UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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CORRELATOS NEUROQUÍMICOS DA ATIVIDADE ANTICONVULSIVANTE DO LINALOL

Lucimar Filot da Silva Brum

Orientadora: Profª Drª: Elaine Elisabetsky

Co-Orientador: Prof. Dr. Diogo Onofre Gomes de Souza

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Aos meus pais, Licino e Lucy,

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SUMÁRIO

RESUMO

Epilepsia é um transtorno neurológico com etiologias diversificadas e provavelmente envolve efeitos na integração de mecanismos inibitórios GABAérgicos e mecanismos excitatórios glutamatérgicos. De fato, alguns fármacos anticonvulsivantes exercem seus efeitos através do aumento da atividade inibitória GABAérgica e/ou através da diminuição da atividade excitatória glutamatérgica.

Linalol é um composto monoterpênico comumente encontrado em óleos essenciais de várias espécies vegetais utilizadas tradicionalmente como remédios caseiros usados no manejo da epilepsia. Linalol é ativo em vários modelos experimentais de convulsões, entre eles, convulsões induzidas por eletrochoque e compostos químicos (pentilenetetrazol, picrotoxina, ácido quinolínico e NMDA) o que sugere sua interação com os sistemas glutamatérgico e/ou gabaérgico.

Com o objetivo de investigar as bases farmacodinâmicas das propriedades anticonvulsivantes do linalol, avaliamos o efeito deste composto em alguns aspectos neuroquímicos dos sistemas glutamatérgico e GABAérgico. Especificamente investigamos: (i) o tipo de inibição induzida por linalol na união específica de L-[^{3}H]glutamato em preparações de membranas de córtex de ratos; (ii) o tipo de inibição induzida por linalol na união específica de [^{3}H]MK 801 em preparações de membranas de córtex de camundongos; (iii) o efeito do linalol na liberação de L-[^{3}H]glutamato em sinaptossomas obtidos a partir de córtex de camundongos; (iv) o efeito do linalol na captação de L-[^{3}H]glutamato em sinaptossomas obtidos a partir de córtex de camundongos; (v) o efeito do linalol na união específica do [^{3}H]muscimol em preparações de membranas de córtex de camundongos. Além disso, investigou-se o efeito

do linalol nas alterações comportamentais e neuroquímicas que acompanham o fenômeno de *kindling* induzido por PTZ.

Os resultados demonstram que o linalol atua como antagonista competitivo da união específica de L-[^{3}H]glutamato e como antagonista não-competitivo da união específica de [^{3}H]MK801. Linalol reduziu significativamente a liberação de glutamato estimulada por potássio sem interferir na liberação basal de glutamato. Linalol inibiu a captação de glutamato. Linalol não interferiu na união de [^{3}H]muscimol. Linalol retardou a expressão comportamental do PTZ-*kindling*, mas não interferiu no aumento da união específica do L-[^{3}H]glutamato que acompanha este fenômeno.

Os resultados mostram que o efeito anticonvulsivante do linalol está relacionado à diminuição da excitabilidade neuronal glutamatérgica. Estes resultados provêm uma base racional para o uso de espécies produtoras de linalol na terapia caseira antiepiléptica e reforçam a validade de se explorar o linalol ou compostos semelhantes como potenciais agentes anticonvulsivantes.

ABSTRACT

Epilepsy is a neurological disorder involving diverse etiologies. It is likely that the various types of epileptic disorders result from deficits in the integration between the inhibitory GABAergic system and the excitatory glutamatergic system. In fact, many anticonvulsant drugs seem to act by increasing GABAergic activity and/or by decreasing the glutamate excitability.

Linalool is a monoterpene compound, commonly found as a major component of essential oils in a diversity of aromatic species traditionally used as home made anticonvulsant recipes. It possesses a broad spectrum of action experimental epilepsy models, including protection against electroshock and chemical induced convulsions (pentylenetetrazol, picrotoxin, quinolinic acid and NMDA), pointing to interaction with the glutamatergic and/or gabaergic systems.

In order to investigate the pharmacodynamic basis of the previously established anticonvulsant activity, we examined the effects of this compound in some neurochemical aspects of the glutamate and GABAergic systems. Specifically we have investigated: (i) the nature of the inhibition of L-[^{3}H]glutamate binding (rat cortex membranes) induced by linalool, (ii) the nature of the inhibition of [^{3}H]MK 801 binding (mice cortex membranes) induced by linalool, (iii) linalool effects on L-[^{3}H]glutamate release at mice cortical synaptosomes, (iv) linalool effects on L-[^{3}H]glutamate uptake at mice cortical synaptosomes, (v) linalool effects on [^{3}H]muscimol binding at mice cortex membranes. In addition, we investigated the effects of linalool on behavioral and neurochemical aspects of PTZ-kindling.

Results indicate that linalool acts as competitive antagonist of L-[^{3}H]glutamate binding and as a non-competitive antagonist of [^{3}H]MK801 binding in mice cortical membranes. Linalool significantly reduced potassium-stimulated glutamate release not interfering with basal glutamate release from mice cortical synaptosomes. Linalool significantly reduced glutamate uptake. Data does not support a direct interaction of linalool with $GABA_A$ receptors. In addition, linalool partially inhibited and significantly delayed the behavioral expression of PTZ-kindling, but did not modify the PTZ-kindling induced increase in L-[^{3}H]glutamate binding.

The available data suggests that anticonvulsant action of linalool is related with a decrease of neuronal glutamatergic excitability. These data provide a rational basis for traditional use of linalool producing species in the management of epilepsy. We suggest that linalool warrants further structure-activity studies in the context of disclosing innovative management strategies for epileptic disorders.

APRESENTAÇÃO

Esta tese é constituída por introdução, artigos publicados, artigos submetidos e a ser submetidos à publicação, discussão, conclusões, perspectivas e bibliografia. O item **1. Introdução** oferece embasamento para o estabelecimento dos objetivos bem como para o desenvolvimento da metodologia empregada nesta tese. O item **2.** é constituído por **Artigos** (material e métodos, resultados e discussão dos resultados encontram-se nos próprios artigos e representam a íntegra deste estudo).

O item **3. Discussão**, contém interpretações e comentários gerais sobre todos os trabalhos publicados ou submetidos. O item **4. Conclusões** representa as conclusões gerais, baseando-se nos objetivos da tese. O item **5. Perspectivas** descreve possibilidades de seqüência do trabalho. O item **6. Referências Bibliográficas** refere-se somente às citações que aparecem na introdução, discussão e perspectivas. As referências bibliográficas que se referem a materiais e métodos, resultados e discussão parcial encontram-se nos artigos em anexo.

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LISTA DE ABREVIATURAS

AMPA - α-amino-3-hidroxi-5-metil-4-isoxazol-propionato

AP5 - Ácido D-2-amino-5-fosfonopentanóico

AP7 - Ácido D-2-amino-7-fosfonoheptanóico

BZD - Benzodiazepínicos

CPPeno - Ácido [3-[(±)-2-carboxipeprazina-4-il]propeno-1-il]-fosfônico

CGP 40116 - Ácido (±)-(E)-2-amino-4-propil-5-fosfopentenóico

CGS 19755 - Ácido [[(±)-2-carboxipiperidina-4-il]metil-4-il]-fosfônico

ECC - Eletrochoque máximo convulsivo

GABA - Ácido gama-aminobutírico

$GABA_A$ - Receptor GABA tipo A

$GABA_B$ - Receptor GABA tipo B

GABA-T - GABA-transaminase

GAD - Descarboxilase do ácido glutâmico

GAT-1 – Transportadores específicos de GABA

GLN - Glutamina

GLU - Glutamato

ICV – Intracerebroventricular

ILAE - International League Against Epilepsy

KA - Ácido caínico

MK 801 - (±)-5-metil-10,11-dihidróxi-5H-dibenzo(a,d)ciclo-hepteno-5,10-imina

NMDA - N-metil-D-aspartato

PTZ - Pentilenotetrazol

SNC - Sistema nervoso central

SSA - desidrogenase semi-aldeído succínica

1. INTRODUÇÃO

A epilepsia é um distúrbio cerebral crônico relativamente comum, com prevalência situada em torno de 0,5 a 1% da população. Caracteriza-se por manifestações recorrentes, clinicamente diversificadas, entre as quais figuram as convulsões (PORTER, 1990; PALMINI & DA COSTA, 1998). Têm sido descritas amplas variações das taxas da prevalência e incidência da epilepsia A incidência de epilepsia em determinada população varia de acordo com a idade, o sexo, a raça, o tipo de síndrome e fatores sócios-econômicos (FERNANDES & SANDER, 1998).

Epilepsias idiopáticas ou primárias são presumidamente de caráter hereditário, não associadas à lesões cerebrais ou anormalidades neurológicas enquanto epilepsias sintomáticas ou secundárias são decorrentes de lesão cerebral (traumatismo, infecção ou crescimento tumoral) ou outros fatores. Outros fatores de risco para a epilepsia incluem o consumo de álcool, uso de heroína, hipertensão, esclerose múltipla e depressão (HESDORFFER & VERITY, 1997). Devido à complexidade da epilepsia com inúmeras causas e os diversos modos de expressão clínica, o manejo terapêutico apropriado do paciente portador de epilepsia requer uma classificação adequada das crises e das síndromes epilépticas (DREIFUSS, 1981; DICHTER, 1997).

O conhecimento do modo de ação dos sistemas neurotransmissores e da neurofisiologia sináptica, tem levado a avanços no tratamento da epilepsia através do desenvolvimento racional de novos fármacos antiepilépticos em contrapartida às estratégias empíricas tradicionalmente empregadas. Entretanto, ainda hoje continua-se elegendo novos fármacos através de embasamento empírico baseado em suas

propriedades anticonvulsivantes testadas frente a vários modelos experimentais de epilepsia (FISHER, 1989; MODY & SCHWARTZKROIN, 1997).

Algumas questões básicas a respeito da epilepsia envolvem aspectos relacionados à neurotransmissão. Primeiro, não está claro se as alterações nos sistemas neurotransmissores são a base da epilepsia ou conseqüência adaptativa de alterações anteriores. Segundo, ainda há dúvidas sobre o papel dos sistemas transmissores na epileptogênese e na expressão das convulsões. Terceiro, não se sabe ao certo quanto a alterações na função sináptica induzidas por fármacos antiepilépticos ou outras intervenções terapêuticas, que contribuem para a supressão das convulsões (MELDRUM, 1995).

A investigação dos eventos celulares e neuroquímicos responsáveis pelos episódios convulsivos geralmente envolve a interação entre mecanismos inibitórios GABAérgicos e mecanismos excitatórios glutamatérgicos (SCHWARTZKROIN & PRINCE, 1980; SNEAD, 1995; NAFFAH-MAZZACORATTI, 1998). Além disso, uma coletânea de dados neuroquímicos e farmacológicos relaciona estes sistemas com o início, propagação e término de diversos tipos de episódios convulsivos epilépticos (BRADFORD, 1995).

1.1. PAPEL DO SISTEMA GABAÉRGICO NA EPILEPSIA.

O ácido gama-aminobutírico (GABA) é o principal neurotransmissor inibitório do sistema nervoso central (SNC). A transmissão inibitória é mediada pela interação do neurotransmissor com receptores específicos *ionotrópicos* ($GABA_A$) ou *metabotrópicos* ($GABA_B$), que diferem entre si fisiológica, farmacológica e bioquimicamente (PAUL, 1995; MACDONALD, 1997b).

A descoberta da existência de sinapses GABAérgicas amplamente distribuídas no SNC levantou a suspeita de que uma possível falha nestes neurônios poderia ser importante na etiologia da epilepsia humana (GALE, 1992; MELDRUM, 1995). Evidências relevantes para esta hipótese incluem relatos da diminuição de neurônios ou terminações nervosas GABAérgicas em foco epiléptico cortical (RIBAK, 1985), redução da neurotransmissão GABAérgica no lobo temporal removido cirurgicamente de pacientes epilépticos (ROUGIER *et al.*, 1989), e baixos níveis de GABA no fluído cérebro-espinhal de pacientes epilépticos (WOOD *et al.*, 1979; MAYNAM *et al.*, 1980; LOSCHER & SIEMES, 1984). Estudos em animais também relatam significante diminuição (aproximadamente 30-50%) nos neurônios e terminações nervosas GABAérgicas (CALLAHAN *et al.*, 1991), bem como uma diminuição de 60-70% nos níveis extracelulares de GABA em amígdala de ratos "abrasados" (*kindleds)* (KAURA *et al.*, 1995).

Por outro lado, o aumento dos níveis de GABA no SNC, seja por inibição da GABA-transaminase (ex. vigabatrina), inibição da captação de GABA (ex. tiagabina) ou através de uma ação agonista em receptores GABA (ex. benzodiazepínicos) promove um efeito anticonvulsivante (MELDRUM, 1989; UPTON, 1994).

Entretanto, a elevação dos níveis de GABA no cérebro com agentes que bloqueiam seu metabolismo (ex. vigabatrina) levando à supressão das convulsões não é *per se* um indicativo do papel causal (etiológico) de uma redução dos níveis tissulares ou diminuição da liberação de GABA endógeno na epilepsia, tornando-se necessário mais evidências de deficiência GABAérgica em cérebros epilépticos (SNODGRASS, 1992; BRADFORD, 1995).

1.2. PAPEL DO SISTEMA GLUTAMATÉRGICO NA EPILEPSIA

Glutamato, o principal neurotransmissor excitatório do SNC de mamíferos, é responsável não somente pela transmissão sináptica rápida, mas também participa na plasticidade e funções cognitivas (GREENAMYRE & PORTER, 1994). A transmissão excitatória glutamatérgica é mediada por vários receptores, os quais podem ser classificados de acordo com suas propriedades farmacológicas e funcionais em *ionotrópicos* [N-metil-D-aspartato (NMDA), α-amino-3-hidróxi-5-metil-4-isoxazol-propionato (AMPA) e ácido caínico (KA)] ou *metabotrópicos* (COTMAN *et al.*, 1995).

Um distúrbio na neurotransmissão excitatória mediada por glutamato, há muito tempo vem sendo postulado como o principal fator na etiologia de pelo menos algumas formas de epilepsia experimental e epilepsia humana (MELDRUM, 1991, 1994; BRADFORD, 1995).

O sistema glutamatérgico excitatório pode estar centralmente envolvido na epilepsia em três níveis: (a) na hiperatividade subconvulsiva crônica que ocorre nos focos epilépticos e que pode ser detectada por eletroencefalograma; (b) na atividade excitatória amplificada que leva ao início da convulsão e ao recrutamento de neurônios adjacentes ao foco e (c) na propagação e generalização da hiperatividade precipitando convulsão generalizada (DINGLEDINE *et al.*, 1986; BRADFORD, 1995).

Vários estudos demonstram o papel do sistema glutamatérgico na epileptogênese e na expressão das convulsões (DINGLEDINE *et al.*, 1990; WIKINSKI & ACOSTA, 1995; OBRENOVITCH *et al.*, 1996). Claramente, a administração intracerebral de glutamato ou seus agonistas (ex. NMDA) causam convulsões (MELDRUM, 1994). O envolvimento do sistema glutamatérgico na epilepsia também tem sido identificado

através do aumento na densidade dos receptores glutamatérgicos no fenômeno de *kindling* (SCHRÖDER *et al.*, 1993; LOSCHER, 1998).

Apesar de raras e controversas, as evidências do envolvimento do sistema glutamatérgico na fisiopatologia da epilepsia humana provêm de análises neuroquímicas de níveis dos aminoácidos em amostras de cérebros removidos cirurgicamente, no plasma ou fluido cérebro-espinhal de pacientes epilépticos (VAN GELDER *et al.*, 1972; PERRY & HANSEN, 1981; BRADFORD, 1995).

Em resumo, a hiperexcitabilidade que caracteriza as vias neuronais, gerando uma atividade epileptiforme ou convulsões, poderia, hipoteticamente, resultar de mudanças na população dos receptores glutamatérgicos e/ou GABAérgicos; ou seja, substancial aumento nos receptores excitatórios (receptores glutamatérgicos) e redução nos receptores inibitórios (receptores GABA) poderiam levar a um estado cronicamente hiperativo resultando em convulsões (BRADFORD, 1995).

1.3. EPILEPSIA HUMANA E MODELOS ANIMAIS

Modelos animais de epilepsia são essenciais para a descoberta de fármacos com ação antiepiléptica bem como para a elucidação de mecanismos neuronais básicos envolvidos na gênese da epilepsia em humanos (BIZIERE & CHAMBON, 1987; ENGEL, 1992; AVANZINI *et al.*, 1997). Além disso, diferentes elementos envolvidos na gênese do episódio epiléptico observados nestes modelos fornecem dados importantes sobre o efeito do fármaco que está sendo testado, mostrando se este atua na iniciação (deflagração) das convulsões ou se previne sua propagação ou generalização (DE DEYN *et al.*, 1992). O quadro 1 relaciona os modelos animais com tipos de epilepsia humana.

Quadro 1. Modelos animais de epilepsia e o Sistema de Classificação do ILAE (1981)*.

Tipo de Epilepsia	Modelo animal
Convulsão parcial simples (aguda)	- Aplicação tópica de convulsivantes: penicilina, bicuculina, estricnina, pilocarpina, lítio-pilocarpina; - ECC
Convulsão parcial simples (crônica)	- Procedimentos cirúrgicos e congelamento cortical local (lesão criogênica)
Convulsão parcial complexa (com generalização secundária)	- *Kindling* (elétrico ou químico) - Microinjeção local de ácido caínico - Cepas de camundongos E1
Convulsão tônico-clônica Generalizada	- Cepas de animais geneticamente manipulados. (fotossensíveis, audiogênicos, etc.) - ECC - Convulsivantes sistêmicos: estricnina, picrotoxina, pentilenotetrazol , pilocarpina, etc.
Crise de ausência generalizada	- Estimulação talâmica, - Penicilina sistêmica, modelos genéticos - Gama-hidroxibutirato sistêmico - Opióides intraventricular.

Adaptada de FISHER (1989) e MODY & SCHWARTZKROIN (1997). * As crises epilépticas são classificadas conforme proposto pela "International League Against Epilepsy" (ILAE) em 1981, que as divide em crises parciais (simples ou complexa) ou generalizadas (ex. crises tônico-clônicas e crises de ausência) (DREIFUSS, 1981).

Uma grande variedade de modelos experimentais em animais têm sido utilizados na pesquisa da epilepsia. Estes variam desde aplicações intracerebrais de óxido de alumínio, cobalto metálico, penicilina, indução de lesões por congelamento local do cérebro a modelos simples e diretos de convulsões induzidas por ECC ou injeção

sistêmica de substâncias convulsivantes (PTZ, picrotoxina, bicuculina, pilocarpina, etc.) (TURSKI *et al.*, 1986; FISHER, 1989; CAVALHEIRO *et al.*, 1996; AL-NOORI *et al.*, 1998).

Na última década tem aumentado o interesse pelo uso do modelo experimental de *kindling (abrasamento)*. *Kindling* refere-se a um processo em que repetidas administrações de um estímulo subconvulsivo, químico ou elétrico, resulta na diminuição do limiar de disparo neuronal, culminando em convulsões generalizadas tônico-clônicas (MASON & COOPER, 1972). O modelo *kindling* permite estudar o efeito de fármacos nos eventos associados ao processo da epileptogênese (atividade antiepileptogênica) bem como aos eventos associados às convulsões (atividade anticonvulsivante) (SATO *et al.*, 1990; SILVER *et al.*, 1991). Outro aspecto de interesse é que este fenômeno permite o estudo de mudanças plásticas a longo prazo na excitabilidade neuronal (FISHER, 1989).

1.4. MECANISMO DE AÇÃO DOS FÁRMACOS ANTIEPILÉPTICOS

O mecanismo de ação dos fármacos antiepilépticos não é inteiramente compreendido já que a própria epileptogênese ainda é, sob alguns aspectos, obscura. Os fármacos antiepilépticos podem afetar o início, a manutenção e a propagação da descarga epiléptica no cérebro (MELDRUM, 1996; PORTER & CHADWICK, 1997).

A história moderna dos fármacos antiepilépticos inicia-se com a introdução do fenobarbital (1912) e os fármacos hoje disponíveis na clínica pertencem a diferentes classes químicas (BRODIE & DICHTER, 1996). Consideram-se, basicamente, três mecanismos gerais de ação da classe de fármacos anticonvulsivantes, a saber:

- aumento da atividade sináptica inibitória (GABA);
- diminuição da atividade sináptica excitatória (glutamato);
- controle da excitabilidade da membrana neuronal e da permeabilidade iônica.

1.4.1. Aumento da atividade sináptica inibitória

Alguns compostos aumentam a inibição mediada por GABA no SNC através dos seguintes mecanismos de ação: (a) agonistas de receptor GABAérgico; (b) inibição da captação de GABA na fenda sináptica; (c) inibição das enzimas responsáveis pela sua degradação e (d) ação em diferentes sítios alostéricos no receptor GABA/BZD (MELDRUM, 1990; UPTON, 1994; PFEIFFER *et al.*, 1996) (Tabela 1, Figura 1).

Tabela 1. Fármacos que aumentam a inibição neuronal mediada por GABA

Fármacos que atuam na liberação, captação ou metabolismo de GABA	
- aumenta a liberação	(? Ácido valpróico)
- diminui a captação	Tiagabina
- inibe o metabolismo	Vigabatrina, (? Ácido valpróico)
Fármacos que atuam no receptor GABA/BZD	
- agonistas	Muscimol
- pró-fármacos	Progabida
- sítio dos barbituratos	Fenobarbital
- sítio dos BZD	Diazepam, Clonazepam, Clobazam
- sítio neuroesteróide	Pregnanolona, Alfaxolona
- outros sítios modulatórios	Clormetiazol, Topiramato, Propofol
	Gamabutirolactona, Loreclezol,
	Losigamona e Felbamato

Adaptado de MACDONALD, 1997a.

