00581
2 1ej

RECEPTORES SINAPTICOS A LOS ACIDOS GLUTAMICO Y ASPARTICO EN LA RETINA

TRABAJO QUE PRESENTA LA
M. en C. ANA MARIA LOPEZ COLOME
PARA OBTENER EL GRADO DE
DOCTOR EN CIENCIAS QUIMICAS (BIOQUIMICA)

UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO
1983.

00581
1983

TESIS CON
FALLA DE ORIGEN

UNAM – Dirección General de Bibliotecas
Tesis Digitales
Restricciones de uso

DERECHOS RESERVADOS ©
PROHIBIDA SU REPRODUCCIÓN TOTAL O PARCIAL

Todo el material contenido en esta tesis esta protegido por la Ley Federal del Derecho de Autor (LFDA) de los Estados Unidos Mexicanos (México).

El uso de imágenes, fragmentos de videos, y demás material que sea objeto de protección de los derechos de autor, será exclusivamente para fines educativos e informativos y deberá citar la fuente donde la obtuvo mencionando el autor o autores. Cualquier uso distinto como el lucro, reproducción, edición o modificación, será perseguido y sancionado por el respectivo titular de los Derechos de Autor.

INTRODUCCION

En el Sistema Nervioso Central (SNC) de los vertebrados, la cantidad de sinapsis excitadoras excede con mucho a la de sinapsis inhibidoras. A pesar de ello, se conoce poco acerca de los neurotransmisores excitadores, sus receptores, las vías que se sirven de estos compuestos como transmisores o su mecanismo de acción, si se comparan con los neurotransmisores inhibidores. Dentro de los compuestos considerados como posibles neurotransmisores excitadores, se encuentran la acetilcolina y los ácidos glutámico y aspártico. Dado que la transmisión ionotrópica en las neuronas del SNC parece estar mediada principalmente por aminoácidos, el glutamato y el aspartato aparecen como los candidatos más viables para llevar a cabo dicha función, ya que ambos se encuentran en alta concentración en el tejido nervioso y llenan gran parte de los criterios fisiológicos (5,6,7,8,18,22,32,42), anatómicos (1,4,9, 12,16,26,39,40,41), bioquímicos (2,3,19,23,24,33,44,45) y farmacológicos (13,14) para ser considerados como neurotransmisores.

Puesto que el fenómeno de la neurotransmisión de tipo químico implica la interacción de la molécula neurotransmisora con su receptor postsináptico en la membrana, la presencia de receptores postsinápticos para un compuesto determinado se ha considerado como un criterio complementario para postular a dicho compuesto como neurotransmisor.

Receptores a glutamato y aspartato en el SNC:

Numerosos estudios indican que el efecto excitador que el L-glutamato y el L-aspartato ejercen sobre la mayor parte de las neuronas del

SNC está relacionado con diferentes tipos de receptores membranales. Los datos que apoyan la existencia de estos receptores provienen tanto de estudios in vivo mediante la aplicación iontoforética de agonistas y antagonistas de la acción de estos aminoácidos excitadores, como de estudios in vitro en los que se ha caracterizado la interacción del glutamato y el aspartato con receptores aislados. La correlación entre estos dos tipos de estudios es importante, ya que en los estudios in vivo es difícil deslindar la interacción con receptores sinápticos de otros fenómenos simultáneos, mientras que los estudios in vitro no permiten discriminar claramente entre los diversos tipos de receptores sinápticos que pueden distinguirse en preparaciones fisiológicas (como sería el caso para los receptores extrasinápticos).

Tanto la caracterización de los receptores a glutamato y aspartato como la identificación de las vías nerviosas que puedieran emplear a estos compuestos como neurotransmisores se facilitaría ampliamente con el descubrimiento de antagonistas específicos de la respuesta postsináptica producida por glutamato o aspartato, ya que los compuestos hasta ahora empleados son en mayor o menor grado inespecíficos y por tanto ineficientes para distinguir entre receptores a glutamato o a aspartato.

