UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

UNIDAD ACADEMICA DE LOS CICLOS PROFESIONAL Y DE POSGRADO
COLEGIO DE CIENCIAS Y HUMANIDADES

INSTITUTO DE FISIOLOGIA CELULAR

"ACCION ANTICONVULSIVANTE DE ANTAGONISTAS DEL RECEPTOR NMDA EN LAS CRISIS CONVULSIVAS INDUCIDAS POR ADMINISTRACION DE 4-AMINOPIRIDINA"

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M. EN C. ALBERTO MORALES VILLAGRAN

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P R E S E N T A

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ACCION ANTICONVULSIVANTE DE ANTAGONISTAS DEL RECEPTOR NMDA EN LAS CRISIS CONVULSIVAS INDUCIDAS POR ADMINISTRACION DE 4-AMINOPIRIDINA

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RESUMEN

La 4-Aminopiridina (4-AP) es una droga que administrada sistémica o intracerebralmente ejerce un efecto convulsivante, e *in vitro* estimula la liberación de neurotransmisores de una manera inespecífica. En este trabajo se estudiaron los efectos biquímicos, conductuales y electrofisiológicos de la 4-AP en ratas de la cepa Wistar, así como el efecto protector de los antagonistas del receptor NMDA sobre las crisis convulsivas inducidas por la 4-AP.

En los estudios bioquímicos se realizaron experimentos en los que se administró la 4-AP a través de cánulas de microdiálisis en el núcleo caudado y se determinó el efecto de ésta sobre la liberación de aminoácidos, cuantificados por cromatografía de líquidos de alta eficiencia. Las perfusiones con 4-AP en el animal despierto produjeron una serie de síntomas cuya severidad fué proporcional a la dosis de 4-AP presente en el medio de perfusión. Las alteraciones se iniciaron con hiperexcitabilidad del animal, que progresaron hasta convulsiones tónico clónicas generalizadas y ocasionalmente la aparición de carreras súbitas. Por lo tanto, la mayoría de los experimentos de liberación de aminoácidos se realizaron con el animal anestesiado. Una dosis de 40mM de 4-AP se utilizó para los estudios en libre movimiento . Las perfusiones que contenían concentraciones entre 20 -75 mM de 4-AP durante 12.5 min resultaron en un aumento masivo de la concentración extracelular de glutamato y más pequeños en la concentración de aspartato y taurina. Solo se encontraron aumentos pequeños de los demás aminoácidos determinados:

glutamina, glicina, alanina y GABA. Por otro lado, la perfusión con una solución concentrada de potasio (100 mM) produjo sólo un aumento discreto en la concentración de glutamato y aspartato, así como una notable disminución en la glutamina. El tetraetilamonio (TEA), otro bloqueador de los canales de potasio, indujo incrementos en las concentraciones de los aminoácidos similares a las de la perfusión con concentraciones elevadas de potasio y en los animales despiertos ni la perfusión con alto potasio ni el TEA afectaron significativamente la conducta motora.

En cuanto a los estudios conductuales y electroencefalográficos, la administración de 4-AP en la corteza cerebral (i.cx) y en el ventrículo cerebral lateral (i.c.v.) produjo diferentes síntomas preconvulsivos, como hiperexcitabilidad, temblores, movimientos masticatorios y salivación, seguidos por convulsiones tónico-clónicas continuas y, después de la inyeccion i.c.v., aparecieron carreras súbitas y convulsiones tónico clónico generalizadas. El patrón conductual persistió por un período que osciló entre 100-150 min. La 4-AP también generó una serie de descargas epileptiformes caracterizadas por espigas aisladas, poliespigas, y complejos punta-onda, que comenzaron unos cuantos segundos después de la administración de 4-AP y permanecieron por más de 2 h (inyección i.c.x) o hasta por 6 h (inyeccion i.c.v.)

El uso de los antagonistas al receptor NMDA ácido (±)-3-(2-carboxi-piperazin-4-yl)-propil-1 fosfónico (CPP), (±) 2-amino-7- heptanoato (AP7) y MK-801 (maleato de dizocilpina), evitaron las alteraciones conductuales producidas por la

administración i.c.v. de 4-AP, particularmente las conductas más severas. Sin embargo, los antagonistas no tuvieron un efecto claro cuando la droga se inyectó i.cx.

Es interesante que tanto conductual como electroencefalográficamente, las crisis inducidas por 4-AP fueron más intensas en la administración i.c.v. que en la i.cx. y asimismo, los efectos protectores de los antagonistas del receptor NMDA. Esto sugiere que tanto la 4-AP y los antagonistas probados difunden más extensamente cuando se administra i.c.v. y alcanzan un mayor número de sinapsis en diferentes regiones del cerebro, mientras que la difusión se restringe cuando los compuestos se inyectan i.cx.

De estos resultados es posible concluir que la liberación excesiva de glutamato inducida por la 4-AP es un factor importante en el mecanismo de acción epileptogénica inducida por esta droga.

ABSTRACT

The systemic or intracerebral administration of 4-aminopyridine induces generalized convulsions. It is also known that 4-AP stimulates the release of neurotransmitters in a calcium dependent manner.

In this work the biochemical, behavioral and electroencephalographic (EEG) effects of 4-AP and protective action of the NMDA receptor antagonists against the seizures convulsions induced by this drug was studied. In biochemical studies the effect of 4-AP on the extracellular concentration of amino acids in rat striatum were evaluated by means of microdialysis and HPLC. The perfusion with 4-AP in awake animals produced behavioral alterations that were proportional to the concentration of the drug present in the medium. All animals showed a series of preconvulsive seizure, including excitability, tremors, salivation and general tonic convulsion, and wild running. Therefore, most microdialysis experiments were carried out in anesthetized rats, and just one dose (40mM) was used for studies in a freely moving animals. Perfusions with 20-75 mM 4-AP during 12.5 min resulted in significant increases of glutamate, aspartate and taurine and slight increments in glutamine, alanine, glycine and GABA.

The perfusions with a high potassium solution produced increases in taurine, glutamate and aspartate, as well as a notable reduction in glutamine. Tetraethylammonium, another potassium channel blocker induced similar effects to that of high potassium, but glutamine concentration was not changed. In awake animals neither high potassium nor tetraetylammonium affected significantly the

motor behavior.

With respect to the behavioral and electroencephalographic studies, the 4-AP administration in cerebral cortex (i.cx.) and lateral ventricle (i.c.v.) produced different preconvulsive symptoms, including excitability, tremors, head nodding, salivation, chewing and rearing, generalized tonic convulsions and occasionally wild running. The behavioral alteration remained for a period from 100-150 min. 4-AP also induced epileptiform discharges, even before the end of injection, and were characterized by high frecuency poly-spikes and spike-wave complex. After about 60 min the discharges were continuos for periods longer than 2 h (i.c.x.) or up to 6 h (i.c.v. injection).

Te use of NMDA receptor antagonists (±)-3-(2-Carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP), (±)-2-amino-7-phosphonoheptanoic acid (AP7) and MK-801 (Dizocilpine maleate), prevented the behavioral and EEG alterations induced by the 4-AP administration, both when administered i.c.v. or i.cx., particularly the most severe alterations. However, the antagonists did not have a clear effect when the drugs injected in the cerebral cortex.

It is interesting that, both behaviorally and electroencephalographically, the seizures induced by 4-AP were more intense when administered i.c.v. than i.cx., and that the former were more clearly antagonized than the later by the NMDA receptor antagonists. This suggests that both 4-AP and the antagonists diffuse extensively when injected i.c.v. and therefore reach a relative large number of synapses in several regions of the brain, whereas the diffusion is restricted when injected i.cx.

It is possible to concluded that excess of glutamate released by 4-AP plays the major role for the epileptogenic action of this drug.

LISTA DE ABREVIATURAS

ACh	Acetilcolina
t-ACPD	Trans-1-aminociclopentano-1,3-dicarboxilato
4-AP	4-Aminopiridina
AMPA	a- amino-3-hidroxi-5-metil-isoxasol-4-propionato
AP5	α-Amino-5-fosofonovalerato
AP7	(±)2-Amino-7-heptanoato
asp	Acido aspártico
CNQX	6-Ciano-7-nitroquinoxalin-2,3-diona
CPD	Cambio paroxístico despolarizante
CPH	Cambio paroxístico hiperpolarizante
CPP	(±)-3-(2-Carboxi-piperazin-4-yl)-propil-1-acido fosfónico
DA	Dopamina
DOM	Acido domoico
glu	glutamato
i.c.	Intracerebral
i.c.v.	Intracerebroventricular
i.cx.	Intracortical
i.p.	Intraperitoneal
IP_3	Trifosfato de inositol
KA	Acido kaínico

MK-801	Maleato de dizocilpina
NMDA	Receptor a N-metil-D-aspartato
no-NMDA	Receptores no NMDA
QA	Acido quiscuálico
SNC	Sistema nervioso central
TEA	Tetraetilamonio

ORGANIZACION DE LA TESIS.

Este trabajo está dividido en tres partes. La primera contiene una revisión sobre los aspectos básicos de la epilepsia, los modelos más utilizados para el estudio de esta enfermedad, así como una revisión actualizada de la literatura, acerca de la participación del glutamato (glu) en el proceso convulsivo, así como de los diferentes receptores para este neurotransmisor excitador. Igualmente se describen los datos más importantes que relacionan a la 4-aminopiridina (4-AP) con la excitabilidad neuronal y los antecedentes inmediatos que llevaron a la realización del presente trabajo.

En la segunda parte se presentan los resultados del trabajo experimental, que están contenidos en dos publicaciones; la primera se titula ***Preferential stimulation of glutamate release by 4-aminopyridine in rat striatum in vivo*** , (en prensa; Neurochemical International) la segunda se titula ***NMDA receptor antagonists protect against seizures induced by intracerebral administration of 4-aminopyridine*** (Se sometará a consideración para publicación a la revista European Journal of Pharmacology). En estos trabajos, se describe también la metodología empleada y se señalan la referencias pertinentes.

La tercera parte es una discusión general del trabajo realizado, que incluye conclusiones y perspectivas.

INTRODUCCION

La epilepsia

La epilepsia es una de las anormalidades neurológicas que más frecuentemente afectan a la población, ya que tiene una incidencia del 0.5 al 1% . Este dato no considera grupos que en algún momento sufren de crisis simples o secundarias a disturbios extracelulares, tales como la supresión súbita de alcohol, hipoxia-isquemia, disturbios electrolíticos, infecciones cerebrales y causas diversas (Fisher, 1982). El término se usa para caracterizar un conjunto de alteraciones conductuales y motoras como la aparición de crisis convulsivas parciales o generalizadas, o no convulsivas como las ausencias.

Fisiológicamente, la epilepsia se caracteriza por la aparición de una despolarización neuronal excesiva, que origina por un lado descargas neuronales de alta frecuencia y, por otro, cambios hiperpolarizantes compensatorios (inhibición) que tratan de regular esta descarga. Además, durante la epilepsia hay un reclutamiento anormal de un conglomerado neuronal al que se denomina foco epiléptico, lo que origina una hipersincronía de las descargas neuronales individuales. Así, la despolarización-hiperpolarización y las descargas neuronales excesivas e hipersincrónicas son la escencia de la actividad convulsiva (Velasco y col. , 1985). La generación de potenciales postsinápticos excitadores en las neuronas del foco epiléptico producen cambios paroxísticos despolarizantes (CPD), que son despolarizaciones repentinas y recurrentes que promueven la generación de

descargas electroencefalográficas, las cuales son seguidas por un período de hiperpolarización o cambio paroxístico hiperpolarizante (CPH) antes de que vuelva a iniciarse un nuevo CPD. La proporción y secuencia alterna entre el CPD y el CPH dan como consecuencia la generación de espigas electroencefalográficas locales en el foco epiléptico y la generación de impulsos que se propagan a otros sitios del cerebro y a los músculos, donde originan respectivamente actividades epileptiformes a distancia y contracciones musculares espasmódicas. Bioquímicamente, existen datos que permiten asociar alteraciones en los diferentes sistemas de neurotransmisión del sistema nervioso central (SNC) con la epilepsia, y que pueden resumirse como un desbalance entre la excitación y la inhibición. Mediante diferentes modelos experimentales de epilepsia se han demostrado alteraciones principalmente en la transmisión sináptica GABAérgica, colinérgica, noradrenérgica y glutamatérgica (Jobe and Laird, 1987). Una disminución en la transmisión GABAérgica y noradrenérgica, o exacerbaciones en la colinérgica y glutamatérgica, parecen ser la causa de crisis en diferentes modelos de epilepsia.

Por otro lado, los núcleos neuronales del SNC no son igualmente susceptibles a los procesos convulsivos, por lo que las alteraciones en los neurotransmisores deben relacionarse con las diferentes estructuras neuronales para entender mejor el fenómeno convulsivo.

Modelos de epilepsia experimental

La limitante natural que existe para estudiar la epilepsia en humanos mediante técnicas invasivas o ensayos farmacológicos, ha propiciado el desarrollo de diversos modelos experimentales de crisis epileptiformes que semejen a la epilepsia en el humano. Existen múltiples preparaciones tanto *in vitro* como *in vivo* que se utilizan para producir crisis convulsivas, las cuales pueden ser recurrentes, episódicas y asociadas con un patrón electroencefalográfico anormal, repetitivo y de alto voltaje y controlables con anticonvulsivantes clínicamente efectivos.

Para el establecimiento de los modelos de epilepsia se han seguido diferentes estrategias. Entre los primeros intentos se han determinado algunas consecuencias neuroquímicas de un estímulo que resulte en crisis convulsivas. Otra manera de estudiar el fenómeno ha sido mediante el estudio de los efectos de drogas antiepilépticas sobre los sistemas de neurotransmisores, y valorar si las alteraciones producidas por estas drogas pudieran reflejar la participación de dichos sistemas. Otra opción es el estudio de animales genéticamente epilépticos, en los cuales se determina si existen alteraciones en los diferentes sistemas de neurotransmisión.

La existencia de un gran número de modelos de epilepsia se debe a que ninguno de ellos es lo suficientemente preciso en la reproducción de la epilepsia clínica. Sin embargo, y a pesar de las limitaciones de muchos modelos, han aportado información valiosa sobre la participación de los diferentes sistemas de neurotransmisión, así como la de los núcleos neuronales que más se han

relacionado con los mecanismos de iniciación, propagación y/o extinción del proceso convulsivo.

A la fecha existen diferentes modelos de epilepsia experimental (≈50) que pueden agruparse de la siguiente manera (Fisher, 1989):

Modelos de crisis parciales simples, agudas

a) Aplicación local de penicilina

b) Estimulación eléctrica aguda

c) Síndrome de abstinencia al GABA

Modelos de crisis parciales simples, crónicas

a) Administración de gel de hidróxido de aluminio

b) Cobalto, zinc, y hierro

c) Daño criogénico

d) Epileptogénesis sistémica focal

Modelos de crisis parciales complejas

a) Administración de ácido kaínico

b) Administración de toxina tetánica

c) Inyección de convulsivantes en el área tempestas

d) Producción de *kindling*

Modelos de crisis tónico clónicas generalizadas

a) Producción de crisis en monos fotosensibles (*Papio papio*)

b) Inducción de crisis por estimulación auditiva a ratones

c) Estudio de ratas genéticamente propensas a la epilepsia

d) Estudio del roedor *Mongolian gerbil* .

e) Producción de crisis por choque eléctrico

f) Aplicación sistémica de convulsivantes: pentilentetrazol, bicuculina, picrotoxina, glutamato monosódico, 4-aminopiridina (4-AP), etc..

Modelos de ausencia generalizada

a) Estimulación talámica

b) administración sistémica de Penicilina

Modelos de estado epiliéptico

a) Administración de litio-pilocarpina

b) Estimulación límbica

c) Aplicación tópica de cobalto, con administración sistémica de ácido homocisteico.

ANTECEDENTES

El gutamato en el proceso convulsivo

El glu está distribuído de manera ubicua en el SNC, ocupa una posición central en el metabolismo del cerebro y ha sido objeto de numerosos estudios en los últimos años. La actividad excitadora del glutamato se demostró inicialmente en el músculo de crustáceos por Kravitz y col. (1970) y posteriormente en el cerebro de mamíferos por administración tópica de este compuesto (Cooper y col. 1982). El glu y otro aminácido con estructura química muy similar, el aspartato (asp) actúan sobre diferentes receptores específicos y particularmente el primero ejerce un papel importante sobre la excitabilidad neuronal.

Se piensa que estos compuestos median la neurotransmisión excitadora rápida en el SNC en condiciones normales (Engelsen, 1986; Fagg y Foster, 1983; Fonnum, 1984) y se les ha relacionado con los mecanismos de iniciación y propagación de crisis convulsivas (Pireda y Gale, 1986; Geddes y col., 1990 y Faingold, 1992).

