ácido quinolínico no transportador vesicular, já que foram capazes de reverter o efeito inibitório observado.
- Este efeito mostrou-se específico dos derivados da guanina, pois derivados da adenina (AMP) e da inosina (IMP) não exerceram nenhum efeito modulatório.
- Estudos *in vivo,* usando modelos experimentais de convulsão, mostraram haver especificidade do ácido quinolínico na estimulação da captação vesicular de glutamato.
- O ácido quinolínico, *in vivo*, inibe a captação vesicular de GABA.
- MK-801, antagonista NMDA, preveniu tanto a ocorrência das convulsões, bem como o estímulo da captação vesicular do glutamato, reiterando a ação do ácido quinolínico via receptores NMDA.
- Os derivados da guanina são efetivos na prevenção da convulsão induzida por ácido quinolínico, e também na modulação de seu efeito sobre o transportador vesicular,

abolindo tanto o efeito estimulatório da captação vesicular de glutamato, como o efeito inibitório da captação vesicular de GABA.

- Também nos estudos *in vivo* o ácido quinolínico estimula a liberação sinaptossomal de glutamato, sem interferir na captação sinaptossomal do mesmo. Quando houve prevenção da convulsão induzida por ácido quinolínico pelos derivados da guanina, este efeito estimulatório não foi observado.

A partir destas conclusões, algumas perspectivas de estudo podem ser desenvolvidas, como:

- Avaliar a ação do ácido quinolínico no efluxo vesicular de glutamato, buscando um melhor entendimento de seu mecanismo de ação.
- Usando modelos *in vivo* e *in vitro*, avaliar a ação do ácido quinolínico na captação de glutamato em fatias de cérebro de ratos, bem como a ação dos derivados da guanina neste mesmo sistema.
- Avaliar a atividade de enzimas antioxidantes, produção de radicais livres e dano mitocondrial em animais submetidos à convulsão induzida por ácido quinolínico, e também naqueles protegidos por derivados da guanina.
- Avaliar o tipo de morte celular, necrose ou apotose, em cérebros de animais submetidos à concentrações convulsivantes de ácido quinolínico.

V. REFERÊNCIAS BIBLIOGRÁFICAS

ANDERSON, C.M. & SWANSON, R.A. Astrocyte Glutamate Transport: Review of Properties, regulation, and Physiological Functions. *GLIA*, 32: 1-14, 2000.

ALEXI, T.; HUGHES, P.E.; FAULL, R.L.M.; WILLIAMS, C.E. 3-Nitropropionic acid´s lethal triplet: cooperative pathways of neurodegeneration. *NeuroReport*, 9: R57-R64, 1998.

ALLEN, N.J.; KARADOTTIR, R.; D. ATTWELL, D. Reversal or reduction of glutamate and GABA transport in CNS pathology and therapy, *Pflugers Arch.* 449: 132-142, 2004.

ARTOLA, A.; & SINGER, W. Long-term potentiation and NMDA receptors in rat visual cortex. *Nature* 330: 649-652, 1987.

BARON B.M., DUDLEY, M.W., MCCARTY, D.R., MILLER, F.P., REYNOLDS, I.J., SCHMIDT, C.J. Guanine nucleotides are competitive inhibitors of N-Methyl-D-Aspartate at its receptor site both *in vitro* and *in vivo*. *J. Pharmacol. Exp. Ther*. 250: 162-169, 1989.

BEAL, M. F.; KOWALL, N. W.; ELLISON, D. W.; MAZUREK, M. F.; SWARTZ, K. J.; MARTIN, J B. Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. *Nature* 321: 168-171, 1986.

BEAL, M. F.; KOWALL, N. W.; SWARTZ, K. J.; FERRANTE, R. J.; MARTIN, J. B. Systemic approaches to modifying quinolinic acid striatal lesions in rats. *J. Neurosci*. 8: 3901-3908, 1988.

BEAR, M.F.; CONNORS, B.W.; PARADISO, M.A. Neuroscience Exploring the Brain. 2.ed., Lippincott Willians & Wilkins, USA, cap. 6, 130-162, 2001.

BELL, M. J.; KOCHANEK, P. M.; HEYES, M. P.; WISNIEWSKI, S. R.; SINZ, E. H.; CLARK, R. S. B.; BLIGHT, A. R.; MARION, D. W.; ADELSON, P. D. Quinolinic acid in the cerebrospinal fluid of children after traumatic brain injury. *Crit. Car. Med.* 27: 493-497, 1999.