Los antagonistas que han resultado más eficaces para bloquear el efecto de estos dos aminoácidos in vivo son el glutamato dietil éster (GDEE) para el ácido glutámico y el D-α-amino adipato (D-αAA) para el ácido aspártico aunque existe el problema de que su acción no es general sino específica para ciertas áreas del SNC (32).

a) Estudios in vivo: Basándose fundamentalmente en la sensibilidad de los receptores al GDEE o bien al DαAA y en la sensibilidad de la interacción farmacológica a la presencia de Mg^{++} y otros cationes divalentes, se ha clasificado a los receptores a aminoácidos acídicos en tres grupos (4 6): 1) Los que son activados por N-metil-D-aspartato (NMDA), ibotenato, D-glutamato y L-homocisteato. La acción excitadora de estos agonistas es antagonizada por DαAA y se inhibe en presencia de concentraciones relativamente bajas de magnesio; 2) los receptores activados por quiscualato cuya respuesta es bloqueada por el GDEE. La excitación provocada por esta interacción no es sensible a los bloqueadores de los receptores a NMDA ni se inhibe por Mg^{++}. Se considera que el L-glutamato y el L-aspartato interactúan respectivamente con los receptores a quiscualato y NMDA, dado que su acción fisiológica es bloqueada por el GDEE y el D-αAA respectivamente. 3) La tercera clase de receptores a aminoácidos acídicos la constituyen aquellos activados por el kainato; no son sensibles al magnesio ni a los bloqueadores de la acción del NMDA, o a la acción del GDEE.

Recientemente se ha descrito una nueva clase de aminoácidos excitadores heterocíclicos derivados del ácido ibotênico, que interactúan específicamente con los receptores de glutamato y aspartato en el SNC (17). El ácido α-amino-5-metil-3-hidroxi-4-isoxasolpropiônico (AMPA) funge como agonista del glutamato y su acción es sensible al GDEE, y el ácido α-amino-5-metil-3-hidroxi-4-isoxazolacético (AMAA) cuya acción excitadora se bloquea por D- AA el cual actúa como agonista específico del aspartato. Estos compuestos pueden ser de utilidad en las

futuras investigaciones relacionadas con el papel fisiológico y el mecanismo de acción del aspartato y el glutamato en el sistema nervioso central.

b) Estudios in vitro: La presencia de receptores sinápticos para los ácidos glutámico y aspártico en regiones específicas del SNC se ha considerado como prueba de su papel como neurotransmisores. Así, en años recientes se ha desarrollado una técnica que permite medir la interacción de los aminoácidos (posibles transmisores) con sus receptores específicos en las membranas (11). Esta técnica permite distinguir a los receptores de captación de alta afinidad de los receptores sinápticos, por la dependencia de sodio de los primeros. Sin embargo, esta técnica no permite distinguir entre los receptores postsinápticos y los extrasinápticos. Para que la interacción de un compuesto con su receptor pueda considerarse fisiológicamente significativa debe caracterizarse desde el punto de vista cinético y farmacológico. El proceso de interacción ligando-receptor debe ser saturable, con una constante de afinidad aparente (K_B) en la gama de nM (alta afinidad). Desde el punto de vista farmacológico, la interacción debe ser estereoespecífica y los compuestos agonistas y antagonistas deben modificar la interacción ligando-receptor en la misma forma en la que modifican la respuesta fisiológica. Aunque se ha descrito receptores de alta afinidad y estereoespecíficos para glutamato y aspartato en diversas áreas del sistema nervioso central, las constantes cinéticas difieren ampliamente como puede observarse en la Tabla I.

La farmacología de estas interacciones corresponde con los estu-

dios electrofisiológicos. Tomando en cuenta los resultados de competencia a nivel del receptor así como la estereoespecificidad de los mismos en este sistema in vitro, se ha clasificado a los receptores de glutamato y aspartato en cuatro clases (20): 1) Los que unen preferentemente ácido glutámico en configuración extendida y que pueden estudiarse usando kainato como ligando. 2) Los que unen preferentemente ácido glutámico en configuración parcialmente plegada, y pueden estudiarse usando L-glutamato como ligando. 3) Los que interactúan principalmente con ácido L-aspártico, y se estudian empleando L-aspartato como ligando. 4) Los que aceptan indistintamente L-aspartato o L-glutamato, pero no interactúan con el kainato; éstos pueden estudiarse empleando D-aspartato como ligando.