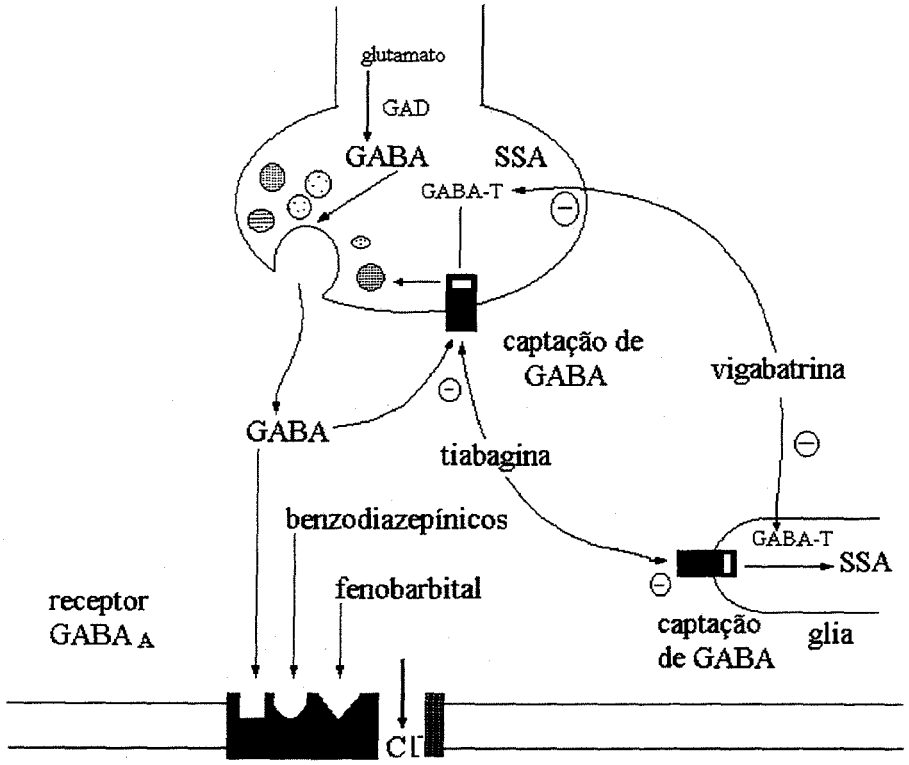

Figura 1. Transmissão GABAérgica e sítios de ação de fármacos anticonvulsivantes (adaptado de UPTON, 1994).

Muscimol é o mais potente agonista $GABA_A$. Já a progabida, além de atividade agonista também atua como pró-fármaco, sendo metabolizada a SL 75102 que apresenta maior atividade agonista nos receptores GABAérgicos (MELDRUM, 1990). O mecanismo pelo qual o ácido valpróico aumenta os níveis de GABA ainda não está totalmente esclarecido. Inicialmente, foi proposto que o mecanismo de ação devia-se à inibição da GABA-transaminase (GABA-T). Posteriormente, verificou-se que o grau de inibição da enzima em doses terapêuticas era insuficiente para justificar a sua ação anticonvulsivante (CHAPMAN *et al.*, 1982). Outras hipóteses sugerem uma possível inibição da semi-aldeído succínica desidrogenase (também responsável pela degradação) ou um aumento da síntese do GABA ou da sua liberação (MELDRUM, 1990; LOSCHER, 1999).

Apesar da gabapentina ter sido desenvolvida para exercer atividade agonista no sistema GABAérgico, diversos estudos têm falhado em demonstrar esta atividade (LOSCHER *et al.*, 1991; BRODIE & DICHTER, 1996; MACDONALD, 1997a).

Os inibidores da captação de GABA, tiagabina e compostos relacionados, são aparentemente específicos para GAT-1 (mediador específico da captação de GABA), promovendo um aumento na concentração extracelular de GABA, o que prolonga o efeito inibitório GABAérgico (SUZDAK & JANSEN, 1995; ADKINS & NOBLE, 1998).

Vigabatrina eleva a concentração de GABA no cérebro através do bloqueio irreversível da GABA-T, enzima responsável pela degradação metabólica do GABA (JUNG *et al.*, 1977; DICHTER & BRODIE, 1996).

O receptor GABA/BZD possui vários sítios alostéricos que regulam a resposta inibitória de GABA. O fenobarbital liga-se a um sítio regulador alostérico no receptor GABA/BZD, aumentando a corrente mediada pelos receptores GABAérgicos por prolongar a abertura dos canais de Cl^- (MACDONALD, 1997a). Os benzodiazepínicos também ligam-se a um sítio regulador alostérico no receptor GABA/BZD, promovendo o aumento da freqüência de abertura do canal sem alterar o tempo de abertura ou condutância de Cl^- induzida por GABA (TWYMAN *et al.*, 1990; MELDRUM, 1996).

Outros compostos que aumentam os efeitos inibitórios de GABA também estão sendo investigados como potenciais anticonvulsivantes. Por exemplo, gamabutirolactona, loreclezol, neuroesteróides, clormetiazol, felbamato, topiramato e losigamona que aumentam atividade inibitória GABAérgica através de ligação a sítios específicos (HOLLAND *et al.*, 1993; PALMER & MCTAVISH, 1993; KOKATE *et al.*, 1994; KUME *et al.*, 1996).

1.4.2. Diminuição da atividade sináptica excitatória

O glutamato é o mais importante neurotransmissor excitatório no SNC, sendo o NMDA o subtipo mais relevante para a gênese e controle da atividade convulsiva (MELDRUM, 1994; CHAPMAN, 1998).

A excitação glutamatérgica pode ser reduzida por agentes que limitam a liberação do glutamato através da estabilização de canais de Na^+ voltagem-dependente pré-sinápticos (interferindo nas descargas repetitivas sustentadas), por agentes que aumentam a captação de glutamato ou que atuem como antagonistas de receptores glutamatérgicos pós-sinápticos (principalmente do receptor NMDA) (CROUCHER *et al.*, 1982; MELDRUM, 1990; ROGAWSKI, 1992; LEPPIK, 1994; UPTON, 1994; MELDRUM, 1996) (Tabela 2, Figura 2).

Tabela 2. Fármacos que diminuem a excitabilidade neuronal mediada por glutamato

Fármacos que atuam na liberação de glutamato	
- diminui a liberação	Lamotrigina, Remacemida, Riluzole
Antagonistas NMDA	
- sítio do glutamato	CPPeno, CGS 19755 (Selfotel), CGP 40116, AP5, AP7
- sítio da glicina	Felbamato, Ácido quinurênico, ACEA 1021, MDL 104653
- bloqueador de canal	Dizocilpina (MK 801), Memantina, Fenciclidina

Adaptado de MACDONALD, 1997a.

O receptor NMDA é permeável aos íons Na^+, K^+ e Ca^{+2} e está sujeito a um bloqueio voltagem-dependente desencadeado pelo Mg^{+2}. Para a ativação do receptor NMDA é necessária a ocupação dos sítios de glutamato e glicina. O receptor NMDA pode

ser bloqueado por antagonistas competitivos, atuando diretamente no sítio do glutamato (CPPeno, CGS 19755, CGP 40116, AP5, AP7) ou da glicina (felbamato, ácido quinurênico, ACEA 1021, MDL 104653) (MCCABE *et al.*, 1993). Antagonistas não-competitivos, que bloqueiam a abertura de canal (fenciclidina, cetamina e MK 801) são potentes anticonvulsivantes, mas têm baixo índice terapêutico, produzindo em animais um aumento da atividade motora e comportamento estereotipado em doses terapêuticas (CARTER, 1994) (Figura 2).

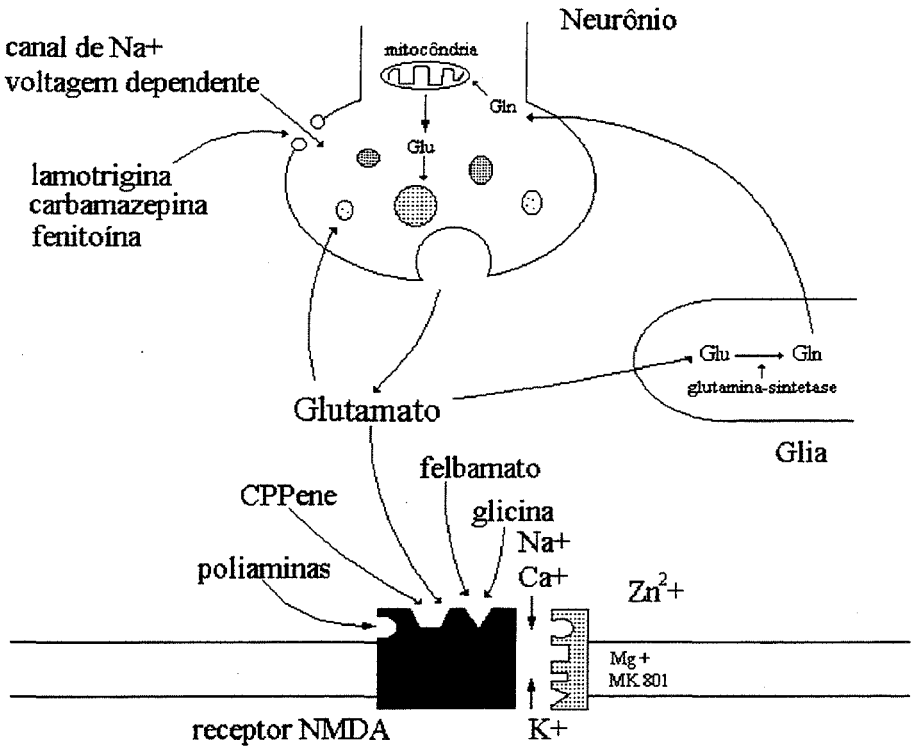

Figura 2. Transmissão glutamatérgica e sítios de ação de fármacos anticonvulsivantes (adaptado de UPTON, 1994).

A excitação glutamatérgica pode também ser reduzida por agentes que diminuem a liberação sináptica de aminoácidos excitatórios, como por exemplo a fenitoína, carbamazepina, lamotrigina, riluzole e remacemida (MILLER *et al.*, 1986; FITTON &

GOA, 1995; MELDRUM, 1996; DAVIES, 1997). O efeito anticonvulsivante da lamotrigina é devido à interação específica com os canais de Na^+ voltagem-dependente, o que resulta em um decréscimo na liberação de glutamato (LEACH *et al.*, 1986; MACDONALD & KELLY, 1994).

Também têm sido avaliados os efeitos de fármacos anticonvulsivantes na atividade da glutamina-sintetase, enzima chave na regulação da transmissão glutamatérgica. Glutamina-sintetase utiliza glutamato captado pela glia para formar glutamina que volta ao neurônio formando glutamato como neurotransmissor (FRASER *et al.*, 1999).

1.4.3. Controle da excitabilidade da membrana neuronal e da permeabilidade iônica

A excitabilidade da membrana neuronal é determinada pelo estado funcional de canais de cálcio, potássio e sódio. Destes, os canais de sódio voltagem-dependente têm sido importante alvo para o desenvolvimento de novos anticonvulsivantes (TAYLOR & MELDRUM, 1995). Os canais de cálcio também são alvo de particular interesse, pois um excessivo influxo de cálcio leva à excitotoxicidade e morte neuronal. Fármacos que atuam em canais iônicos naturalmente modificam a excitabilidade de neurônios normais ou epilépticos (MELDRUM, 1997). Potencialmente tais fármacos poderiam restabelecer a normalidade da excitabilidade de neurônios epilépticos; caso isso não ocorra, eles poderiam bloquear o início e/ou propagação e generalização de fenômenos convulsivos (MELDRUM, 1997). A modulação de canais de sódio e a inibição das descargas repetidas de alta freqüência (diminuindo a excitabilidade celular e propagação axonal de impulsos nervosos) parecem ser responsáveis pela atividade anticonvulsivante da carbamazepina, fenitoína e oxcarbazepina (WAMIL *et al.*, 1994; MELDRUM, 1996).

A supressão das crises de ausência obtida com etossuximida, trimetadiona, zonisamida e ácido valpróico é atribuída ao bloqueio de canais de cálcio de baixo limiar (tipo T) (ROGAWSKI & PORTER, 1990; MACDONALD, 1997a). Há várias evidências sugerindo que outros fármacos usados na terapêutica antiepiléptica atuem em canais de cálcio pré-sinápticos (STEFANI *et al.*, 1996). À exceção da flunarizina, outros bloqueadores de canais de cálcio utilizados na prática clínica (como anti-hipertensivos) são desprovidos de ação antiepiléptica, já que a maioria não atravessa eficazmente a barreira hematoencefálica ou não tem seletividade requerida para determinado canal de cálcio (MACDONALD, 1997a).

Têm sido avaliados vários agentes com efeitos sistêmicos e atividade seletiva para canais de cálcio tipo N, P ou Q (DICKIE & DAVIES, 1992). O interesse nestes canais de cálcio está relacionado ao efeito regulatório na liberação de neurotransmissores (VERHAGE *et al.*, 1994). A possibilidade da utilização de facilitadores da abertura de canais de potássio como anticonvulsivantes também tem sido extensivamente avaliada em alguns fármacos anti-hipertensivos (cromacalina, minoxidil, diazóxido e pinacidil) (WESTON & EDWARDS, 1992).

1.5. ESTRATÉGIAS PARA O DESENVOLVIMENTO E IDENTIFICAÇÃO DE NOVOS FÁRMACOS ANTIEPILÉPTICOS

Embora a maioria dos pacientes epilépticos sejam convenientemente tratados com os fármacos ora disponíveis, entre 20-30% dos pacientes não têm suas crises adequadamente controladas (MATTSON, 1994).

A maioria dos fármacos antiepilépticos clinicamente efetivos, introduzidos desde 1938, têm sido identificados através de modelos experimentais *in vivo*, principalmente

convulsões induzidas por ECC ou PTZ (KRALL *et al.*, 1978; SWINYARD & KUPFERBERG, 1985) em cérebros normais. Não obstante, a ampla utilização destes modelos animais, desenvolvidos há décadas, leva à identificação de compostos de eficácia clínica semelhante aos já existentes e também fornecem pouca informação a respeito de seus mecanismos de ação. Considerando a necessidade de se desenvolver fármacos ativos em pacientes refratários à terapia anticonvulsivante padrão, torna-se essencial o desenvolvimento de novos modelos experimentais para a identificação de compostos com mecanismos de ação inovadores (HEINEMANN *et al.*, 1994; KUPFERBERG & SCHMUTZ, 1997).

É também de interesse para clínica uma possível interferência com o processo epileptogênico. Apesar da administração crônica de fármacos antiepilépticos ser efetiva no controle de crises convulsivas, não há nenhum dado clínico disponível sugerindo que a administração destes fármacos tenha algum impacto na epileptogênese (PORTER & MELDRUM, 1997).

A hipótese de que alguns eventos responsáveis pela epileptogênese, como por exemplo, movimento de íons (Na^+, K^+, Ca^{+2} e Cl^-), liberação, interação com receptores pós-sinápticos e recaptação de neurotransmissores inibitórios e excitatórios, estejam anormais em cérebro de epilépticos é corroborada por dados obtidos de animais *kindled* (MODY, 1993) e pacientes com epilepsia (DURING & SPENCER, 1993; DURING *et al.*, 1995). Consequentemente, para a identificação de compostos que atuem na epileptogênese torna-se necessário o estudo de tais compostos em cérebros epilépticos (por exemplo, cepas de camundongos DBA/2 ou Frings).

Neste contexto, o modelo de *kindling* também é bastante apropriado devido às alterações induzidas no canal iônico e na função do receptor (MODY, 1993). Entretanto,

devido ao tempo necessário para a indução do fenômeno, este modelo não tem sido usado como *screening* primário e sim num estágio posterior na caracterização de compostos anteriormente identificados através de outros modelos experimentais (MODY, 1993).

Novas metodologias para a identificação de potenciais agentes terapêuticos (geralmente métodos *in vitro*), são baseadas nos mecanismos básicos da propagação e inibição de convulsões (NOEBELS, 1996; KUPFERBERG & SCHMUTZ, 1997; TAYLOR & MARKS, 1998). Estes métodos não oferecem informações além do mecanismo de ação específico (como por exemplo, aspectos farmacocinéticos ou toxicidade do composto), mas assumem que o mecanismo está diretamente relacionado à epilepsia humana e não levam à identificação de compostos com mecanismos de ação inovadores (KUPFERBERG & SCHMUTZ, 1997).

1.6. PERFIL ANTICONVULSIVANTE DO LINALOL

Em todo o mundo, a medicina tradicional faz uso de uma grande variedade de plantas como fonte de remédios (SAMUELSON, 1989; SONNEN, 1997). Na busca de novos fármacos, a pesquisa envolvendo plantas medicinais tem sido considerada uma abordagem profícua (FARNSWORTH, 1990, 1994; BUM *et al.*, 1996). Uma estratégia para o estudo de plantas medicinais é a etnofarmacologia, que pode ser definida como "a exploração científica interdisciplinar dos agentes biologicamente ativos, tradicionalmente empregados ou observados pelo homem" (HOLMSTEDT & BRUHN, 1983).

A etnofarmacologia é uma área de pesquisa interdisciplinar, sendo fundamental no seu estudo a interação de grupos de fitoquímica e farmacologia, bem como o aporte de botânicos, antropólogos e bioquímicos para a realização de um trabalho completo (ELISABETSKY, 1987; ELISABETSKY & WANNMACHER, 1993). O percentual de

fármacos que são descobertos a partir de abordagem etnofarmacológica tem se mostrado superior ao percentual obtido através de triagem cega de plantas selecionadas ao acaso (FARNSWORTH, 1994). No Brasil, a pesquisa etnofarmacológica é bastante promissora para a descoberta de novas fontes de medicamentos a partir da flora nativa, principalmente na Amazônia, onde índios e caboclos são fontes óbvias de conhecimento sobre plantas medicinais (ELISABETSKY, 1987; COELHO DE SOUZA & ELISABETSKY, 1999).

Entre 1981 e 1991, uma pesquisa etnofarmacológica realizada com caboclos na Amazônia objetivou a busca de psicofármacos em espécies vegetais utilizadas na prática médica tradicional daquela região (ELISABETSKY & SETZER, 1985).

Foram identificadas quatro espécies vegetais tradicionalmente usadas no preparo de uma fórmula "antiepiléptica". Esse anticonvulsivante caseiro é obtido da extração mecânica da seiva de folhas de *Ruta graveolens* L (Rutaceae), folhas de *Cissus sicyoides* Spreng (Vitaceae), folhas de *Aeollanthus suaveolens* Spreng (Labiatae) e sementes de *Sesamum indicum* L. (Pedaliaceae). Esta preparação mostrou-se ativa frente a modelos animais de convulsão (ELISABETSKY & SANTANA, 1984), e o estudo separado de cada espécie componente da mistura mostrou que o sumo de *A. suaveollens*, conhecida popularmente como "Catinga-de-Mulata", quando administrado intraperitonealmente, protege os animais de convulsões induzidas por PTZ (ELISABETSKY & SANTANA, 1984).

Estudos fitoquímicos monitorados pelo resultado da avaliação farmacológica demonstraram que a atividade anticonvulsivante de *A. suaveollens* está associada ao óleo essencial. A análise cromatográfica (SOUZA *et al.*, 1993) identificou quatro componentes principais (linalol, delta decanolactona, delta-decen-2-lactona e acetato de

linalil) (vide estruturas em anexo 1). Desses componentes, apenas o linalol mostrou-se ativo em modelos experimentais de epilepsia *in vivo* (ELISABETSKY *et al.*, 1995b) e *in vitro* (ELISABETSKY *et al.*, 1995a).

Linalol (dl-3,7-dimetil-3-hidroxi-1,6-octadieno) é um composto monoterpênico presente em óleos essenciais aromáticos de várias espécies vegetais usadas como sedativas do SNC, incluindo *A. suaveollens* (ELISABETSKY *et al.*, 1995b; COELHO DE SOUZA & ELISABETSKY, 1999).

A avaliação psicofarmacológica *in vivo* de linalol demonstrou que este composto possui atividade depressora do SNC, resultando em efeitos hipnóticos, diminuição da temperatura corporal, proteção a convulsões induzidas por PTZ, ECC (ELISABETSKY *et al.*, 1995b), picrotoxina (BARROS & ELISABETSKY, 1996b), ácido quinolínico (icv) (MARASCHIN *et al.*, 1996), aumento da latência para convulsões induzidas por NMDA (BARROS & ELISABETSKY, 1996a), mas não proteção a convulsões induzidas por estricnina (BARROS & ELISABETSKY, 1996b). Em experimentos *in vitro*, linalol inibiu a união específica de [^{3}H]glutamato (40 nM) em membranas corticais de ratos (ELISABETSKY *et al.*, 1995a). Apesar de evidências que sugerem efeitos modulatórios na transmissão glutamatérgica e/ou GABAérgica associadas à atividade anticonvulsivante do linalol, estes mecanismos ainda não estão totalmente esclarecidos.

Devido a importância do papel inibitório do GABA, bem como o aumento na excitabilidade neuronal via sistema glutamatérgico na geração, propagação e manutenção da atividade epileptiforme, é fundamental que conheçamos a ação do linalol em eventos relacionados à transmissão glutamatérgica e/ou GABAérgica.

1.7. OBJETIVOS

O objetivo principal desta Tese foi investigar as bases farmacodinâmicas das propriedades anticonvulsivantes do linalol.

Foram estabelecidos os seguintes objetivos:

1. Avaliar o efeito do linalol no sistema glutamatérgico:
    a) através do estudo de união específica do L-[^{3}H]glutamato em preparações de membranas de córtex de ratos;
    b) através do estudo de união específica de [^{3}H]MK 801 em preparações de membranas de córtex de camundongos;
    c) verificar se o linalol modula a liberação de L-[^{3}H]glutamato em sinaptossomas obtidos a partir de córtex de camundongos.
    d) verificar se o linalol modula a captação de L-[^{3}H]glutamato em sinaptossomas obtidos a partir de córtex de camundongos.
2. Avaliar o efeito do linalol no sistema GABAérgico através do estudo de união específica do [^{3}H]muscimol em preparações de córtex de camundongos.
3. Avaliar o efeito do linalol nas alterações comportamentais e neuroquímicas que acompanham o fenômeno de *kindling* induzido por PTZ.
4. Comparar o efeito do linalol nas alterações comportamentais e neuroquímicas que acompanham o fenômeno de *kindling* induzido por PTZ com um anticonvulsivante clássico, o fenobarbital.