La participación del glu en la epilepsia puede considerarse desde dos puntos de vista: la producción de crisis por glutamato o bien la de glutamato por crisis. Con relación al primer postulado existe evidencia sustancial de que la sobreactivación de receptores a aminoácidos excitadores (particularmente el receptor a NMDA) en el SNC puede inducir crisis convulsivas. La administración de glu o de agonistas del

receptor NMDA en ciertas áreas del cerebro producen crisis convulsivas (Croucher y Bradford, 1989; Stone y Javid, 1983; Piredda y Gale, 1986; Mc Allister, 1993), mientras que el bloqueo de estos receptores protege contra las convulsiones en diferentes modelos de inducción de crisis (Akaike y Himori, 1993; Dingledine, 1990; Faingold, 1992; Fragoso-Veloz y Tapia, 1992; Meldrum, 1992 y McAllister, 1993).

Con respecto a la producción de glutamato por crisis, la primera evidencia fué reportada por Van Gelder y col. (1980) y Janjua y col. (1989), quienes describieron niveles elevados de glu en el plasma de pacientes con epilepsia primaria generalizada. En estudios más recientes, se ha encontrado que la concentración de glu está significativamente elevada en el líquido cefalorraquídeo de pacientes epilépticos (Kälviäinen y col., 1993). Asimismo, mediante estudios realizados a través de microdiálisis intracerebral en pacientes con epilepsia intratable, se observó un aumento considerable en la concentración del glu en el foco epiléptico durante las crisis (During y Spencer, 1993). De estos resultados surge la duda de si este aumento en la concentración de glu está asociado a la liberación sináptica o al recambio metabólico, ya que sólo una mínima parte del contenido total se relaciona con la transmisión sináptica (Prusiner, 1981). Sin embargo, existen estudios en modelos experimentales de epilepsia en los que se muestra que la concentración elevada de glu se puede relacionar con los procesos convulsivos, por ejemplo, la liberación de glu aumenta en la neocorteza de gato en la región del foco epiléptico que se induce mediante la administración de cobalto (Dodd y Bradford, 1976; Dodd y col., 1980 y Koyama, 1972). Asimismo, esta liberación es en cierto modo

proporcional a la intensidad de las espigas (Koyama, 1972). De igual manera, el glu está también relacionado con descargas epileptiformes en la corteza cerebral aislada del gato (Koyama y Jasper, 1977).

Por otro lado, Lehman (1987) identificó un aumento significativo en la concentración extracelular de glu, medido por microdiálisis en el hipocampo, después de inducir crisis por administración intraamigdalar de folato. En el modelo de inducción de crisis convulsivas por administración de pilocarpina, Millan y col. (1993), determinaron que la concentración extracelular de asp y glu en el hipocampo, medida por microdiálisis, aumentaron en en porcentajes significativos (alrededor de 150-180 %), por efecto de la administración de esta droga.

En contraste, en algunos estudios en los que se indujeron crisis con ácido kaínico (KA) o bicuculina en conejos, se encontró poco o ningún cambio en la concentración de glu (Lehman y col., 1985). Similarmente, Bruhn y col. (1993) encontraron que en crisis inducidas por la administración de KA en la amígdala, la concentración extracelular de glu en el hipocampo no se alteró incluso en períodos de actividad convulsiva intensa.

Receptores a aminácidos excitadores

Las diferentes acciones del neurotransmisor glu, que van desde la acción excitadora rápida hasta la regulación de los procesos del desarrollo, se llevan a cabo a través de la activación de diferentes clases de receptores (Seeburg , 1993).

Los receptores a glu pueden clasificarse en dos grupos: el primero forma un canal que permite el paso de iones, por lo que se les denomina receptores ionotrópicos. Los canales de estos receptores poseen características diferenciales de activación e inactivación, así como diferentes conductancias y permeabilidades a los iones. El segundo tipo de receptores, denominado metabotrópicos se acopla a una proteína G, que activa a una fosfolipasa C, la cual produce segundos mensajeros como el diacilglicerol y trifosfato de inositol (IP_3), que a su vez generan diversos procesos bioquímicos y fisiológicos. La activación de estos receptores posiblemente se relaciona con fenómenos de plasticidad sináptica (Sladeczek y col., 1988).

El primer tipo de receptores ionotrópicos se ha subdividido en dos categorias:

a) Receptores a N-metil-D-aspartato (NMDA)

b) Receptores no-NMDA, (AMPA y KAINATO) (Watkins y col.,1990).

Esta clasificación se realizó con base en las características farmacológicas

Mediante técnicas de secuenciación y clonación, se ha logrado identificar la estructura básica de los diferentes receptores a glu. Hollmann y col., (1989) clonaron una subunidad proteica y funcional de un receptor a glu, denominada GluR1, y desde entonces se han clonado otras 27 en diferentes especies (Hollman y Heinemann, 1994). Los genes que codifican para estas cadenas se agrupan en diferentes familias y conforman la gama extensa de receptores a glu y no todos corresponden a genes diferentes sino que algunas resultan de modificaciones postranscripcionales (ver la siguiente fig).

SUBUNIDADES PROTEICAS DE LOS RECEPTORES AL GLUTAMATO

RECEPTOR	SUBUNIDADES
NMDA	NR1 NR2A-NR2D
AMPA	GLUR1-GLUR4
KAINICO baja afinidad	GLUR5-GLUR7
KAINICO alta afinidad	KA1 KA2
METABOTROPICO	mGLUR1-mGLUR6
GLUTAMATO	DGLURI DGLURII drosofila LYmGLUR serpiente
"ORPHAN"	DELTA 1 DELTA 2
OTROS	
PROTEINAS QUE UNEN GLUTAMATO	GBP
PROTEINAS QUE UNEN KAINICO	KBP-pollo KBP-rana

Los receptores ionotrópicos a glu se forman por el agregado de cinco subunidades proteicas. El receptor NMDA posee características funcionales diferentes de otros receptores a glu, como una desensibilización rápida y una modulación negativa por Mg^{++} en concentraciones fisiológicas, que depende del voltaje (Davis y Watkins, 1977; Mayer y col., 1984) por lo que parece ser funcional sólo cuando la membrana se despolariza. La activación de este receptor no solo permite la entrada de sodio sino también y principalmente de Ca^{++} (McDermott y col., 1986), que puede iniciar diferentes procesos bioquímicos y fisiológicos. Asimismo, se modula positivamente por la glicina, que funciona como coagonista de este receptor. (Johnson y Ascher, 1987). Tiene además otros sitios de unión: el de las poliaminas, cuya acción puede ser positiva o negativa sobre la actividad del receptor; el del Zn^{++}, que posiblemente regula el tiempo y probabilidad de apertura del canal, efectos que son independientes del voltaje y que se registran en concentraciones similares a las requeridas para la inhibición de la respuesta a NMDA (Christine y Choi; 1990;Westbrook, 1987); y recientemente, se descubrió otro sitio de unión al que se une el glutatión con modulación de oxido reducción (Sucher y Lipton, 1991).

Molecularmente, el receptor a NMDA puede ser reconstituído como una entidad heteromérica formada de dos tipos de subunidades, la subunidad NR1, clonada por primera vez por Moriyoshi y col. (1991) y otra, del grupo de subunidades NR2A-NR2D (Kutsuwada y col., 1992; Monyer y col., 1992). La subunidad NR1 puede presentarse en diferentes formas alternas de expresión o de modificación

postranscripcional que le proporcionan propiedades farmacológicas muy particulares (Hollmann M. y col., 1993). La subunidad está formada por un polipéptido de 938 aminoácidos y a pesar de su baja similitud con los receptores no-NMDA (25-29%), presenta una estructura muy similar a la de canales iónicos ligados al receptor, como son: una región extracelular grande en el amino terminal, con varios sitios de glucosilación, 4 segmentos hidrofóbicos (TMI-TMIV), una región intracelular grande entre los segmentos TMIII y TMIV y otro segmento extracelular en el carboxilo terminal. Una característica distintiva en comparación con los receptores AMPA/KA, es la presencia de un determinante funcional en el segmento TMII (que se encuentra parcialmente inmerso en la mebrana en forma de horquilla), poseé una asparagina en lugar de glutamina, que le confiere la característica de permeabilidad al Ca^{++} y bloqueo por Mg^{++} (Burnashev y col., 1992; Sakurada y col., 1993). Este determinante también lo poseen las subunidades NR2. La subunidad NR1 puede autoensamblarse, y cuando se expresa en ovocitos de *Xenopus* , muestra las características y propiedades del canal del receptor a NMDA. Sin embargo, cuando se ensambla con otra subunidad NR2, las corrientes activadas por glutamato y NMDA se incrementan en varios órdenes de magnitud, por lo que parece ser que la entidad heteromérica funciona con una mejor eficiencia. Es importante señalar que los homómeros de las subunidades NR2 no parecen formar canales-receptor por sí mismos, por lo que a estas subunidades se les puede considerar como moduladoras, ya que el receptor a NMDA muestra distintas características según la subunidad NR2 a la que se ensamble NR1.

La estimulación excesiva de receptores a glu, particularmente la del tipo NMDA, produce hiperexcitación que conduce a diversas alteraciones patológicas. A este fenómeno se le denomina excitotoxicidad, término que fué acuñado por Olney (1969).

Dentro los receptores no-NMDA, se encuentra el receptor a AMPA, denominado así por los efectos farmacológicos que tiene el principal agonista, el ácido α-amino-3-hidroxi-5-metil-4-isoxasol propiónico (AMPA). Este compuesto activa canales con una cinética rápida y en la mayoría de las neuronas estos canales poseen una baja permeabilidad al Ca^{++} y alta al Na^{+} y al K^{+} (Iino y col., 1990). Farmacológicamente, estos receptores se activan por otros agonistas del glu, como son principalmente los ácidos quiscuálico (QA), domoico (DOM) y el kaínico.

Mediante estudios de unión, se ha encontrado que tanto el AMPA como el KA activan al mismo receptor, aunque dada la distribución de los sitios de unión para KA y AMPA en el cerebro, se llegó a la conclusión de que los receptores a AMPA y KA son entidades diferentes (Monagahan y col., 1989). Los receptores a AMPA, al igual que los NMDA, se encuentran ampliamente distribuídos en el SNC (Monaghan y col., 1984b; Nielsen y col., 1988, 1990) y parecen mediar la despolarización rápida en la mayoría de las sinapsis excitadoras del SNC.

Los canales receptores a AMPA pueden ser reconstituídos por expresión o coexpresión de las subunidades denominadas GluR1-GluR4. Estas subunidades están formadas por aproximadamente 900 aminoácidos y existen dos formas que se generan por un corte postranscripcional: "flip" y "flop".

Presentan características de un receptor canal como el receptor de acetilcolina (Ach), de GABA y de Glicina. Al igual que el receptor NMDA, presentan 4 regiones hidrofóbicas. La subunidad GluR2 juega un papel importante con respecto a su contribución al receptor AMPA, ya que en entidades heteroméricas determina las propiedades características del receptor natural, como son la linearidad en la relación corriente-voltaje. De manera similar a las subunidades del receptor NMDA, en el segundo segmento hidrofóbico de esta subunidad existe un determinante funcional (arginina) que disminuye la permeabilidad al Ca^{++}. Si se introduce este aminoácido en las subunidades GluR1,GluR3 y GluR4 (que poseen glutamina), se produce un canal con una relación lineal corriente -voltaje y disminuye la permeabilidad al Ca^{++} (Seeburg, 1993; Hollman y Heinemann, 1994).

Otro receptor ionotrópico es el receptor a KA, que aunque como se mencionó anteriormente se parece mucho al receptor a AMPA, es capaz de inducir respuestas independientes de las mediadas por éste. Es permeable al Na^+ y al $K+$ pero no al Ca^{++}, aunque las respuestas producidas por los agonistas son muy complejas. Las subunidades GluR5-GluR7 forman receptores de baja afinidad a KA, mientras que los de alta afinidad se forman por la subunidades KA1 ó KA2. Mediante estudios de hibridización *in situ* , se ha encontrado que estas 5 subunidades forman un mosaico de expresión complejo en el cerebro de rata, lo cual indica que este receptor está involucrado en todas la redes neuroanales del SNC. Las corrientes producidas por agonistas de este receptor se observan en las conformaciones homoméricas de las subunidades GluR5 y GluR6, más no con GluR7, y la expresión de las subunidades

KA1 ó KA2 solas no genera canales funcionales (Werner y col., 1991; Herb, 1992 y Sakimura y col., 1992). Sin embargo, cuando se combinan con las subunidades GluR5 ó GluR6 forman receptores con propiedades que se diferencian de las formadas por los homómeros con las subunidades GluR5 ó GluR6.

RECEPTOR METABOTROPICO

La primera evidencia de que un receptor a glu está acoplado a segundos mensajeros y no sólo a canales iónicos que median despolarizaciones rápidas de la membrana, se obtuvo de los estudios realizados por Sladeczek y col. (1985). En estos estudios se observó que el QA y el glu estimularon el recambio del IP_3 en cultivos de neuronas del estriado de ratón, mientras que el NMDA y el KA fueron menos efectivos. Posteriormente Sugiyama y col. (1985), proporcionaron una evidencia directa que asoció la respuestas producidas por el glu, con la movilización de Ca^{++}, que se induce por el IP3.

Farmacológicamente el receptor metabotrópico es distinto de los otros receptores a los aminoácidos excitadores. El QA y glu son agonistas, mientras que el NMDA y el AMPA no lo son y no responde a antagonistas como el 2-amino-5-fosofonovalerato (AP5) o al 6-ciano-7-nitroquinoxalin-2,3-diona (CNQX). Aunque el trans-ACPD no produce la respuesta más potente, parece ser el más específico como agonista para los recpectores metabotrópicos.

Houamed y col., 1991 y Masu y col., 1991, mediante técnicas de clonación caracterizaron el primer receptor metabótropico mGluR1. Este receptor no tiene secuencia similar a la de otros receptores que se acoplan a proteínas G, por lo que se

considera a los receptores metabotrópicos como una clase nueva de proteínas que inducen la producción de segundos mensajeros.

Houamed y col., 1991; Masu y col., 1991; Abe y col., 1992; Nakanishi, 1992; Tanabe y col., 1992; Nakajima y col., 1993; Okamoto y col., 1994 , a través de técnicas similares de biología molecular descubrieron una familia de genes relacionados, a los que se denominó consecutivamente mGluR2-mGluR7, y recientemente se descubrio otro receptor denominado mGluR8 (Duvoisin y col., 1995). Los 8 receptores son proteínas de entre 854 y 1170 aminoácidos, formadas por segmento amino terminal de ≈ 550 residuos extracelular, otro de 7 regiones transmembranales, características de las proteínas receptoras acopladas a una proteína G, y un dominio carboxilo terminal intracelular en el cual existen numerosos sitios para fosforilación.

4-Aminopiridina y sus efectos en la transmisión sináptica

La 4-AP es una droga que cuando se administra por vía intraperitoneal (i.p.) intracerebral (i.c.) es capaz de inducir crisis convulsivas en diferentes especies de animales, tales como ratones (Pasantes-Morales y Arzate, 1981; Tapia, 1982), ratas (Pasantes-Morales y col. 1987), perros, pollos, caballos e inclusive el hombre (Glover, 1982; Milhaly y col. 1990; Spyker y col. 1980).

El patrón de crisis convulsivas inducido por administración i.p. de 4-AP en la rata, es muy similar al que se produce por administración i.c. de KA (Ben-Ari y col.,

1985), caracterizado por la aparición de un período convulsivo largo, con una etapa inicial de hiperexcitación, seguido de convulsiones clónicas, convulsiones tónico clónicas y muerte durante una convulsión tónica en algunos casos, mientras que los animales que sobreviven presentan movimientos clónicos alternados frecuentemente con contracciones tónicas de mediana intensidad (Fragoso-Veloz y col., 1990). De manera similar a la administración sistémica, la 4-AP ejerce un poderoso efecto convulsivante cuando se administra intracerebralmente, ya sea en el hipocampo (Fragoso-Veloz y col., 1990) o en el ventrículo cerebral lateral de la rata (Gandolfo y col, 1989).

Los efectos epileptogénicos de la 4-AP han sido estudiados en rebanadas cerebrales in vitro. La aplicación de 4-AP en preparaciones de hipocampo produce actividad epileptiforme interictal en neuronas del área CA3 (Voskyul y Albus, 1985). De manera similar, Gean (1990) mostró que la 4-AP es capaz de inducir descargas epileptiformes en neuronas de la amígdala cerebral de rata.

En preparaciones de la placa neuromuscular, la 4-AP se ha utilizado como herramienta para estudiar la función sináptica colinérgica, y se ha encontrado que este compuesto aumenta los potenciales postsinápticos excitadores (Lundh y col. 1976; Molgo y col 1977), la excitabilidad eléctrica de la membrana y la actividad mecánica espontánea (Arzate y col., 1984) y prolonga la duración del potencial de acción (Van Bogaert y Snyders, 1982). Mediante estudios bioquímicos y farmacológicos en estas preparaciones se encontró que la 4-AP potenció la liberación de Ach inducida por estimulación eléctrica (Kim y col., 1980). Este compuesto

elimina el efecto inhibidor de la toxina botulínica (Lund y col.,1977) y de algunos antibióticos sobre la liberación de este neurotransmisor (Uchiyama y col,1981), inhibe ligeramente la actividad de la acetilcolinesterasa (Davidson y col., 1988) y ejerce un efecto potenciador de la recaptura de colina (Buyukuysal y Wurtman, 1989). Algunos estudios *in vivo* permitieron confirmar el efecto estimulador que posee la 4-AP sobre la transmisión colinérgica en la unión neuromuscular, ya que la parálisis flácida inducida por rojo de rutenio se antagoniza efectivamente por la adminstración de 4-AP (Tapia, 1982).