Como puede concluirse de los datos expuestos, el ácido glutámico y el ácido aspártico reunen muchas de las características anatómicas, químicas, fisiológicas y farmacológicas para ser considerados como transmisores ionotrópicos excitadores en el sistema nervioso central. Sin embargo, la comprobación absoluta de esta función así como la distinción entre las vías glutamatérgias y las aspartatérgicas probablemente tendrá que esperar al descubrimiento de antagonistas específicos de su acción post-sináptica.

II. Receptores a glutamato y aspartato en la retina:

La retina de los vertebrados está constituída por cinco diferentes tipos de neuronas dispuestas en tres capas celulares. En la primera se encuentran los fotorreceptores, en la segunda los somas de las

células bipolares, horizontales y amacrinas, y en la tercera las células ganglionares. Intercaladas entre estas capas celulares, se encuentran dos zonas sinápticas. En la zona plexiforme externa, establecen contacto las terminales de las células bipolares y horizontales (dirigidas hacia el exterior) con las terminaciones procedentes de los fotorreceptores. En la zona plexiforme interna, establecen contacto sináptico las células bipolares y amacrinas con las células ganglionares, cuyas terminaciones eferentes forman el nervio óptico. Las células gliales o de Müller, atraviesan la retina verticalmente en todo su espesor (10).

El impulso nervioso que se genera en la capa de los receptores, es transmitido en sentido vertical a través de las células bipolares y ganglionares hasta salir por el nervio óptico hacia centros superiores de integración. El impulso es modulado en sentido horizontal por las interneuronas inhibidoras horizontales y amacrinas. Se ha propuesto que la substancia química (neurotransmisor) que transmite el impulso nervioso de los fotorreceptores hacia las capas internas de la retina debe ser un compuesto excitador. Trifonov (43) ha postulado que en la oscuridad, este compuesto excitador se libera continuamente de la terminal sináptica del fotorreceptor; al incidir la luz sobre la retina, los flujos iónicos en el fotorreceptor se modifican y el neurotransmisor se dejaría de liberar. Esta hipótesis se apoya principalmente en estudios de tipo fisiológico, en los que numerosos investigadores han registrado la actividad de células post-sinápticas a los fotorreceptores. La identidad del compuesto neurotransmisor en cuestión se

desconoce aún, y nosotros tratamos de contribuir información a este respecto, usando la retina del pollo como modelo.

Se han postulado numerosas sustancias como transmisores a nivel de los fotorreceptores, pero los candidatos más viables parecen ser el ácido glutámico y el ácido aspártico (29). La evidencia más consistente que apoya el papel del glutamato y el aspartato como neurotransmisores, procede de estudios fisiológicos en los que se ha demostrado que los efectos producidos por estos compuestos cuando son aplicados exógenamente a la retina, semejan en todo los efectos producidos por el transmisor liberado naturalmente por estas células (21,35,47) tanto en su acción excitadora sobre neuronas determinadas como en la modificación de su efecto por agonistas y antagonistas farmacológicos (15,35,38).

Desde el punto de vista bioquímico, aunque la evidencia para postular al glutamato y al aspartato como neurotransmisores en la retina es más escasa, se ha podido demostrar que existe un sistema de captación específico, de alta afinidad y dependiente de la presencia de sodio en el medio, en terminales sinápticas de ambas capas plexiformes de la retina (25,34). Algunos estudios apoyan particularmente al aspartato como el transmisor de los fotorreceptores, ya que su liberación continua en la oscuridad disminuye notablemente cuando se ilumina la retina (31). Otros en cambio, postulan que el glutamato y no el aspartato podría ser el transmisor liberado por el fotorreceptor, pues la aplicación del ácido 2-amino-4-fosfonobutírico, análogo del glutamato bloquea la transmisión sináptica en las vías ON de la retina (38). Por

otra parte, estudios en retinas lesionadas con ácido kaínico han llevado a la proposición de que mientras el glutamato podría ser el transmisor de los bastones, el aspartato podría ser el transmisor utilizado por los conos (48).