BENNETT, M. K.; & SCHELLER, R. H. A molecular description of synaptic vesicle membrane trafficking. *Annu. Rev. Biochem*. 63: 63-100, 1994.

BETTLER, B.; KAUPMANN, K.; MOSBACHER, J.; GASSMANN, M. Molecular structure and physiological functions of GABA(B) receptors, *Physiol Rev*. 84 : 835-867, 2004.

BOECK, C.R.; GANZELLA, M.; LOTTERMANN, A.; VENDITE, D. NMDA Preconditioning Protects against Seizures and Hippocampal Neurotoxicity Induced by Quinolinic Acid in Mice. *Epilepsia* 45: 745-750, 2004.

BOLE, D.G.; HIRATA, K.; UEDA, T. Prolonged depolarization of rat cerebral synaptosomes leads to na increase vesicular glutamate content. *Neurosc. Lett*. 322: 17-20, 2002.

BORDELON, Y.M.; CHESSELET, M.F.; NELSON, D.; WELSH, F.; ERECINSKA, M. Energetic Dysfunction in Quinolinic Acid-Lesioned Rat Striatum. *J. Neurochem*. 69: 1629-1639,1997.

BOURNE, H.R.; SANDERS, D.A.; McCORMICK, F. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* 348:125-131,1990.

BURGOS, J.S., BARAT, A., SOUZA, D.O., RAMÍREZ, G. Guanine nucleotides protect against kainate toxicity in an ex vivo chick retinal preparation. *FEBS Lett*. 430:176-180, 1998.

CHOI, D.W. & ROTHMAN, S.M. The role of glutamate neurotoxicity in hypoxic-isquemic neuronal death. *Ann. Rev. Neurosci*. 13: 171-182, 1990.

COLLINGRIDGE, G. L.; & LESTER, R. A. Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Rev*. 40: 14-209, 1989.

CONN, P.J. & PIN, J.P. Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol*. 37: 205-237, 1997.

COTMAN, C.W.; KAHLE, J.S.; MILLER, S.E.; ULAS, J.; BRIDGES, R.J. ecxitatory Amino Acid Neurotransmission. EM: *Psychopharmacology: The Fourth Generation of Progress*, eds.: Bloom, F.E. & Kupfer, D.J. Raven Press, New York, pp. 75-85, 1995.

DANBOLT, N.C. Glutamate uptake. *Progress in Neurobiology*. 65: 1-105, 2001.

De ROBERTIS, E. D. P.; Arnaiz, G. R. L.; SALGANICOFF, L.; IRALDI, A. P.; ZIEHER, L. M. Isolation of synaptic vesicles and structural organization of the acetylcholine system within brain nerve endings. *J. Neurochem*. 10: 255-235, 1963.

DINGLEDINE, R.; McBAIN, C. J.; & McNAMARA, J. O. Excitatory amino acid recepos in epilepsy. *TiPS* (Special Report) 49-53,1991.

DINGLEDINE, R. & McBAIN, C.J. Excitatory amino acid transmitters. EM: *Basic Neurochemistry: molecular, cellular and medical aspects*, eds.: Siegel, G.J.; Agranoff, B.W.; Albers, R.W. & Molinoff, P.B. Raven Press, New York, pp. 367-387, 1994.

EDMONDS, B.; GIBB, A.J.; COLQUHOUN, D. Mechanisms of activation of glutamate receptors and time course of excitatory synaptic currents. *Annu. Rev. Physiol*. 57: 495-519, 1995.

ERICKSON, J. B.; FLANAGAN, E. M.; CHANG, S. Y.; SALTER, M.; REINHARD, J. F., Jr. The neurotoxin quinolinic acid: differential modulation of brain and blood levels. *Soc. Neurosci. Abstr.* 18: 443, 1992.

FONNUM, F. Glutamate: a neurotransmitter in mammalian brain. *J. Neurochem.* 42: 1-11, 1984.

FOSTER, A. C.; WHITE, R. J.; SCHWARCZ, R. Synthesis of quinolinic acid by 3-hydroxyanthranilic acid oxygenase in rat brain tissue in vitro. *J. Neurochem*. 47: 23-30,1986.