En preparaciones cerebrales tales como sinaptosomas de ratón, produce un aumento en la liberación espontánea de GABA, glu y Ach de una manera dependiente de Ca^{++} (Tapia y Sitges, 1982; Tapia y col., 1985), mientras que en rebanadas y sinaptosomas de estriado induce un aumento sobre la liberación espontánea de dopamina (DA) y Ach (Arzate y col., 1986; Dolezal y Tucek, 1983). La aplicación intraestriatal de 4-AP a través de cánulas de microdiálisis, induce aumentos similares en la liberación de DA y Ach (Damsma y col., 1988).

Los estudios *in vitro* han permitido observar que el efecto de la 4-AP sobre la liberación de neurotransmisores es de amplio espectro, es decir, independiente de la sustancia mediadora, tipo de sinapsis o especie (Thesleff, 1980). Mediante estudios electrofisiológicos se ha demostrado que la 4-AP bloquea la corriente transitoria de potasio (I_A) y/o de inactivación lenta (I_D), lo cual conduce a una prolongación del potencial de acción a través de un retardo en la fase de repolarización membranal, fenómeno que mantiene los canales de Ca^{++} sensibles al voltaje abiertos (Rogawski y

Barker, 1983) y que podrían explicar la inducción del aumento en la liberación de neurotransmisores. Sin embargo, existen evidencias que demuestran que la 4-AP aumenta la corriente de Ca^{++} (Agoston y col., 1983; Burley y Jacobs, 1981; Gibson y Manger, 1988), lo que podría regular la actividad de ciertas proteínas de la membrana involucradas en la liberación de neurotransmisores (Heemskerk y col., 1987).

OBJETIVOS:

Con base en los antecedentes que relacionan al glu en la participación de los fenómenos convulsivos, fueron planteados los siguientes objetivos con el propósito de evaluar la participación del glu en las crisis convulsivas inducidas por la administración de 4-AP.

OBJETIVO GENERAL: Determinar la participación del glutamato en las crisis convulsivas inducidas por 4-AP.

OBJETIVOS PARTICULARES:

1.-Evaluar el efecto de diferentes concentraciones de 4-AP sobre la liberación endógena de aminoácidos transmisores y particularmente de glutamato en el núcleo caudado del cerebro de rata, mediante el uso de la técnica de microdiálisis y cromatografía de líquidos de alta eficiencia.

2.- Determinar las alteraciones conductuales y electroencefalográficas producidas por la administración de 4-AP en el núcleo caudado, corteza cerebral y ventrículo lateral.

3.- Evaluar el efecto anticonvulsivante de los antagonistas al receptor tipo NMDA, sobre las alteraciones conductuales y electroenefalográficas producidas por la administración de 4-AP en las regiones antes mencionadas.

RESULTADOS

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PREFERENTIAL STIMULATION OF GLUTAMATE RELEASE BY 4-AMINOPYRIDINE IN RAT STRIATUM *IN VIVO*

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Abstract—The potassium channel blocker 4-aminopyridine (4-AP) is a potent convulsant drug which, *in vitro*, stimulates the release of neurotransmitter amino acids. We have studied the effect of 4-AP *in vivo* on the extracellular concentration of amino acids in rat striatum, by means of microdialysis and HPLC. Perfusion with 4-AP in the awake animal produced intense motor alterations, including barrel turning and running fits. Therefore, most microdialysis experiments were carried out in anesthetized rats. Perfusion with 20–75 mM 4-AP for 12.5 min resulted in a massive increase in extracellular glutamate (up to 20-fold), smaller increases in aspartate and taurine (up to 10-fold) and slight increments in glutamine, alanine, glycine and GABA. In contrast, perfusion with 100 mM K^+ produced, mainly, an increment in taurine (7-fold) and modest increases in glutamate and aspartate (100–300%), as well as a notable decrease in glutamine. Tetraethylammonium (TEA, 120 mM) perfusion induced taurine and glutamate elevations similar to those after high K^+, but glutamine was not affected. In unanesthetized rats, perfusion with 40 mM 4-AP induced changes in extracellular amino acids similar to those observed under anesthesia. In these animals neither high K^+ nor TEA affected significantly the motor behavior. The results suggest that an enhancement of glutamatergic synaptic transmission, rather than a general depolarizing action, is an important factor in the neuronal hyperexcitability induced by 4-AP, which is consistent with the previously demonstrated inhibition of its convulsant effect by glutamate receptor antagonists.

The systemic administration of 4-aminopyridine (4-AP) induces generalized tonic convulsions in man and other species (Schafer *et al.*, 1973; Fragoso-Veloz *et al.*, 1990), and the microinjection of the drug in the rat substantia nigra reticulata (Tapia and Flores-Hernández, 1990) or the CA1 region of the hippocampus (Fragoso-Veloz *et al.*, 1990; Fragoso-Veloz and Tapia, 1992) produces intense motor alterations, including circling behavior, limbic type seizures and wet-dog shakes.

It is also known that 4-AP stimulates the release of neurotransmitters in several preparations, including the neuromuscular junction (Thesleff, 1980), brain slices (Dolezal and Tucek, 1983; Hu *et al.*, 1991) and synaptosomes (Tapia and Sitges, 1982; Tapia *et al.*, 1985; Tibbs *et al.*, 1989). Among the neurotransmitters released by 4-AP is glutamate, and in view of the well established role of excitatory amino acid synapses in convulsive and excitotoxic mechanisms (Dingledine *et al.*, 1990), it is possible that the excess glutamate released may be involved in the excitatory effects of the drug. In support of this hypothesis, it has been found that *N*-methyl-D-aspartate (NMDA) receptor antagonists notably protect against both seizures produced by the i.p. injection of 4-AP and wet-dog shakes induced by the intrahippocampal microinjection of the drug (Fragoso-Veloz and Tapia, 1992). Furthermore, non-NMDA receptor antagonists block the 4-AP-induced epileptiform burstings in amygdala (Gean *et al.*, 1990) and hippocampus (Perreault and Avoli, 1991) slices.

One important and missing point in the above hypothesis is the study of glutamate release under the action of 4-AP *in vivo*, and this was the aim of the present investigation. The effect of 4-AP on the extracellular concentration of glutamate and other amino acids was studied in the striatum of awake and anesthetized rats, using a microdialysis procedure. The striatum was chosen because it receives abundant glutamatergic fibers from the cerebral cortex and because its comparatively large size ensures that the amino

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acids collected in the dialysis perfusate arose only from that region. For comparative purposes and to gain information on the possible mechanism of action of 4-AP, the effect of a depolarizing potassium concentration and of tetraethylammonium (TEA), another K^+ channel blocker, was also studied.

EXPERIMENTAL PROCEDURES

Before implantation, each microdialysis cannula (exposed membrane 0.5 mm diameter and 2 mm length, obtained from BAS, West Lafayette, IN, U.S.A.) was flushed with distilled water for 30 min at a flow rate of 5 μl/min. The recovery of 4-AP from the dialysis membrane was assessed by perfusing probes (2 μl/min) immersed in standard solutions of 4-AP in Krebs–Ringer medium, and measuring spectrophotometrically the concentration of the drug in the perfusate (Biessels and Agoston, 1987). The calculated recovery of 4-AP was 11.3% ± 0.28 ($n = 3$).

Adult male Wistar rats (195 ± 5 g) were used. They were handled in accordance with the Rules for Research in Health Matters (Mexico), with approval of the local Animal Care committee. Animals were anesthetized with halothane in oxygen and secured in a Kopf stereotaxic frame with the incisor bar positioned at −3.3 mm. Microdialysis cannulas were implanted into the right striatum (A ±0.8 mm from bregma, L 2.8 and V 5.1 from dura; Paxinos and Watson, 1986) and the rats were maintained under low anesthesia (0.5% halothane) throughout the experiment, except in some animals, as described below.

The implanted cannulas were perfused with an oxygenated Ringer–Krebs medium of the following composition (in mM): NaCl, 118; K_2PO_4, 1.25; KCl, 4.0; $MgSO_4$, 1.17; $NaHCO_3$, 25; $CaCl_2$, 2.2; glucose, 10; pH 7.4. The perfusion rate was 2 μl/min, using a BAS microinjection pump. When a high potassium (100 mM) or magnesium (20 mM) concentration was tested, or when 4-AP or TEA was added to the medium, the NaCl concentration was reduced in order to maintain isoosmolarity. The 20 mM Mg^{2+} medium was Ca^{2+}-free and was used for assessing the Ca^{2+}-dependency of the effects. After a 60 min equilibration period, 25 μl (12.5 min) consecutive fractions of the perfusate were continuously collected. The first three fractions were used to determine the basal extracellular levels of the amino acids, and the medium was then changed for the experimental medium to be tested, which was perfused during the time periods indicated in Results.

The amino acid content of the 25 μl perfusate fractions was determined by HPLC after *o*-phthaldialdehyde derivatization, as previously described (Salazar *et al.*, 1994; Massieu *et al.*, 1995). The recovery of amino acids from the dialysis probes varied from 6.2% for glutamate to 11.1% for taurine, as previously reported (Massieu *et al.*, 1995). Statistical analyses were carried out by ANOVA and group comparison *post hoc* tests.

In some experiments the microdialysis perfusion was carried out in freely moving rats, using the BAS setup designed for this purpose. The implanted cannulas were cemented to the skull, the anesthesia was discontinued and drugs were perfused in the awake animals. Due to the motor effects of 4-AP perfusion, microdialysis under these conditions was carried out only with a 40 mM concentration of the drug and the animals had to be manually restrained during the most intense periods of motor abnormalities.

RESULTS

Behavioral observations

Perfusion with 4-AP (75 mM during one 12.5 min period) in unanesthetized animals produced a variety of severe motor alterations, including hyperactivity, frequent rearing, intense ipsilateral barrel turning, salivation, jumps followed by running fits and tonic–clonic convulsions, which lasted for at least 3 h and were more intense between 40 and 80 min after drug administration. Lower doses of 4-AP (10–50 mM for 12.5 min) produced less intense motor alterations, and convulsions did not occur. In contrast, when 120 mM TEA was perfused during 12.5 min only occasional rearing and barrel turning occurred and these effects disappeared in less than 35 min, and perfusion with the high potassium medium during two consecutive 12.5 min periods did not induce any behavioral alteration. In view of these behavioral effects, in most experiments the microdialysis procedure was carried out in anesthetized rats, as described under ???

EXPERIMENTAL PROCEDURES

Extracellular amino acids

The concentration of the amino acids in the striatum dialysate was similar in the first three basal fractions collected and therefore their values were averaged. With the notable exception of glutamate, the extracellular basal levels in the anesthetized ($n = 5$) and awake animals ($n = 4$) were very similar. Values were as follows, without correction for recoveries from the dialysis membrane (μM, means ± SEM; the first figure refers to the anesthetized rats): aspartate, 0.42 ± 0.08 and 0.31 ± 0.03; glutamate, 0.55 ± 0.01 and 8.1 ± 0.57; glutamine, 12.7 ± 1.21 and 14.3 ± 0.46; glycine, 2.65 ± 0.23 2.74 ± 0.11; taurine, 1.5 ± 0.14 and 2.5 ± 0.01 and alanine 2.57 ± 0.16 and 2.4 ± 0.01; basal GABA concentration was too low to be reliably detected under both experimental conditions.

Preliminary experiments showed that perfusion with a 100 mM KCl medium during one fraction period (12.5 min) produced only slight changes in the concentration of some amino acids, and that clear effects were observed when the K^+-depolarization was extended to two fraction periods. In order to compare, in the same animal, the effect of 4-AP with that of K^+-depolarization, the experimental paradigm used was to perfuse with a high potassium medium for 25 min and, after the amino acids had returned to basal levels, to perfuse with a 4-AP containing medium during one fraction period (Fig. 1). In some experiments this procedure was reversed (i.e. 4-AP medium was perfused before the high potassium medium), and the results were very similar to those described below, indicating that previous K^+-depolarization does not modify the action of 4-AP nor vice versa.

Results are shown in Figs 1–3 as percent of the basal

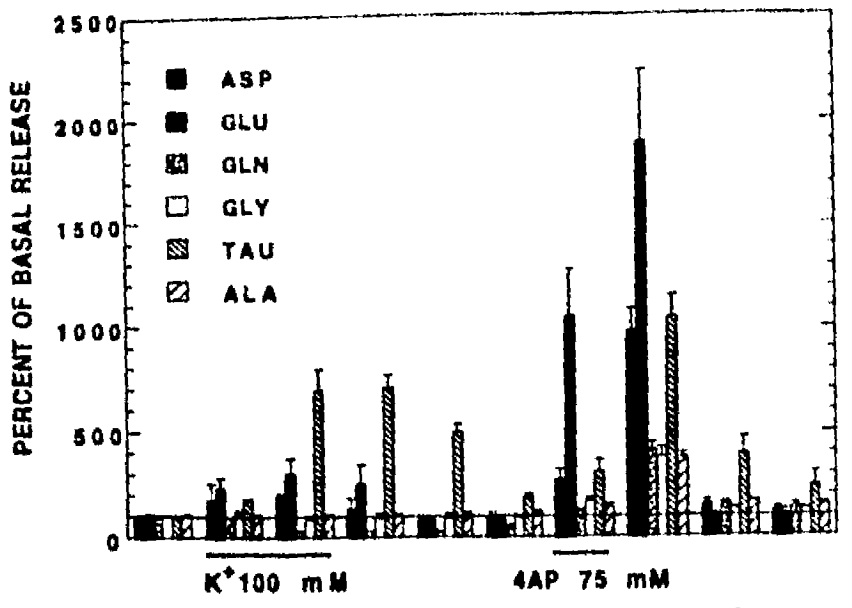

Fig. 1. Changes with time in the concentration of extracellular amino acids, induced by microdialysis perfusion with 100 mM K^+ for 25 min followed by perfusion with 75 mM 4-AP for 12.5 min, in anesthetized rats. Each set of bars corresponds to one 12.5 min fraction (25 μl). 100% basal release is the average of the first three fractions collected. Mean values ± SEM for 7 rats. See legend of Fig. 2 for statistical significance of the changes.

extracellular concentration of amino acids. In the anesthetized animals K^+-depolarization resulted in moderate elevations in extracellular aspartate and glutamate (100–300%), a remarkable increase in taurine (>600%) and a notable decrease (up to 75%) in glutamine. A peak concentration of 1.21 ± 0.29 μM GABA was detected under these conditions, whereas glycine and alanine did not change significantly. With the exception of glutamine, amino acid levels returned to approximate basal values after 25 min of perfusion with a normal medium. A similar stimulation of amino acids release by high K^+ was observed in the awake rats, except that some delay occurred due to the necessarily longer tubing required (Figs 1 and 3); in these animals the stimulated GABA release reached a 1.13 ± 0.13 μM concentration.

The effect of 4-AP was remarkably different from that of K^+-depolarization. As shown in Fig. 1, 75 mM 4-AP for only 12.5 min induced a massive release of glutamate (20-fold peak) and a 10-fold peak release of aspartate and taurine. Extracellular glutamine, glycine and alanine levels were also increased, but to a much smaller extent (about 4-fold peak). The releasing action of 4-AP occurred more rapidly than after high K^+ and it was transient. Immediately after perfusion with the drug all amino acids increased (glutamate release was already about 10-fold), peak levels were attained in the next fraction and they returned to nearly basal levels in the next two fractions. As shown in Fig. 3, strikingly similar results to those in anesthetized rats, including the decrease in glutamine, were obtained in the awake animals, in spite of the fact that a lower dose of 4-AP (40 mM) had to be used because of the intense motor hyperactivity. GABA concentration was also similar (1.53 ± 0.34 μM). The only exception was taurine release, which was considerably less stimulated than under anesthesia.

Dose–response curves of the peak releasing effect of 4-AP in the anesthetized rats are shown in Fig. 2, as compared to the peak action of K^+-depolarization (note the different ordinate scales in this figure). Perfusion with 10 mM 4-AP resulted in comparatively slight increases of all amino acids measured, and a dose-dependent response was observed for glutamate, aspartate and taurine. Glutamine, glycine and alanine were less affected by the drug. Glutamate was by far the most profoundly affected amino acid followed by aspartate and taurine. In contrast, K^+-depolarization showed the highest stimulatory effect on taurine, besides its notable decreasing effect on extracellular glutamine. GABA concentration was not affected by 10 mM 4-AP and increased to 1.09 ± 0.02, 1.03 ± 0.02 and 2.21 ± 0.3 μM by 20, 40 and 75 mM 4-AP, respectively. The first two values are similar to that observed after K^+-depolarization.