Puesto que la existencia de receptores sinápticos para glutamato y aspartato en la retina apoyaría su función como neurotransmisores, hemos estudiado y caracterizado los receptores póstsinápticos para estos compuestos en la retina de pollo. Hemos encontrado que estos receptores de glutamato y aspartato son muy semejantes entre sí pero difieren en algunas de sus propiedades cinéticas y farmacológicas lo cual nos permite suponer que se trata de dos poblaciones de receptores, ambas sinápticas.

TABLA I

Comparación de las constantes cinéticas obtenidas para la unión del L-Glutamato con su receptor

Preparación	Rango de concentración	K_B (uM)	Capacidad del sistema (nmol/mg proteína)
Corteza cerebral de rata* (membranas sinápticas)	0.4 - 8.7 uM	a) 4.0 b) 8.3	0.2 0.028
Cerebro completo de rata* (membranas sinápticas)	0.1 - 30 uM	a) 0.18 b) 2.08	4.44 - -
Glicoproteínas de cerebro de rata*		0.85	65.5
Proteolípido de membranas aisladas de corteza cerebral de rata. *	0.06 - 250 uM	a) 0.3 b) 5.0 c) 55.0	0.53 32.0 166.0
Cerebelo de rata* (membranas sinápticas)	0.001 - 1.8 uM	0.744	0.073
Cerebelo de gato+ (membranas sinápticas	0.023 - 16 uM	a) 0.33 b) 1.8	0.015 0.065
Complejos sinápticos** de cerebro de rata	0.010 - 1.0 uM	0.453	0.090
Retina de pollo++ (membranas sinápticas)	0.001 - 2.0 uM	0.55	0.020

* Ref. 13
** Ref. 14
\+ y ++ estos trabajos.

Neurochemical Research, Vol. 6, No. 9, 1981

HIGH-AFFINITY BINDING OF L-GLUTAMATE TO CHICK RETINAL MEMBRANES

A. M. LÓPEZ-COLOMÉ

Centro de Investigaciones en Fisiología Celular
Universidad Nacional Autónoma de México
Apartado Postal 70-600
México 20, D. F. México

Accepted

Binding of L-[^{3}H]glutamate to membranes from whole chick retina and from subcellular fractions enriched with photoreceptor terminals (P_1), or terminals from the inner plexiform layer (P_2) was studied. Na^+-dependent and Na^+-independent binding to these membranes was demonstrated. Na^+-independent binding was stereospecific. Kinetic analysis of the binding process indicated a single high-affinity system (K_B = 0.55 μM) with a capacity of approximately 20 pmoles/mg protein in all the membrane fractions. [^{3}H]Glutamate binding to P_1 and P_2 fractions was effectively displaced by several structural analogues of glutamate. Glutamate diethyl-ester appreciably displaced binding, whereas kainic acid did not displace bound glutamate. Data indicate the binding of [^{3}H]glutamate to physiologically relevant receptors in the chick retina.

INTRODUCTION

L-Glutamate is likely to be a major excitatory transmitter in the mammalian central nervous system (1, 2) exerting its actions through specific membrane receptors (3). In the retina, it has been suggested that glutamate and/or aspartate could be the excitatory transmitter substance released from the photoreceptor terminals (4). It has been postulated that an excitatory neurotransmitter must be released continuously from the photoreceptor terminals in the dark, maintaining bipolar and horizontal cells depolarized. Upon light stimulation, the inward sodium current would be blocked, causing the hyperpolarization of photoreceptors and the arrest

0364-3190/81/0900-1019$03.00/0 © 1981 Plenum Publishing Corporation

of the excitatory neurotransmitter release (5). There is inferential evidence, that the hyperpolarizing response of Horizontal Cells to stimulation by light might be due to the decrease of an excitatory transmitter substance. The identity of this excitatory transmitter is unknown, but in the vertebrate retina it is unlikely to be acetylcholine or catecholamines, since these are not present in the outer retinal layers (6), and acetylcholine has been shown to be without effect when applied to the Horizontal Cells of the carp retina (7).