2. ARTIGOS CIENTÍFICOS

2.1. CAPÍTULO 1 – ELISABETSKY, E., SILVA BRUM, L.F., SOUZA, D.O. Anticonvulsant properties of linalool in glutamate-related seizure models. *Phytomedicine* 6(2): 107-113, 1999.

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Anticonvulsant properties of linalool in glutamate-related seizure models

E. Elisabetsky[1,2], L. F. Silva Brum[1,2] and D. O. Souza[2]

[1]Laboratório de Etnofarmacologia, Departamento de Farmacologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.
[2]Curso de Pós-Graduação em Ciências Biológicas-Bioquímica, Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Brazil.

Summary

In order to investigate the pharmacodynamic basis of the previously-established anticonvulsant properties of linalool, we examined the effects of this compound on behavioral and neurochemical aspects of glutamate expression in experimental seizure models. Specifically, linalool effects were investigated to determine its inhibition of (i) L-[^{3}H]glutamate binding at CNS (central nervous system membranes), (ii) N-methyl-D-aspartate (NMDA)-induced convulsions, (iii) quinolinic acid (QUIN)-induced convulsions, and the behavioral and neurochemical correlates of PTZ-kindling. The data indicate that linalool modulates glutamate activation expression *in vitro* (competitive antagonism of L-[^{3}H]glutamate binding) and *in vivo* (delayed NMDA convulsions and blockage of QUIN convulsions). Linalool partially inhibited and significantly delayed the behavioral expression of PTZ-kindling, but did not modify the PTZ-kindling-induced increase in L-[^{3}H]glutamate binding.

Key words: linalool, anticonvulsants, glutamate, NMDA, PTZ-kindling

Introduction

Epilepsy is a disease with many apparent etiologies linked to alterations in inhibitory or excitatory neurotransmitter systems (Kozikowski, 1993; Obrenovitch et al., 1996). Regarding the latter, it has been established that epilepsy may be caused at least in part by overstimulation of glutamate receptors (Lipton and Rosemberg, 1994). Despite the recognition of the physiological importance of the glutamatergic system and the therapeutic potential of agents affecting glutamate receptors (Meldrum and Garthwait, 1990), only a few of the currently available drugs that modify excitatory neurotransmission (e.g., remacenide, lamotrigine, felbamate and topiramate) have an acceptable therapeutic index (Thomas, 1995; Meldrum, 1996). Since 25 to 30 percent of patients continue to have seizures despite optimal therapy (Dichter and Brodie, 1996), there is clearly a need for the development of additional antiepileptic drugs (Meldrum, 1997).

Linalool is a monoterpene compound, found commonly as a major component of essential oils in aromatic species. Several linalool-producing species are used in traditional medicine, including *Aeollanthus suaveolens* Spreng (Libiatae) (Elisabetsky et al., 1995b) used in a home made anticonvulsant remedy throughout the Brazilian Amazon (Elisabetsky and Setzer, 1985; Kainer and Duryea, 1992).

Psychopharmacological *in vivo* evaluation of linalool revealed that this compound has dose-dependent marked sedative effects in the Central Nervous System (CNS). These include protection against pentylenetetrazol (PTZ, s.c. 88mg/kg) and transcorneal electroshock (ECC)-induced convulsions, as well as hypnotic and hypothermic properties (Elisabetsky et al., 1995b). Initial neurochemical analysis showed that linalool had an inhibitory effect on glutamate binding in rat cortex membranes (Elisabetsky et al., 1995a).

In order to clarify further the modes of glutamatergic modulation relevant to linalool's profile as an anticon-

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vulsant, we considered it of interest to detail the nature of linalool effects on L-[^{3}H]glutamate binding at CNS membranes, and to examine the effect of linalool on subcutaneous (s.c.) N-methyl-D-aspartate (NMDA)-induced convulsions, on intracerebroventricular (i.c.v.) quinolinic acid (QUIN)-induced convulsions, and on the PTZ-kindling model (both behaviorally and on glutamate changes that accompany the kindling phenomenon).

Materials and Methods

Drugs and reagents

Diazepam, NMDA, QUIN, tween 80, propylene glycol (PPG), linalool (dl-3,7-dimethyls-3-hydroxy-1,6-octadiene, 95–97 % purity), and phenobarbital were acquired from Sigma; MK-801 was obtaints from RBI. Glutamate was purchased from Merck and L-[^{3}H]glutamate from Amersham. Linalool was suspended in tween (1 %); diazepam was suspended in PPG (40 % solution); all other drugs were dissolved in distilled water.

Animals

Male adult Wistar rats and male albino mice, strain CF-1, from Instituto de Pesquisas Biológicas were used throughout the study. The animals were kept on a 12-hr light/dark cycle, at 12 ± 1°C, with free access to food (Nuvilab CR1) and water. All procedures were carried out according to institutional policies on experimental animal handling.

Effects of linalool an L-[^{3}H]glutamate binding on rat cortex

- *Membrane Preparation*

Membranes were prepared as described by Elisabetsky et al (1995a). Rat cerebral cortex was homogenized in 20 vol. (vol:weight) of 0.32M sucrose containing 10mM Tris/HCl buffer, pH 7.4, and 1mM $MgCl_2$. The homogenate was centrifuged twice at 1,000g for 15min and the final pellet discarded. Both supernatants were pooled and centrifuged at 27,000g for 15min. The supernatant was discarded and the resulting pellet was lysed (20:1 vol:weight) for 30min in 10mM Tris/HCl buffer, pH 7.4. The lysed pellet was washed three times with lysing buffer (20:1 vol:weight) by centrifuging at 27,000g for 15min. Supernatants were discarded and membranes of the final pellet were used for the binding experiments. All steps were carried out at 4°C.

- *Binding of L-[^{3}H]glutamate*

For measurement of L-[^{3}H]glutamate binding, membranes (100µg of protein) were incubated at 30°C for 15min in 50mM Tris/HCl buffer, pH 7.4, in various L-[^{3}H]glutamate concentrations (final volume of 0.5ml). The reaction was interrupted by cooling the tubes and further centrifugation for 20min at 27,000g. The supernatant was discarded. The walls of the tubes and the surface of the pellets were quickly and carefully rinsed with cold distilled water. The pellets were processed for radioactivity measurement. In order to determine specific binding of L-[^{3}H]glutamate, each experiment was processed with parallel control tubes containing L-[^{3}H]glutamate in the presence of 1000 times the concentration of non-labeled glutamate. Specific binding was defined as the difference of L-[^{3}H]glutamate binding between tubes without (total binding) and with (non-specific binding) non-labeled glutamate.

- *Protein measurement*

Protein was measured according the method of Lowry et al. (1951).

- *Inhibition curve*

Inhibition curve studies were carried out by incubating 100µg membrane protein with 1000nM L-[^{3}H]glutamate at 30°C for 15min in 50mM Tris/HCl buffer, pH 7.4, in the presence of linalool (0.1; 0.3; 3 or 5mM).

- *Saturation and competition curve*

Saturation and competition curves were produced by incubating L-[^{3}H]glutamate at increasing concentrations (40–3000nM) with 100µg membrane protein at 30°C for 15min in 50mM Tris/HCl buffer, pH 7.4, in the absence (control curve) or presence of linalool (0.3 and 1mM).

- *Statistical analysis*

Results were compared by means of two way ANOVA for competition curves. Maximum number of L-[^{3}H]glutamate binding sites (B_{max}) and equilibrium dissociation constant (K_d) were calculated by Scatchard plot. IC_{50} was calculated by linear regression.

NMDA-induced convulsions

The method used by Czuczwar et al. (1985) was followed. Mice were subcutaneously (s.c.) injected with the experimentally determined ED_{97} (270mg/kg) of NMDA. Saline, tween, MK-801 (2mg/kg) and linalool (350mg/kg) were injected intraperitonially (i.p.) 30 minutes before NMDA, and with diazepam and PPG, 45 minutes before seizure induction. Animals were individually placed in Plexiglas chambers (20x20x20cm) and observed for the occurrence of clonic seizures with loss of righting reflex. The latency to onset of first seizure with loss of righting reflex were noted. Percentage of seizuring animals was analyzed by means of the Fisher Exact test. Latency data were analyzed by means of ANOVA, followed by Duncan's test.

Quinolinic-acid induced convulsions

The surgery and i.c.v. infusion procedures were adapted from Izquierdo et al. (1992). Mice were anesthetized with sodium pentobarbital (60mg/kg, i.p.) and placed in the stereotaxic apparatus. The skin of the skull was removed and a 27 gauge, 7mm guide cannula was placed 1.0mm above the lateral brain ventricle, 1mm lateral from midline an 1mm posterior to bregma. Through a 2-mm hole made at the cranial bone, the cannula was implanted 1.5mm ventral to the superior surface of the skull, and fixed with jeweler acrylic cement. After 48h, i.c.v. infusions were performed. 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 1.0mm beyond the guide cannula and was therefore localized in the lateral brain ventricle. Animals (n=8) received a 4 microliter (µl) i.c.v. infusion of tween (1 %), 17mM MK-801 or linalool 15, 30, and 45mM and, 5min later, another infusion of the same volume of quinolinic acid (9.2mM). Mice were observed for 10 minutes in Plexiglas chambers for presence of wild running, clonic convulsions, tonic seizures (or both) lasting more than 5 seconds (Hallak et al., 1993). In order to ascertain correct cannula position, methylene blue (µl) was injected through the cannula; animals not showing contrast in the lateral brain ventricle were discarded. Statistical comparisons were accomplished by means of the Fisher Exact test.

PTZ-induced kindling in mice

- *Behavior*

The method has been detailed elsewhere (Silva et al. 1998). Mice (8 weeks old at the start of the experiment) were divided in five groups (n=10). Each treatment consisted of two drug administrations, repeated once every third day, for a total of 6 treatments. The first drug administration was an oral administration as follows: Group I, saline (phenobarbital vehicle); Group II, 15mg/kg phenobarbital; Group III, tween (linalool vehicle); Group IV, 2.2g/kg linalool; and Group V, 2.5g/kg linalool. Thirty minutes after the oral treatment both groups received (s.c.) 60mg/kg of PTZ; controls were treated identically to experimental groups, except that both injections consisted of saline solution. Following each PTZ injection, animals were placed individually in acrylic chambers for 30 minutes and observed for clonic convulsions lasting more than 3 seconds.

- *Neurochemistry*

L-[^{3}H]glutamate binding to naive mice cortex membranes was established. Twenty-four hours after the last injection (PTZ or saline), mice were decapitated, the brains were rapidly removed, and the membranes were prepared as described previously. For measurement of L-[^{3}H]glutamate binding, studies were performed as described above using 60nM of L-[^{3}H]glutamate, a concentration close to the K_d value. Differences in percentage of animals presenting seizures with ≥3 seconds were evaluated by Fisher's Exact test. Binding values were compared by means of one way ANOVA followed by Duncan test.

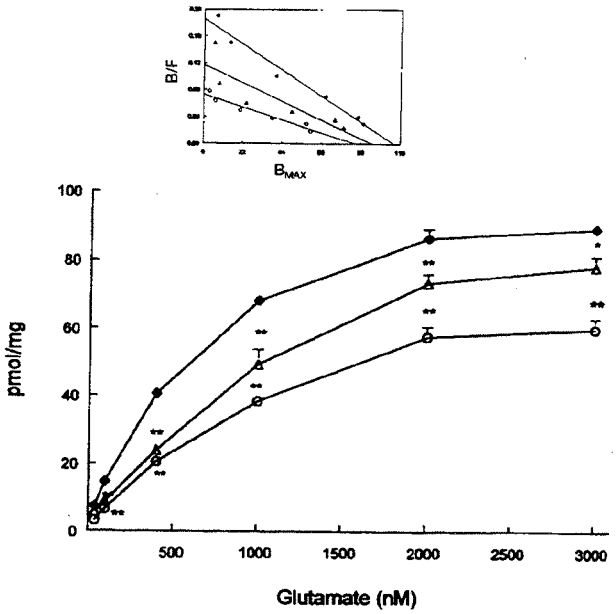

Fig. 1. Inibition curves and Scatchard plot (inset) of L-[^{3}H]glutamate binding to rat cortex membranes in the presence of linalool. Membranes were incubated with increasing concentrations of L-[^{3}H]glutamate (40–3000nM) in the absence (◆) or presence of linalool 0.3mM (△) or 1.0 mM (○). B = Bound; B/F = Bound/Free. Each point represents the mean ± SEM of four separate experiments determined in triplicate.
* = $p < 0.05$, ** = $p < 0.01$, ANOVA.

Results

Under the conditions used in this study, binding of L-[^{3}H]glutamate to cortex membranes from control rats is similar to previously published studies (Rubin et al. 1997). There was no difference in L-[^{3}H]glutamate binding (1000nM) using either water (68.1 ± 1.3 pmol/mg of protein) or tween at its maximum concentration (66.7 ± 1.1pmol/mg of protein).

L-[^{3}H]glutamate binding decreases with increasing concentrations of linalool and the inhibition is dose-dependent, with binding abolished at 5mM (IC_{50}0.57 ± 0.05mM, correlation coefficient = 0.97). Saturation

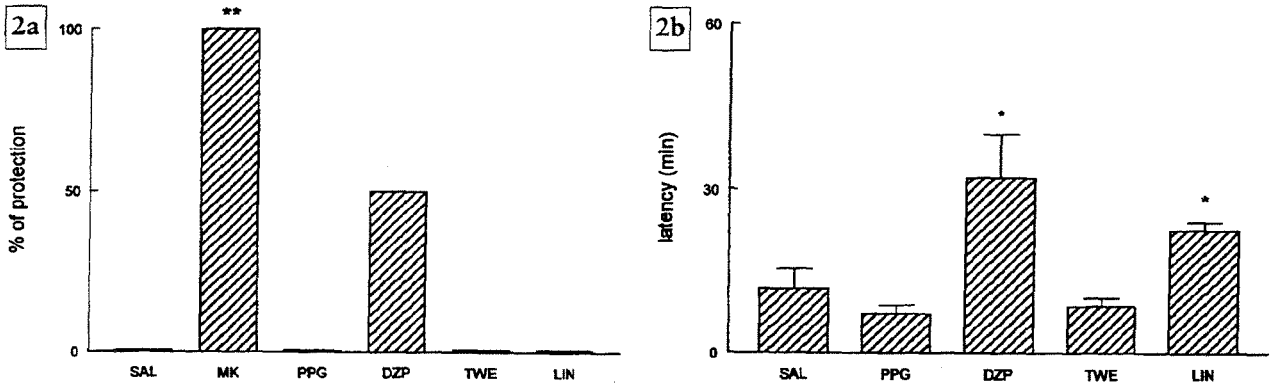

Fig. 2a. Effect of MK 801, diazepam and linalool on subcutaneous NMDA-induced seizures. **2a:** Percentage of protection of MK-801 (MK, 2mg/kg), diazepam (DZP, 30mg/kg), linalool (LIN, 350mg/g), and respective vehicles (SAL = saline, PPG = propylene glycol and TWE = tween) against NMDA (270mg/kg, s.c.) induced seizures. ** = $p < 0.01$, Fisher's Exact Test. **2b:** Latency (min) for onset of NMDA induced clonic convulsions. MK-801 (MK, 2mg/kg), diazepam (DZP, 30mg/kg), linalool (LIN, 350mg/kg), and respective vehicles (SAL = saline, PPG = propylene glycol and TWE = tween). * = $p < 0.05$, ** = $p < 0.01$, ANOVA.

curves without and with linalool (0.3 and 1mM) are presented in Fig. 1. Linalool significantly increased K_d with no significant alteration in B_{max}. The following B_{max} and K_d values were obtained: 107.3 ± 3.7pmol/mg and 0.58 ± 0.04μM in the absence of linalool; 95.8 ± 5.5pmol/mg and 0.83 ± 0.05μM ($p < 0.05$) with 0.3mM of linalool; and 94.9 ± 4.9pmol/mg and 1.4 ± 0.16μM ($p < 0.01$) with 1mM of linalool.

Subcutaneous NMDA induced a known pattern of altered behavior preceding the occurrence of seizures (Czuzwar et al., 1985). All control animals had clonic seizures with loss of righting reflex, whereas only a few animals presented a tonic component. Figure 2a shows that MK-801 afforded complete protection against NMDA-induced convulsions ($p < 0.01$). Diazepam protected only 50 % subjects, whereas linalool was ineffective. Figure 2b shows that both diazepam and linalool significantly delayed the onset of NMDA-induced seizures ($p < 0.01$).

Quinolinic acid induces seizures in all animals treated with saline of tween (Figure 3). MK-801 (17mM) afforded complete protection against quinolic acid-induced convulsions. As described previously (Hallak et al., 1993) MK-801 at this dosage also induced tremors, ataxia, incoordination, and loss of balance. Linalool dose dependently inhibited QUIN-induced convulsions, affording complete protection with 45mM ($p < 0.01$). ED_{50} was estimated at 16.9 (0.8–24.5)mM.

The effects of linalool and phenobarbital on PTZ-kindling are presented in Figure 4. 100 % of control (saline and tween) animals were fully kindled after the third treatment (Fig. 4a). Treatments resulted in delay and protection of kindling: 60 % ($p < 0.05$) of linalool treated animals were fully kindled after the fourth treatment and 40 % ($p < 0.01$) of phenobarbital were kindled after the sixth treatment.

L-[^{3}H]glutamate binding was measured 24 hours after the completion of the sixth treatment. Under the incubation conditions used in the study, binding of L-[^{3}H]glutamate to cortex membranes from naive (no PTZ) was characterized by B_{max} = 146.6 ± 5.1 and K_d = 1417.8 ± 210 (n=4) companable to previously published studies (Silva et al., 1998). Fig. 4b shows that neither linalool nor phenobarbital prevented the increase of L-[^{3}H]glutamate binding induced by PTZ-kindling.

Discussion

Seizures are often proposed to result from an imbalance of excitatory/inhibitory neuronal activities, following increased glutamatergic excitation or reduced GABAergic inhibition. Experimental seizures can be induced by activation of and suppressed by inhibition of glutamate receptors (Obrenovitch et al., 1996). Glutamate antagonists (acting at NMDA or non-NMDA receptor subtypes) have been shown to possess anticonvulsant properties in several animal models (Meldrum, 1992).

This study examined linalool antagonism to behavioral and neurochemical expressions of glutamate transmission. In glutamate binding to rat cortex membranes, the inhibitory effect of linalool was found to be of a competitive nature, with increasing K_d and maintenance of B_{max} with increasing concentrations of linalool. Regarding direct glutamate activation, both s.c.

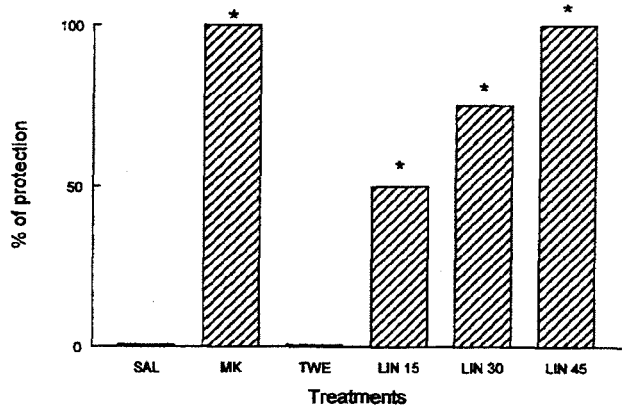

Fig. 3. Effect of MK-801 (MK) and linalool (LIN) on Quinolinic Acid-induced (QUIN) seizures. Mice received a 4µl i.c.v. infusion of tween (TWE), 17mM MK801 (MK), or 15, 30 and 45mM of Linalool (LIN) followed 5 minutes later by a second infusion of 4µl of QUIN (9.2mM). Animals were observed for 10min. after QUIN. Results are presented as percentage of protection against QUIN-induced seizures. * = $p < 0.01$, Fisher's Exact Test.

NMDA- and i.c.v. Quin-induced convulsions were modified by linalool treatment.

As previously reported (Czuczwar et al., 1985) subcutaneous administration of NMDA clearly induced signs of excitotoxicity, including myoclonic jerks, circling behavior, mild salivation, tail biting, and wild running which eventually preceded the appearance of full seizure activity. As in previous findings (Hallak et al., 1993) clonic seizures were effectively blocked by MK801 in doses that also induce typical behavioral changes, such as stereotypic head movements, tendency to circle continuously, ataxia, and loss of balance. Diazepam (30mg/kg) controlled 50 % of the NMDA-induced seizures. This is a dose almost one hundred times greater than that of Diazepam ED_{50} (0.36mg/kg, 0.3–0.4) against PTZ-induced convulsions (Barros, Elizabetsky, 1996); at this dose diazepam also induced intense depressant effects, with animals losing righting reflex and showing markes muscular relaxation. Linalool at 350mg/kg, a dose larger that its ED_{50} on PTZ-induced convulsions (243mg/kg, 186.2–304.2), did not protect against NMDA-induced seizures; at this dose linalool induced ataxia and loss of righting reflex. It is noteworthy that linalool shared with diazepam the ability to substantially increase the latency for NMDA-induced convulsions (Figure 2b). Given the high doses of established and newer anticonvulsants necessary for full protection in this model, the ability to increase latency can be regarded as an useful criteria for detecting meaningful modulation of glutamatergic modulation.