In some experiments the effect of 75 mM 4-AP and of high K^+ was studied in the absence of Ca^{2+} and in the presence of 20 mM Mg^{2+} in the medium. The basal amino acid levels did not differ significantly from those observed with the calcium-containing medium. As shown in Fig. 2, no significant reduction in the releasing action of 4-AP on any amino acid (including GABA, not shown) was observed under these conditions. With high potassium, the stimulated release of glutamate (Fig. 2) and GABA (not shown) decreased by about 75 and 85%, respectively, whereas the effect on the other amino acids was not modified by Ca^{2+} omission.

It was interesting to compare the effect of 4-AP with that of TEA, another K^+ channel blocker. As shown in Fig. 4, perfusion with 120 mM TEA during one fraction period did not modify the extracellular levels of aspartate, glutamine, glycine or alanine, and induced a slight increase of glutamate (88%) and a nearly 6-fold increase in taurine. Subsequent K^+-depolarization in the same animals resulted in changes very similar to those already described.

DISCUSSION

The systemic, intrahippocampal or intracerebroventricular injection of 4-AP in the rat results in seizures and other signs of motor hyperexcitability (Schafer *et al.*, 1973; Gandolfo *et al.*, 1989; Fragoso-Veloz *et al.*, 1990; Bagetta *et al.*, 1992; Fragoso-Veloz and Tapia, 1992). Although, in general, the striatum is not considered an epileptogenic region, the infusion of 4-AP through the microdialysis probes in awake rats induced severe motor alterations, such as ipsilateral barrel turning and, with the highest dose used, even running fits and tonic–clonic seizures. Considering the recovery of the drug through the dialysis membrane (11.3%), the total amount of the drug theoretically reaching the striatum after 12.5 min of perfusion with the highest concentration used is 210 nmol. Convulsions and/or motor abnormalities such as as wet-dog shakes have been observed after the bolus injection of 100–300 nmol in the lateral ventricle (Gandolfo *et al.*, 1989) or 2–100 nmol in the hippocampus (Fragoso-Veloz *et al.*, 1990; Bagetta *et al.*, 1992; Fragoso-Veloz and Tapia, 1992). Our results show that the intensity of the behavioral effects of 4-AP was in general directly related to the dose used, whereas perfusion with 100 mM K^+ for 25 min was without behavioral effects and TEA, at a considerably

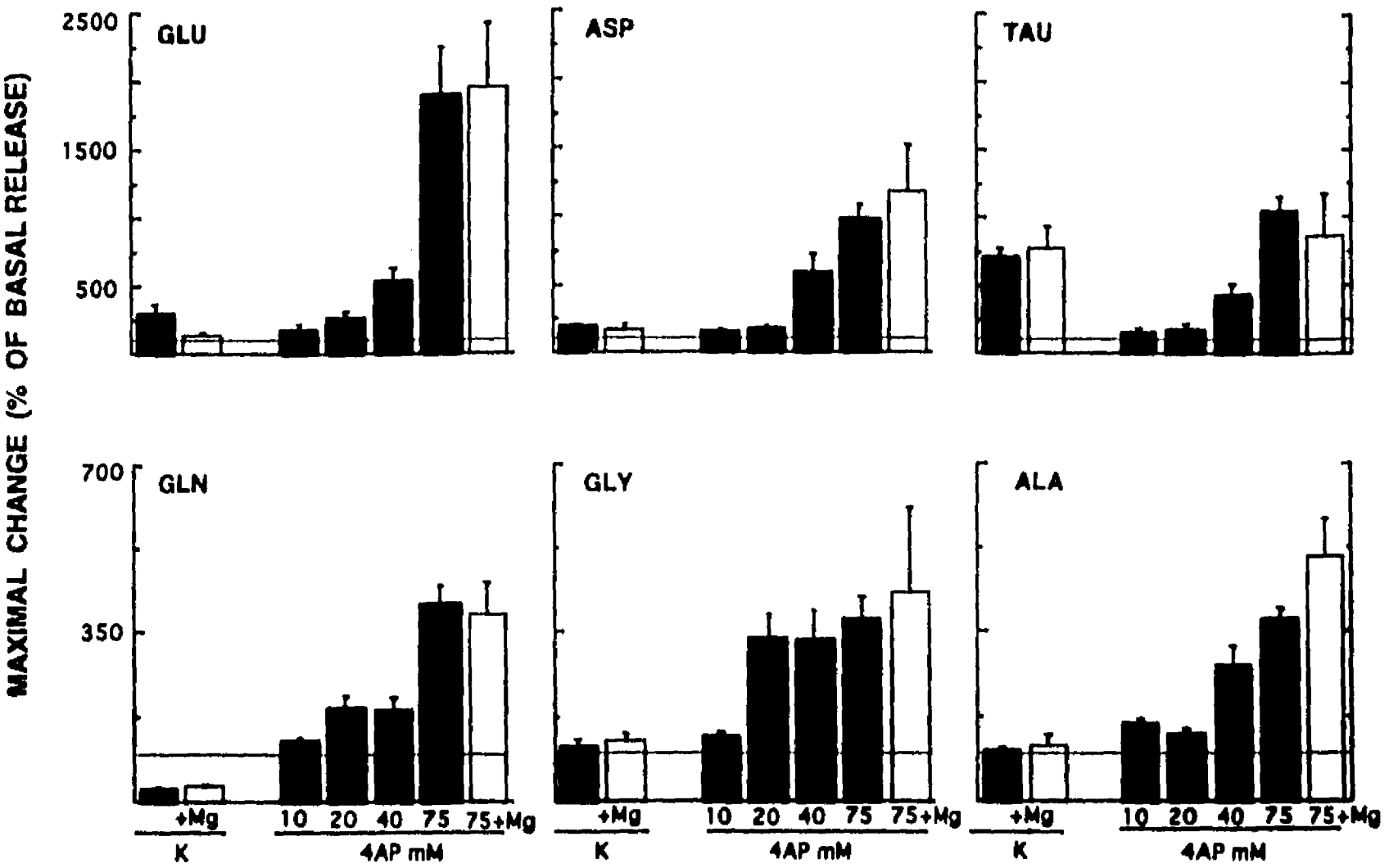

Fig. 2. Peak percent changes in the levels of extracellular amino acids induced by 100 mM K^+ and by different concentrations of 4-AP. The experiments were as in Fig. 1 and the peak value in each case was used. The empty bars in each panel show the effect of high K^+ and of 75 mM 4-AP in the absence of Ca^{2+} and in the presence of 20 mM Mg^{2+} in the perfusion medium. Note the different ordinate scales. Mean values ± SEM for 7–8 rats, except for high K^+ plus Mg^{2+} ($n = 5$). Changes induced by high potassium, except for glycine and alanine, and changes induced by all 4-AP concentrations, except for aspartate and glycine with 10 mM, were statistically significant (at least $P < 0.05$). Changes induced by 40 mM or higher 4-AP concentration, except for taurine, were statistically different from those induced by high potassium (at least $P < 0.05$). Glutamate stimulation by high potassium in the presence of calcium was statistically different from that in its absence ($P < 0.05$).

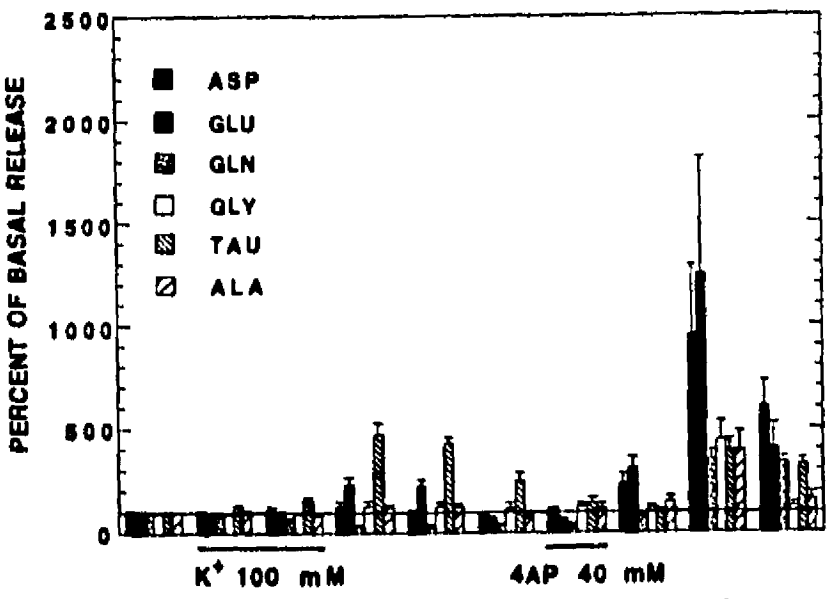

Fig. 3. Changes with time in the concentration of extracellular amino acids, induced by 100 mM K^+ and 40 mM 4-AP in freely moving rats. Details as for Fig. 1. Mean values ± SEM for 4 animals.

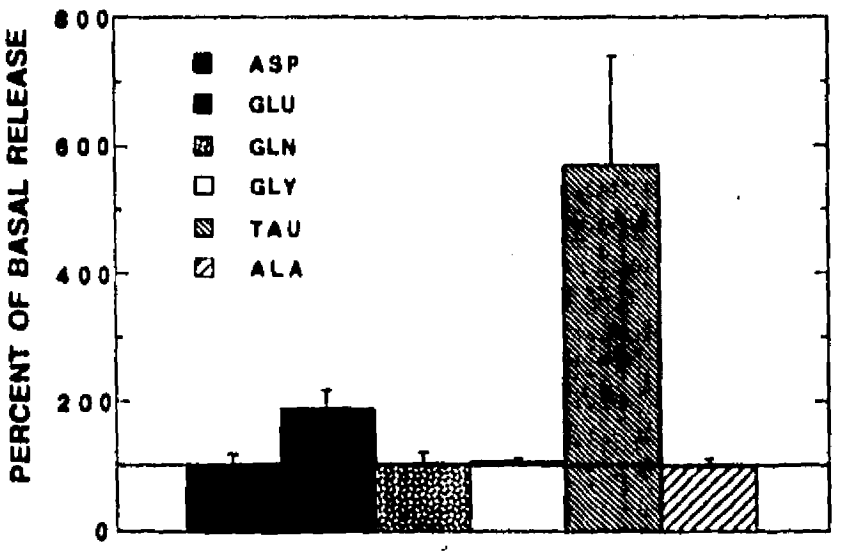

Fig. 4. Peak percent changes in the levels of extracellular amino acids induced by 120 mM TEA in anesthetized rats. Mean values ± SEM for 4 animals. Changes in glutamate and taurine were statistically significant ($P < 0.05$).

higher dose than 4-AP, produced only weak and transient motor alterations.

The behavioral effects of 4-AP can be correlated with its releasing action on the excitatory amino acid neurotransmitters. In fact, the more notable effect of

4-AP was on the extracellular levels of aspartate and particularly glutamate and, in parallel with the intensity of motor alterations, the concentration of these amino acids increased with the 4-AP dose. That the anesthesia used did not influence the action of the drug on the release of these amino acids is shown by the experiments in the freely moving rats, in which 40 mM 4-AP produced effects similar to those of 75 mM in the anesthetized animals. In this respect, it is interesting that the basal release of glutamate was approximately 15-fold higher in the former than in the latter, whereas the other amino acids did not differ.

The hyperexcitation produced by 4-AP could also be accounted for by its blocking effect on voltage-dependent K^+ channels, which could result in prolonged depolarization. Two lines of evidence argue against the latter possibility and support an increase of glutamate release as the main factor involved in the 4-AP-induced hyperexcitability. Firstly, depolarization with high potassium and injection of TEA, another K^+ channel blocker which acts, at least partially, on the same type of K^+ channels blocked by 4-AP (Castle *et al.*, 1989), affected only slightly, or not at all, glutamate and aspartate release, and, as already discussed, did not produce motor hyperexcitability. Secondly, NMDA receptor antagonists protect against seizures induced by the systemic or intracerebral administration of 4-AP (Gandolfo *et al.*, 1989; Fragoso–Veloz and Tapia, 1992; Morales–Villagrán, Ureña–Guerrero and Tapia, submitted), whereas they fail to protect against convulsions produced by certain K^+ channel blockers, such as α-dendrotoxin or mast cell-degranulating peptide (Gandolfo *et al.*, 1989).

It is difficult to offer an explanation for the comparatively small increases in extracellular concentration of glutamine, glycine and alanine induced by 4-AP, which did not occur with high K^+ or TEA. Neither these changes, nor the large increase of glutamate, can be ascribed to cell damage, because they were reversible after only a few minutes of reperfusion with basal medium. Furthermore, they were readily reproduced independently of whether a previous high K^+ medium was applied and, conversely, the changes induced by the latter were also very similar before and after 4-AP perfusion in the same animal.

The notable decrease of extracellular glutamine induced by high potassium, in both anesthetized and awake rats, has been previously described in some brain regions, and it has been interpreted as supporting the view that glutamine is an important precursor of the neurotransmitter pool of glutamate (Young and Bradford, 1986; Paulsen and Fonnum, 1989; Sved and Curtis, 1993). However, our data with 4-AP are not consistent with this interpretation, since this drug induced a much higher glutamate increase than K^+-depolarization and also some increase in glutamine. The only amino acid whose release was increased similarly by 4-AP, TEA and high K^+ was taurine. This is not surprising, since it has been shown, both *in vitro* and *in vivo*, that the extracellular concentration of this amino acid increases with a variety of stimulations, the most important being a change in osmolarity, although the physiological significance of this increase is not clear (Wade *et al.*, 1988; Martin, 1992; Dutton, 1993). Such a change does not seem probable in our experiments, since isoosmolarity was always maintained. Our finding that the stimulation of taurine release by 4-AP was lower in awake rats might indicate an anesthesia-related phenomenon. However, since such a difference was not observed with high K^+ this explanation is clearly not sufficient. Differences in the releasing effects of 4-AP and TEA have also been described in hippocampal slices, where the former notably stimulates the basal release of noradrenaline while even high concentrations of TEA fail to do so (Hu *et al.*, 1991).

It has been shown in synaptosomes that, differently from high K^+, the transmitter releasing action of 4-AP requires only a minimal concentration of external Ca^{2+} and is not affected by tetrodotoxin, which has been interpreted as a direct effect of 4-AP on the Ca^{2+}-dependent release process (Tapia and Sitges, 1982; Tapia *et al.*, 1985; Fragoso–Veloz *et al.*, 1990). It is then not surprising that its effect *in vivo* was not blocked by the calcium-free medium, because one should expect that the relatively high concentration of this cation in the extracellular fluid is enough to permit the stimulatory action of 4-AP, in spite of the presence of Mg^{2+}. Consistent with this interpretation, it has been shown that in the neuromuscular junction 4-AP is capable of stimulating transmitter release in the presence of high Mg^{2+} concentrations (Lundh, 1978). In contrast, the effect of high K^+ on glutamate and GABA, in agreement with other work (Paulsen and Fonnum, 1989; Sved and Curtis, 1993; Lawrence and Jarrott, 1994), was considerably reduced by Ca^{2+} omission.

In conclusion, it is possible to correlate the releasing action of 4-AP on glutamate with the motor hyperexcitation produced by this drug. In view of the abundance of corticofugal glutamatergic nerve endings in the striatum, this conclusion implies that the action of 4-AP *in vivo* is predominantly at the synaptic level, although an action at other sites cannot be discarded, since other amino acids were also released. The glutamate releasing effects of TEA or high potassium,

albeit most probably also occurring at nerve endings, seem to be insufficient to induce hyperexcitation, in spite of the depolarization produced by these agents. The possibility that the 4-AP-induced increase in extracellular glutamate may result in neuronal damage is presently being investigated in our laboratory.

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PREFERENTIAL STIMULATION OF GLUTAMATE RELEASE BY 4-AMINOPYRIDINE IN RAT STRIATUM IN VIVO

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Abbreviations: 4-AP, 4-aminopyridine; NMDA, N-methy-D-aspartic acid; TEA, tetraethylammonium

Running title: Glutamate release by 4-aminopyridine in vivo

ABSTRACT

The potassium channel blocker 4-aminopyridine (4-AP) is a potent convulsant drug which in vitro stimulates the release of neurotransmitter amino acids. We have studied the effect of 4-AP in vivo on the extracellular concentration of amino acids in rat striatum, by means of microdialysis and HPLC. Perfusion with 4-AP in the awake animal produced intense motor alterations, including barrel turning and running fits. Therefore, most microdialysis experiments were carried out in anesthetized rats. Perfusion with 20-75 mM 4-AP during 12.5 min resulted in a massive increase in extracellular glutamate (up to 20-fold), smaller increases in aspartate and taurine (up to 10-fold) and slight increments in glutamine, alanine, glycine and GABA. In contrast, perfusion with 100 mM K^+ produced mainly an increment in taurine (7-fold) and modest increases in glutamate and aspartate (100-300%), as well as a notable decrease in glutamine. Tetraethylammonium (TEA, 120 mM) perfusion induced taurine and glutamate elevations similar to those after high K^+, but glutamine was not affected. In unanesthetized rats, perfusion with 40 mM 4-AP induced changes in extracellular amino acids similar to those observed under anesthesia. In these animals neither high K^+ nor TEA affected significantly the motor behavior. The results suggest that an enhancement of glutamatergic synaptic transmission, rather than a general depolarizing action, is an important factor in the neuronal hyperexcitability induced by 4-AP, which is consistent with the previously demonstrated inhibition of its convulsant effect by glutamate receptor antagonists.