FOSTER, A. C.; GILL, R.; WOODRUFF, G. N. Neuroprotective effects of MK-801 in vivo: selectivity and evidence for delayed degeneration mediated by NMDA receptor activation. *J. Neurosci*. 8: 4745-4754, 1988.

FRANCISCO, S.R.S.; ROMÃO, L.F.; MOURA-NETO, V.; TASCA, C.I. Trophic Effects of GMP and Guanosine on Cerebellar Neuron-Astrocyte Coculture. *J. Neurochem.* 90 (Suppl. 1): CP6-02,75, 2004.

FRIZZO, M.E.S.; LARA, D.R.; DAHM, K.C.S.; PROKOPIUK, A.S.; SWANSON, R.; SOUZA, D.O. Activation of glutamate uptake by guanosine in primary astrocyte cultures. *NeuroReport* 12: 879-881, 2001.

FRIZZO, M.E.S., LARA, D.R., PROKOPIUK, A.S., VARGAS, C.R., SALBEGO, C.G., WAJNER, M., SOUZA, D.O. Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. *Cell. Mol. Neurobiol*. 22: 353-63, 2002.

FRIZZO, M.E.S., SOARES, F.A., DALL'ONDER, L.P., LARA, D.R., SWANSON, R.A., SOUZA, D.O. Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. *Brain Res*. 972: 84-89, 2003.

FYKSE, E. M.; & FONNUM, F. Amino acid neurotransmission: dynamics of vesicular uptake. *Neurochem. Res*. 21: 1053-1060, 1996.

GHOLSON, R. K.; UEDA, I.; OGASAWARA, N.; HENDERSON, L. M. The enzymatic conversion of quinolinic acid to nicotinic acid mononucleotide in mammalian liver. *J. Biol. Chem*. 239: 1208-1214, 1964.

GHOLSON,R. K. The pyridine nucleotide cycle. *Nature* 212: 933-934, 1966.

HEPLER, J.R. & GILMAN, A.G. Gproteins. *Trends Biochem. Sci*. 17: 383-387, 1992.

HEYES, M. P. Hypothesis: a role for quinolinic acid in the neuropathology of glutaric aciduria type I. *Can. J. Neurol. Sci*. 14: 441-443, 1987.

HEYES, M. P.; & MARKEY, S. P. Quantification of quinolinic acid in rat brain, whole blod, and plasma by gas chromatography and negative chemical ionization mass spectrometry: effects of systemic L-tryptophan administration on brain and blood quinolinic acid concentrations. *Anal. Biochem.* 174: 349-359, 1988.

HEYES, M. P.; RUBINOW, D.; LANE, C.; MARKEY, S. P. Cerebrospinal fluid quinolinic acid concentrations are increased in acquired immune deficiency syndrome. *Ann. Neurol*. 26: 275-277, 1989.

HEYES, M. P.; BREW, B.; MARTIN, A.; MARKEY, S. P.; PRICE, R. W.; BHALLA, R. B.; SALAZAR, A. Cerebrospinal fluid quinolinic acid concentrations are increased in acquired immune deficiency syndrome. *Adv. Exp. Med. Biol*. 294: 687-690, 1991a.

HEYES, M. P.; BREW, B. J.; MARTIN, A.; PRICE, R. W.; SALAZAR, A. M.; SIDTIS, J. J.; YERGEY, J. A.; MOURDIAN, M. M.; SADLER, A. E.; KEILP, J.; RUBINOW, D.; MARKEY, S. P. Quinolinic acid in cerebrospinal fluid and serum in HIV-1 infection: relationship to clinical and neurologic status. *Ann. Neurol*. 29: 202-209, 1991b.

HEYES, M. P.; SAITO, K.; MARKEY, S. P. Human macrophages convert L-tryptophan into the neurotoxin quinolinic acid. *Biochem. J.* 283: 633-635, 1992a.

HEYES, M. P.; SAITO, K.; CROWLEY, J. S.; DAVIS, L. E.; DEMITRACK, M. A.; DER, M. et al. Quinolinic acid and kynurenine pathway metabolism in inflammatory and non-inflammatory neurological disease. *Brain* 115: 1249-1273, 1992b.

HEYES, M. P.; ACHIM, C. L.; WILEY, C. A.; MAJOR, E. O.; SAITO, K.; MARKEY, S. P. Human microglia convert L-tryptophan into the neurotoxin quinolinic acid. *Biochem. J*. 320: 595-597, 1996.