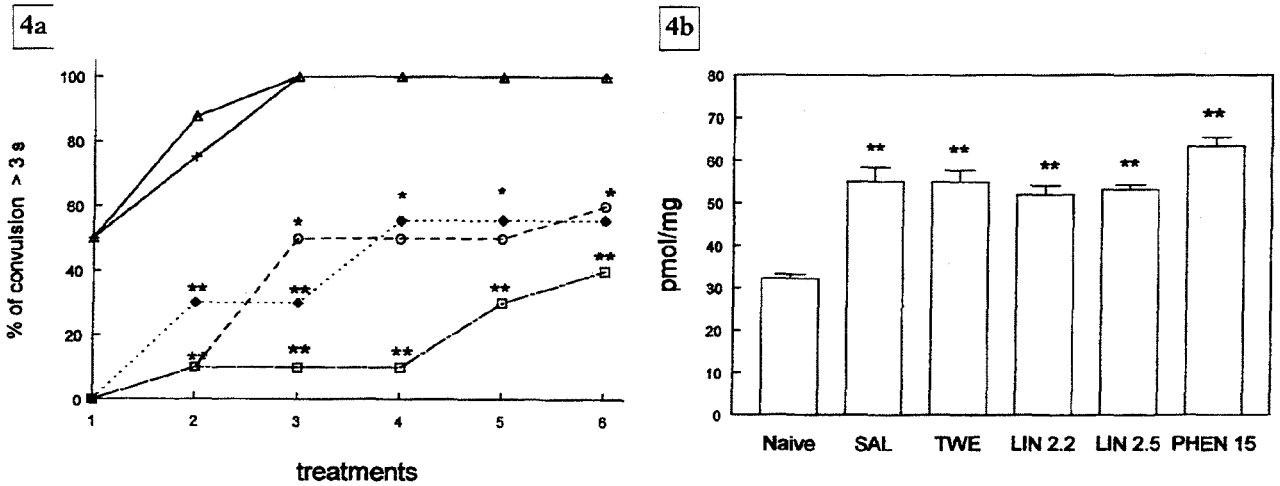

Fig. 4. Effect of linalool on PTZ-induced kindling in mice. **4a:** Percentage of kindled animals treated with saline (△), Tween (+), linalool 2.2g/kg (○), linalool 2.5g/kg (◆) and phenobarbital 15mg/kg (□) on treatment days. * = $p < 0.05$, **) $p < 0.01$, Fisher Exact Test. **4b:** Effects of saline (SAL), tween (TWE), linalool 2.2g/kg (LIN 2.2), linalool 2.5g/kg (LIN 2.5), and phenobarbital (PHEN) 15mg/kg on L-[^{3}H]glutamate binding of cortical membranes of mice submitted to PTZ-kindling. Naive mice did not receive PTZ. Values are expressed as pmol/mg of protein. Each point represents the mean ± SEM of three separate experiments determined in triplicate. ** = $p < 0.01$, ANOVA.

Quinolinic acid (QUIN) is a NMDA agonist, synthesized in neuronal and glial cells with an uncertain role in epilepsy. Intracerebral administration of MK 801 and linalool were effective in protecting against i.c.v. Quin-induced convulsions. Although less potent than MK801, linalool activity was clearly dose-dependent in antagonizing the effect of this NMDA endogenous agonist.

PTZ-kindling is a process whereby repeated administration of subconvulsive doses of this compound leads to the development of behavioral convulsive activity (Cain, 1989). Increases in glutamate release and receptor density in target neuron population after electrical and PTZ-induced kindling have been reported (Wu et al., 1990), being understood as possible substrates of kindling-associated neuronal changes (Schröder et al, 1993). Kindling therefore allows for evaluating the ability of drugs to modify neuronal plasticity associated with long-term alterations in neural excitability. Our results indicate that linalool partially inhibited and significantly delayed the behavioral expression of PTZ-kindling. Nevertheless, the core modifications of glutamate binding levels that accompany kindling were not modified by linalool or phenobarbital.

Although a specific site for linalool's modulating effect on glutamate receptors cannot be determined by this study, our data shows that linalool modulates glutamate activation *in vitro* (L-[^{3}H]glutamate binding) and *in vivo* (NMDA and QUIN). Nevertheless, considering the above detailed L-[^{3}H]glutamate binding data, the inhibition of PTZ-kindling behavioral effects obtained with linalool is to be attributed to mechanisms other than glutamate blockade. Because PTZ convulsions are also related to inhibition of gabaergic transmission (Giorgi et al., 1991), linalool's effects on this system can not be ruled out. This hypothesis is reinforced by preliminary data showing protection against picrotoxin-induced convulsions (Barros and Elisabetsky, 1996).

For diseases that result from complex processes involving more than a single neurotransmitter system, ameliorative or curative effects are rarely obtained with a single drug (Kozikovisky, 1993). The availability of a single anticonvulsant with multiple mechanisms of action may be of interest for a variety of reasons, including ease of titration, lack of drug-drug interactions, and reduces potential for pharmacodynamic tolerance (White, 1997). Considering that linalool is a major component of plant-based anticonvulsant remedies used in traditional medicinal systems, and the indication of multiple mechanisms of action relevant for decreasing neuronal excitability, further investigation on linalool as a potential anticonvulsant drug are desired. The available data provide a rational basis for traditional use of linalool-producing species against specific diseases affecting the central nervous system.

Acknowledgements

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Address

E. Elisabetsky, Caixa Postal 5072, 90041–970, Porto Alegre, RS, Brazil
Phone/Fax: (55) 51 316–3121; E-mail: elisasky@vortex.ufrgs.br

2.2. CAPÍTULO 2 – SILVA BRUM, L.F., ELISABETSKY, E., SOUZA, D.O. Effects of linalool on [^{3}H]MK801 and [^{3}H]muscimol binding in mice cortex membranes. Submetido ao *Phytotherapy Research* (02/08/1999).

EFFECTS OF LINALOOL ON [^{3}H]MK801 AND [^{3}H]MUSCIMOL BINDING IN MICE CORTEX MEMBRANES.

L. F. Silva Brum[1,2], E. Elisabetsky[1,2,3] and D. Souza[2]

1- Laboratório de Etnofarmacologia, Departamento de Farmacologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

2- Curso de Pós-Graduação em Ciências Biológicas-Bioquímica, Departamento de Bioquímica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Brazil.

3- Address reprint request to: Elaine Elisabetsky, Caixa Postal 5072, 90041-970, Porto Alegre, RS, Brazil. Phone/FAX: (051) 316-3121. , elisasky@vortex.ufrgs.br

RUNNING TITLE: Effects of linalool on glutamate and GABA binding.

ABSTRACT

Linalool is a monoterpene compound reported to be a major component of essential oils of several aromatic species. Several linalool-producing species are used in traditional medical systems for sedative purposes, including to interrupt and prevent seizures. Previous studies in mice revealed that linalool modulates glutamatergic (competitive antagonism of L-[^{3}H]glutamate binding, delayed ip NMDA-induced convulsions and blockade of icv QUIN-induced convulsions) and gabaergic transmission (protection against pentylenetetrazol and picrotoxin induced convulsions). To further clarify the anticonvulsive mechanisms of linalool, we studied the effects of linalool on binding of [^{3}H]MK801 (NMDA antagonist) and [^{3}H]muscimol ($GABA_A$ agonist) to mice cortex membranes. Linalool shows a dose dependent non-competitive inhibition of [^{3}H]MK801 binding (IC_{50} = 2.97 mM) and no effects on [^{3}H]muscimol binding. Data suggest that linalool anticonvulsant mode of action include direct interaction with NMDA receptor complex. Data does not support direct interaction of linalool with $GABA_A$ receptors, although changes in GABA-mediated neuronal inhibition or effects on GABA release and uptake can not be ruled out.

KEY WORDS: linalool, anticonvulsants, NMDA, glutamate, GABA, muscimol, binding, MK801.

INTRODUCTION

Considering that epilepsy apparently results from complex processes involving more than a single neurotransmitter system (Bradford,1995; Obrenovitch *et al.*, 1996), and given that 15% of epileptics are refractory to even polypharmacy treatments, the development of anticonvulsants with multiple mechanisms of action has been considered of great interest (White, 1997).

Linalool is a monoterpene compound, commonly found as major component of essential oil of several aromatic species, many of which are used traditionally as sedatives (Elisabetsky and Setzer, 1985; Elisabetsky *et al.*, 1995). Psychopharmacological evaluation of linalool in mice revealed that this compound have dose-dependent marked sedative effects at the Central Nervous System (CNS), including protection against pentylenetetrazol(PTZ), picrotoxin and transcorneal electroshock-induced convulsions, hypnotic, and hypothermic properties (Elisabetsky *et al.*, 1995; Barros and Elisabetsky, 1996). We also reported that linalool modulates glutamate activation expression *in vitro* (competitive antagonism of L-[^{3}H]glutamate binding) and *in vivo* (delayed sc *N*-methyl-D-aspartate convulsions and blockade of icv quinolinic acid convulsions) (Elisabetsky *et al.*, 1999). In addition, linalool significantly impaired the behavioral expression of PTZ-kindling, although not modifying the associated increase in L-[^{3}H]glutamate in cortical membranes (Elisabetsky *et al.*,1999). These data suggest that the anticonvulsant properties of linalool involve multiple mechanisms of action affecting different neurotransmitter systems.

It is widely accepted that excitatory amino acids glutamate and aspartate, and the inhibitory amino acid, γ-amino butyric acid (GABA) are involved in the basic mechanisms of epileptogenesis and epileptic seizures (Bradford, 1995). Indeed, impairment of GABA-

mediated inhibition (as caused by bicuculline) and enhanced excitability of NMDA receptors (as caused by NMDA agonists) could be the mechanism responsible for the development of epileptic seizures (Curtis *et al.*, 1970; Czuczwar *et al.*, 1985; Lipton and Rosemberg, 1994).

The purpose of this study was to further clarify the roles of glutamate and GABA in the anticonvulsant profile of linalool, by investigating its effects on [^{3}H]MK801, an NMDA antagonist, and [^{3}H]muscimol, a GABA agonist binding to central nervous system membranes.

EXPERIMENTAL PROCEDURE

Drugs and reagents. (±)Linalool was purchased from Aldrich Chemical Co. (catalog number L260-2) and solubilized in Tween (80 Polisorbate, 25%). MK801 was acquired from RBI whereas Glutamate, GABA and Glycine were purchased from Merck. [^{3}H]MK801 was purchased from Du Pont-NEN Products and [Methylamine-3H]muscimol from Amersham Life Science.

Animals: Male adult albino mice, strain CF-1, from Instituto de Pesquisas Biológicas (Porto Alegre, Brazil) were used throughout the study. The animals were kept on a 12 light/dark cycle, at 22 ± 1 °C, with free access to food (Nuvilab CR1) and water. All procedures were carried out according to institutional policies on experimental animals handling.

[^{3}H] MK801 binding

Membrane Preparation: Membranes were prepared as described by Emanuelli *et al.*, 1998. Mice cerebral cortex were homogenized (20:1 v:w) in 0.32 M sucrose containing 5 mM Tris/HCl buffer (pH 7.4). All steps were carried out at 4°C. The homogenate was

centrifuged twice at 1,000g for 15 min and the final pellet discarded. Both supernatants were pooled and centrifuged at 27,000 g for 15 min. The supernatant was discarded and the pellet was lysed (20:1 v:w) for 30 min in 5 mM Tris/HCl buffer (pH 7.4). The lysed pellet was washed three times with lysing buffer (20:1 v:w) by centrifuging at 27,000 g for 15 min. Supernatants were discarded and the final pellet was frozen at –70°C for at least 24 h. On the day of binding assay, the membranes were rapidly thawed in a water bath (37°C), homogenized with 3 volumes of 5 mM Tris/HCl (pH 7.4), and centrifuged at 27,000 g for 15 min. The resulting pellet was resuspended in the same buffer, pré-incubated at 37°C for 30 min and centrifuged at 27,000 g for 15 min. The pellet was washed three times in 3 volumes of the 5 mM Tris/HCl (pH 7.4), and centrifuged at 27,000 g for 15 min. The final pellet was resuspended in the same buffer in order to yield a protein concentration of 1-2 mg/ml and was used for binding assays. Protein concentration was measured according to method of Lowry *et al.*, 1951.

Binding of [^{3}H]MK-801: Binding assay was based on the method of Piggott *et al.*, 1992. Briefly, 200 µg of homogenate protein was incubated in 5 mM Tris/HCl buffer (pH 7.4) at 25°C for 1h, containing 1 to 15 nM [^{3}H]MK-801 in the presence of glutamate (50 µM) and glycine (30 µM) in a final volume of 0.5 ml. Non-specific binding was defined as binding which occurred in the presence of 17,000 times of unlabelled MK-801. After incubation, membranes were filtered under reduced pressure through a Whatman GF/B filters (pre-wetted in 5 mM Tris/HCl buffer), and rinsed rapidly with 3x5 ml ice-cold buffer. The filters were deposited in vials and radioactivity measurement in cintilador Beckmam (RackBeta 1217).

Inhibition curve: Inhibition curve studies were carried out by incubating 200 µg membrane protein with 2 nM [^{3}H]MK801 at 25°C for 1h in 5 mM Tris/HCl buffer (pH

7.4) containing glutamate (50 μM) and glycine (30 μM) in the presence of linalool 0.1; 0.3; 1 ; 3 or 5 mM.

Saturation and competition curve: Saturation and competition curves were produced by incubating [^{3}H]MK801 at increasing concentrations (1 - 15 nM) with 200 μg membrane protein at 25°C for 1h in 5 mM Tris/HCl buffer (pH 7.4), containing glutamate (50 μM) and glycine (30 μM) in the absence (control curve) or presence of linalool 1 and 3 mM.

[^{3}H]muscimol binding

Membrane Preparation: Membranes were prepared as described by Zukin *et al.*, 1974. The final crude synaptic membrane pellet was resuspended in distilled water and centrifuged five times at 48,000 x g for 20 min and the last pellet was ressuspended in 50 mM Tris/Citrate buffer (pH 7.1) and frozen at -30°C for at least 18 hr. The frozen pellets were resuspended in distilled water, maintained at 25° C for 20 min, centrifuged at 48,000 x g for 10 min and the pellets were suspended in 0.05 M Tris/Citrate buffer (pH 7.1; 4°C). Protein concentration was measured according to method of Lowry *et al.*, 1951.

Binding of [^{3}H]muscimol: Binding assay was based on the method of Demasi *et al.*, 1996. Briefly, 300 μg of homogenate protein was incubated in 50 mM Tris/Citrate buffer (pH 7.1) at 4°C for 30 min, containing 12 nM [^{3}H]muscimol in a final volume of 1.0 ml. Non-specific binding was defined as binding which occurred in the presence of 1 mM GABA unlabelled. Incubations were terminated by rapid filtration under vacuum through a Whatman GF/B filters (pre-wetted in poliethylamine), and rinsed rapidly with 3x5 ml ice-cold buffer. The filters were dried and deposited in vials and radioactivity measurement in cintilador Beckmam (RackBeta 1217).

Inhibition curve: Inhibition curve studies were carried out by incubating 300 μg membrane protein with 12 nM [^{3}H]muscimol at 4°C for 30 min in 50 mM Tris/Citrate buffer (pH 7.1) in presence of linalool 0.3; 1 ; or 3 mM.

Statistical analysis. Results were compared by means of one way ANOVA for inhibition and competition curves. Maximum number of binding sites (B_{max}) and equilibrium dissociation constant (K_d) were calculated by Scatchard plot. IC_{50} was calculated by linear regression.

RESULTS

Binding of [^{3}H]MK801 decreases with increasing linalool concentrations (Figure 1). This inhibition is dose-dependent, attaining 75% of inhibition at 5 mM (IC_{50} 2.97 ± 0.13 mM, correlation coefficient = 0.98). There was no difference in [^{3}H]MK801 binding (2 nM) using either water (0.65 ± 0.08 pmol/mg of protein) or tween, linalool diluent, at its maximum concentration (0.64 ± 0.09 pmol/mg of protein). Figure 2 shows saturation curves without and with linalool (1 and 3 mM). Linalool significantly decreased B_{max} with no significant alteration in K_d. The following B_{max} and K_d values were obtained: 1.39 ± 0.13 pmol/mg and 3.4 ± 0.1 nM in the absence of linalool; 1.05 ± 0.02 pmol/mg ($p < 0.05$) and 3.0 ± 0.2 nM with 1.0 mM of linalool; and 0.77 ± 0.02 pmol/mg ($p < 0.01$) and 3.4 ± 0.2 nM with 3 mM of linalool. Linalool concentrations ranging from 0.3 to 3.0 mM did not alter equilibrium binding of [^{3}H]muscimol to $GABA_A$ receptor complex in mice cerebral cortical membranes (Table 1).

DISCUSSION

Abnormal expression or enhanced function of glutamate receptor subtypes are central to various acquired forms of epilepsy. (McNamara, 1994; Meldrum, 1994). Notably the NMDA subtype glutamate receptor is though to play key roles on epileptic phenomena, including genesis of burst firing (Dingledine *et al.*, 1986), initiation and propagation of seizures (Dingledine *et al.*, 1990; Bradford, 1995). Evidences from animal studies indicated that NMDA antagonists possess anticonvulsant activity, among other neuronal protective properties (Dingledine *et al.*, 1991; Rogawski, 1992).This body of data arouse interest in possible therapeutic applications of excitatory amino acid antagonists (Meldrum, 1992).

NMDA receptors have distinct amino acid recognition sites, including one associated to ion channels. MK801 (dizocilpine), a potent anticonvulsant, binds to this site blocking the channel in its open state (Wong and Kemp, 1991), although marked psychotomimetic effects prevent its use in clinical practice. The dose dependent non-competitive inhibition of MK801 binding brought about by increasing linalool concentrations suggests that at least part of linalool anticonvulsant action might be mediated via direct interaction with NMDA receptor complex.

Studies of the role played by GABA in human epilepsy and in animal model of epilepsy have indicated that increment of GABAergic neurotransmission may control epileptic seizures (Meldrum, 1975; 1989). In fact, many anticonvulsants drugs appear to act by increasing the levels of activity in GABAergic systems (Holland *et al.*, 1992; Suzdak and Jansen, 1995). The effects of linalool against picrotoxin and PTZ induced convulsions raised the hypothesis of gabaergic involvement in its mode(s) of action. Results obtained through the binding assay does not support direct interaction with $GABA_A$ receptors,

although changes in GABA-mediated neuronal inhibition or effects on GABA release and uptake can not be ruled out.

In light of the need for additional pharmacotherapies for epilepsy, with distinct pharmacodynamic basis and of the alleged anticonvulsive usefulness of linalool bearing plants by users of several traditional medical systems, a meaningful anticonvulsive profile of linalool has been produced (Elisabetsky *et al.*, 1995; Barros and Elisabetsky, 1996; Elisabetsky *et al.*, 1999). Experimental data revealed an interesting heterogeneous mode of action as well as suggesting a somewhat narrow safety margin when the oral or intraperitoneal routes are used. Considering the recent clinical reports that indicate the usefulness of essential oils in the management of epileptic disorders (Betts *et al.*, 1995; Lis-Balchin, 1997), the evidence of sedative effect through inhalation (Buchbauer *et al.*, 1991) and the significant absorption of essential oil components through various modes of administration (Jager *et al.*, 1992; Jirovetz *et al.*, 1992), we suggest that linalool warrants subsequent structure-activity studies in the context of disclosing innovative management strategies for epileptic disorders.

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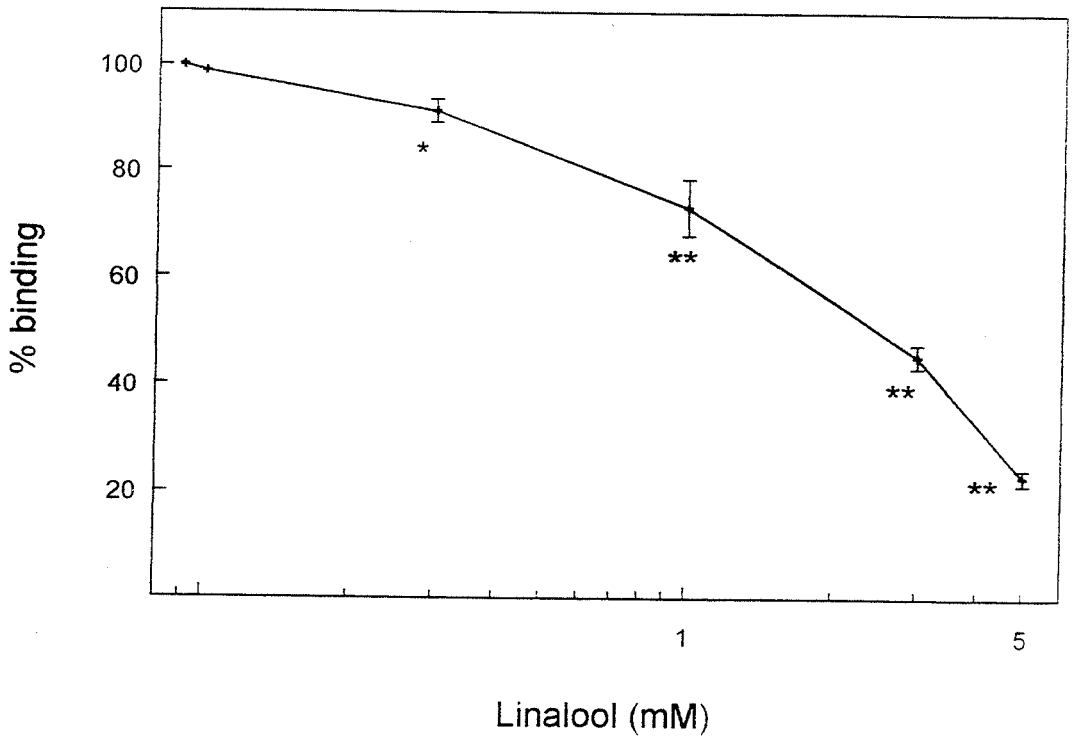

Figure 1. Dose-response curve of linalool effect on [^{3}H]MK 801 (2 nM) binding. Values are expressed as pmol/mg of protein. Each point represents the mean ± SEM of three separate experiments determined in triplicate. * = $p < 0.05$ and ** = $p < 0.01$, ANOVA.