INTRODUCTION

The systemic administration of 4-aminopyridine (4-AP) induces generalized tonic convulsions in man and other species (Schafer *et al.*, 1973; Fragoso-Veloz *et al.*, 1990), and the microinjection of the drug in the rat substantia nigra reticulata (Tapia and Flores-Hernández, 1990) or the CA1 region of the hippocampus (Fragoso-Veloz *et al.*, 1990; Fragoso-Veloz and Tapia, 1992) produces intense motor alterations, including circling behavior, limbic type seizures and wet-dog shakes.

It is also known that 4-AP stimulates the release of neurotransmitters in several preparations, including the neuromuscular junction (Thesleff, 1980), brain slices (Dolezal and Tucek, 1983; Hu *et al.*, 1991) and synaptosomes (Tapia and Sitges, 1982; Tapia *et al.*, 1985; Tibbs *et al.*, 1989). Among the neurotransmitters released by 4-AP is glutamate, and in view of the well established role of excitatory amino acid synapses in convulsive and excitotoxic mechanisms (Dingledine *et al.*, 1990), it is possible that the excess glutamate released may be involved in the excitatory effects of the drug. In support of this hypothesis, it has been found that N-methyl-D-aspartate (NMDA) receptor antagonists notably protect against both seizures produced by the i.p. injection of 4-AP and wet-dog shakes induced by the intrahippocampal microinjection of the drug (Fragoso-Veloz and Tapia, 1992). Furthermore, non-NMDA receptor antagonists block the 4-AP-induced epileptiform burstings in amygdala (Gean *et al.*, 1990) and hippocampus (Perreault and Avoli, 1991) slices.

One important and missing point in the above hypothesis is the study of glutamate release under the action of 4-AP in vivo, and this was the aim of the present investigation. The effect of 4-AP on the extracellular concentration of glutamate and other amino acids was studied in the striatum of awake and anesthetized rats, using a microdialysis procedure. The striatum was chosen because it receives abundant glutamatergic fibers from the cerebral cortex and because its comparatively large size ensures that the amino acids collected in the dialysis perfusate arised only from that region. For comparative purposes and to gain information on the possible mechanism of action of 4-AP, the effect of a depolarizing potassium concentration and of tetraethylammonium (TEA), another K^+ channel blocker, was also studied.

EXPERIMENTAL PROCEDURES

Before implantation, each microdialysis cannula (exposed membrane 0.5 mm diameter and 2 mm length, obtained from BAS, West Lafayette, IN, USA) was flushed with distilled water for 30 min at a flow rate of 5 µl/min. The recovery of 4-AP from the dialysis membrane was assessed by perfusing probes (2 µl/min) immersed in standard solutions of 4-AP in Krebs-Ringer medium, and measuring spectrophotometrically the concentration of the drug in the perfusate (Biessels and Agoston, 1987). The calculated recovery of 4-AP was 11.3% ± 0.28 (n = 3).

Adult male Wistar rats (195 ± 5 g weight) were used. They were handled in accordance with the Rules for Research in Health Matters (Mexico), with approval of the local Animal Care committee. Animals were anesthetized with

halothane in oxygen and secured in a Kopf stereotaxic frame with the incisor bar positioned at -3.3 mm. Microdialysis cannulas were implanted into the right striatum (A +0.8 mm from bregma, L 2.8 and V 5.1 from dura; Paxinos and Watson, 1986) and the rats were maintained under low anesthesia (0.5% halothane) throughout the experiment, except in some animals, as described below.

The implanted cannulas were perfused with an oxygenated Ringer-Krebs medium of the following composition (in mM): NaCl 118, K_2PO_4 1.25, KCl 4.0, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.2, glucose 10, pH 7.4. The perfusion rate was 2 µl/min, using a BAS microinjection pump. When a high potassium (100 mM) or magnesium (20 mM) concentration was tested, or when 4-AP or TEA was added to the medium, the NaCl concentration was reduced in order to maintain isoosmolarity. The 20 mM Mg^{2+} medium was Ca^{2+}-free and was used for assessing the Ca^{2+}-dependency of the effects. After a 60 min equilibration period, 25 µl (12.5 min) consecutive fractions of the perfusate were continuously collected. The first three fractions were used to determine the basal extracellular levels of the amino acids, and the medium was then changed for the experimental medium to be tested, which was perfused during the time periods indicated in Results.

The amino acid content of the 25 µl perfusate fractions was determined by HPLC after *o*-phthaldialdehyde derivatization, as previously described (Salazar *et al.*, 1994; Massieu *et al.*, 1995). The recovery of amino acids from the dialysis probes varied from 6.2% for glutamate to 11.1% for taurine, as previously

reported (Massieu *et al.*, 1995). Statistical analyses were carried out by ANOVA and group comparison post hoc tests.

In some experiments the microdialysis perfusion was carried out in freely moving rats, using the BAS setup designed for this purpose. The implanted cannulas were cemented to the skull, the anesthesia was discontinued and drugs were perfused in the awake animals. Due to the motor effects of 4-AP perfusion, microdialysis under these conditions was carried out only with a 40 mM concentration of the drug and the animals had to be manually restrained during the most intense periods of motor abnormalities.

RESULTS

Behavioral observations

Perfusion with 4-AP (75 mM during one 12.5 min period) in unanesthetized animals produced a variety of severe motor alterations, including hyperactivity, frequent rearing, intense ipsilateral barrel turning, salivation, jumps followed by running fits and tonic-clonic convulsions, which lasted for at least three h and were more intense between 40 and 80 min after drug administration. Lower doses of 4-AP (10-50 mM during 12.5 min) produced less intense motor alterations, and convulsions did not occur. In contrast, when 120 mM TEA was perfused during 12.5 min only occasional rearing and barrel turning occurred and these effects disappeared in less than 35 min, and perfusion with the high potassium medium during two consecutive 12.5 min periods did not induce any behavioral alteration. In view of these behavioral effects, in most experiments the microdialysis procedure was carried out in anesthetized rats, as described under

Materials and Methods.

Extracellular amino acids

The concentration of the amino acids in the striatum dialysate was similar in the first three basal fractions collected and therefore their values were averaged. With the notable exception of glutamate, the extracellular basal levels in the anesthetized (n = 5) and awake animals (n = 4) were very similar. Values were as follows, without correction for recoveries from the dialysis membrane (μM, means ± SEM; the first figure refers to the anesthetized rats): aspartate, 0.42 ± 0.08 and 0.31 ± 0.03; glutamate, 0.55 ± 0.01 and 8.1 ± 0.57; glutamine, 12.7 ± 1.21 and 14.3 ± 0.46; glycine, 2.65 ± 0.23 2.74 ± 0.11; taurine, 1.5 ± 0.14 and 2.5 ± 0.01 and alanine 2.57 ± 0.16 and 2.4 ± 0.01; basal GABA concentration was too low to be reliably detected under both experimental conditions.

Preliminary experiments showed that perfusion with a 100 mM KCl medium during one fraction period (12.5 min) produced only slight changes in the concentration of some amino acids, and that clear effects were observed when the K^+-depolarization was extended to two fraction periods. In order to compare in the same animal the effect of 4-AP with that of K^+-depolarization, the experimental paradigm used was to perfuse with a high potassium medium during 25 min and, after the amino acids had returned to basal levels, to perfuse with a 4-AP containing medium during one fraction period (Fig. 1). In some experiments this procedure was reversed (i.e. 4-AP medium was perfused before the high potsassium medium), and the results were very similar to those described below, indicating that previous K^+-depolarization does not modify the

action of 4-AP nor viceversa.

Results are shown in Figs. 1-3 as percent of the basal extracellular concentration of amino acids. In the anesthetized animals K^+-depolarization resulted in moderate elevations in extracellular aspartate and glutamate (100-300%), a remarkable increase in taurine (>600%) and a notable decrease (up to 75%) in glutamine. A peak concentration of 1.21 ± 0.29 μM GABA was detected under these conditions, whereas glycine and alanine did not change significantly. With the exception of glutamine, amino acid levels returned to approximate basal values after 25 min of perfusion with a normal medium. A similar stimulation of amino acids release by high K^+ was observed in the awake rats, except that some delay occurred due to the necessarily longer tubing required (Figs. 1 and 3); in these animals the stimulated GABA release reached a 1.13 ± 0.13 μM concentration.

The effect of 4-AP was remarkably different from that of K^+-depolarization. As shown in Fig. 1, 75 mM 4-AP during only 12.5 min induced a massive release of glutamate (20-fold peak) and a 10-fold peak release of aspartate and taurine. Extracellular glutamine, glycine and alanine levels were also increased, but to a much smaller extent (about 4-fold peak). The releasing action of 4-AP occurred more rapidly than after high K^+ and it was transient. Immediately after perfusion with the drug all amino acids increased (glutamate release was already about 10-fold), peak levels were attained in the next fraction and they returned to nearly basal levels in the next two fractions. As shown in Fig. 3, strikingly similar results to those in anesthetized rats, including the decrease in glutamine, were obtained

in the awake animals, in spite of the fact that a lower dose of 4-AP (40 mM) had to be used because of the intense motor hyperactivity. GABA concentration was also similar (1.53 ± 0.34 μM). The only exception was taurine release, which was considerably less stimulated than under anesthesia.

Dose-response curves of the peak releasing effect of 4-AP in the anesthetized rats are shown in Fig. 2, as compared to the peak action of K^+-depolarization (note the different ordinate scales in this figure). Perfusion with 10 mM 4-AP resulted in comparatively slight increases of all amino acids measured, and a dose-dependent reponse was observed for glutamate, aspartate and taurine. Glutamine, glycine and alanine were the less affected by the drug. Glutamate was by far the amino acid most profoundly affected, followed by aspartate and taurine. In contrast, K^+-depolarization showed the highest stimulatory effect on taurine, besides its notable decreasing effect on extracellular glutamine. GABA concentration was not affected by 10 mM 4-AP and increased to 1.09 ± 0.02 μM, 1.03 ± 0.02 μM and 2.21 ± 0.3 μM by 20, 40 and 75 mM 4-AP, respectively. The first two values are similar to that observed after K^+-depolarization.

In some experiments the effect of 75 mM 4-AP and of high K^+ was studied in the absence of Ca^{2+} and in the presence of 20 mM Mg^{2+} in the medium. The basal amino acid levels did not differ significantly from those observed with the calcium-containing medium. As shown in Fig. 2, no significant reduction in the releasing action of 4-AP on any amino acid (including GABA, not shown) was observed under these conditions. With high potassium, the stimulated release of glutamate (Fig. 2) and GABA (not shown) decreased by about 75% and 85%,

respectively, whereas the effect on the other amino acids was not modified by Ca^{2+} omission.

It was interesting to compare the effect of 4-AP with that of TEA, another K^+ channel blocker. As shown in Fig. 4, perfusion with 120 mM TEA during one fraction period did not modify the extracellular levels of aspartate, glutamine, glycine or alanine, and induced a slight increase of glutamate (88%) and a nearly 6-fold increase in taurine. Subsequent K^+-depolarization in the same animals resulted in changes very similar to those already described.

DISCUSSION

The systemic, intrahippocampal or intracerebroventricular injection of 4-AP in the rat results in seizures and other signs of motor hyperexcitability (Schafer *et al.*, 1973; Gandolfo *et al.*, 1989; Fragoso-Veloz *et al.*, 1990; Bagetta *et al.*, 1992; Fragoso-Veloz and Tapia, 1992). Although in general the striatum is not considered an epileptogenic region, the infusion of 4-AP through the microdialysis probes in awake rats induced severe motor alterations, such as ipsilateral barrel turning and, with the highest dose used, even running fits and tonic-clonic seizures. Considering the recovery of the drug through the dialysis membrane (11.3%), the total amount of the drug theoretically reaching the striatum after 12.5 min of perfusion with the highest concentration used is 210 nmoles. Convulsions and/or motor abnormalities such as as wet-dog shakes have been observed after the bolus injection of 100-300 nmoles in the lateral ventricle (Gandolfo *et al.*, 1989) or 2-100 nmoles in the hippocampus (Fragoso-Veloz *et al.*, 1990; Bagetta *et al.*, 1992; Fragoso-Veloz and Tapia, 1992). Our results

show that the intensity of the behavioral effects of 4-AP was in general directly related to the dose used, whereas perfusion with 100 mM K^+ during 25 min was without behavioral effects and TEA, at a considerably higher dose than 4-AP, produced only weak and transient motor alterations.

The behavioral effects of 4-AP can be correlated with its releasing action on the excitatory amino acid neurotransmitters. In fact, the more notable effect of 4-AP was on the extracellular levels of aspartate and particularly glutamate and, in parallel with the intensity of motor alterations, the concentration of these amino acids increased with the 4-AP dose. That the anesthesia used did not influence the action of the drug on the release of these amino acids is shown by the experiments in the freely moving rats, in which 40 mM 4-AP produced effects similar to those of 75 mM in the anesthetized animals. In this respect, it is interesting that the basal release of glutamate was approximately 15-fold higher in the former than in the latter, whereas the other amino acids did not differ.

The hyperexcitation produced by 4-AP could also be accounted for by its blocking effect on voltage-dependent K^+ channels, which could result in prolonged depolarization. Two lines of evidence argue against the latter possibility and support an increase of glutamate release as the main factor involved in the 4-AP-induced hyperexcitability. First, depolarization with high potassium and injection of TEA, another K^+ channel blocker which acts, at least partially, on the same type of K^+ channels blocked by 4-AP (Castle *et al.*, 1989), affected only slightly, or nothing at all, glutamate and aspartate release, and, as already discussed, did not produce motor hyperexcitability. Second, NMDA

receptor antagonists protect against seizures induced by the systemic or intracerebral administration of 4-AP (Gandolfo *et al.*, 1989; Fragoso-Veloz and Tapia, 1992; Morales-Villagrán, Ureña-Guerrero and Tapia, submitted), whereas fail to protect against convulsions produced by certain K^+ channel blockers, such as α-dendrotoxin or mast cell-degranulating peptide (Gandolfo *et al.*, 1989).

It is difficult to offer an explanation for the comparatively small increases in extracellular concentration of glutamine, glycine and alanine induced by 4-AP, which did not occur with high K^+ or TEA. Neither these changes nor the large increase of glutamate can be ascribed to cell damage, because they were reversible after only a few min of re-perfusion with basal medium. Furthermore, they were readily reproduced independently of whether a previous high K^+ medium was

applied and, conversely, the changes induced by the latter were also very similar before and after 4-AP perfusion in the same animal.

The notable decrease of extracellular glutamine induced by high potassium, in both anesthetized and awake rats, has been previously described in some brain regions, and it has been interpreted as supporting the view that glutamine is an important precursor of the neurotransmitter pool of glutamate (Young and Bradford, 1986; Paulsen and Fonnum, 1989; Sved and Curtis, 1993). However, our data with 4-AP are not consistent with this interpretation, since this drug induced a much higher glutamate increase than K^+-depolarization and also some increase in glutamine. The only amino acid whose release was increased similarly by 4-AP, TEA and high K^+ was taurine. This is not surprising, since it has been shown, both in vitro and in vivo, that the extracellular concentration of this amino acid increases with a variety of stimulations, the most important being a change in osmolarity, although the physiological significance of this increase is not clear (Wade *et al.*, 1988; Martin, 1992; Dutton, 1993). Such a change does not seem probable in our experiments, since isoosmolarity was always maintained. Our finding that the stimulation of taurine release by 4-AP was lower in awake rats might indicate an anesthesia-related phenomenon. However, since such a difference was not observed with high K^+ this explanation is clearly not sufficient. Differences in the releasing effects of 4-AP and TEA have also been described in hippocampal slices, where the former notably stimulates the basal release of noradrenaline while even high concentrations of TEA fail to do so (Hu *et al.*, 1991).

It has been shown in synaptosomes that, differently from high K^+, the transmitter releasing action of 4-AP requires only a minimal concentration of external Ca^{2+} and is not affected by tetrodotoxin, which has been interpreted as a direct effect of 4-AP on the Ca^{2+}-dependent release process (Tapia and Sitges, 1982; Tapia *et al.*, 1985; Fragoso-Velz *et al.*, 1990). It is then not surprising that its effect in vivo was not blocked by the calcium-free medium, because one should expect that the relatively high concentration of this cation in the extracellular fluid is enough to permit the stimulatory action of 4-AP, in spite of the presence of Mg^{2+}. Consistently with this interpretation, it has been shown that in the neuromuscular junction 4-AP is capable of stimulating transmitter release in the presence of high Mg^{2+} concentrations (Lundh, 1978). In contrast, the effect of high K^+ on glutamate and GABA, in agreement with other work (Paulsen and Fonnum, 1989; Sved and Curtis, 1993; Lawrence and Jarrott, 1994), was considerably reduced by Ca^{2+} omission.