The evidence for postulating glutamate or aspartate as neurotransmitter candidates in the retina is mainly indirect, and is derived from electrophysiological studies, which clearly establish their capacity for depolarizing horizontal, bipolar, and amacrine cells (4, 8, 9). On the other hand, glutamate and aspartate have been found mainly localized in the external layers of the retina (10). A high-affinity, sodium-dependent uptake mechanism shared by glutamate and aspartate has been characterized in the rat retina (11, 12) as well as in synaptosomal fractions from the chick retina (13).

Recently, Neal et al. (14) have demonstrated in a superfusion system, the arrest of aspartate release following illumination of the rabbit retina, which backs up aspartate as the transmitter at this level. Based on studies of kainic acid lesions in the Goldfish retina, it has been proposed that while glutamate could be the transmitter released by rods: cones could release aspartate as the transmitter (15). Recently, pharmacological evidence has been provided as to propose glutamate as the neurotransmitter of the ON pathways in the retina (16).

The presence of specific postsynaptic receptors for a substance paralleling the distribution of its physiological effects, has been considered as evidence for the compound to play a neurotransmitter role. Binding of highly labeled ligands to membrane preparations is now a well established technique for the direct study of drug-receptor interactions, and has been applied extensively to the study of neurotransmitters and their physiological receptors. The binding of amino acids to synaptic membranes has been largely studied for GABA (17–21) and glycine (22, 23), which are considered as major neurotransmitter candidates in the CNS. In these studies, it has been established that the binding of amino acids to synaptic membranes in the absence of sodium involves an interaction with postsynaptic receptor sites, while binding to pre and postsynaptic uptake sites is a temperature and sodium-dependent phenomenon (17–23). Using these techniques, postsynaptic receptors for L-glutamate have been characterized in brain homogenates (24), synaptic membranes (25), isolated membrane glycoproteins (26), and proteolipid membrane fractions (27), as well as, in cerebellar membranes (28, 29).

We have, therefore, investigated the binding of L-[^{3}H]glutamate to membranes from the whole chick retina, as well as, from subcellular fractions enriched with photoreceptor terminals (P_1) and conventional synaptosomes from the inner plexiform layer (P_2) (30), in an attempt to demonstrate the interaction of glutamate with its postsynaptic receptor in the retina.

EXPERIMENTAL PROCEDURE

Membrane Preparation. Retinas free from pigment epithelium, were obtained from 4–6 week old chicks and osmotically shocked by homogenization in 20 volumes (w/v) of double distilled water in a glass-glass homogenizer. The homogenate was placed on ice for 15 min and the membranes were pelleted by centrifugation at 45,000*g* at 4°C for 20 min, washed twice with the same volume of water and recentrifuged. The final pellet was resuspended by homogenization in Krebs Tris buffer pH 7.4, without glucose for binding assays. Primary subcellular fractions were isolated as previously described (31) from retinas homogenized in 0.32 M sucrose containing 10^{-4} M $MgSO_4$. The crude nuclear fraction (P_1) and the crude synaptosomal fraction (P_2) were osmotically disrupted by homogenization in 20 vol of water, and membranes were obtained as described for whole retina.

Binding Assay. Essentially, binding assays were carried out following the procedure described by Enna & Snyder (21), except that Krebs Tris buffer was used instead of Krebs Citrate buffer. Na^+-dependent binding was measured in freshly prepared membranes; the membrane pellet for these assays was resuspended in Krebs Tris buffer (118 mM NaCl; 1,2 mM KH_2PO_4; 4.7 mM KCl; 2.5 mM $CaCl_2$; 1.17 mM $MgSO_4$; 26 mM Tris-HCl) at pH 7.4 without glucose. Assays for sodium-independent binding were carried out in membranes frozen at −20°C for a period between one and two weeks. Frozen membranes, unless stated otherwise, were incubated 30 min at 37°C in 50 volumes (w/v) of Krebs Tris buffer pH 7.4 without NaCl in the presence of 0.05% Triton-X-100 (final concentration) prior to the binding assay. Membranes were recovered by centrifugation and resuspended in Krebs-Tris buffer in which NaCl was omitted or substituted by sucrose.