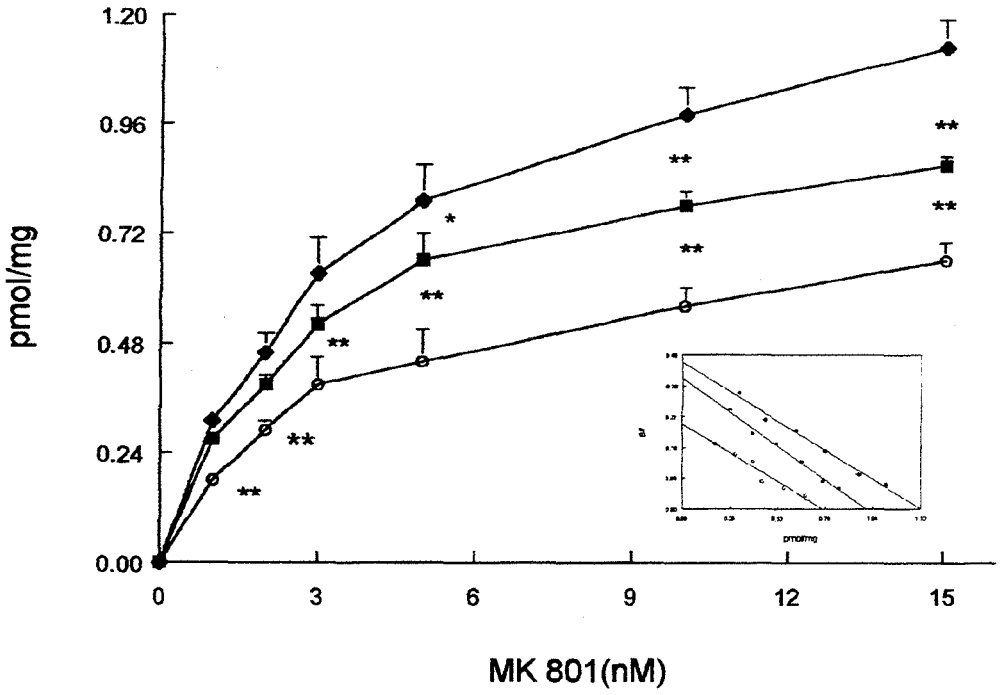

Figure 2. Inhibition curves and Scatchard plot (inset) of [^{3}H]MK801 binding to mice cortex membranes in the presence of linalool. Membranes were incubated with increasing concentrations of [^{3}H]MK 801 (1-15 nM) in the absence (♦) or presence of linalool 1 mM (■) or 3 mM (○). B=Bound; B/F=Bound/Free. Each point represents the mean ± SEM of three separate experiments determined in triplicate. * = $p < 0.05$ and ** = $p < 0.01$, ANOVA.

Table 1. Effect of linalool on binding of [^{3}H]muscimol in mice cerebral cortical membranes.

Treatment	pmol/mg of protein
Control	1.32 ± 0.02
Linalool 0.3 mM	1.31 ± 0.05
1.0 mM	1.32 ± 0.03
3.0 mM	1.34 ± 0.02

Membranes were incubated on [^{3}H]muscimol (12 nM) in the absence or presence of linalool. Values are expressed as pmol/mg of protein. Results are expressed as mean ± SEM of three separate experiments determined in triplicate.

2.3. CAPÍTULO 3 – SILVA BRUM, L.F., EMANUELLI, T., SOUZA, D.O., ELISABETSKY, E. Effects of linalool on glutamate release and uptake on mice cortical synaptosomes. Em fase de revisão pelos autores para ser submetido a *Phytomedicine*

EFFECTS OF LINALOOL ON GLUTAMATE RELEASE AND UPTAKE ON MICE CORTICAL SYNAPTOSOMES

L. F. Silva Brum[1,2], Emanuelli, T.[2,3], D. O. Souza[2] and E. Elisabetsky[1,2,4]

1- Laboratório de Etnofarmacologia, Departamento de Farmacologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

2- Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Brazil.

3- Departamento de Tecnologia e Ciência dos Alimentos, CCR, Universidade Federal de Santa Maria, Santa Maria, Brazil

4- Address reprint request to: Elaine Elisabetsky, Caixa Postal 5072, 90041-970, Porto Alegre, RS, Brazil. Phone/FAX: (55) 51 316-3121. elisasky@vortex.ufrgs.br

ABSTRACT

Linalool, a monoterpene compound prevalent in essential oil of plant species traditionally used as sedatives, has been characterized as anticonvulsant in several experimental models. The mechanism of action includes inhibition of glutamate binding to brain cortical membranes. In this study, we investigated the effect of linalool on [^{3}H]glutamate release (basal and potassium-induced) and uptake on mice cortical synaptosomes. Linalool significantly reduced potassium-stimulated glutamate release as well as glutamate uptake, not interfering with basal glutamate release. The data suggests that linalool anticonvulsant activity could involve inhibition of seizuring activity by limiting further glutamate release from depolarizing neurons.

KEY WORDS: linalool, anticonvulsants, glutamate, uptake, release

INTRODUCTION

Although the majority of epileptic patients are adequately treated with well established anticonvulsant drugs, the need for more effective drug treatments, as well as true antiepileptic approaches, remains as a genuine necessity for as much as one forth of affected patients. Linalool, a monoterpene commonly found in essential oils, is present in several traditional medical remedies used for sedative and anticonvulsant purposes (Elisabetsky *et al.*, 1995). It possesses a broad spectrum of action in mice experimental epilepsy models, including protection against pentylenetetrazol, picrotoxin, and electroshock induced convulsions (Elisabetsky *et al.*, 1995; Barros and Elisabetsky, 1996).We also reported that linalool interferes with glutamate function *in vivo,* delaying sc NMDA-induced convulsions and blocking i.c.v. quinolinic acid convulsions (Elisabetsky *et al.*, 1999). Relevant to its mechanism of action, linalool behaves as a competitive antagonist of L-[^{3}H]glutamate binding, and as a non-competitive antagonist of [^{3}H]MK801 (NMDA antagonist) binding in brain cortical membranes pointing to a modulation of glutamatergic transmission (Elisabetsky *et al.*, 1999; Silva Brum *et al.*, 1999).

The excitatory glutamatergic system has long been postulated to be involved in the etiology of at least some forms of human and experimental epilepsy (Bradford, 1995). In fact, the NMDA receptor complex is though to play key roles on epileptic phenomena, including genesis (Dingledine *et al.*, 1986), initiation and propagation of seizure activity (Dingledine *et al.*, 1990; Chapman, 1998). Considering that seizures in experimental models can be induced by activation and suppressed by inhibition of glutamate receptors, particularly NMDA (Rogawiski, 1992; Dichter, 1997), it has been proposed that a high

extracelular glutamate level subsequent to excessive release and/or altered glutamate uptake is epileptogenic (Lipton and Rosenberg, 1994).

In order to further clarify the pharmacodynamic basis of linalool anticonvulsant properties, we investigated the effects of linalool on both release (basal and potassium-induced) and uptake of [^{3}H]glutamate from synaptosomes obtained from mice cerebral cortex.

EXPERIMENTAL PROCEDURE

Drugs and reagents. (±)Linalool (dl-3,7-dimethyls-3-hydroxy-1,6-octadiene, 95-97% purity) was purchased from Aldrich Chemical Co. (catalog number L260-2) and solubilized in Tween (80 Polisorbate, 25%). Glutamate was purchased from Merck and L-[^{3}H]glutamate from Amersham.

Animals: Male adult albino mice, strain CF-1, from Instituto de Pesquisas Biológicas (Porto Alegre, Brazil) were used throughout the study. The animals were kept on a 12 light/dark cycle, at 22 ± 1 °C, with free access to food (Nuvilab CR1) and water. All procedures were carried out according to institutional policies on experimental animals handling.

[^{3}H]Glutamate release: Cortical synaptosomes were prepared on a discontinuous Percoll gradient according to Migues *et al.*, 1999. Protein concentration was measured according to the method of Lowry *et al.*, 1951. The synaptosomal preparation was incubated in HBSS (Hepes buffered salt solution, mM composition: HEPES 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 12, $CaCl_2$ 1.0) for 15 min at 37°C in the presence of [^{3}H] glutamate (Amersham, specific activity 53 Ci/mmol, final concentration 5 x 10^{-7} mM). Aliquots of labeled synaptosomes (1.4 mg protein) were centrifuged at

16,000 g for 1 min. Supernatants were discarded and the pellets were washed four times in HBSS, and centrifuged at 16,000 g for 1 min. The final pellets were resuspended in HBSS and incubated for 1 min at 37°C in the following conditions: absence of linalool (blank), tween (25%), linalool (1.0 and 3.0 mM in tween 25%) in the absence (basal glutamate release) or presence of high depolarizing medium (40 mM KCl), for potassium-stimulated glutamate release measurements. Incubation was completed by centrifuging (16,000 g, 1 min), and supernatants and pellets separately resuspended with milli-Q water. Radioactivity counting was processed in WALLAC scintillation counter. The released [^{3}H] glutamate was calculated as a percentage of the total amount of radiolabel present at start of the incubation period.

[^{3}H]Glutamate uptake: Cortical synaptosomes were prepared on a discontinuous Percoll gradient according to Nagy & Delgado-Escueta, 1984. Protein concentration was measured according to the method of Lowry *et al.*, 1951. The synaptosomal preparation was washed in 15 mM Tris/Acetate and 0.3 M sucrose buffer (pH 7.4), centrifuged at 27,000 g for 20 min. The final pellet was resuspended in 15 mM Tris/Acetate and 0.3 M sucrose buffer, and incubated for 1 min at 37°C in HBSS (Hepes buffered salt solution, mM composition: HEPES 24, NaCl 119, KCl 2.1, $MgSO_4$ 1.08, KH_2PO_4 1.08, glucose 10.8, $CaCl_2$ 0.9) loaded with [^{3}H]glutamate (final concentration 1 μM) in the following conditions: absence of linalool (blank), tween (25%), in the presence of linalool (0.1 - 3.0 mM in tween 25%). The reaction was stopped by filtration through GF/B filters, followed by 3 washes with 3 ml of ice-cold 15 mM Tris/Acetate and 155 mM $AcNH_4^+$ buffer. Radioactivity retained on the filters was measured by WALLAC scintillation counter. Specific glutamate uptake was calculated as the difference between the uptake

performed in the incubation medium described above and the uptake performed in similar incubation medium without Na^+.

Measurement of the Lactate Dehydrogenase (LDH): In order to evaluate the integrity of the synaptosomes after incubation with linalool (1.0 and 3.0 mM) for 1 min, the LDH activity in the incubation medium was assayed spectrophotometrically using an assay kit (Doles Reagentes, Brazil).

Statistical analysis: Statistical significance was assessed by ANOVA followed by Duncan's multiple range test.

RESULTS

Figure 1 shows the effects of linalool on [^{3}H]glutamate release: linalool (1.0 and 3.0mM) had no effect on the basal [^{3}H]glutamate release (Fig.1A) but inhibited (31.3%) the potassium-stimulated [^{3}H]glutamate release ($p < 0.01$) (Figure 1B). Linalool significantly decreased the [^{3}H]glutamate uptake by mice cortical synaptosomes in a dose-dependent manner, as shown in Figure 2 (IC_{50}=1.50 ± 0.08 mM). Synaptosomes incubated with linalool (3.0 mM) for up to 15 min showed no significant leakage of the cytosolic marker LDH (data not shown).

DISCUSSION

The essential role of glutamate transmission in seizure phenomena has been thoroughly documented (Bradford, 1995; Chapman, 1998). There are several potential sites at which glutamatergic excitation can be modulated eventually attenuating epileptic phenomena (Dingledine et al., 1991; Meldrum, 1992; Dichter, 1997). Nevertheless, in practice only two approaches appear to be of consequence in the suppression of seizures: to act post-

synaptically on receptors to decrease glutamate-induced excitability, and to decrease extracelular glutamate availability (by inhibition of glutamate release or increment of its uptake) (Meldrum, 1996).

In this study, we investigated the effect of linalool in both release (basal and potassium-induced) and uptake of [^{3}H]glutamate by synaptosomes of mice cerebral cortex. Results showed that linalool did not interfere with basal glutamate release but significantly reduced glutamate release potassium-stimulated from synaptosomes. Linalool notably inhibited neuronal glutamate uptake.

Basal glutamate release is related to the release of cytoplasm glutamate, mainly due to the inversion of the membrane glutamate transporters (consequent to the dilution of preloaded synaptosomes in a glutamate free medium). Potassium-stimulated glutamate release concern glutamate stored in vesicles, in addition to cytoplasm glutamate. The pattern of restraint glutamate release induced by linalool may be of relevance to its anticonvulsant properties: glutamate release would be restrained while seizure is taking place, therefore interfering with seizure maintenance and spreading.

NMDA blockade is known to result in inhibition of glutamate release (Srinivasan *et al*, 1995) and linalool behave as NMDA antagonist (Silva Brum *et al*., 1999). Nevertheless, the methodology here used evaluates glutamate extrusion directly by NMDA free synaptosomes. Therefore, NMDA antagonism is unlikely to explain the observed inhibition of glutamate release. Drugs known to inhibit glutamate release through mechanisms other than NMDA inhibition include lamotrigine, phenytoin, carbamazepine (Leach *et al*., 1986).

Linalool markedly (around 90%) inhibited the uptake of glutamate. Such inhibition could lead to an increase in extracelular levels of glutamate which facilitates seizure.

The fact that this frame is in contrast with the anticonvulsant profile of linalool in vivo, suggests that the effects of linalool on glutamate uptake is not a relevant event in vivo. In fact, it has been difficult to evaluate the actual impact of increases on extracelular glutamate level on glutamate transmission. Massieu *et al.*, (1995) showed that accumulation of extracelular glutamate by inhibition of its uptake is not sufficient for inducing neuronal damage *in vivo.* Moreover, seizures are not necessarily accompanied by increased extracelular glutamate levels (Obrenovitch *et al*, 1996), although increased levels certainly contribute to seizure activities. In view of the *in vivo* anticonvulsant profile established for linalool in mice and its traditional use in humans (Elisabetsky *et al.*, 1995; Elisabetsky *et al.*, 1999), it is fair to conclude that inhibition of neuronal uptake seems to be irrelevant for its overall anticonvulsant activity.

Activation of NMDA receptors results on glutamate release and further initiates a positive feedback resulting on more glutamate release (Srinivasan *et al*, 1995). Previous studies indicated that linalool activity involves NMDA receptors (Elisabetsky et al, 1999; Silva Brum et al., 1999). Concerning glutamate transmission, it can therefore be argued that the anticonvulsant efficacy of linalool, is likely to be consequent to its effect on NMDA receptors complemented by a inhibitory effect on glutamate release.

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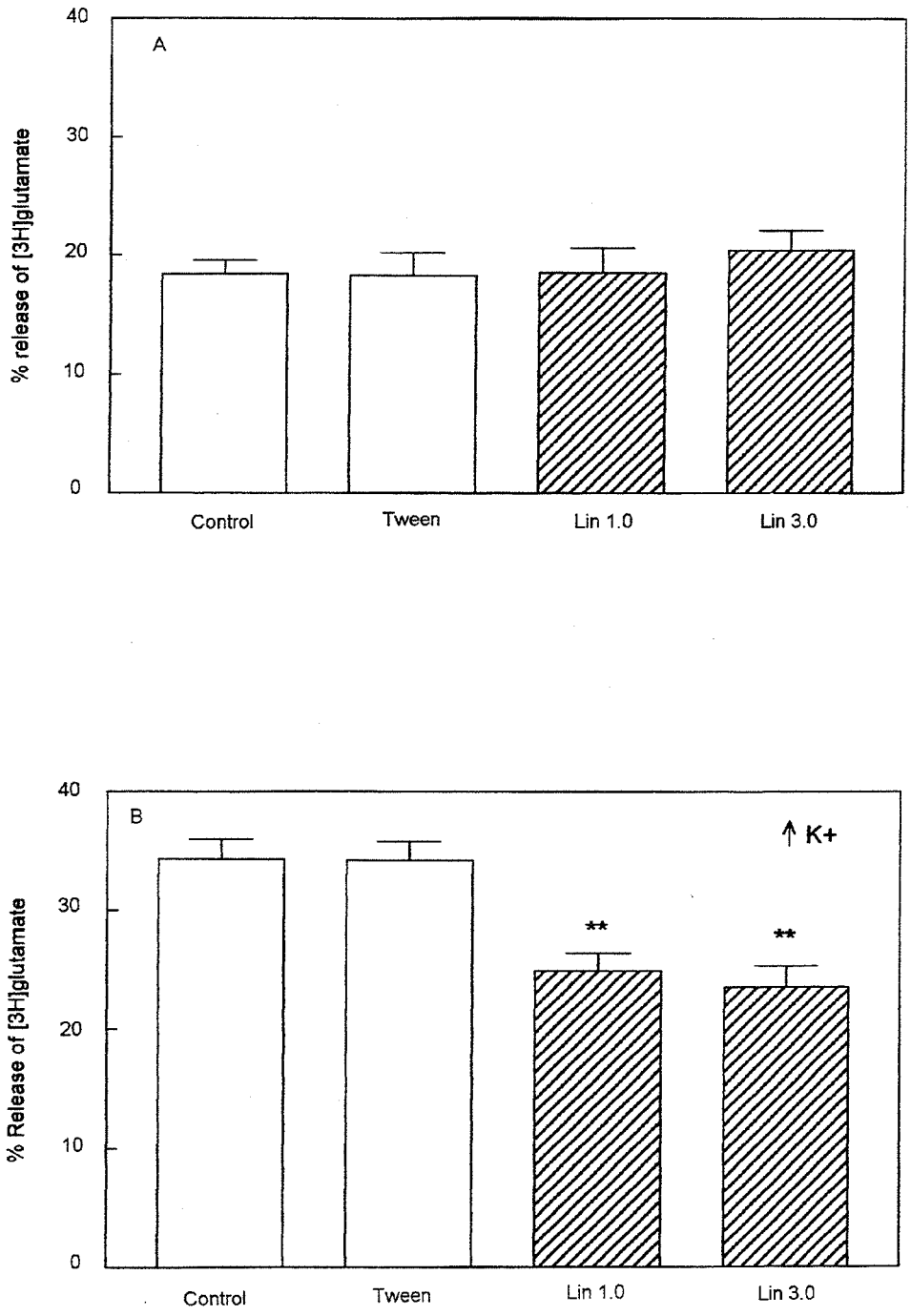

Figure 1. Effects of linalool on the release of [^{3}H]glutamate from mice cortical synaptosomes. (A) basal release and (B) potassium-stimulated release. Glutamate release is expressed as a percentage of synaptosomes total radioactivity content. Data are mean ± SEM from 4 independent experiments performed in triplicate. ** = $p<0.01$, ANOVA/Duncan.

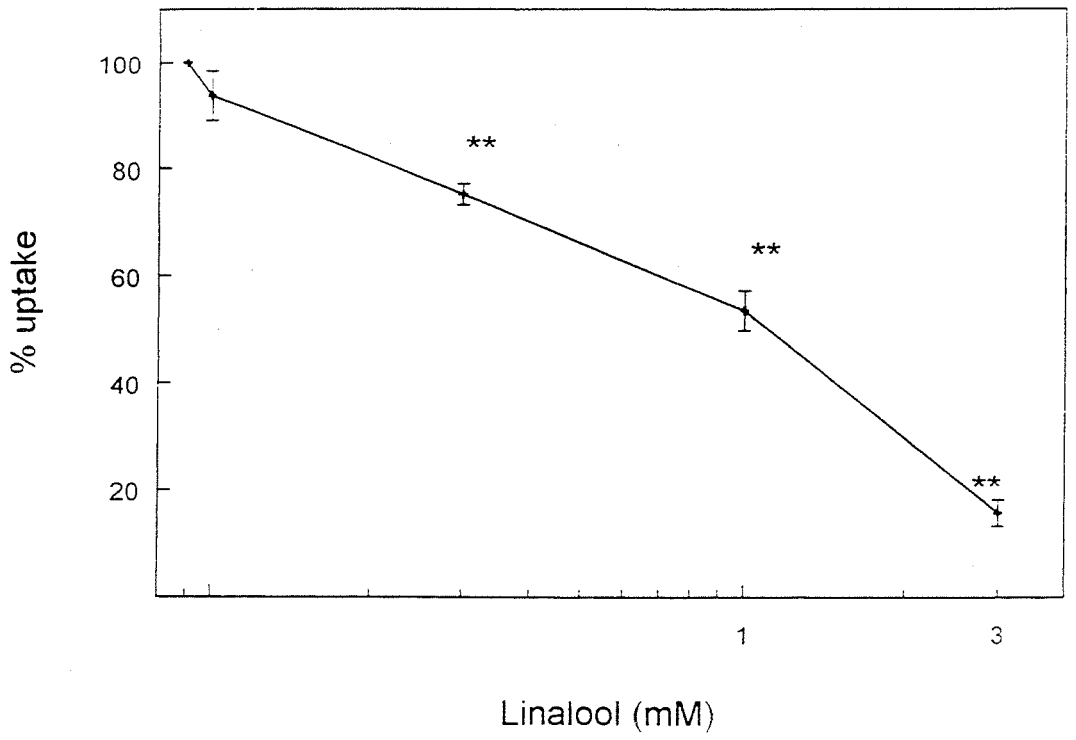

Figure 2. Effects of linalool on [^{3}H]glutamate uptake. Synaptosomes were incubated in the absence or presence of linalool (0.1, 0.3, 1.0 and 3.0 mM). [^{3}H]glutamate uptake is expressed as pmol/min/mg of protein. Data are mean ± SEM from 4 independent experiments performed in triplicate. * = $p<0.05$ and ** = $p<0.01$, ANOVA/Duncan.