ISQUITH, A. J.; & MOAT, A. G. Biosynthesis of nicotinic acid by *Clostridium butyricum*. *Biochem. Biophys. Res. Commun*. 22: 565-571, 1966.

IZQUIERDO, I. Pre- and postsynaptic events in long-term potentiation in memory. *FASEB J*. 8: 1139-1145, 1994.

ITO, M. Long-term depression. *Ann. Rev. Neurosci*. 12: 85-102, 1989.

JOHNSTON, G.A.R. Glutamate uptake and its possible role in neurotransmitter inactivation. EM: *Glutamate: Transmitter in the Central Nervous System*, eds.:

Roberts, P.J., Storm-Mathisen, J., Johnston, G.A.R. Wiley, Chichester, pp. 77-87, 1981.

KATZ, B. The release of neural transmitter substances. Thomas, Springfield, L. 1969

KOMMERS, T.; RODNIGHT, R.; OPPELT, D.; OLIVEIRA, D.; WOLFCHUK, S. The mGluR stimulating GFAP phosphorilation in immature hippocampal slices has some properties of group II receptor. *NeuroReport*. 10: 2119-2123, 1999.

LAFON-CAZAL. M.; PIETRL, S.; CULCASI, M.; BOCKAERT, J. NMDA-dependent superoxide production and neurotoxicity. *Nature* 364: 535-537, 1995.

LAPIN, I. P. Stimulant and convulsant effects of kynurenines injected into brain ventricles in mice. *J. Neural Trans*. 42: 37-43, 1978.

LARA, D.R., SCHMIDT, A.P., FRIZZO, M.E.S., BURGOS, J.S., RAMIREZ, G., SOUZA, D.O.. Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. *Brain Res*. 912: 176-180, 2001.

LEWIS, S.M.; LEE, F.S.; TODOROVA, M.; SEYFRIED, T.N.; UEDA, T. Synaptic vesicle glutamate uptake in epileptic (EL) mice. *Neurochem, Int.* 31: 581-585, 1997.

LIPTON, S. A.; & ROSENBERG, P. A. Mechanisms of disease. Excitatory amino acids as a final common pathway for neurological disorders. *The New Eng. J Med*. 330: 613-622, 1994.

LIU, Y.; & EDWARDS, R. H. The role of vesicular transport proteins in synaptic transmission and neural degeneration. *Ann. Rev. Neurosci*. 20: 125-156, 1997.

LUCAS, D. r.; NEWHOUSE, J. P. The toxic effect of sodium L- glutamate on the inner layers of the retina. *Acta Ophthalmol*. 58: 193-201, 1957.

MAEDA, K.; KANEDA, H.; WHETSELL, W.O.; TAMMINGA, C.A. Neurochemical and metabolic consequences of elevated cerebrospinal fluid quinolinic acid concentrations in rat brain. *Neurosc. Res*. 29: 303-309, 1997.

MALCON, C.; ACHAVAL, M.; KOMLUS, S.; PARTATA, W.; SAURESSIG, M.; RAMÍREZ, G.; SOUZA, D. O. GMP protects against quinolinic acid-induced loss of NADPH diaphorase-positive cells in the rat striatum *Neurosci. Lett*. 225: 145-148, 1997.

MARAGAKIS, N.J. & ROTHSTEIN, J.D. Glutamate transporters: animal models to neurologic disease. *Neurobiology of Disease*. 15:461-473, 2004.

MARTIN, D.C.; PLAGENHOEF, M.; ABRAHAM, J.; DENNISON, R.L.; ARONSTAM, R.S. Volatile anesthetics and glutamate activation of N-meyhyl-D-aspartate receptors. *Biochem. Pharmacol*. 49: 809-817, 1995.

MAYCOX, P. R.; HELL, J. W. ; JAHN, R. Amino acid neurotransmission- spotlight on synaptic vesicles. *Trens Neurosci*. 13: 83-87, 1990.

MELDRUM, B.; & GARTHWAITE, J. Excitatory amino acid neurotoxicity and neurodegenerative disease. *TiPS* (Special Report) 54-62, 1991.

MICHAELIS, E.K. Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. *Prog. Neurobiol*. 54:369-415, 1998.