In conclusion, it is possible to correlate the releasing action of 4-AP on glutamate with the motor hyperexcitation produced by this drug. In view of the abundance of corticofugal glutamatergic nerve endings in the striatum, this conclusion implies that the action of 4-AP in vivo is predominantly at the synaptic level, although an action at other sites cannot be discarded, since other amino acids were also released. The glutamate releasing effects of TEA or high potassium, albeit most probably occurring also at nerve endings, seem to be insufficient to induce hyperexcitation, in spite of the depolarization produced by these agents. The possibility that the 4-AP-induced increase in extracellular

glutamate may result in neuronal damage is presently being investigated in our laboratory.

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FIGURE LEGENDS

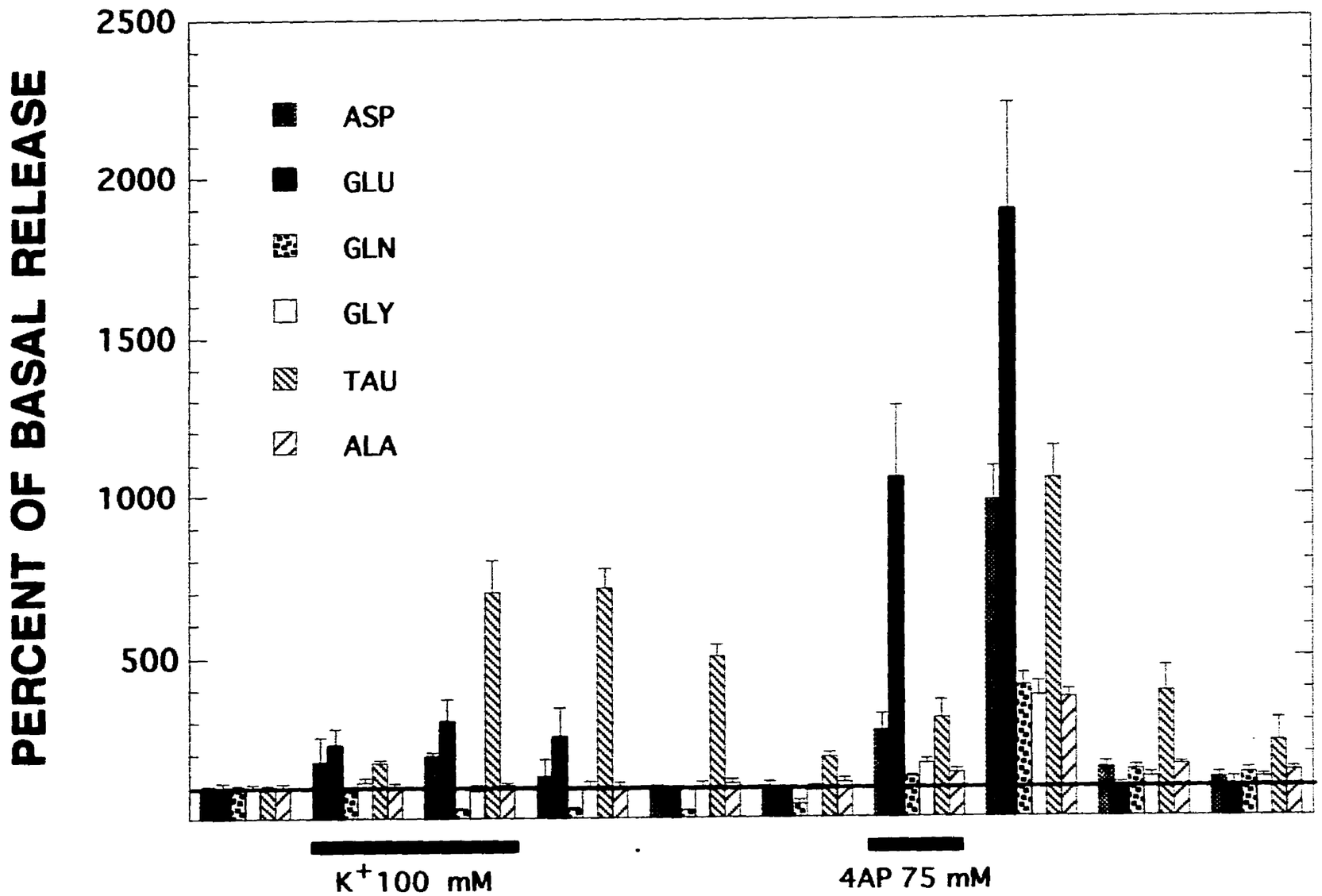
Fig. 1. Changes with time in the concentration of extracellular amino acids, induced by microdialysis perfusion with 100 mM K^+ during 25 min followed

by perfusion with 75 mM 4-AP during 12.5 min, in anesthetized rats. Each set of bars corresponds to one 12.5 min fraction (25 μl). One hundred percent basal release is the average of the first three fractions collected. Mean values ± SEM for 7 rats. See legend of Fig. 2 for statistical significance of the changes.

Fig. 2. Peak percent changes in the levels of extracellular amino acids induced by 100 mM K^+ and by different concentrations of 4-AP. The experiments were as in Fig. 1 and the peak value in each case was used. The empty bars in each panel show the effect of high K^+ and of 75 mM 4-AP in the absence of Ca^{2+} and in the presence of 20 mM Mg^{2+} in the perfusion medium. Note the different ordinate scales. Mean values ± SEM for 7-8 rats, except for high K^+ plus Mg^{2+} (n = 5). Changes induced by high potassium, except for glycine and alanine, and changes induced by all 4-AP concentrations, except for aspartate and glycine with 10 mM, were statistically significant (at least $P<0.05$). Changes induced by 40 mM or higher 4-AP concentration, except for taurine, were statistically different from those induced by high potassium (at least $P<0.05$). Glutamate stimulation by high potassium in the presence of calcium was statistically different from that in its absence ($P<0.05$).

Fig. 3. Changes with time in the concentration of extracellular amino acids, induced by 100 mM K^+ and 40 mM 4-AP in freely moving rats. Details as for Fig. 1. Mean values ± SEM for 4 animals.

Fig. 4. Peak percent changes in the levels of extracellular amino acids induced by 120 mM TEA in anesthetized rats. Mean values ± SEM for 4 animals. Changes in glutamate and taurine were statistically significant ($P<0.05$).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2000
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GLN
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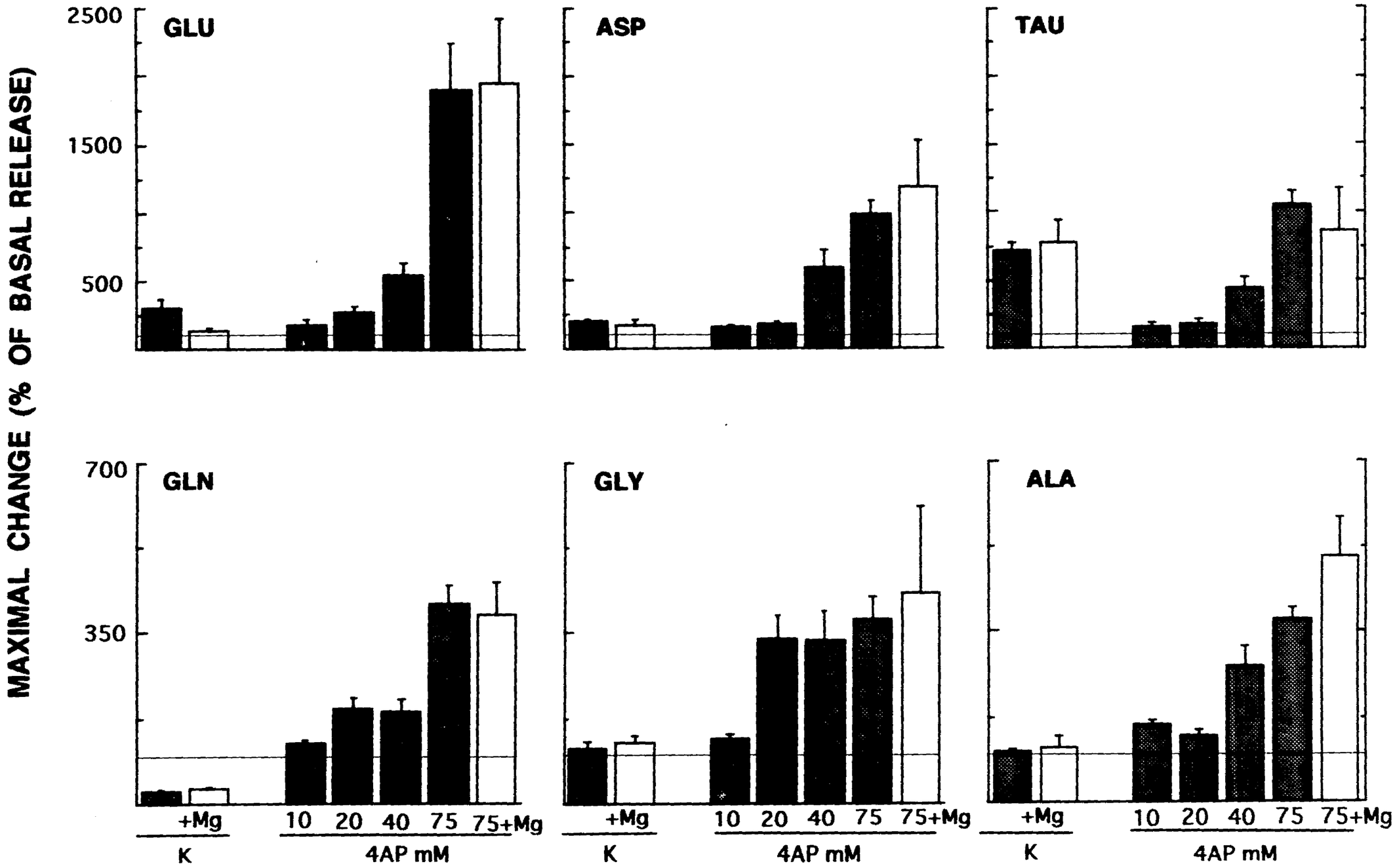
ALA
K+100 mM
4AP 75 mM


MAXIMAL CHANGE (% OF BASAL RELEASE)
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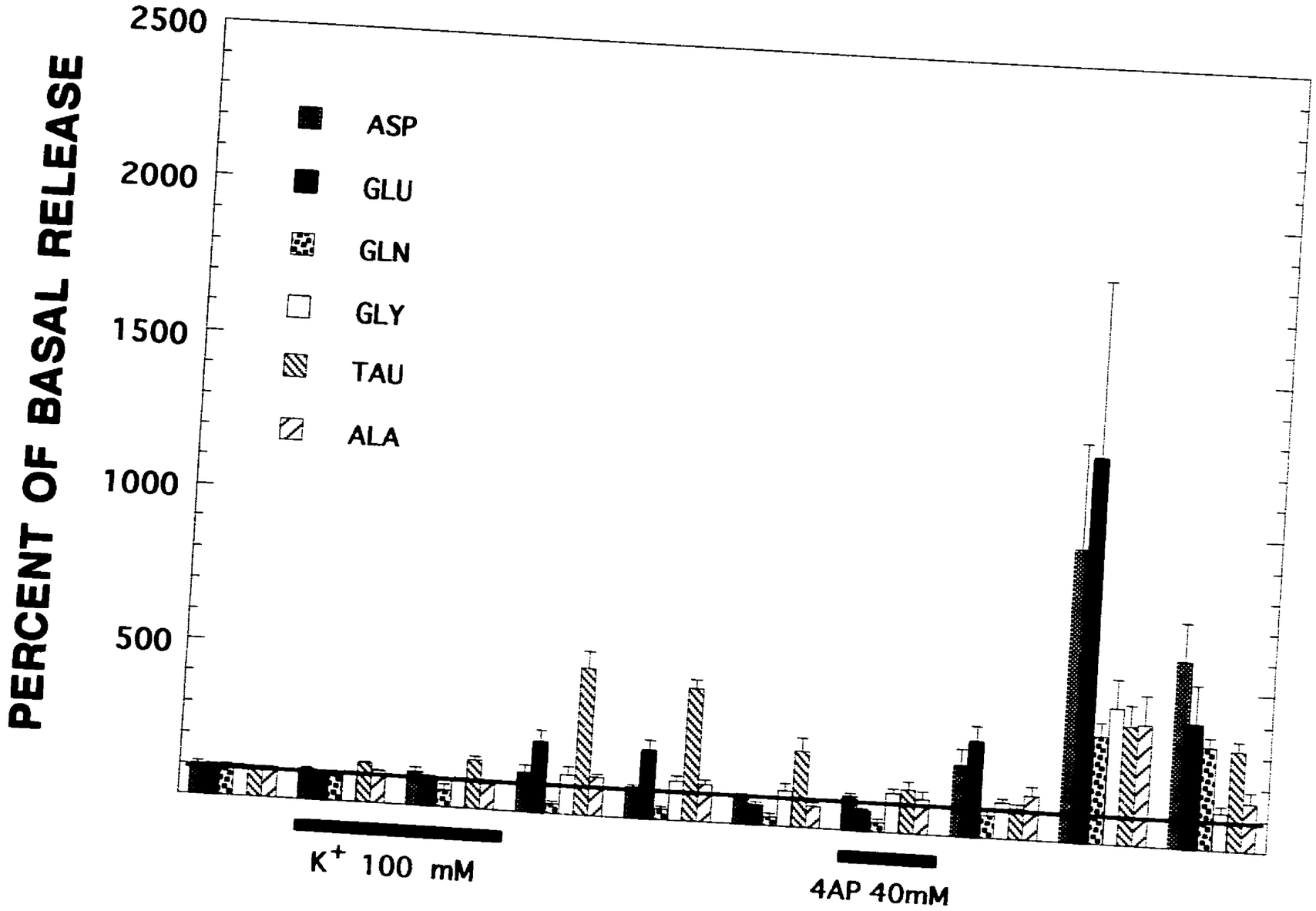
10 20 40 75 75+Mg
K
4AP mM


PERCENT OF BASAL RELEASE
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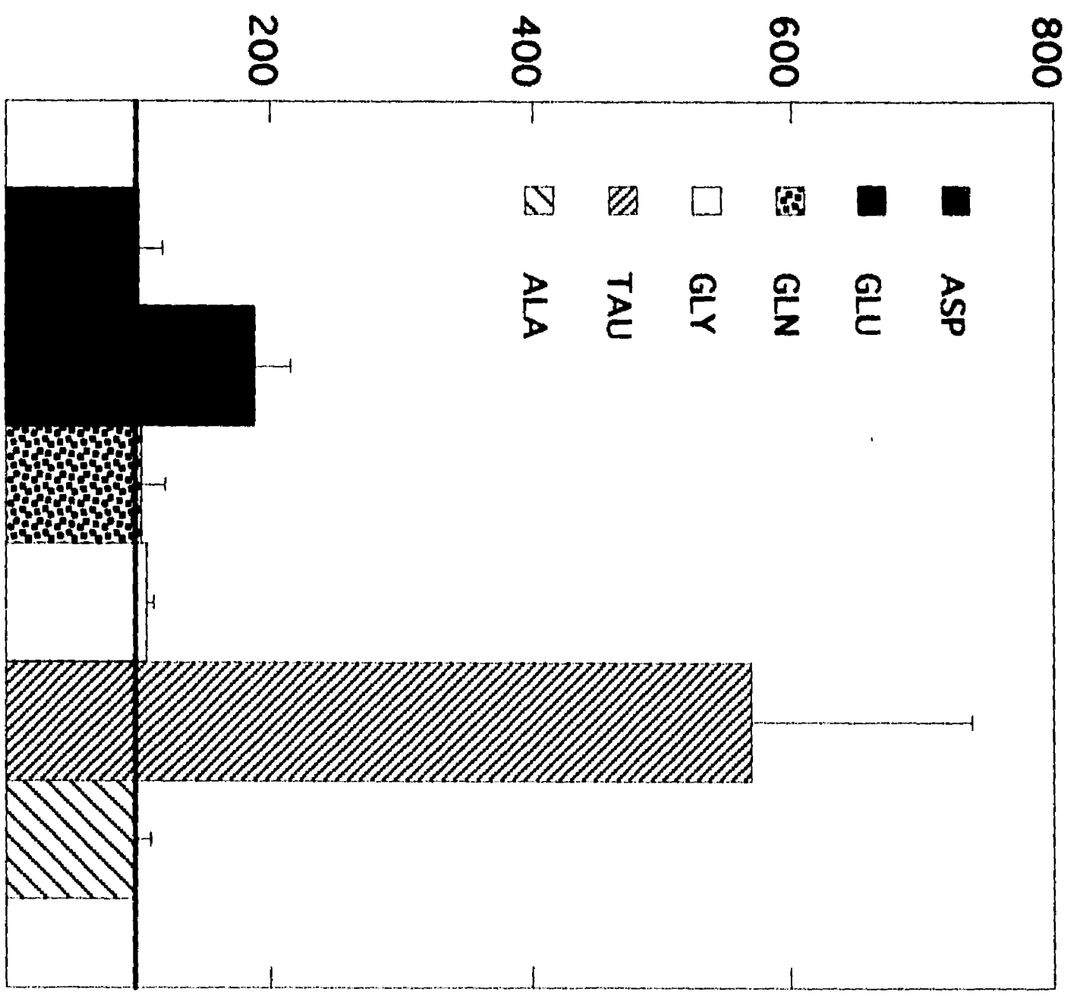
K+ 100 mM
4AP 40mM