2.4. CAPÍTULO 4 – SILVA, L.F., PEREIRA, P., ELISABETSKY, E. A neuropharmacological analysis of PTZ-induced kindling in mice. *General Pharmacology* 31(1): 47-50, 1998.

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A Neuropharmacological Analysis of PTZ-Induced Kindling in Mice

*Lucimar F. da Silva, Patrícia Pereira and Elaine Elisabetsky**

LABORATÓRIO DE ETNOFARMACOLOGIA, DEPTO. DE FARMACOLOGIA, AND CURSO DE PÓS GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS—BIOQUÍMICA, UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL, CAIXA POSTAL 5072, 90041-970, PORTO ALEGRE, RS, BRAZIL [E-MAIL: elisasky@vortex.ufrgs.br]

ABSTRACT. 1. Glutamate seems to play a central role in epilepsy, and kindling is considered the most useful experimental model in revealing plastic changes associated with epileptic features.

2. The aim of this study was to optimize pentylenetetrazol (PTZ)-kindling conditions in mice and analyze glutamatergic changes associated with this phenomena.

3. A significant increase (85.7%) in seizuring animals was observed after four PTZ administrations, with all subjects presenting full seizures after five administrations.

4. PTZ kindling, but not acute seizure, significantly increased (169.8%) the specific binding of [^{3}H]glutamate in the cerebral cortex.

5. The development of PTZ-induced kindling in mice was prevented by the coadministration of phenobarbital or diazepam.

6. This study indicates that mice can be used in a reliable model of PTZ-induced kindling and that, as in rats, the kindling increases the specific [^{3}H]glutamate binding in the cerebral cortex, therefore allowing for screening new drugs that can interfere in the plastic changes believed to underlie epileptic phenomena. GEN PHARMAC 31;1:47–50, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Binding, kindling, mice, pentylenetetrazol, glutamate, anticonvulsants

INTRODUCTION

Kindling has been defined as the process whereby repeated applications of subconvulsive electrical or chemical stimuli to animals lead to an increase in convulsive activity, resulting in generalized seizures (Goddard, 1967; Goddard *et al*, 1969; Schöder *et al.*, 1993). The kindling-induced enhanced seizure activity is understood to indicate a long-lasting alteration of the neuronal excitability, which can be regarded as suggestive of neuronal plasticity (Cain, 1989). As an epilepsy experimental model associating neuronal plasticity and seizures, kindling is unique in providing opportunities to study the ability of drugs to modify these progressive changes. Such ability may be due to the underlying mechanisms of drug action on seizure development (Albertson *et al.*, 1981).

Electrical kindling in rats has become a widely employed technique for studying seizure mechanisms and is considered to be a useful experimental seizure model. An increased susceptibility of rats to pentylenetetrazol (PTZ) seizures after repeated injections of this drug was first observed in 1941 (Sacks and Glaser, 1941). PTZ kindling therefore provides a means for studying a persistent decrease in seizure thresholds, although this method has been much less utilized than the electrical kindling. There are few studies using chemical kindling in mice (Karler *et al.*, 1984, 1989; Piredda *et al.* 1986). Earlier investigators questioned the soundness of PTZ-kindling in mice, suggesting age as a major factor in decreasing seizure threshold (Craig and Colasanti, 1989). Advantages in working with mice rather than rats include the possibility of testing compounds available in limited amounts, such as in the study of natural products or large screening programs or both (Kupferberg, 1989; Swinyard *et al.* 1986).

Concerning the transmitter systems in neuronal plasticity, much attention has been focused on excitatory amino acids, especially on glutamate. Under pathological conditions such as acute (hypoxia, ischemia, hypoglycemia and seizures) and chronic (neurodegenerative diseases) insults, an excessive release of glutamate results in neural injury and cell death (Lipton and Rosemberg, 1994). Studies concerning the neural excitotoxicity have indicated that glutamate plays crucial roles in the initiation, spread and maintenance of epileptic activity (Dingledine *et al.*, 1990; Meldrum, 1994).

In this study, we determined conditions to optimize PTZ kindling in mice. We evaluated the effects of age and those of diazepam and phenobarbital on seizure threshold. In addition, the changes in glutamate receptors in the kindling phenomenon was accessed by determining glutamate-specific binding at cortex membranes of PTZ-kindled mice in comparison with naive and acutely treated animals.

METHODS

Animals

Male SR-1 mice, 25–30 g, were used throughout the experiments. They were housed at 20±2°C, maintained on a 12-hr light–dark cycle, with food and water *ad libitum*. All procedures were carried out according to institutional policies on experimental animal handling.

Behavioral studies

PTZ KINDLING. Animals (8 weeks old at the start of the experiment) were divided into three groups. Each treatment consisted of two drug administrations. All groups received saline intraperitoneally and, 30 min later, one of the following subcutaneous injections: (group I) saline, (group II) 40 mg/kg PTZ and (group III) 60 mg/kg

*To whom correspondence should be addressed.
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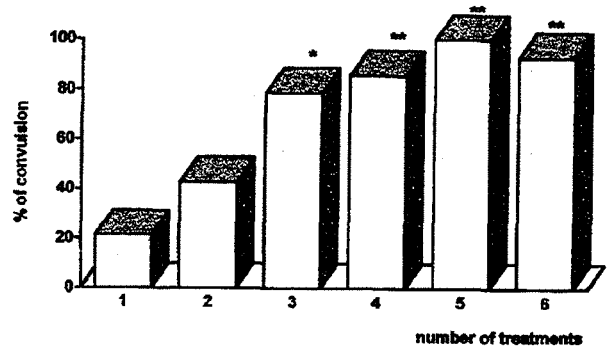

FIGURE 1. Percentage of mice presenting seizures with score ≥3 after repeated PTZ (60 mg/kg, SC) administration, $n=14$. *=$P<0.05$ and **=$P<0.01$, Fisher.

PTZ. These treatments were repeated once every third day, in a total of six treatments. Injections (0.1 ml/10 g) were given between 7:00 and 11:00 A.M.

After each PTZ injection, animals were placed in acrylic observation chambers for 30 min, and behavioral seizure activity was rated according to the following scale [adapted from Ito *et al.* (1977)]: 0, no convulsive behavior; 1, playing piano; 2, clonic forelimb convulsions lasting less than 3 sec; 3, clonic forelimb convulsions lasting more than 3 sec; 4, generalized convulsions with tonic extension episodes and full status epilepticus; 5, death. Kindling was considered to be established if the animal presented seizures of rate ≥3.

EFFECTS OF ANTICONVULSANTS ON PTZ KINDLING. Animals (8 weeks old at the start of the experiment) were divided into four groups ($n=10$–14). As in the first experiment, each treatment consisted of two drug administrations, repeated once every third day, in a total of six treatments. The first IP injection was as follows: Group I, 10 mg/kg phenobarbital; Group II, saline (phenobarbital vehicle); Group III, 0.5 mg/kg diazepam; Group IV, propylenoglycol (PPG, diazepam vehicle). Thirty minutes after the IP treatment, all groups received 60 mg/kg of PTZ subcutaneously. Epileptic behavior was evaluated as above.

EFFECT OF AGE ON SEIZURE THRESHOLD TO PTZ. The effect of age on the seizure threshold was evaluated by using animals 3, 8, 11, 18 and 28 weeks of age. Animals received saline intraperitoneally and, 30 min later, a single dose of PTZ (60 mg/kg SC). Seizures were evaluated as in the kindling experiment.

Binding studies

MEMBRANE PREPARATION. Twenty-four hours after the last injection (PTZ or saline), animals were decapitated, and the brains were rapidly removed. The cerebral cortex was dissected and used for the biochemical studies. Membranes were prepared as described elsewhere (Elisabetsky *et al.*, 1995). The cerebral cortices were homogenized (20:1 v:w) in 0.32 M sucrose containing 10 mM Tris/KCl buffer, pH 7.4, and 1 mM $MgCl_2$. All steps were carried out at 4°C. The homogenate was centrifuged twice at 1,000*g* for 15 min and the final pellet discarded. Both supernatants were pooled and centrifuged at 27,000*g* for 15 min. The supernatant was discarded and the pellet was lysed (20:1 v:w) for 30 min in 10 mM Tris/HCl buffer, pH 7.4. The lysed pellet was washed three times with lysing buffer (20:1 v:w) by centrifuging at 27,000*g* for 15 min. Supernatants were discarded and the final pellet was used for the experiments.

Binding of [^{3}H]glutamate

For measurement of the specific binding of [^{3}H]glutamate, membranes (100 μg of protein) were incubated at 30°C for 15 min in 50 mM Tris/KCl buffer, pH 7.4, and 40 nM of [^{3}H]glutamate, in a final volume of 0.5 ml. The reaction was interrupted by cooling the tubes and a further centrifugation for 20 min at 27,000*g*. The supernatant was discarded. The walls of the tubes and the surface of the pellets were quickly and carefully rinsed with cold distilled water. The pellets were processed for radioactivity measurement in cintilador Beckman. To determine the specific binding of [^{3}H]glutamate, each experiment was processed with parallel control tubes containing [^{3}H]glutamate, in the presence of 1,000 times of the amount of non-labeled glutamate. Specific binding was defined as the difference in [^{3}H]glutamate binding between tubes without (total binding) and with (nonspecific binding) unlabeled glutamate in excess concentration.

To evaluate changes in glutamate binding associated with PTZ-induced kindling, we studied acute and chronically PTZ treated animals. To compare chronically applied PTZ (kindled mice) with acutely induced convulsions, mice were given a single subconvulsive dose (60 mg/kg of PTZ, SC) or a single convulsive dose (88 mg/kg PTZ, SC). Only mice presenting score ≥ 3 convulsions were selected for binding assays, always carried out 24 hr after the last PTZ administration.

RESULTS

PTZ kindling

The behavioral seizure activity as established by PTZ-induced kindling in mice can be seen in Figure 1. With a single PTZ (60 mg/kg) injection 21.4% of the animals presented seizures with score ≥ 3. A significant increase in the percentage of seizuring animals occurred after the third PTZ injection. Repeated administration of saline alone or PTZ 40 mg/kg failed to induce convulsions with scores ≥3 at any point of the study (data not shown).

Effects of anticonvulsants on PTZ kindling

The ability of anticonvulsants to counteract PTZ kindling is shown at Figure 2. The development of PTZ-induced kindling was prevented by coadministration of phenobarbital (10 mg/kg) and diazepam (0.5 mg/kg).

Effect of age on seizure threshold to PTZ

Figure 3 shows the effect of age on PTZ seizure threshold in mice. Figure 3a shows that a significant increase in the percentage of seizuring animals with acute adminsitration occurs only after 28 weeks.

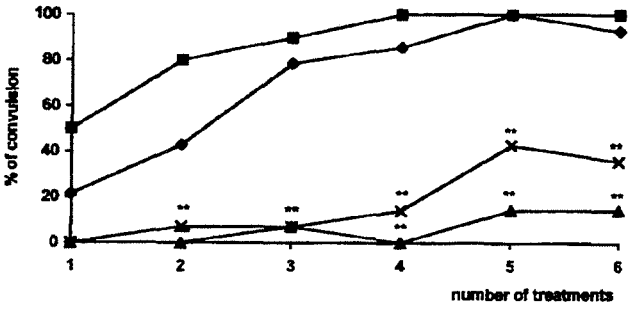

FIGURE 2. Protective effects of phenobarbital [▲](diluted in saline), saline alone [◆] and diazepam [×](diluted in propyleneglycol) propyleneglycol alone [■] on PTZ kindling in mice. $n=10$–14. **=$P<0.01$, Fisher compared with respective control.

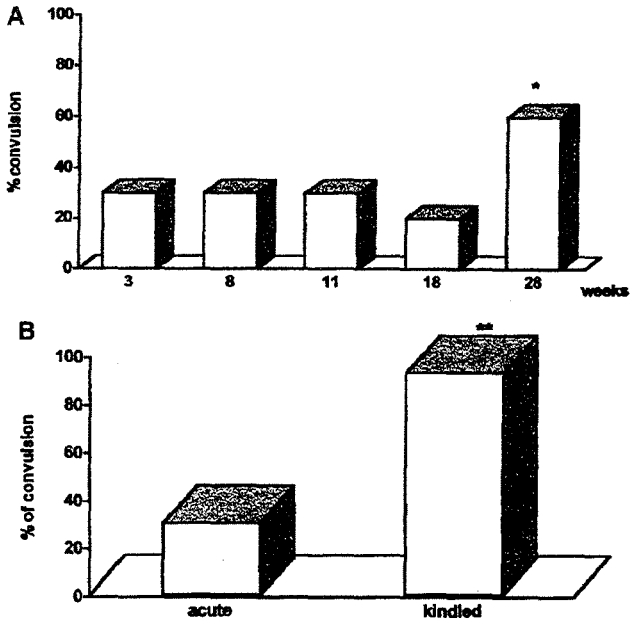

FIGURE 3. Effect of age on PTZ-induced convulsions. (a) Percentage of seizuring animals acutely treated with PTZ (60 mg/kg, SC). *=$P<0.05$, Fisher. (b) Percentage of 11-week-old mice presenting convulsions after acute or chronic (kindled) PTZ (60 mg/kg, SC) administration. **=$P<0.01$, Fisher.

Figure 3b presents the percentage of seizuring mice at 11 weeks of age, acutely or chronically (kindled) treated with PTZ.

Binding studies

Table 1 presents L-[^{3}H]glutamate-specific binding (pmol/mg protein) in the cortex membrane of PTZ acutely treated or kindled mice. At a ligand concentration of 40 nM, the specific L-[^{3}H]glutamate binding to the cerebral cortex membrane of kindled mice was significantly increased compared with groups treated with saline and with the group of convulsing animals acutely treated with PTZ.

DISCUSSION

PTZ kindling is a procedure of chemical kindling in which the repeated administration of this compound causes a progressive increase in the excitability of the central nervous system so that the threshold for generalized convulsions decreases over time (Corda *et al.*, 1990). Therefore, repeated administrations of otherwise subconvulsant doses lead to generalized seizures. The neurophysiological basis and neurochemical correlates of the kindling phenomena still lack complete elucidation. PTZ-induced kindling has been reported to be associated with a reduction in γ-aminobutyric acid-mediated neurotransmission in the central nervous system (Corda *et al.*, 1990; Giorgi *et al.*, 1991). Numerous studies have shown that *N*-methyl-D-aspartate subtype of glutamate receptors plays an important role in experimental epilepsy and kindling (McNamara *et al.*, 1988; Morimoto, 1989; Vezzani *et al.*, 1988).

Here we reported that repeated administration of 60 mg/kg of PTZ effectively induces kindling in mice (Fig. 1). Like that reported by Craig and Colasanti (1989), our study detected an age-dependent decrease in PTZ threshold. Nevertheless, the effect of age was not detected in mice younger than 18 weeks, and it became significant only after 28 weeks of age. Because significant expression of kindled is seen already with 11-week old animals (Fig. 3a and b), this study contradicts previous considerations in regard to the use of mice as reliable subjects for PTZ kindling (Craig and Colasanti, 1989). In conclusion, our data show a correlation between the behavioral expression of kindling and an increse in [^{3}H]glutamate binding in mice cortex membranes, as previously reported for rats (Schöder *et al.*, 1993).

Kindling in mice can play an important role in the context of screening new drugs that cold interfere with the plastic changes believed to underlie epileptic phenomena.

TABLE 1. Effect of PTZ kindling, acute convulsive PTZ (88.0 mg/kg, SC) and acute subconvulsive PTZ (60 mg/kg, SC) on L-[^{3}H]glutamate-specific binding in cortex membrane of mice

Treatment	*n*	L-[^{3}H]glutamate binding (pmol/mg protein)
Naive	6	1.16 ± 0.04
PTZ kindling	6	3.13 + 0.62**
PTZ acute 60 mg/kg	6	1.45 ± 0.04
PTZ acute 88 mg/kg	3	1.59 ± 0.23

Binding assays performed 20 h after the last drug administration. Data expressed as mean ± SE. **P < 0.01, analysis of variance.

This work was supported by grants from CNPq.

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2.5. CAPÍTULO 5 – SILVA BRUM, L.F., ELISABETSKY, E. Antiepileptogenic properties of phenobarbital on PTZ-Kindling: Behavior and neurochemical analysis. Submetido ao *Pharmacology Biochemical Behavior*

Antiepileptogenic Properties of Phenobarbital on PTZ-Kindling: Behavior and Neurochemical Analysis.

L. F. Silva Brum[1,2] and E. Elisabetsky[1,2,3]

1- Laboratório de Etnofarmacologia, Departamento de Farmacologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 90050-170, Brazil.

2- Curso de Pós-Graduação em Ciências Biológicas-Bioquímica, Departamento de Bioquímica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

3- Mailing address: Elaine Elisabetsky, Caixa Postal 5072, 90041-970, Porto Alegre, RS, Brazil. Phone/FAX: (051) 316-3121, elisasky@vortex.ufrgs.br

Running head: Phenobarbital as antiepileptogenic

ABSTRACT

Kindling provides a suitable means for quantifying epileptogenesis as well as investigating neurochemical changes associated with neuronal plasticity that leads to seizuring conditions. The aim of this paper was to investigate antiepileptogenic properties of phenobarbital, focusing on the neurochemical changes associated with PTZ-kindling in mice. Phenobarbital (10 mg/kg) significantly diminished the severity of seizures induced by PTZ-kindling. PTZ-kindling was associated with an increase in glutamate binding sites (B_{max} 196.6 ± 10.2 pmol/mg in comparison to control B_{max} 137.7 ± 17.0 pmol/mg). In addition, phenobarbital prevented the kindling-induced increase in binding sites (B_{max} 133.7 ± 11.4 pmol/mg). These data reveals an interesting capability of phenobarbital in interfering with the establishment of both the behavioral expression and associated neurochemical changes of the kindling process, which may be important in the context of preventing epileptogenesis.

Key Words: antiepileptogenic, glutamate binding, kindling, pentylenetetrazol, phenobarbital

INTRODUCTION

Pharmacotherapy of epilepsy utilizes chronic administration of anticonvulsants with the intent to prevent the occurrence of convulsive seizures. Nevertheless, no effective prophylaxis or pharmacotherapeutic cure of epilepsy is currently available (17, 29). Efforts to intervene in the development of epilepsy - epileptogenesis - are in place in the case of patients with brain injury (i.e., through administration of phenytoin and/or carbamazepine) (8, 15, 26). It has been argued that preventing epileptogenesis would obviate the need for chronic anticonvulsant drug, and that the use of drugs which inhibit the progression of an epileptic condition would be preferable for patients where epilepsy arises months after initial brain injury (22).

Specific knowledge about epileptogenesis as initiated by trauma, fever, or genetic factors may share new light regarding methods for prophylaxis. Investigations with various animal models have provided experimental evidences for the heterogeneity of events inherent to epileptogenesis; in addition, useful data were provided in clarifying the putative mode, mechanisms and sites of action of chemical agents on epileptogenesis and seizures (23-25, 27). Kindling, an *in vivo* chronic model of epilepsy in which an epileptic state gradually develops, provides an appropriate approach for quantifying epileptogenesis (16, 22).

Pentylenetetrazol(PTZ)-kindling refers to a process whereby the repeated administration of subconvulsive doses of this compound lead to a progressive decrease in seizure threshold, resulting in generalized seizures (9). Once established, such decrease in seizure threshold appears to be permanent for the life of the animal (11, 22). Kindling therefore allows for evaluating neuronal plasticity associated with long term alterations in neural excitability (3), and PTZ-kindling has been associated with a permanent increase

in glutamate binding (18, 19).

Phenobarbital has long been used in the clinic for its antiseizure properties and its therapeutic effectiveness in suppressing the spread of epileptiform seizure activity in the human brain (5, 13). Phenobarbital is relatively nonselective and efficacious in various animal models, inhibits clonic-tonic seizures evoked by electroshock or PTZ, and is able to suppress both electrophysiological and behavioral effects induced by electrical or chemical kindling in rats (1, 30). Phenobarbital also appears to exhibit antiepileptogenic activity on amygdala-kindling in rats, although at doses associated with ataxia and lethargy (22). In addition, we have recently reported that phenobarbital prevents the development of PTZ-induced kindling in mice (21).

The aim of this paper was to investigate the neurochemical changes associated with phenobarbital activity on PTZ-kindling in mice, in the context of the antiepileptogenic and anticonvulsant properties of phenobarbital.

MATERIALS AND METHODS

***Animals*:** Male CF1 mice, 25-30 g, were used throughout the experiments. Mice were kept at 20 ± 2°C, maintained on a 12-hr light-dark cycle, with food and water "*ad libitum*". All procedures were carried out according to institutional policies on the handling of experimental animals.

Drugs and reagents: Phenobarbital was purchased from Sigma. L-[^{3}H]Glutamate was purchased from Amersham. Glutamate was purchased from Merck. All other reagents were of analytical grade.

Behavioral Studies: The procedure is reported in detail elsewhere (21). Animals (8 weeks old at the start of the experiment) were divided into three groups. Each treatment consisted of two drug administrations repeated once every third day, in a total of six

treatments (16 days). Animals were given i.p. injections of 10 mg/kg phenobarbital (Group I; n=11) or its vehicle (saline solution; group II; n=12) 30 minutes prior to convulsive stimuli (60 mg/kg of PTZ, s.c.). Following each PTZ injection, mice were placed individually in acrylic observation chambers for 30 minutes and behavioral seizures rated according to the following scale: (0) no convulsive behavior; (1) *playing piano*; (2) clonic forelimb convulsions lasting less than 3 seconds; (3) clonic forelimb convulsions lasting more than 3 seconds; (4) generalized convulsions with tonic extension episodes and full status epilepticus. Kindling was considered to be established if the animal presented clonic convulsions lasting more than 3 seconds. A control group (Group III; n=12) consisted of seizure naive mice that were treated as experimental groups I and II, except that both injections consisted of saline solution.