MOLZ, S.; DECKER, H.; OLIVEIRA, I.J.L.; SOUZA, D.O.; TASCA, C.I. Neurotoxicity Induced by Glutamate in Glucose-Deprived Rat Hippocampal Slices is Prevented by GMP. *Neurochem. Res*. 30: 83-89, 2005.

MONAGHAN, D. T.; BRIDGES, R. J.; COTMAN, C. W. The excitatory amino acid receptors *Annu. Rev. Pharmacol. Toxicol*. 29: 365-402, 1989.

MORONI, F.; LOMBARDI, G.; CARLA, V.; MONETI, G. The excitotoxin quinolinic acid is present and unevenly distributed in the rat brain. *Brain Res*. 295: 352-355, 1984a.

MORONI, F.; LOMBARDI, G.; MONETI, G.; ALDINO, C. The excitotoxin quinolinic acid is present in the brain of several mammals and its cortical content increases during the aging process. *Neurosci. Lett*. 47: 51-55, 1984b.

MORONI, F.; LOMBARDI, G.; CARLA, V.; LAL, S.; ETIENNE, P.; NAIR, N. P. Increase in the content of quinolinic acid in cerebrospinal fluid and frontal cortex of patients with hepatic failure. *J. Neurochem*. 47: 1667-1671, 1986.

MORRIS, J.A. & MALBON, C.C. Physiological regulation of G protein-linked signaling. *Physiol. Rev*. 79: 1373-1430, 1999.

NICHOLS, D. G.; & ATWELL, D. The release and uptake of xcitatory amino acids. *Trens Neurosci*. 11: 462-468, 1990.

NICHOLS, D. G. Ion channels and the regulation of neurotransmitter glutamate release. *Biochem. Soc. Transac*. 21: 53-58, 1993.

NISHIZUKA, Y.; & HAYAISHI, O. Studies on the biosynthesis of nicotinamide adenine dinucleotide. *J. Biol. Chem*. 238: 3369-337, 1963.

OBRENOVITCH, T.P.; URENJAK, J.; JAY, T.M. Excitotoxicity in neurological disorders- the glutamate paradox. *Int. J. Devl. Neuroscience*. 18:281-287, 2000.

OLIVEIRA, I.J.L.; MOLZ, S.; SOUZA, D.O.; TASCA, C.I. Neuroprotective effect of GMP in hippocampal slices submitted to na in vitro model of ischemia. *Cell. Mol. Neurobiol*. 22: 335-344, 2002.

OLNEY, J. W. Cytotoxic lesions obeity and other disturbances in mice treated with monosodium glutamate. *Science* 164: 719-721, 1969.

OLNEY, J. W.; HO, O. L.; RHEE, V. Cytotoxic effects of acidic and sulpher containing amino acids on the intant mouse central nervous system. *Exp. Brain Res*. 14: 61-76, 1971.

OLNEY, J. W. Neurotoxicity of excitatory amino acids. In: McGEER, E. G.; OLNEY, J. W.; McGEER, P. L. eds. *Kainic acid as a tool in neurobiology*. New York: Raven Press, 95-121, 1978.

OLNEY, J. W. Excitotoxic amino acids and neuropsychiatric disorders. *Ann. Rev. Pharmacol. Toxicol.* 30: 47-71, 1990.

OZAWA, S.; KAMIYA, H.; TSUZUKI, K. Glutamate receptors in the mammalian central nervous system. *Progress in Neurobiology* 54: 581-618, 1998.

PAZ, M.M., RAMOS, M., RAMIREZ, G., SOUZA, D.O. Differential effects of guanine nucleotides on kainic acid binding and on adenylate cyclase activity in chick optic tectum *FEBS Lett*. 355: 205-208, 1994.

PEARSON, S. J.; & REYNOLDS, G. P. Determination 3-hidroxykynurenine in human brain and plasma by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr*. 565: 436-440, 1991.

PELLEGRINO-GIAMPIETRO, D. E.; CHERICI, G.; ALESTANI, M; CARIA, V.; MORONI, F. Excitatory amino acid release and free radical formation may cooperative

in the genesis of ischemia-induced neuronal damage. *J. Neurosci*. 10: 1035-1041, 1990.

PRICE, D. L. New order from neurological disorders. *Nature* 399: A3-A5, 1999.