PERCENT OF BASAL RELEASE
200
400
600
800
ASP
GLU
GLN
GLY
TAU
ALA

PROTECTION BY NMDA RECEPTOR ANTAGONISTS AGAINST SEIZURES INDUCED BY INTRACEREBRAL ADMINISTRATION OF 4-AMINOPYRIDINE

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Abstract

The effects of NMDA receptor antagonists on the convulsant action of the administration of 4-aminopyridine (4-AP) in the rat lateral cerebral ventricle (i.c.v. injection) and motor cerebral cortex (i.cx. injection) were studied. 4-AP administration in both regions induced various preconvulsive symptoms, such as salivation, tremors, chewing and rearing, followed by continuous clonic convulsions and, only after i.c.v. injection, running fits and generalized tonic convulsions. This behavioral pattern appeared 5-9 min after administration of 4-AP and persisted for 100-150 min. 4-AP also generated epileptiform EEG discharges characterized by isolated spikes, poly-spikes and spike-wave complexes, which began some seconds after administration of 4-AP and were present for more than 2 h. The NMDA receptor antagonists (±)-3-(2-carboxy-piperazin-4-yl)-propyl-1-phosphonic acid (CPP), (±)-2-amino-7-phosphono-heptanoic acid (AP7) and dizocilpine maleate (MK-801) partially prevented the behavioral alterations induced by i.c.v. 4-AP, particularly the tonic convulsions, but had little effect on those produced by i.cx. 4-AP. These antagonists also delayed the appearance of EEG epileptiform discharges, reduced its amplitude, frequency and duration, and blocked their propagation to other cortical regions after i.cx. 4-AP. These results, together with previous data showing that 4-AP stimulates the release of glutamate from nerve endings, suggest that an excessive glutamatergic neurotransmission involving NMDA receptors is implicated in 4-AP-induced seizures.

Key words: 4-Aminopyridine; NMDA receptor antagonists; Seizures; Excitatory amino acid neurotransmission

1. Introduction

The convulsant action of the systemic administration of the potassium channel blocker 4-aminopyridine (4-AP) in several species is well known [7,18]. It has also been reported that the microinjection of the drug in the rat substantia nigra reticulata [19] or the CA1 region of the hippocampus [1,7,8] produces EEG seizures and intense motor alterations, including circling behavior, limbic type seizures and wet-dog shakes.

Although the mechanism of its convulsant action has not been determined, it is known that 4-AP stimulates the release of neurotransmitters, including glutamate, in several preparations such as the neuromuscular junction [13,22], brain slices [5,12] and synaptosomes [20,21,23]. Furthermore, we have recently found by a microdialysis procedure in vivo that glutamate is by far the amino acid predominantly released by 4-AP in the striatum, whereas tetraethylammonium, another potassium channel blocker, has only a weak effect [Morales-Villagrán and Tapia, submitted]. Therefore, and in view of the well established role of excitatory amino acid synapses in convulsive and excitotoxic mechanisms [4,14], we have postulated that the excess glutamate released may be involved in the excitatory effects of 4-AP. With respect to this hypothesis, it has been found that N-methyl-D-aspartate (NMDA) receptor antagonists notably protect against both seizures produced by the i.p. injection of 4-AP and wet-dog shakes induced by the intrahippocampal microinjection of the drug [8]. Furthermore, non-NMDA receptor antagonists block the 4-AP-induced epileptiform burstings in amygdala and hippocampus slices [10,17]. In order to

better understand the possible role of excessive glutamate release in the hyperexcitation produced by 4-AP, in the present communication we have studied the effect of several NMDA receptor antagonists on the behavioral and electroencephalographic alteration produced by intracerebroventricular and intracortical administration of this drug.

2. Materials and methods

2.1. Materials

Adult male Wistar rats, weighing 200-250 g were used throughout. They were maintained at constant temperature (18-22 °C) and humidity (40%) under controlled light-dark cycles (12/12 h) and with free access to food and water. 4-AP and direct blue 15 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). (±)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), (±)-2-amino-7-phosphonoheptanoic acid (AP7) and (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) were from Research Biochemicals Int. (Natick, MA, USA).

2.2. Surgical procedures

The rats were anesthetized with halothane-oxygen mixture and secured in a Stoelting stereotaxic frame with the incisor bar positioned at -3.3 mm. They were implanted with guide cannulas for drug injection into the lateral ventricle (i.c.v. administration) or on the motor cerebral cortex (i.cx. administration). Coordinates were: A 0.8, L 1.7 and V 3.5 for lateral ventricle and P 2.0, L 3.0 and V 1.5 for motor cerebral cortex, respectively [16]. The cannula tip was located 1 mm above the injection zone, and it was sealed with a dummy injection cannula

until the time of drug administration. For EEG recording in i.c.v.-injected rats, in addition to the cannula animals were bilaterally implanted with copper bipolar electrodes (0.25 mm diameter, 0.1 mm tip, 0.25 mm interelectrode distance) in the motor cerebral cortex (coordinates as above) and hippocampus (P 3.2, L 2.0 and V 3.5). For EEG recording after i.cx. administration, the cannula guide served as an electrode, in addition to bilaterally implanted epidural electrodes consisting of stainless steel screws fixed to the skull. The reference electrode was located over the frontal sinus. Screw electrodes were positioned at frontal A 4 and L 2, premotor A 0 and L 3, motor P 2 and L 3 and occipital P 5 and L 3 coordinates. The electrodes were attached to a multipin socket anchored to the skull with dental acrylic cement. Amplification and recording of electrical activity were carried out using a Grass polygraph (paper speed 15 mm/s). Electrical activity was monitored during one min every five min, starting 30 min before drug injection (basal record), until the epileptiform discharges had disappeared.

2.3. *Drug administration*

Some rats were not implanted with guide cannulas nor electrodes but were anesthetized and injected i.c.v. with 4-AP (75 nmol in 5 μl, infused in a period of 2-3 min) through a needle attached to a 10 μl syringe. The anesthesia was then discontinued and behavior was evaluated by continuous observation during 2-3 h (until motor abnormalities disappeared), as soon as the animals woke up (about 10 min after injection). Unanesthetized rats implanted with guide cannulas received 75 nmol of 4-AP i.c.v. through the cannulas, one week after

surgery, and were used for EEG recording. The i.cx. 4-AP administration was in all cases through the guide cannula (75 nmol in one μl).

The following NMDA receptor antagonists were tested against both the i.c.v. and the i.cx. 4-AP administration, at the doses and routes of administration indicated: CPP, 10 nmol, coinjected i.c.v. or i.cx. with 4-AP; AP7, 10 nmol coinjected i.c.v. or i.cx. with 4-AP; MK-801, 10 nmol coinjected i.c.v. or i.cx. with 4-AP and, furthermore, 0.2 mg/kg i.p. 30 min before 4-AP.

All drugs were dissolved in isotonic saline containing 6 mg/ml of direct blue 15 to locate the injection site. This dye did not produce any effect per se, nor affected the action of the drugs used. At the end of the experiment rats were decapitated and the brain was removed and sliced. The location of both the electrodes and injection sites were verified by gross histological examination.

3. Results

3.1. Intracerebroventricular injections

3.1a. Behavioral observations

The i.c.v. administration of 75 nmol 4-AP produced a series of preconvulsive symptoms including hyperexcitability, tremors, head nodding, salivation, chewing and rearing. After these symptoms, all treated animals examined showed convulsive behavior characterized by bursts of forepaw clonic jerks, wild running and continuous clonic convulsions (status epilepticus), which usually lasted for more than 30 min. Furthermore, 85% of the animals showed generalized tonic convulsions (GTC), which occurred during the status epilepticus. The behavioral changes appeared at 9.3 ± 1.0 min after treatment

and lasted for 147 ± 8.0 min (n = 13).

The NMDA receptor antagonists CPP, AP7 and MK-801 prevented or diminished the intensity of the behavioral alterations produced by i.c.v. 4-AP. The most notable protective effect was that, with the exception of one rat treated with AP7, status epilepticus, wild running and GTC were totally prevented. In contrast, all animals receiving the NMDA receptor antagonists showed numerous wet-dog shakes, which were never observed with 4-AP alone (Table 1).

3.1b. EEG recordings

The i.c.v. administration of 4-AP induced epileptiform discharges in all regions recorded, but were persistently much more intense in the ipsilateral motor cortex. The discharges appeared immediately, even before the end of the injection period, and were characterized by high frequency poly-spikes and spike-wave complexes which were of low amplitude at the beginning of the effect. Their amplitude increased with time, reaching a maximum at about 30 min. During the first 10-20 min the average duration of discharges was 40 s with interictal periods of approximately one min. After about 60 min the discharges became continuous for periods longer than 4 h, even when the behavioral alterations had ceased, and then gradually disappeared.

A notable potection against 4-AP-induced EEG seizures was observed in some, but not all rats treated with the NMDA receptor antagonists. When protection occurred, a significant delay in the latency of appearance of the discharges was observed (25-150 s). Furthermore, as exemplified in Fig. 1, the

frequency, amplitude and duration of the discharges were also considerably reduced. In no case the discharges became continuous and their duration did not excede 20 s. As indicated in the legend to Fig. 1, with CPP, AP7 and i.p. MK-801 protection was observed in at least 50% of the animals, whereas with i.c.v. MK-801 protection was observed in all rats treated. However, even in the less protected rats, with all antagonists the spike amplitude decreased and the duration of the continuous discharge was reduced to less than 1.5 h, although the maximum spike frequency did not change.

3.2. Intracortical injections

3.2.a. Behavioral observations

The i.cx. administration of 4-AP produced a pattern of preconvulsive symptoms similar to that induced by the i.c.v. injection, except that weak contralateral circling behavior and occasional wet-dog shakes were also observed. An important difference was that running fits and GTC did not occur and only 2/7 animals showed status epilepticus. Motor alterations appeared 4.4 ± 0.6 min after injection and lasted for 107 ± 6.5 min.

At the doses indicated in Table 1, neither of the NMDA receptor antagonists tested exerted a significant protection against the behavioral effects of i.cx. 4-AP, with the exception of salivation, which was clearly reduced (results not shown, 7 rats were tested with each antagonist).

3.2.b. EEG recordings

Epileptiform discharges were observed in all electrode derivations, starting about 14 s after the i.cx. administration of 4-AP. The discharges were

characterized by isolated spikes, repetitive spikes and spike-wave complexes. Discharge amplitude increased with time and reached a maximum at about 30 min. Differently from the i.c.v. administration, the EEG epileptiform pattern was continuous from the time of its appearance and lasted for more than 2 h.

As indicated in Fig. 2, in contrast to the lack of protection against the i.cx. 4-AP behavioral effects, in at least 65% of the rats all NMDA receptor antagonists tested produced a notable delay in the latency of appearance of the discharge, and in these animals the discharges were not continuous but lasted for 12-14 s and were interrupted by interictal periods of about 12 s during the first 30 min. After this time, the duration of the interictal periods gradually increased with time, until the disappearance of the discharges. In addition, the propagation of the epileptiform activity to the contralateral hemisphere, and sometimes even to other ipsilateral regions, was prevented (Fig. 2).

4. Discussion

The results of the present study extend previous work from this laboratory, in which it was shown that NMDA receptor antagonists, but not non-NMDA receptor antagonists, are good anticonvulsants against seizures produced by the systemic administration of 4-AP and protect against wet-dog shakes induced by the intrahippocampal administration of the drug [8]. In fact, both behaviorally and electroencephalographically, the three NMDA receptor antagonists tested, particularly MK-801, prevented or reduced the intensity of the hyperexcitability symptoms induced by 4-AP, both when administered i.c.v. or i.cx. These results, therefore, support the hypothesis that an excess of glutamate release stimulated

by the drug is an important factor in the mechanism of its epileptogenic effect. Further support for such hypothesis is provided by our recent finding that the infusion of 4-AP through a microdialysis probe in vivo induces a massive release of glutamate, whereas other amino acids are much less affected [Morales-Villagrán and Tapia, submitted]. It is well known that in vitro 4-AP stimulates the basal (not depolarization-dependent) release of neurotransmitters in a Ca^{2+}-dependent manner [5,12,13,20-23].

It is interesting that, both behaviorally and EEG, the seizures induced by 4-AP were more intense when administered i.c.v. than i.cx., and that the former were more clearly antagonized than the latter by the NMDA-receptor antagonists. This suggests that both 4-AP and the antagonists diffuse extensively when injected i.c.v. and therefore reach a relatively large number of synapses in several regions of the brain, whereas their diffusion is restricted when injected i.cx. Consistently with this interpretation, we have previously found that the antagonists are effective against the convulsive activity of 4-AP only when injected i.c.v. or i.p. (in the case of MK-801), but not when coinjected with 4-AP in the CA1 hippocampal area [8].

Other potassium channel blockers, such as α-dendrotoxin or mast-cell degranulating peptide, also induce seizures when administered i.c.v. or into the CA1 area of the hippocampus. However, in contrast to 4-AP, their effects are not antagonized by NMDA receptor antagonists [1,9] and, conversely, some L-type channel blockers are good anticonvulsants against α-dendrotoxin but they fail to protect, or even potentiate, the effects of 4-AP [6,7,9,24]. This suggests that a

generalized depolarizing action of 4-AP, which is probably shared by the other blockers, is not the main factor involved in its convulsant action.

4-AP has been used in the treatment of several neurological disorders, such as miasthenia gravis, Eaton-Lambert syndrome, multiple sclerosis, Alzheimer disease and spinal cord injury [2,11,15,25]. The benefit of this treatment is probably due to the restoration of current conduction by 4-AP in demyelinated axons, as a consequence of the blockade of potassium channels, or to the increased release of acetylcholine at neuromuscular junctions, but one of the risks of 4-AP treatment is that a slight overdose might lead to convulsions [2,3,11]. Our findings indicate that this dangerous side effect is due to increased glutamatergic neurotransmission at central synapses, and suggest that a combined treatment with 4-AP and NMDA receptor antagonists might reduce the risk of convulsions and other excitatory actions of the drug and therefore facilitate its therapeutic use.

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Table 1

Main behavioral effects of the i.c.v. administration of 4-AP (75 nmol) and protection by NMDA receptor antagonists. With the exception of systemic MK-801 (last column), 10 nmol of each antagonist was coinjected i.c.v. with 4-AP

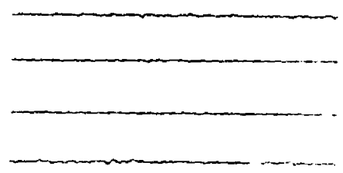
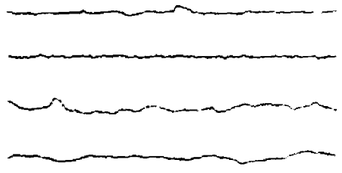
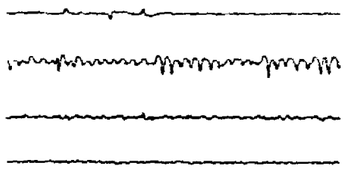
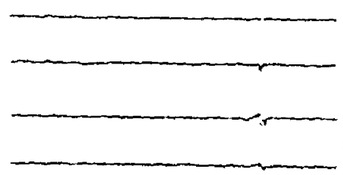
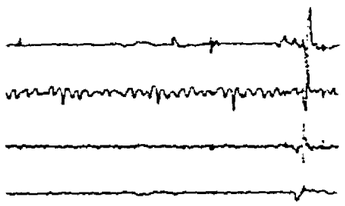
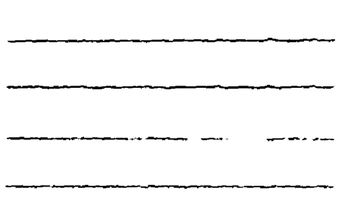
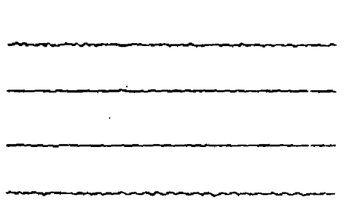
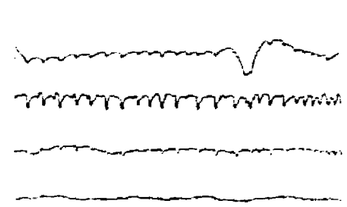
Symptom	4-AP alone	+ CPP	+ AP7	+ MK-801	+ MK-801 i.p. (0.2 mg/kg)
Wet-dog shakes (number)	0/13 (0)	6/6 (39±9)	7/7 (122±37)	6/6 (73±5)	7/7 (129±27)
Salivation	13/13	4/6	6/7	0/6	0/7
Clonic convuls. (number)	12/13 (5.5±1.2)	6/6 (5.0±1.0)	7/7 (5.0±1.2)	5/6 (3.6±1.2)	4/7 (3.5±1.7)
GTC (number)	7/13 (5.3±1.8)	0/6 (0)	1/7 (3)	0/6 (0)	0/7 (0)

Figures are No. of rats showing the symptom/No. of rats treated. In parentheses it is shown the mean total number of times that the symptom occurred in a single animal ± SEM.