Following the 6th treatment (day 16), with fully developed kindling, animals were given a 2-week rest. A further PTZ (60 mg/kg, s.c.) administration was given alone on day 31, and mice were observed as above. Twenty-four hours after the last testing day mice were decapitated, brains rapidly removed and cerebral cortices dissected and used for the [^{3}H]glutamate binding assay.

Neurochemical studies

Membrane Preparation: Membranes were prepared as described by Emanuelli et al., 1998 (6). The cerebral cortices were homogenized (20:1 v:w) in 0.32 M sucrose containing 10 mM Tris/HCl buffer (pH 7.4) for [^{3}H]glutamate binding and 1 mM $MgCl_2$. All steps were carried out at 4°C. The homogenate was centrifuged twice at 1,000 x g for 15 min and the final pellet discarded. Both supernatants were pooled and centrifuged at 27,000 x g for 15 min. The supernatant was discarded and the pellet was lysed (20:1 v:w) for 30 min in 10 mM Tris/HCl buffer (pH 7.4). The lysed pellet was washed three times

with lysing buffer (20:1 v:w) by centrifuging at 27,000 x g for 15 min. Supernatants were discarded and the final pellet was frozen at –70°C for at least 24 h. On the day of binding assay, the membranes were rapidly thawed in a water bath (37°C), homogenized with 3 volumes of 10 mM Tris/HCl (pH 7.4), and centrifuged at 27,000 x g for 15 min. The resulting pellet was resuspended in the same buffer, pre-incubated at 37°C for 30 min and centrifuged at 27,000 x g for 15 min. The pellet was washed three times in 3 volumes of the 10 mM Tris/HCl (pH 7.4), and centrifuged at 27,000 x g for 15 min. The final pellet was resuspended in the same buffer in order to yield a protein concentration of 1-2 mg/ml and was used for binding assays. Protein concentration was measured according to method of the Lowry et al., 1951 (10).

Binding of [^{3}H]glutamate: For measurement of [^{3}H]glutamate binding, studies were carried out using ligand concentrations in the range from 40 to 3000 nM. [^{3}H]glutamate was incubated with 100 μg of membrane in 50 mM Tris/HCl buffer (pH 7.4) at 30°C for 15 min, in a final volume of 0.5 ml. The reaction was interrupted by centrifugation for 20 min at 27,000 x g. The supernatant was discarded. The walls of the tubes and the surface of the pellets were quickly and carefully rinsed with cold distilled water. The pellets were processed for radioactivity measurement in cintilador Beckman scintilation counter. In order to determine specific binding of [^{3}H]glutamate, each experiment was processed with parallel control tubes containing [^{3}H]glutamate in the presence of 1,000 times the amount of non-radioactive glutamate. Specific binding was defined as the difference of [^{3}H]glutamate binding between tubes without (total binding) and with (non-specific binding) an excess amount of unlabeled glutamate.

Statistical Methods: Differences in percentage of mice presenting seizures with duration ≥ 3 seconds were evaluated by Fisher's Exact test. Differences in seizure scores were

evaluated by non-parametric Kruskal-Wallis analysis. Statistical significance was defined as $p \leq 0.05$. Dissociation constant (K_d) and maximal number of specific binding sites (B_{max}) values were estimated by Scatchard analysis. Binding values were compared by means of two way ANOVA followed by Duncan test.

RESULTS

Phenobarbital significantly inhibited the development of PTZ-kindling (figure 1) ($p<0,01$, Fisher). The mean seizure score at day 16 was: zero in naive animals, 3.3 ± 0.2 in control mice and 1.5 ± 0.3 in mice treated with phenobarbital ($p<0,01$, Kruskal-Wallis) (figure 1A). As shown in Figure 1, two weeks after completion of kindling the induced epileptic status persisted in untreated subjects. Likewise, the antiepileptogenic effect of phenobarbital remained significant ($p<0,05$, Fisher). Regarding scores on day 31, the saline-treated group's mean score was 3.4 ± 0.2 whereas the phenobarbital treated group's mean score was 1.8 ± 0.3 ($p<0,01$, Kruskal-Wallis).

Under the incubation conditions used in this study binding of [^{3}H]glutamate to cortex membranes from naive mice is similar to previously published studies (21), a B_{max} value of 137.7 ± 17.0 pmol/mg a K_d value of 916.3 ± 96 nM. PTZ-kindling significantly increased [^{3}H]glutamate binding (B_{max} 196.6 ± 10.2 pmol/mg; $p<0,01$) without affecting K_d (953.9 ± 26.0 nM). Co-administration of 10 mg/kg phenobarbital prevented the PTZ-induced increase in [^{3}H]glutamate binding (B_{max} 133.7 ± 13.0 pmol/mg) without interfering with its affinity (K_d 914.2 ± 81.0 nM) (Figure 2).

DISCUSSION

Our data confirm previous suggestions from electrically induced kindling in rats (22) that phenobarbital (10 mg/kg) holds potent antiepileptogenic and anticonvulsant effects. In the case of PTZ-kindling in mice, phenobarbital is effective in a dose that does not

induce ataxia or loss of righting reflex.

Kindling is an accepted model of experimental epileptogenesis and reflects an altered activity of the excitatory glutamatergic synaptic processes. Supporting previous data (18, 21), the density of glutamate binding sites in the cortex was enhanced in response to PTZ-kindling. Pretreatment with phenobarbital (10 mg/kg) downregulated the PTZ-induced enhancement of glutamate binding to control levels (naive animals) (Figure 2).

The cellular mechanism through which phenobarbital exerts this antiepileptogenic action is uncertain. As with other drugs, an enhancement of inhibitory synaptic transmission (20, 30) and/or inhibition of excitatory synaptic transmission (12) may be relevant. Impairment of GABAergic inhibition is also thought to play a crucial role in the processes underlying epileptogenesis in the PTZ-kindling model (4,7), and the well known effects of phenobarbital in facilitating GABAergic transmission is likely to contribute to its antiepileptogenic effect in this model.

It is noteworthy that controlled randomized trials show that prophylactic administration of phenytoin and carbamazepine failed to inhibit the development of posttraumatic epilepsy (8, 14, 26, 31), drugs that also failed to inhibit development of kindling in animals (2, 16, 22, 28). If epileptogenesis is dependent upon neural modification common to PTZ-kindling in mice and some forms of human epilepsy, the properties of phenobarbital reported here may have meaningful implications for treating epilepsy.

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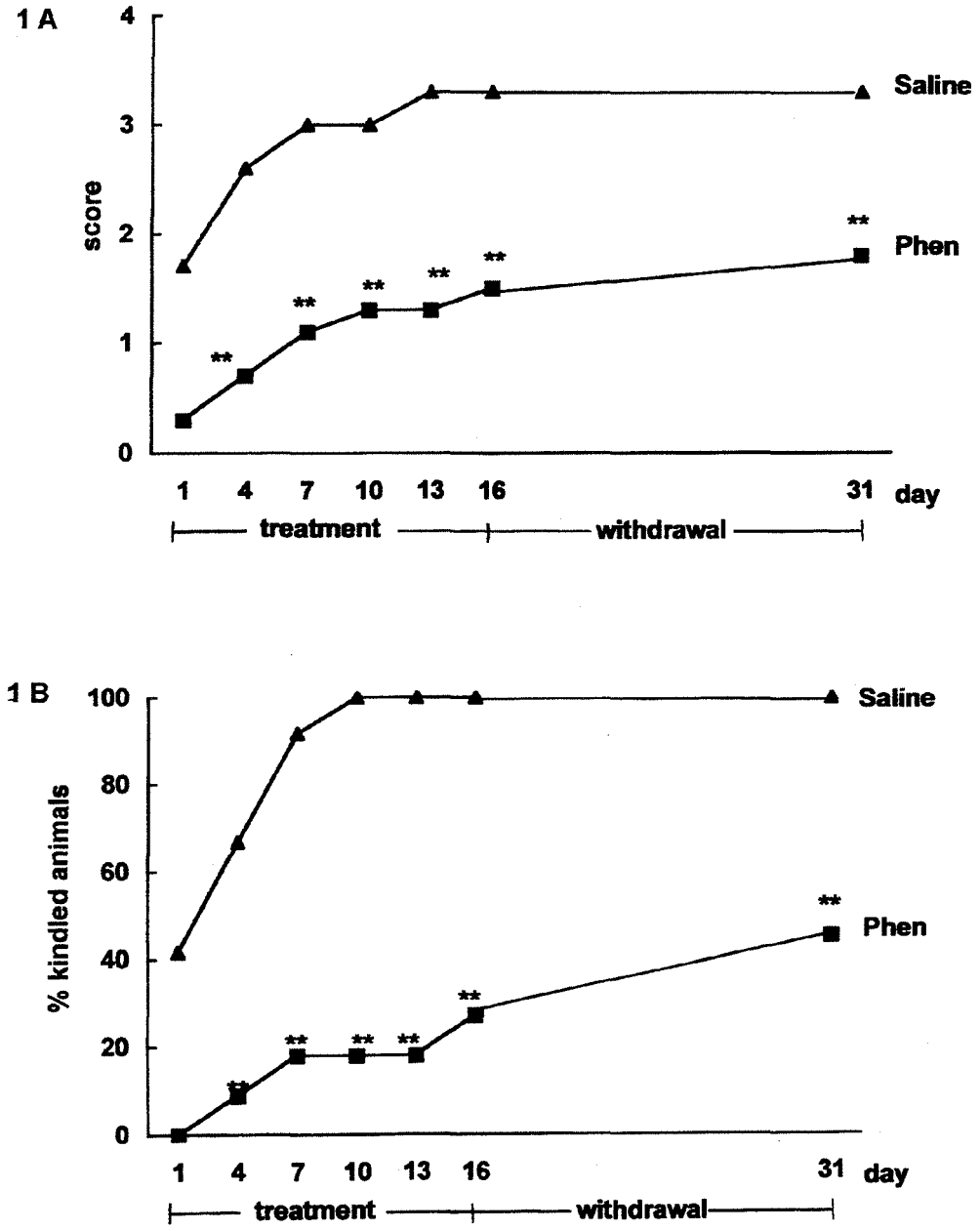

Figure 1. Mean seizure score (1A) and percentage of kindled animals (figure 1B) of saline (▲) and phenobarbital (■) groups during kindling procedure (days 1-16) and after two weeks withdrawal (day 31). ** $p < 0,01$, Fisher test.

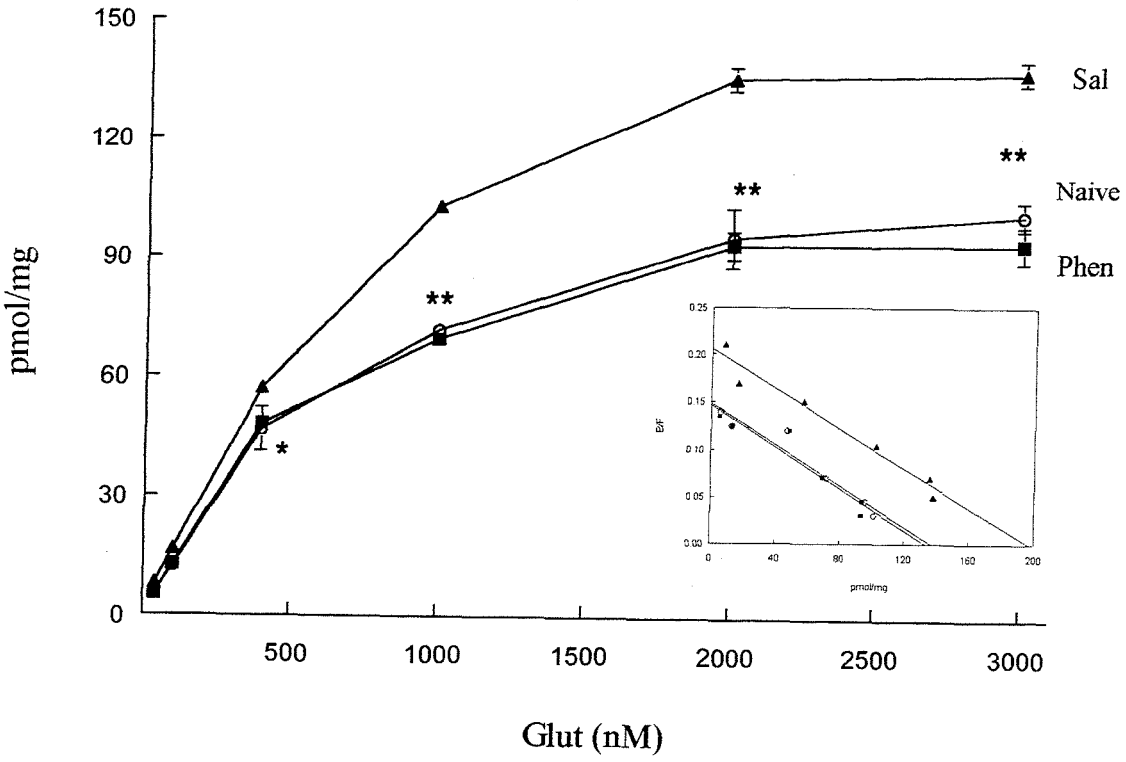

Figure 2. Effect of saline (▲) and 10 mg/kg phenobarbital (■) on L-[^{3}H]glutamate binding (40-3000nM) of cortical membranes of mice submitted to PTZ-kindling. Control (○) mice did not receive PTZ. Values are expressed as pmol/mg of protein. Each point represents the mean ± S.E.M. of three separate experiments determined in triplicate (pooled tissues from 3-4 mice for each membrane preparation). Statistical analysis (ANOVA followed by Duncan test) compared binding levels of saline group. *$p<0.05$, **$p<0.01$.

3. DISCUSSÃO

Baseando-se nos eventos fisiopatológicos responsáveis pela epilepsia e no conhecimento dos mecanismos de ação dos fármacos anticonvulsivantes clássicos, o desenvolvimento racional de novos fármacos anticonvulsivantes tem resultado na introdução de novos fármacos com atividade específica nos sistemas transmissores inibitórios ou excitatórios envolvidos na epileptogênese (SABERS & GRAM, 1996).

Estudos clínicos demonstram que 70% dos pacientes epilépticos têm suas crises satisfatoriamente controladas empregando tratamento monoterápico com fármacos anticonvulsivantes clássicos (PALMINI, 1993; WIESER, 1994). Em torno de 15% dos pacientes são refratários à monoterapia e conseguem melhoras no controle das crises epilépticas com a combinação de fármacos anticonvulsivantes. No entanto, a terapia geralmente apresenta mais efeitos colaterais (SCHMIDT & GRAM, 1995). Outros 15% dos pacientes permanecem refratários ao tratamento com os fármacos anticonvulsivantes disponíveis, ou o tratamento é pouco efetivo devido à intolerância à medicação (MATTSON, 1994). O desenvolvimento de novos fármacos anticonvulsivantes com maior eficácia e menores efeitos colaterais tem sido, portanto, um constante desafio.

Linalol é ativo em vários modelos *in vivo* preconizados para a triagem primária visando a identificação de novos fármacos antiepilépticos (SWINYARD & KUPFERBERG, 1985). Entre eles podemos citar convulsões induzidas por ECC e compostos químicos (PTZ, picrotoxina, ácido quinolínico, NMDA). Estas propriedades indicam que a administração do linalol eleva o limiar para convulsão, e/ou previne a sua propagação, provavelmente por mecanismos envolvendo a modulação do sistema

GABAérgico e/ou glutamatérgico (ELISABETSKY *et al.*, 1995ab; BARROS & ELISABETSKY, 1996a; MARASCHIN *et al.*, 1996).

Baseados na relevância da diminuição do efeito inibitório do sistema GABAérgico, bem como do aumento na excitabilidade neuronal via sistema glutamatérgico na geração, propagação e manutenção da atividade epileptiforme (BRADFORD, 1995), esta tese objetivou investigar os modos de interação do linalol com estes sistemas transmissores. Investigou-se o efeito do linalol (a) na união específica de L[^{3}H]glutamato, [^{3}H]MK 801 (antagonista NMDA) e [^{3}H]muscimol (agonista $GABA_A$) em membranas corticais; (b) efeito do linalol na liberação e captação de L[^{3}H]glutamato em sinaptossomas de córtex de camundongos e (c) o efeito do linalol no PTZ-*kindling*.

Os resultados apresentados nesta tese demonstram que: a) uma diminuição na transmissão excitatória glutamatérgica (através da inibição da liberação de glutamato e/ou bloqueio de receptores glutamatérgicos pós-sinápticos) está envolvida na atividade anticonvulsivante do linalol; b) o linalol não interfere diretamente na união de [^{3}H]muscimol ao receptor $GABA_A$; c) o linalol retarda a expressão comportamental induzida por PTZ-*kindling* sem interferir nas alterações neuroquímicas que acompanham este fenômeno; d) o fenobarbital, mas não o linalol (dados não publicados), apresenta efeito antiepileptogênico no que permite a avaliação pelo modelo de PTZ-*kindling* em camundongos.

O papel do sistema glutamatérgico, principalmente do receptor NMDA, nos mecanismos básicos da epileptogênese, bem com na expressão das convulsões, tem sido amplamente discutido (MELDRUM, 1994; BRADFORD, 1995; FOUNTAIN & LOTHMAN, 1995; CHAPMAN, 1998). Estudo previamente realizado em nosso laboratório já havia demonstrado uma atividade inibitória, porém sem a caracterização do

tipo de antagonismo do linalol na união específica de L[^{3}H]glutamato (40 nM) em membranas de córtex de ratos (ELISABETSKY *et al.*, 1995a).

No estudo agora realizado para a caracterização do tipo de antagonismo, demonstramos que o linalol exerce um antagonismo competitivo na união específica de L[^{3}H]glutamato (Cap. 1). Linalol mostrou um efeito inibitório de forma dose-dependente na união específica de L[^{3}H]glutamato (1000 nM), apresentando efeito máximo (100% de inibição) na maior concentração utilizada (5,0 mM). Observamos ainda que o linalol aumenta significativamente o K_d sem interferir significativamente no $B_{máx}$ da união específica de L[^{3}H]glutamato, o que caracteriza um antagonismo do tipo competitivo (Cap. 1).

Devido à importância do receptor NMDA na epilepsia, investigamos a atividade do linalol no receptor NMDA marcado com [^{3}H]MK 801 (antagonista não-competitivo do receptor NMDA). Linalol inibiu de forma dose-dependente a união especifica de [^{3}H]MK 801 (2 nM) (IC_{50} = 2.97 ± 0.13 mM) (Cap. 2) e através da análise de Scatchard nas curvas de saturação, observamos que o linalol promoveu uma diminuição significativa do $B_{máx}$ sem alterar o K_d, o que caracteriza um antagonismo não-competitivo. Portanto, os resultados demonstrando uma inibição da união específica de L[^{3}H]glutamato e [^{3}H]MK 801 por linalol indicam que o bloqueio de receptores glutamatérgicos pós-sinápticos deve estar relacionado ao efeito anticonvulsivante do linalol (anexo 2).

Além da reconhecida importância do receptor NMDA na epilepsia, também tem sido proposto que um aumento no nível de glutamato extracelular, seja por uma liberação excessiva e/ou inadequada captação de glutamato, seria epileptogênico (LIPTON & ROSENBERG, 1994). Neste contexto e com o objetivo de melhor caracterizar o efeito do linalol na transmissão glutamatérgica, investigamos o efeito do linalol na liberação de

[^{3}H]glutamato (basal e estimulada por potássio) e na captação de [^{3}H]glutamato em sinaptossomas de córtex de camundongos (modelo de liberação e captação de glutamato a partir de terminais sinápticos intactos) (Cap. 3). Observamos que o linalol não interfere na liberação basal de glutamato (glutamato citosólico) mas demonstra um efeito inibitório na liberação de glutamato estimulada pelo aumento da concentração extracelular de potássio (provavelmente do compartimento vesicular) (Cap. 3). A concentração de glutamato armazenado em vesículas sinápticas (100 mM) é 10 vezes maior que a normalmente encontrada no citosol de terminais pré-sinápticos (10 mM); a liberação do glutamato vesicular, estimulada pelo aumento da concentração extracelular de potássio, é resultante da abertura de canais de Ca^{+2}-voltagem dependente (NICHOLLS, 1989). Portanto, o efeito inibitório do linalol na liberação do glutamato durante a despolarização induzida por potássio é relevante para seu efeito anticonvulsivante, pois é relatado uma elevação na liberação de glutamato - provavelmente liberada do compartimento vesicular- estimulada por potássio, em condições patológicas tais como hipóxia ou epilepsia (NICHOLLS & ATTWELL, 1990; THOMAS, 1995). É lícito supor que a manutenção e/ou propagação da atividade epiléptica seja inibida por este mecanismo de ação (anexo 2).

Até o momento não está totalmente esclarecido o mecanismo pelo qual o linalol interfere na liberação de glutamato durante a despolarização induzida por potássio. Em 1995, SRINIVASAN e colaboradores relacionaram o efeito inibitório de FPL 12495AA e MK801 na liberação de glutamato estimulada por potássio como possível resultado do bloqueio de receptores NMDA, pois a ativação do receptor NMDA por ligantes endógenos desencadeia um *feedback* positivo a nível pré-sináptico resultando no estímulo da liberação do glutamato. Consequentemente, através do bloqueio da ativação do receptor

NMDA por FPL 12495AA e MK801 esta estimulação não ocorreria. No presente estudo, demonstramos que o linalol, além de inibir a liberação de glutamato estimulada por potássio (Cap. 3) também interfere na união específica de [^{3}H]MK 801, sugerindo uma interação com receptores NMDA (Cap. 2). Portanto, é possível que o efeito inibitório do linalol na liberação de glutamato estimulada por potássio seja resultado do bloqueio de receptores NMDA.