RATHBONE, M.P., MIDDLEMISS, P.J., GYSBERGS, J.W., ANDREW, C., HERMA, M.A.R., REE, J.K., CICCARELLI, R., DI IORIO, P., CACIAGLI, F. Trophic effects of purines in neurons and glial cells. *Prog. Neurobiol*. 59: 663-690, 1999.

REGNER, A.; RAMÍREZ, G.; BELLÓ-KLEIN, A.; SOUZA, D.O. Effects of guanine nucleotides on glutamate-induced chemiluminescence in rat hippocampal slices submitted to hypoxia. *Neurochem, Res*. 23: 523-528, 1998.

REINHARD, J. F., Jr.; JANSEN, M.; ERICKSON, J. B.; CHANG, S. Y.; FLANAGAN, E. M. Control of quinolinic acid biosynthesis in THP-1 cells. *Soc. Neurosci. Abstr.* 19: 1346, 1993.

REYNOLDS, N. C., Jr.; LIN, W.; CAMERON, C. M.; ROERIG, D. L. Differential responses of extracellular GABA to intrastriatal perfusions of 3-nitropropionic acid and quinolinic acid in the rat. *Brain Res*. 778: 140-149, 1997.

RIOS, C.; & SANTAMARIA, A. Quinolinic acid is a potent lipid peroxident in rat brain homogenates. *Neurochem. Res*. 16: 1139-1143, 1991.

ROBINSON, M. B.; & DOWD, L. A. Heterogeneity and functional properties of subtypes os sodium-dependent glutamate transporters in the mammalian central nervous system. *Adv. Pharmacol*. 37: 69-115, 1997.

ROTHSTEIN, J.D.; DYKES-HOBERG, M.; PARDO, C.A.; BRISTOL, L.A.; JIN, L.; KUNCL, R.W.; KANAI, Y.; HEDIGER, M.A.; WANG, Y.; SCHIELKE, J.P.; WELTY, D.F. Knockout of Glutamate Transporters Reveals a Major Role for

Astroglial Transport in Excitotoxicity and Clearence of Glutamate. *Neuron*, 16: 675-686, 1996.

RUDNICK, G. Bioenergetics of neurotransmitter transport. *J. Bioenerg. Biomemb*. 30: 73-185, 1998.

SAITO, K.; CHEN, C. Y.; MASANA, M.; CROWLEY, J. S.; MARKEY, S. P.; HEYES, M. P. 4-Chloro-3-hydroxyanthranilic acid, 6-chlorotryptophan and norharmane attenuate quinolinic acid formation by interferon-γ-stimulated monocytes (THP-1 cells). *Biochem. J*. 291: 1-14, 1993b.

SÁNCHEZ-PRIETO, J. & GONZÁLEZ, P. Occurence of a large Ca^{+2}-independent release of glutamate during anoxia in isolated nerve terminals (synaptosomes). *J. Neurochem*. 50:1322-1324, 1998.

SANTAMARIA, A.; GALVÁN-ARZATE, S.; LISY, V.; ALI, S.F.; DUHART, H.M.; OSORIO-RICO, L.; RIOS, C.; STASTNY, F. Quinolinic acid induces oxidative stress in rat braun synaptosomes. *NeuroReport*, 12: 871-874, 2001.

SCHMIDT, A.P., LARA, D.R., MARASCHIN, J.F., PERLA, A.S., SOUZA, D.O. Guanosine and GMP prevent seizures induced by quinolinic acid in mice. *Brain Res*. 864: 40-43, 2000.

SCHWARCZ, R.; & KOHLER, C. Differential vulnerability of central neurons of the rat to quinolinic acid. *Neurosci. Lett*. 38: 85-90, 1983.

SHARMA, K. N.; PAL, V.; SAWHNEY, M. P. Mixed ligand complexes of nickel (II) and cadmium (II) with quinlinic acids as primary ligand and glycine and valine as second ligand. *J. Inst. Chem*. 61: 149, 1989.

SOARES, F.A., SCHMIDT, A.P., FARINA, M., FRIZZO, M.E., TAVARES, R.G., PORTELA, L.V., LARA, D.R., SOUZA, D.O. Anticonvulsant effect of GMP depends on its conversion to guanosine. *Brain Res*. 1005: 182-186, 2004.