Figure legends:

Fig. 1. Representative examples of the effect of the NMDA receptor antagonists on the discharges induced by the i.c.v. administration of 4-AP in the right lateral ventricle. Each set of records show the basal activity, the activity immediately after injection of 4-AP, and at 30 and 60 min afterwards, as indicated. In each set, records 1 and 2 are left and right motor cerebral cortex, respectively, and 3 and 4 left and right hippocampus, respectively. Except for the last column (MK-801 administered i.p. 30 min before 4-AP), 10 nmol of the NMDA receptor antagonists were coinjected i.c.v. with 4-AP. The convulsant action of 4-AP was observed in 4 rats; protective effects by the antagonists were observed in 4 of 8 treated rats for CPP, 4 of 7 for AP7, 6 of 6 for i.c.v. MK-801, and 3 of 6 rats for i.p. MK-801. Scale: horizontal bar = 2s; vertical = 840 μvolts.

Fig. 2. Representative examples of the effect of NMDA receptor antagonists on the discharges induced by the i.cx. administration of 4-AP in the right cortex. In each set of records, traces 1-4 correspond to the left frontal, premotor, motor and occipital cortex, respectively, and traces 5-8 to the equivalent right cortices. Other details as in Fig. 1. Note that the NMDA receptor antagonists blocked the propagation of the epileptiform activity to the contralateral hemisphere. The convulsant action of 4-AP was observed in 4 rats; protective effects by the antagonists were observed in 4 of 6 treated rats for CPP, 5 of 7 for AP7, 4 of 4 for i.c.v. MK-801, and 4 of 6 rats for i.p. MK-801. Scale: horizontal bar = 2 s; vertical = 600 μvolts.

4-AP alone	+ CPP	+ AP7	+ MK-801	+ MK-801 i.p. (0.2 mg/kg)
BASAL				
1				
2				
3				
4				
INJECTION				
				
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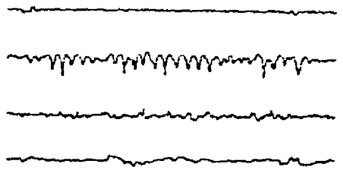
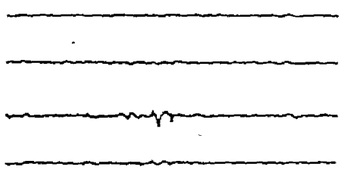
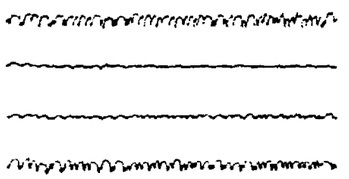
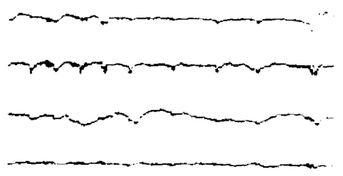
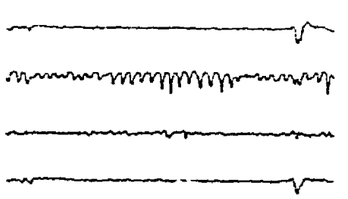
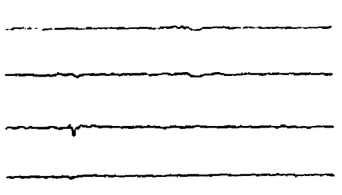
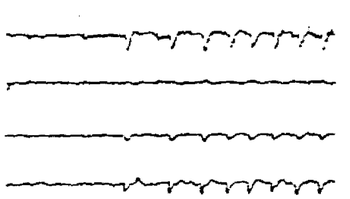
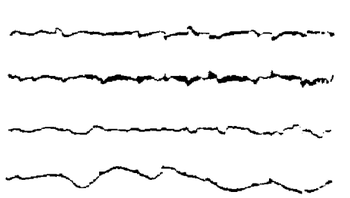
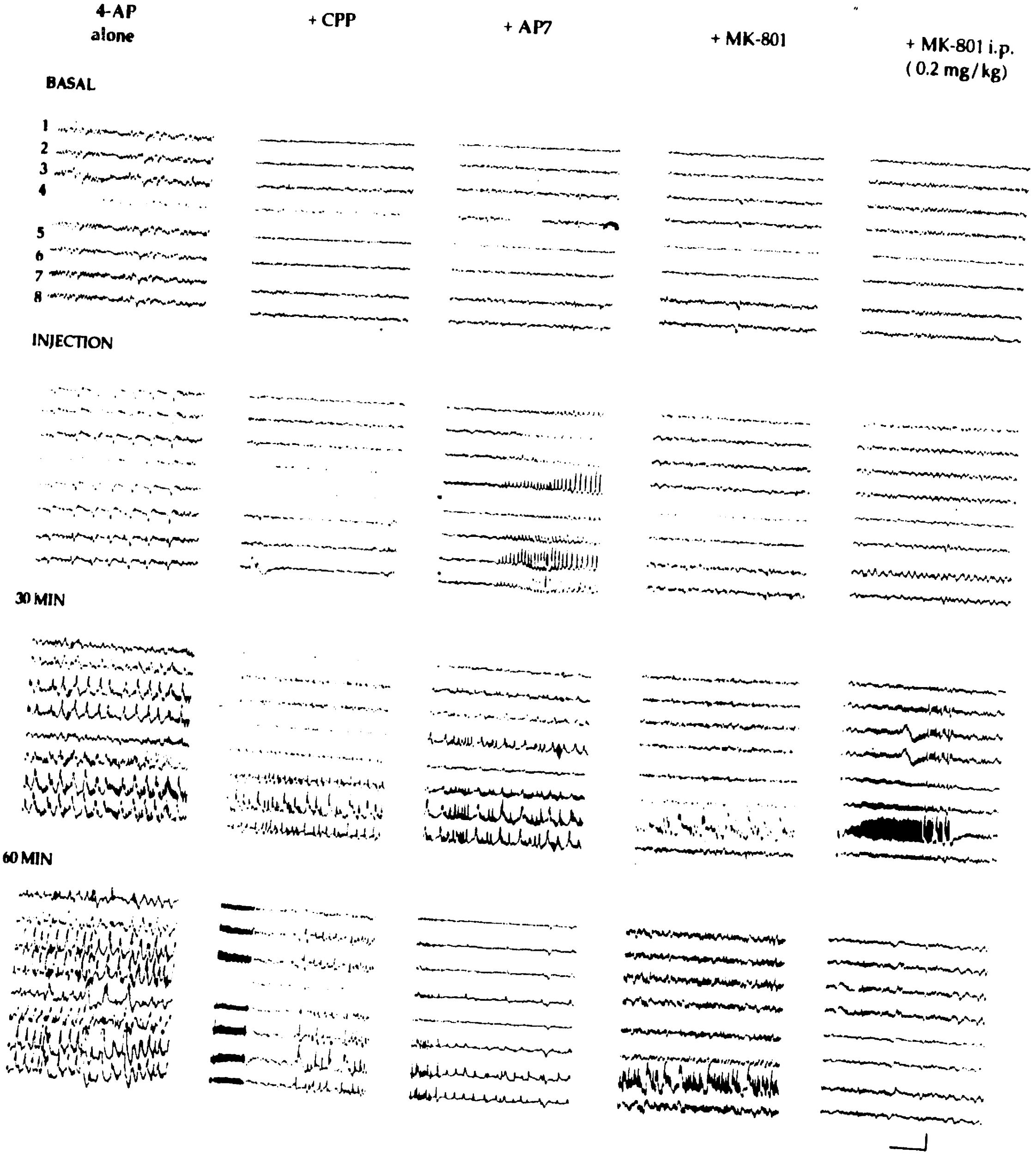
				
60 MIN				
				



DISCUSION

En el presente trabajo, se abordaron algunos principios básicos de la epilepsia en el modelo de crisis convulsivas inducidas por 4-AP. En el primer trabajo se presentan los resultados de las infusiones por 4-AP a través de cánulas de diálisis en el núcleo caudado y se observa de manera general, que aunque esta región no se considera epileptogénica, la 4-AP es capaz de inducir crisis convulsivas que varían en intensidad y frecuencia de acuerdo con la dosis administrada. Los resultados más relevantes de este estudio se relacionan con la liberación de glutamato observada. Esta liberación fue significativamente más alta que la de los demás aminoácidos evaluados. La explicación más probable es que dicho aumento se deba a la abundancia de terminales glutamatérgicas en esta región, más que a un efecto específico sobre las sinapsis glutamatérgicas.

Se han iniciado estudios para tratar de corroborar esta hipótesis, mediante decorticación de animales adultos con el objeto de producir una denervación de las fibras corticoestriatales, y así disminuir las sinapsis glutamatérgicas para valorar de nuevo los efectos tanto conductuales como bioquímicos de la 4-AP, con el mismo esquema de estudio. Es necesario señalar que el cuerpo estriado recibe una inervación de prácticamente toda la corteza, por lo que el área a eliminar sería muy grande.

De los resultados obtenidos en los estudio de liberación endógena, se puede sugerir que la sobrexcitación que se produce por la administración 4-AP, se debe a la

liberación excesiva de glu. Esta conclusión se apoya fuertemente en el hecho de que las alteraciones motoras producidas por la 4-AP son bloqueadas por. antagonistas del receptor NMDA.

En los experimentos realizados con el animal en libre movimiento, se observó que la concentración extracelular incrementó al inicio de la actividad convulsiva. A pesar de que la concentración de glu se relacionó con las alteraciones conductuales, la actividad convulsiva más severa se presentó aún cuando la concentración del aminoácido había regresado al nivel basal. Estos resultados sugieren que la concentración elevada de glu que se induce por la administración de 4-AP, da lugar al inicio de la actividad convulsiva y que la propagación de ésta involucra otros núcleos cerebrales y otros neurotransmisores, probablemente también glu.

Con respecto al mecanismo de acción de la 4-AP, se ha propuesto que sus efectos son principalmente debidos al efecto bloqueador que tiene esta droga sobre los canales de potasio sensibles al voltaje, particularmente sobre aquellos canales que se activan por despolarización y se inactivan lentamente, esto es, la corriente (I_D) o los que se abren rápidamente y de manera transitoria, después de un estímulo despolarizante (I_A), que de manera general retardan el período de repolarización y con esto se aumenta la cantidad de transmisor liberado. Un mecanismo alterno por el cual la 4-AP aumenta posiblemente la cantidad de neurotransmisor liberado, se relaciona con una facilitación de la acción del calcio sobre la liberación, ya que se sabe que este compuesto es capaz de inducir una liberación de neurotransmisores,

incluidos los aminoácidos, en ausencia de un estímulo despolarizante (Tapia y col., 1982; Tapia 1985), aunque no se puede descartar por completo el primer mecanismo. Los datos obtenidos en la presente tesis apoyan esta última hipótesis, ya que el tetraetilamonio (TEA), que posee un efecto similar al de la 4-AP sobre las corrientes de potasio, no produjo los efectos conductuales y bioquímicos que los observados con las infusiones de 4-AP. Como puede observarse en los resultados del primer trabajo, no fué posible demostrar *in vivo* la dependencia del calcio de la liberación, ya que al eliminar el calcio del medio de perfusión, se obtuvo una liberación significativa de los aminoácidos por efecto de la 4-AP. La explicación más probable es que la concentración extracelular presente en el tejido sea suficiente para facilitar el efecto de la 4-AP, ya que en preparaciones *in vitro* , se ha encontrado que la 4-AP requiere solamente de concentraciones mínimas de calcio para ejercer sus efectos sobre la transmisión sináptica (Tapia y col., 1982; Tapia y col., 1985).

La disminución en la concentración extracelular de glutamina que se indujo por la solución concentrada de potasio se ha observado en algunas regiones del cerebro y se relacionó con el hecho de que la glutamina es el precursor de glu, con lo que se sugiere que la liberación de glu depende del deposito disponible de glutamina (Paulsen y Fonnum ,1989; Sved y Curtis, 1993 y Young y Bradford, 1986). Sin embargo los datos presentados aquí no son consistentes con esta hipótesis, ya que la 4-AP indujo un incremento mayor, tanto de glu como de glutamina, que los producidos por la solución de potasio alto.

La taurina extracelular se incrementó de manera similar con 4-AP, TEA y alto

potasio. Previamente se ha encontrado que la la concentración extracelular de este aminoácido se aumenta con una variedad de estímulos, particularmente cuando se altera la osmolaridad (Dutton, 1993; Martin, 1922; Wade y col., 1988). Sin embargo nuestros resultados no pueden atribuirse a este hecho, ya que la osmolaridad fué controlada durante todo el desarrollo experimental, en cada uno de los tipos de estímulos utilizados.

Como se mencionó en la introducción, el exceso de glu extracelular se ha asociado a daño celular, por lo que se inició un estudio en el que se pretende evaluar si la 4-AP es capaz de inducir daño celular como consecuencia de la liberación excesiva de glu que produce. Es conveniente señalar que si la 4-AP produce daño neuronal, éste se lleva a cabo en etapas posteriores, ya que como se mencionó anteriormente, las neuronas responden de manera similar a un segundo estímulo de 4-AP.

La concentración excesiva de glu parece producir un daño selectivo sobre somas neuronales, respeta los axones de paso y terminales nerviosas, por lo que es de esperarse que la transmisión glutamatérgica no se dañe, sino mas bien la gran cantidad de neuronas GABAérgicas de proyección o bien las interneuronas colinérgicas que existen en el estriado. En experimentos realizados por Massieu y col. (1995), en los que se bloqueó la recaptura de glu con el inhibidor pirrolidin dicarboxilato, se encontró que la concentración excesiva de glu inducida por este compuesto no era suficiente para producir daño celular, quizá porque esta acumulación no ocurre cerca de los receptores a glu. Por lo tanto resulta interesante

evaluar si la 4-AP es capaz de inducir daño celular por poseer efectos principalmente sobre las terminales nerviosas presinápticas.

La administración de 4-AP en las diferentes áreas estudiadas en el presente trabajo produjo crisis límbicas con un período convulsivo largo y una latencia relativamente corta, que se inicia con una hiperreactividad del animal, seguida por mioclonos, temblores, movimientos masticatorios, salivación intensa, sacudidas de perro mojado, levantamiento del cuerpo sobre las patas traseras y caída ipsilateral al sitio de inyección (*rearing*), convulsiones clónicas y tónico-clónicas, que desaparecen lentamente, para regresar a un estado conductual casi normal. Algunos de los patrones de conducta mencionados anteriormente varian levemente, lo cual depende de la región donde se administre la droga. Sin embargo, es importante señalar que el "rearing" se presenta independientemente de la región en la que se administre la 4-AP, ventrículo lateral, corteza motora, área tempestas (datos no incluídos en el presente trabajo) y núcleo caudado, lo cual indica que posiblemente la estimulación de las regiones estudiadas incide sobre un mismo circuito neuronal responsable de la expresión de este patrón convulsivo.

Los datos presentados en el segundo trabajo muestran que los antagonistas del receptor NMDA son buenos anticonvulsivantes contra las crisis producidas por la administración intracerebral de 4-AP, que amplían los resultados previamente obtenidos cuando la 4-AP se administra sistémicamente (Fragoso-Veloz y col., 1992). Los tres antagonistas al NMDA probados en este estudio, y particularmente el MK-801, evitaron o redujeron la intensidad de las convulsiones inducidas por la 4-AP

cuando se administró i.c.v. o i.cx. Estos resultados, por lo tanto, apoyan la hipótesis planteada en el primer trabajo. Es interesante que tanto las crisis conductuales como electroencefalográficas son más intensas cuando la 4-AP se administra i.c.v. que cuando se administra en la corteza, y asimismo los efectos protectores de los antagonistas al NMDA son más evidentes. Esto sugiere que tanto la 4-AP como los antagonistas se difunden más extensamente cuando son inyectados i.c.v. y por lo tanto alcanzan un número relativamente más grande de sinapsis en varias regiones del cerebro, mientras que la difusión se restringe cuando se inyectan i.cx.

En conclusión, los resultados presentes en este trabajo, señalan que el glu liberado *in vivo* está implicado en el proceso convulsivo producido por la administración de 4-AP. Sin embargo, deberán realizarse estudios posteriores para conocer con más detalle la participación del glu, así como la de otros neurotransmisores en este modelo de epilepsia.

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