For the binding assay, 2 ml of the membrane suspension (0.5–1.0 mg protein) were incubated on ice for 10 min in the presence of 20 nM L-[^{3}H]glutamate or 40 nM [^{3}H]kainic acid in the absence (control) or the presence of unlabeled L-glutamate (1 mM final concentration), or an equivalent concentration of the compound under test. The reaction was stopped by centrifugation at 45,000*g* at 4°C for 20 min.

After decanting the supernatant, the membrane pellets containing bound radioactivity were superficially rinsed twice with 5 ml of double distilled water and solubilized in 0.2 ml of NCS (Tissue solubilizer, Amersham). Bound radioactivity was determined by the addition of 10 ml of Tritosol (32) followed by liquid scintillation spectrometry. All samples were kept 12 hr in the dark before counting. Radioactivity was corrected for quenching, background, and counting efficiency. Calculation of the specific binding was made by subtraction of the non-specific binding component, which persisted in the presence of 1 mM unlabeled compound, from the total binding in the absence of unlabeled glutamate. Protein was measured by the method of Lowry et al. (33).

Materials. L-[^{3}H]glutamate (43–46 Ci/m mol) and [^{3}H]kainic acid (7.8 Ci/m mol) were obtained from New England Nuclear Corp., Boston, Massachusetts. NCS (Tissue Solubilizer) was from Amersham-Searle Corp., Arlington Hills, Illinois. All common analytical agents and drugs were from Sigma Chemical Co., St. Louis, Missouri.

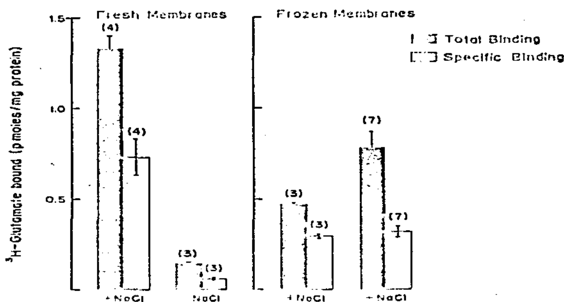

FIG. 1. Sodium-dependence of L-[^{3}H]glutamate binding to fresh and frozen membranes from whole retina. Membranes were obtained as described in the text. Assays were conducted in the presence or the absence of 118 mM NaCl. Membranes were incubated in the presence of 20 nM [^{3}H]glutamate, and specific binding was measured by displacement of bound glutamate with cold glutamate (1 mM final concentration). Values are the means ± SEM of the number of separate experiments included in parentheses. All experiments were performed in triplicate.

RESULTS

Binding of L-[^{3}H]Glutamate to Whole Retinal Membranes. The presence of specific receptors for L-glutamate was assayed in membranes obtained from whole retina homogenates. Binding to freshly prepared membranes in the presence of 118 mM NaCl, which represents mainly an interaction with transport sites was determined (20, 34). Specific binding under this condition was 0.75 pmoles/mg protein, which represents 56% of total binding, which was 1.33 pmoles/mg protein (Figure 1).

In frozen and thawed membranes in the absence of sodium, in which interaction with postsynaptic sites should be more apparent (17–21), total binding to the membranes was 0.78 pmoles/mg protein, and specific binding was 0.32 pmoles/mg protein. These membranes were previously incubated with Triton-X-100, which slightly enhances binding (see below).

Na^+-Dependence of L-glutamic Acid Binding. Binding to uptake sites, which is prior to transport itself, seems to be absolutely sodium-dependent, while binding to postsynaptic receptors shows no requirement for sodium, and in the case of glutamate, is even inhibited by the presence

of sodium (35). In an attempt to clarify the nature of binding to retinal membranes, the Na^+-dependence of binding to fresh and frozen membranes from whole retina was measured. Whereas, binding to fresh membranes was almost abolished in the absence of sodium, binding to frozen membranes was even slightly enhanced in the absence of this ion (Figure 1).