STONE, T. W.; & PRKINS, M. N. Quinolinic acid: a potent, endogenous excitant at amino acid receptors in CNS. *Eur. J. Pharmacol*. 72: 411-412, 1981.

STONE, T.W. Kynurenines in the CNS: from endogenous obscurity to therapeutic importance. *Progress in Neurobiology*. 64: 185-218, 2001.

SUCHER, N.J.; AWOBULUYI, A.; CHOI, Y.B.; LIPTON, S.A. NMDA receptors: from genes to channels. *Trends Pharmacol*. Sci. 17:348-355, 1996.

SZATKOWSKI, M.; BARBOUR, B. & ATTWELL, D. Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature.* 348: 443-446, 1990.

TABB, J. S.; KISH, P.E.; VAN DYKE, R.; UEDA, T. Glutamate transport into synaptic vesicles. *J. Biol. Chem*. 267: 15412-15418, 1992.

TANAKA, K. Functions of glutamate transporters in the brain. *Neuroscience Research*, 37: 15-19, 2000.

TASCA, C.I.; WOFCHUK, S.T.; SOUZA, D.O.; RAMÍREZ, G.; RODNIGHT, R. Guanine nucleotides inhibit the stimulation of GFAP phosphorylation by glutamate. *NeuroReport,* 6: 249-252, 1995.

TASCA, C.I.; CARDOSO, L.F.; MARTINI, L.H.; RAMÍREZ, G.; SOUZA, D.O. Guanine nucleotides inhibit cAMP accumulation induced by metabotropic glutamate receptors activation. *Neurochem. Res.* 23: 183-188, 1998.

TASCA, C.I.; CARDOSO, L.F.; SOUZA, D.O. Effects of guanine nucleotides on adenosine and glutamate modulation of cAMP levels in optic tectum slices from chicks. *Neurochem. Int*. 34: 213-220, 1999.

TASCA, C.I.; SANTOS, T.G.; TAVARES, R.G.; BATTASTINI, A.M.O.; ROCHA, J.B.T.; SOUZA, D.O. Guanine derivatives modulate L-glutamate uptake into rat brain synaptic vesicles. *Neurochem. Int*. 44: 423-431, 2004.

TAVARES, R.G.; TASCA, C.I.; SANTOS, C.E.S.; WAJNER, M.; SOUZA, D.O.; DUTRA-FILHO, C.S. Quinolinic acid inhibits glutamate uptake into synpatic vesicles from rat brain. *NeuroReport* 11: 249-253, 2000.

VINADÉ, E.R., SCHMIDT, A.P., FRIZZO, M.E.S., IZQUIERDO, I., ELIZABETSKY, E., SOUZA, D.O. Chronically administered guanosine is anticonvulsant, amnesic and anxiolytic in mice. *Brain Res*. 977: 97-102, 2003.

VINADE, E.R., IZQUIERDO, I., LARA, D.R., SCHMIDT, A.P., SOUZA, D.O. Oral administration of guanosine impairs inhibitory avoidance performance in rats and mice. *Neurobiol. Learn. Mem*. 81: 137-143, 2004.

VINADÉ, E.R., SCHMIDT, A.P., FRIZZO, M.E.S., PORTELA, L.V., SOARES, F.A., SCHWALM, F.D., ELISABETSKY, E., IZQUIERDO, I., SOUZA, D.O. Effects of Chronic Administered Guanosine on Behavioral Parameters and Brain Glutamate Uptake in Rats. *Journal of Neurosci. Res*. 79: 248-253, 2005.

WAXHAM, M. N. Neurotransmitter receptors In:ZIGMOND, M. J.; BLOOM, F. E.; LANDIS, S. C.; ROBERTS, J. L.; SQUIRE, L. R. eds. *Fundamental Neuroscience.* San Diego: Academic Press, 235-267, 1999.

WOLFENSBERGER, M.; AMSLER, U.; CUENOD, M.; FOSTER, A. C.; WHETSELL, W. O. J.; SCHWARCZ, R. Identification of quinolinic acid in rat and human brain tissue. *Neurosci. Lett*. 41: 247-252, 1983.

WOLOSKER, H.; SOUZA, D. O.; de MEIS, L. Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient. *J. Biol. Chem*. 271: 11726-11731, 1996.

YING, W. A new hypothesis of neurodegenerative diseases: the deleterious network hypothesis. *Med. Hypotheses* 46: 307-313, 1996.