Em relação à captação de [^{3}H]glutamato, observamos que o linalol inibiu de forma dose-dependente a captação de [^{3}H]glutamato (IC_{50}=1.50 ± 0.08 mM) em sinaptossomas de córtex de camundongos (Cap. 3). Linalol, na concentração de 3,0 mM, inibiu a captação de [^{3}H]glutamato em aproximadamente 90%. Consequentemente, poderíamos esperar um aumento significativo do glutamato extracelular. Considerando que a captação de glutamato pelas células gliais é mais importante que a captação neuronal para a manutenção de baixos níveis de glutamato extracelular (1μM) (NICHOLLS & ATTWELL, 1990), torna-se difícil avaliar o real impacto deste efeito do linalol no aumento dos níveis de glutamato extracelular ou na transmissão glutamatérgica. Neste contexto, MASSIEU *et al.*, (1995) demonstraram que o acúmulo de glutamato extracelular resultante da inibição de sua captação não é suficiente para induzir danos neuronais *in vivo*. De fato, fenômenos convulsivos não são necessariamente resultantes de, ou sequer acompanhados por, aumento dos níveis de glutamato extracelular (OBRENOVITCH *et al.*, 1996), embora níveis elevados certamente contribuam para tal. Considerando-se o perfil anticonvulsivante do linalol, estabelecido através de modelos *in vivo* com camundongos e seu uso tradicional em humanos (ELISABETSKY *et al.*, 1995b; BARROS & ELISABETSKY, 1996a), pode-se

inferir que a inibição da captação neuronal do glutamato não seja relevante para a atividade anticonvulsivante do linalol (anexo 2).

Devido à relevância e à ampla distribuição do sistema inibitório GABAérgico, e baseados no efeito protetor do linalol contra convulsões induzidas por PTZ e picrotoxina (modelos associados à redução dos mecanismos inibitórios mediados pelo GABA), levantamos a hipótese de um possível envolvimento do sistema GABAérgico no seu mecanismo de ação. Os resultados obtidos através do estudo da união específica do [^{3}H]muscimol (agonista do receptor $GABA_A$) (Cap. 2) não evidenciaram uma interação direta do linalol com receptor $GABA_A$. A modulação em outros sítios alostéricos do receptor GABA/BZD, bem como atuação na liberação, recaptação ou metabolismo de GABA não podem ser descartadas.

A farmacoterapia da epilepsia é sintomática, na medida em que os fármacos antiepilépticos disponíveis inibem convulsões mas não são efetivos na profilaxia ou cura da epilepsia (WILLMORE, 1997). Logo, os antiepilépticos são prescritos para prevenir a recorrência de crises. Em alguns pacientes fármacos antiepilépticos são administrados com o objetivo de interferir na epileptogênese (WILLMORE, 1997). O exemplo desta medida profilática inclui a administração de fármacos antiepilépticos (ex. fenitoína ou carbamazepina) a pacientes com traumatismo craniano (RAPPORT & PENRY 1972; 1973) ou a pacientes que se submeteram a intervenções cirúrgicas neocorticais (WANG *et al.*, 1994). Mas, apesar do amplo uso clínico de fármacos antiepilépticos no controle de crises convulsivas, não há nenhum dado clínico disponível sugerindo que a administração destes fármacos tenha algum impacto na epileptogênese (WILLMORE, 1997).

Kindling é um modelo crônico *in vivo*, no qual a epilepsia desenvolve-se de forma gradual e permite avaliar atividade antiepileptogênica (interferência no desenvolvimento

da epilepsia) e anticonvulsivante (supressão de convulsões generalizadas) de fármacos antiepilépticos (SATO *et al.*, 1990; SILVER *et al.*, 1991). Devido à hipótese de semelhanças entre a epileptogênese no modelo *kindling* e algumas formas de epilepsia em humanos, os dados obtidos neste modelo experimental permitem uma seleção racional de possíveis fármacos que possam atuar na prevenção bem como na atenuação da progressão da epilepsia em humanos. Neste trabalho, primeiramente otimizamos o modelo de *kindling* induzido por pentilenetetrazol (PTZ-*kindling*) em camundongos (Cap. 4). Injeções repetidas de dose subconvulsivante de PTZ (60 mg/kg, s.c.) a cada 3 dias, totalizando 6 administrações, promoveram uma diminuição no limiar para convulsões culminando em convulsões generalizadas (*status epilepticus*). Os resultados também demonstraram que nos camundongos, semelhante ao verificado em ratos (SCHRÖDER *et al.*, 1993), o PTZ-*kindling* promoveu alterações neuroquímicas que resultam em uma maior ligação específica de [^{3}H]glutamato em membranas corticais (maior $B_{máx.}$). Portanto, o modelo mostrou-se adequado para a triagem de compostos que possam interferir tanto nas alterações comportamentais como nas alterações plásticas relacionadas ao fenômeno de PTZ-*kindling*.(Cap. 4).

Avaliamos o efeito da linalol (2,2 e 2,5 g/kg) administrado por via oral no PTZ-*kindling*. Linalol retardou o desenvolvimento de PTZ-*kindling*, sem interferir contudo, no aumento da união específica de L[^{3}H]glutamato induzido por este fenômeno (Cap. 1). A habilidade do linalol em retardar o desenvolvimento do PTZ-*kindling* sugeria um potencial efeito antiepileptogênico além do efeito anticonvulsivante verificado em outros modelos de convulsão (ELISABETSKY *et al.*, 1995b). No entanto, um efeito antiepileptogênico não foi corroborado nem pelas alterações neuroquímicas associadas ao PTZ-*kindling* e nem pela manutenção do *status epilepticus* após um período de 15 dias

sem qualquer tratamento (dados não publicados, vide anexo 3). Contudo, pode-se argumentar que o antagonismo do linalol à transmissão glutamatérgica resulta em uma maior dificuldade no estabelecimento do *kindling*.

Cabe notar que, tanto por via i.p como por via oral, as DE_{50} de linalol nos modelos de convulsão química e eletricamente induzida são inferiores às DL_{50} (ELISABETSKY *et al.*, 1995b). As doses usadas no modelo de *kindling* guardam relação com a curva dose-efeito obtida experimentalmente em PTZ, e que equivalem às DE_{80} e DE_{90}, respectivamente. Linalool apresenta-se como óleo aromático e volátil (PM = 154,3g, densidade específica 0,87, o da Sigma com grau de pureza 97%), do qual 1µl equivale a 1mg.

A título de validação do desenho experimental e para comparação com linalol, investigamos o efeito do fenobarbital no desenvolvimento do PTZ-kindling (Cap. 1). Fenobarbital (15 mg/kg) administrado oralmente retardou o desenvolvimento de PTZ-*kindling*, contudo sem interferir no aumento da união específica de L[^{3}H]glutamato induzido por este fenômeno (Cap. 1). A ausência de efeito antiepileptogênico do fenobarbital neste experimento provavelmente está relacionada à absorção parcial do fármaco quando da administração do PTZ. O efeito do fenobarbital administrado oralmente com uma modificação no desenho experimental usado, onde a administração do PTZ fosse 1-2h ao invés de 30min pós fenobarbital, requer esclarecimento.

No capítulo 4 demonstramos que fenobarbital administrado intraperitonealmente (10 mg/kg) de fato retarda o desenvolvimento do kindling induzido por PTZ em camundongos. Este dado acrescenta-se ao estudo de SILVER *et al.*, (1991), demostrando que fenobarbital (40 mg/kg; i.p.) exerce efeito antiepileptogênico no modelo de *kindling* elétrico em amígdala ratos.

Investigamos também o efeito antiepileptogênico do fenobarbital (10 mg/kg) administrado intraperitonealmente no modelo de PTZ-*kindling* em camundongos (Cap. 5). Fenobarbital (10 mg/kg) significativamente diminuiu a severidade das convulsões induzidas por PTZ-*kindling* e também inibiu o aumento da união específica de L[^{3}H]glutamato induzido pelo PTZ-*kindling*. Duas semanas após encerrados os tratamentos, não se verifica o *status epilepticus* total nos animais, demonstrando que de fato não houve estabelecimento do fenômeno de *kindling*. Após este período, 100% dos animais controle (grupo salina) ainda apresentam convulsões generalizadas enquanto apenas 40% dos animais tratados com fenobarbital (10 mg/kg; i.p.) apresentam convulsões (Cap. 5). Estes dados corroboram outros dados da literatura que sugerem uma capacidade do fenobarbital em interferir com o estabelecimento das alterações plásticas que acompanham o fenômeno de PTZ-*kindling*. Essa conclusão pode ser importante no contexto de prevenção do estabelecimento da epilepsia.

As estratégias utilizadas para a descoberta de novos princípios ativos na flora incluem a randômica, quimiotaxonômica e etnofarmacológica (BALICK, 1990). A etnofarmacologia, através do somatório das informações tradicionais com os dados farmacológicos, consegue um resultado consideravelmente mais eficaz que a busca randomizada de novas drogas na flora mundial (BALICK, 1990). Além disso, uma outra vantagem na abordagem etnofarmacológica é a de que a seleção de espécies medicinais já parte da experiência em humanos, o que aumenta as chances de biodisponibilidade do(s) princípio(s) ativo(s) e, consequentemente, aumenta as probabilidades de uso clínico (ELISABETSKY, 1999). Portanto, estudos demonstrando as propriedades anticonvulsivante do linalol, tradicionalmente usado como anticonvulsivante caseiro pelos caboclos da Amazônia, reforçam a importância do estudo etnofarmacológico como

fonte promissora de novos compostos farmacologicamente ativos (ELISABETSKY & SANTANA, 1984; SOUZA *et al.*, 1993; ELISABETSKY *et al.*, 1995b).

Considerando a importância do sistema glutamatérgico, principalmente do receptor NMDA, nos mecanismos básicos da epileptogênese, bem com na expressão das convulsões, a demonstração de que o linalol interage com o sistema glutamatérgico por inibição de receptores pós-sinápticos (inibição da união específica de L[^{3}H]glutamato e [^{3}H]MK 801) (Cap. 1 e 2) ou diminuição na liberação de L[^{3}H]glutamato (Cap. 3), justifica a utilização popular de plantas produtoras de linalol no manejo de crises convulsivas por caboclos da Amazônia, bem como reforça a validade de se explorar o linalol ou compostos semelhantes como potenciais agentes anticonvulsivantes.

4. CONCLUSÕES

- Linalol inibe de forma dose-dependente a união específica do [^{3}H]glutamato (1000 nM) em membranas de córtex de ratos. Linalol (0,3 e 1,0 mM) aumenta o K_d sem interferir significativamente no $B_{máx}$ nas curvas de saturação de L[^{3}H]glutamato, o que caracteriza um antagonismo do tipo competitivo.
- Linalol inibe de forma dose-dependente a união específica do [^{3}H]MK 801 (2 nM) em membranas de córtex de camundongos. Linalol (1,0 e 3,0 mM) diminui o $B_{máx}$ sem interferir significativamente no K_d nas curvas de saturação de L[^{3}H]MK 801, o que caracteriza um antagonismo do tipo não-competitivo.
- O bloqueio de receptores glutamatérgicos pós-sinápticos, do subtipo NMDA, está envolvido nas propriedades anticonvulsivantes do linalol.
- Linalol (1,0 e 3,0 mM) inibe a liberação de glutamato estimulada por potássio sem interferir na liberação basal de glutamato, em sinaptossomas de córtex de camundongos.
- Linalol inibe de forma dose-dependente a captação de [^{3}H]glutamato (IC_{50}=1.50±0.08 mM) (Cap. 3) em sinaptossomas de córtex de camundongos.
- Linalol (0.3 a 3.0 mM) não interfere na união específica de [^{3}H]muscimol (12 nM) ao receptor $GABA_A$ em membranas de córtex de camundongos.
- Linalol retarda a expressão comportamental do PTZ-*kindling*, mas não interfere no aumento da união específica do [^{3}H]glutamato em membranas de córtex de camundongos induzido pelo PTZ-*kindling*.

- Linalol interfere na transmissão glutamatérgica, seja por inibição de receptores pós-sinápticos (inibição da união específica de L[^{3}H]glutamato e [^{3}H]MK 801) ou pela diminuição na liberação de L[^{3}H]glutamato.
- O efeito anticonvulsivante do linalol está relacionado ao efeito inibitório na transmissão glutamatérgica.

5. PERSPECTIVAS

Nos últimos anos, têm sido significantes os avanços no tratamento medicamentoso da epilepsia. Por um lado, novas formulações de vários anticonvulsivantes clássicos, bem como o desenvolvimento racional de novos fármacos, têm proporcionado a inserção de medicamentos de melhor eficácia e menor toxicidade, o que, consequentemente, resultou em uma maior adesão ao tratamento. Por outro, nas triagens clínicas para testar novos fármacos anticonvulsivantes, tem-se levado em questão a importância do tratamento a longo prazo no controle das convulsões, bem como o papel da monoterapia ou o emprego da politerapia racional (RAMSAY, 1993; SABERS & GRAM, 1996).

O desenvolvimento racional de novos fármacos anticonvulsivantes é resultante do avanço nos conhecimentos dos eventos fisiopatológicos responsáveis pela epilepsia e do melhor entendimento dos mecanismos de ação de fármacos anticonvulsivantes. Portanto, torna-se possível desenvolver fármacos que apresentam efeito específico excitatório ou inibitório em sistemas transmissores específicos. Além disso, a politerapia *racional* têm sido prescrita em uma proporção significativa de pacientes epilépticos (RICHENS, 1995).

O conceito de politerapia *racional* baseia-se no fato que *a combinação de alguns fármacos anticonvulsvantes pode resultar em uma eficácia supra-aditiva (sinergismo) e uma toxicidade infra-aditiva (antagonismo), resultando num melhor perfil eficácia/toxicidade;, ou seja, efetividade* (SCHMIDT & GRAM, 1995). De fato, estudos

em animais ou clínicos envolvendo a associação entre etossuximida e ácido valpróico comprovam tal hipótese (ROWAN *et al.*, 1983; BOURGEOIS, 1988).

Considerando o ponto de vista teórico, a politerapia *racional* poderia envolver (a) fármacos com mecanismos de ações complementares (por exemplo, associação entre um fármaco que diminua a excitabilidade glutamatérgica e outro que estimule a atividade inibitória gabaérgica) (ROBINSON *et al.*, 1993); (b) fármacos que atuam no mesmo sistema neurotransmissor, mas por diferentes mecanismos (LEACH & BRODIE, 1994). Linalol interfere na excitabilidade glutamatérgica através da modulação da liberação de glutamato e por inibir a interação do glutamato com seus receptores, em particular o receptor NMDA, subtipo envolvido na gênese da epilepsia. Portanto, seu mecanismo de ação vem de encontro ao contexto atual de terapia antiepiléptica.

Por outro lado, é cada vez maior a procura por tratamentos alternativos, principalmente por produtos naturais, por pacientes epilépticos resistentes/refratários aos métodos convencionais de tratamento (DANESI & ADETUNJI, 1994; SONNEN, 1997). Além disso, relatos clínicos recentes indicam o benefício do uso de óleos essenciais no manejo de fenômenos epilépticos (BODEN *et al.*, 1990; BETTS *et al.*, 1995; BETTS, 1996; LIS-BALCHIN, 1997). De fato, há evidências de absorção de óleos essenciais através de várias vias de administração (JAGER *et al.*, 1992; JIROVETZ *et al.*, 1992). Portanto, embasados no uso tradicional de plantas produtoras de linalol como remédios anticonvulsivantes, indicativo de biodisponibilidade e segurança, e no efeito modulatório do linalol na excitabilidade glutamatérgica, pode-se delinear experimentos pré-clínicos envolvendo a associação entre linalol/lamotrigina ou linalol/vigabatrina. Sugerimos também que linalol é adequado para estudos de estrutura-atividade no sentido de descobrir estratégias inovadoras para o manejo de distúrbios epilépticos.

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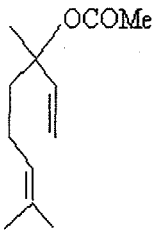
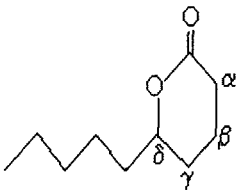
Anexo 1

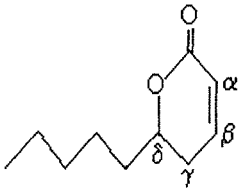
Linalol



acetato de linalil



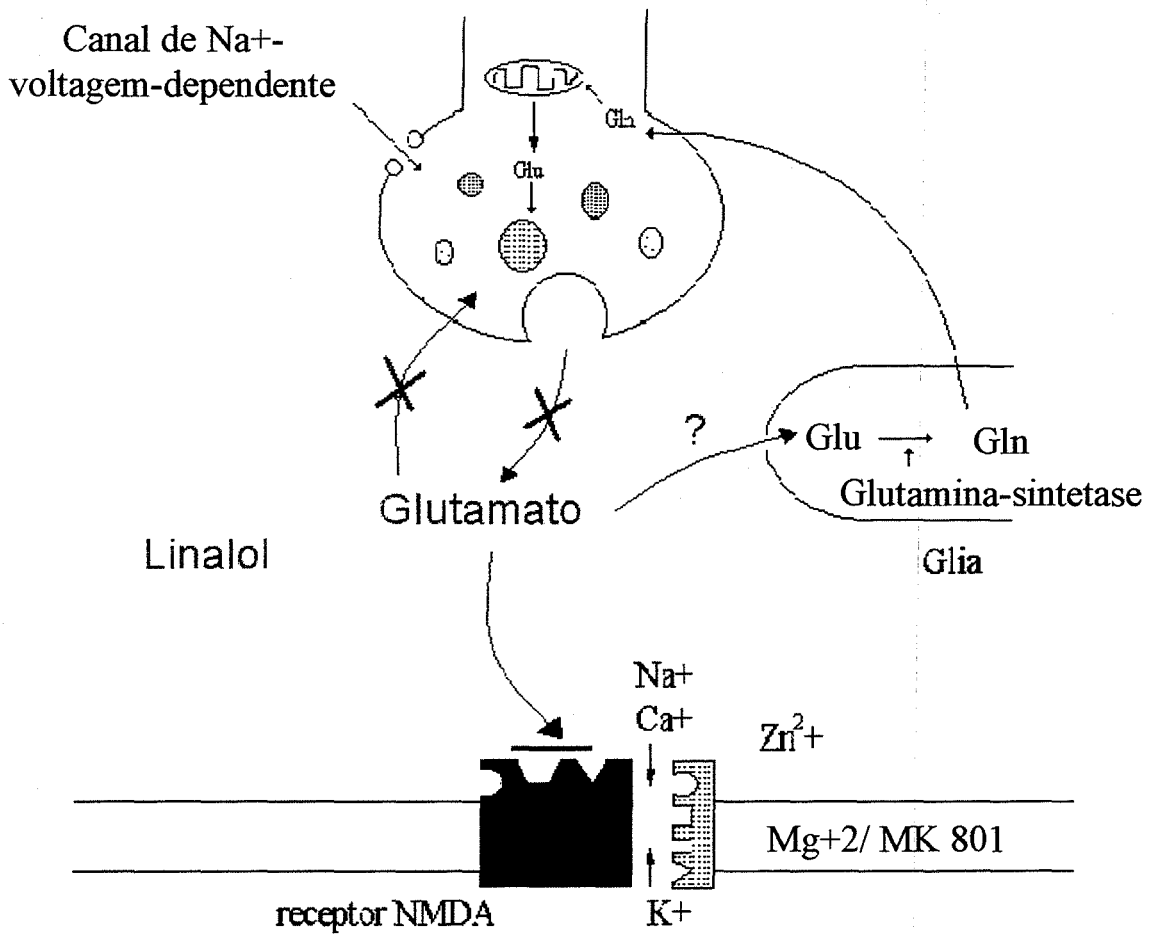
delta-decanolactona



delta-decen-2-lactona

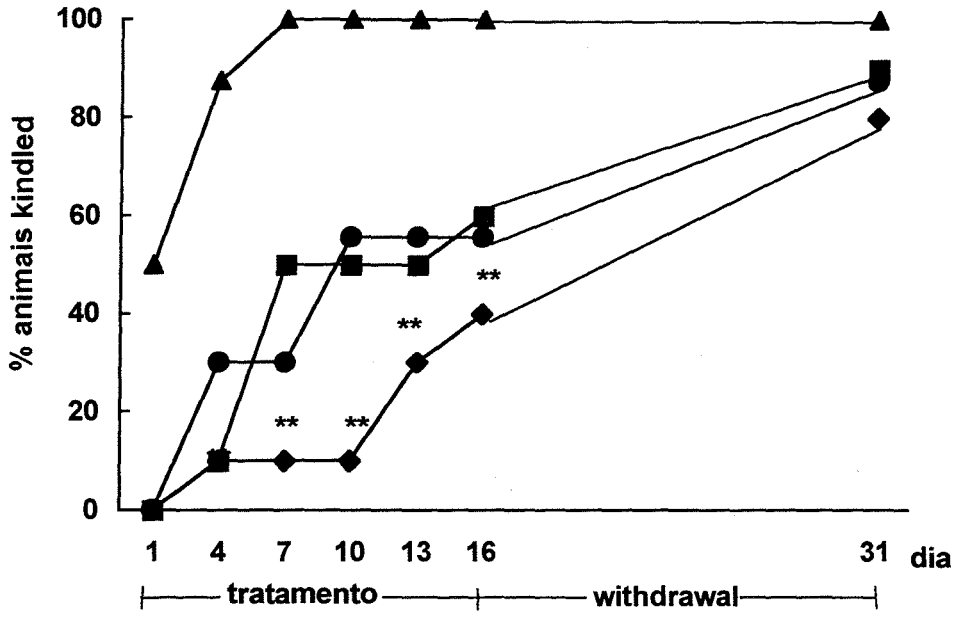
Estruturas químicas dos princípais constituintes do éleo essencial de *Aeolanthus suaveolens* .

Anexo 2



Sítios de ação do linalol na transmissão glutamatérgica.

ANEXO 3



Percentagem de animais em *status epilepticus* (kindled) do grupo salina (▲), linalol (2,2 g/kg) (■), linalol (2,5 g/kg) (●) e fenobarbital (15 mg/kg)(♦) durante o tratamento (1-16 dias) e após duas semanas de encerrado o tratamento (31° dia). ** $p < 0,01$, Teste de Fisher.