Binding to Subcellular Fractions. Subcellular distribution of L-glutamate binding (20 nm) as displaced by 1 mM cold glutamate was determined in the presence and absence of physiological sodium concentration. Binding to fresh membranes in the presence of sodium was equally distributed in the membranes from the P_1 fraction, which contains the photoreceptor terminals and very few conventional nerve endings, and the P_2 fraction, which contains only conventional synaptosomes from the inner plexiform layer.

Binding to frozen membranes in the absence of sodium was slightly higher in the P_2 fraction, as compared to the P_1 fraction (Table I).

Effect of Triton X-100. Triton treatment has been reported to enhance specific binding in some preparations (20), probably due to unmasking of receptor sites, or removal of inhibitory elements (36). However, in other cases Triton has been reported to decrease specific binding of glutamate (37, 38). In order to determine the sensitivity of retinal glutamate receptors, we measured binding to fresh and frozen membranes from subcellular fractions in the presence and absence of Triton. In fresh membranes (with Na^+) Triton decreased binding, whereas, in frozen membranes (without Na^+) it even enhanced specific glutamate binding (Figure 2).

Binding of [^3H]Kainic Acid. Kainic acid has been considered by some authors as a possible tool for mapping glutamanergic pathways (39, 40). Although characteristics of kainic acid receptor binding were studied by Coyle et al. in the whole retina (41), we tried to determine if kainate receptors could be restricted to, or concentrated in, membranes from the inner or the outer plexiform layer, and if this distribution paralleled glutamate receptors distribution.

Kainic acid binds specifically to freshly obtained retinal membranes in the presence of sodium, as well as, to frozen membranes in the absence of sodium. Values obtained were with 0.05 pmoles/mg protein, which are about 10 times lower than those obtained for glutamic acid under the same conditions. Although non-specific binding was higher in the presence of sodium, specific binding remained unchanged in both conditions. In all cases, 1 mM glutamate displaced [^3H]kainate bound 100%.

Kinetics of L-[^3H]Glutamate Binding to Membranes From Whole Retina and Subcellular Fractions. The saturation curve for L-glutamate binding was studied incubating the membranes from either whole retina or sub-

TABLE I

SUBCELLULAR DISTRIBUTION OF Na^+-DEPENDENT AND Na^+-INDEPENDENT BINDING OF L-[^{3}H]GLUTAMATE

Membrane preparation	[^{3}H]glutamate binding (pmoles/mg protein)					
	Fresh membranes + NaCl			Frozen membranes − NaCl		
	Total	Nonspecific	Specific	Total	Nonspecific	Specific
Whole retina	1.33 ± 0.085 (4)	0.58 ± 0.051 (4)	0.75 ± 0.120 (4)	0.776 ± 0.090 (7)	0.456 ± 0.074 (7)	0.320 ± 0.035 (7)
P_1	0.627 ± 0.011 (3)	0.247 ± 0.016 (3)	0.329 ± 0.045 (3)	0.452 ± 0.05 (5)	0.238 ± 0.047 (5)	0.246 ± 0.026 (5)
P_2	0.449 ± 0.033 (3)	0.171 ± 0.010 (3)	0.241 ± 0.038 (3)	0.448 ± 0.025 (3)	0.258 ± 0.041 (3)	0.190 ± 0.035 (3)

Membranes were prepared as described in Experimental Procedure. [^{3}H]Glutamate (20nM) was displaced with 1 mM cold glutamate. Results are expressed as the means ± SEM of the number of experiments indicated in parentheses.

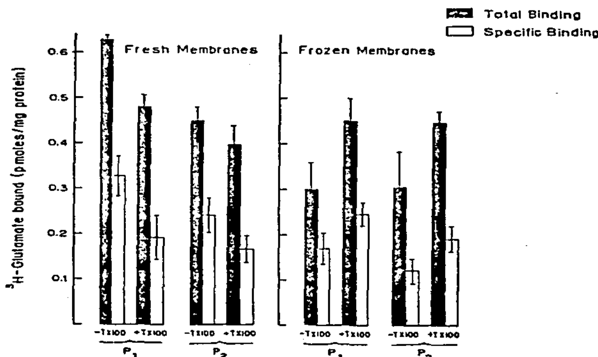

FIG. 2. Effect of Triton-X-100 on L-[^{3}H]glutamate binding to membranes from subcellular fractions. Subcellular retinal fractions and membranes from these fractions were obtained as described in Experimental Procedure. With fresh membranes, assays were conducted in the presence of 118 mM NaCl and with frozen membranes, in the absence of this ion. Prior to the assay, membranes were incubated 30 min at 37°C in the presence or absence of 0.05% Triton-X-100. Membranes were recovered by centrifugation, washed once and then resuspended in Krebs Tris medium for the binding assay. Results are the means ± SEM of 3–5 separate experiments performed in duplicate.

cellular fractions in the presence of increasing concentrations of [^{3}H] glutamate from 0.001 to 2.0 μM. In all three cases, binding showed saturation kinetics with a K_B of 0.555 μM. Maximum capacity of the system varied between 19 pmoles/mg protein for membranes from whole retina (Figure 3), and 15–22 pmoles/mg protein for membranes from P_1 and P_2 fractions, respectively (Figure 4). Double reciprocal plots (Figures 3, 4), as well as, Scatchard analysis (not shown), revealed a single component of the binding system, (see Figure 5).

Displacement of L-[^{3}H]Glutamate Binding by Structurally Related Compounds. The specificity of L-glutamate binding to membranes from retinal subcellular fractions was tested using 1 mM concentration of different analogues as displacers in the binding assay. Table II shows that L-glutamic acid was the most potent displacer of bound glutamate. L-aspartate, L-cysteic acid, and L-cysteine sulfinic acid displaced about 90%

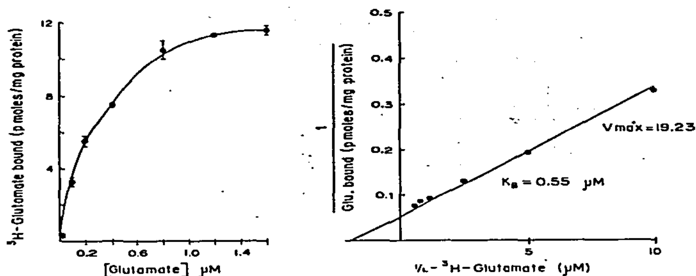

FIG. 3. Saturation of specific Na^{+}-independent [^{3}H]glutamate binding to membranes from whole chick retina. Frozen membranes preincubated with Triton were resuspended in Krebs Tris medium without NaCl. Membranes were incubated in the presence of increasing concentrations of [^{3}H]glutamate. Linear plot (left) and Double Reciprocal plot (right) revealed a single high affinity component. Each point represents the mean ± SEM of 3–5 experiments performed in triplicate.

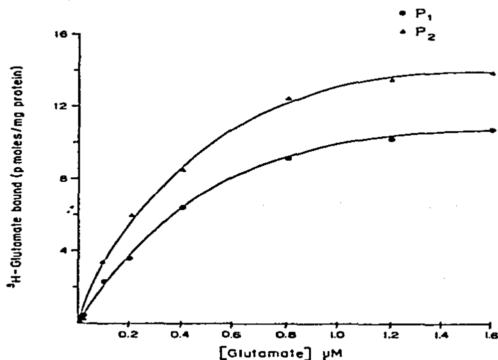

FIG. 4. Saturability of specific Na^{+}-independent [^{3}H]glutamate binding to retinal subcellular fractions. Experiments were performed as described in Figure 3. [^{3}H]Glutamate binding was investigated over a glutamate concentration range of 0.001–2.0 μM. Results are means of 3–6 separate experiments performed in triplicate, which varied less than 10%. ●, P_1 fraction: ▲, P_2 fraction.

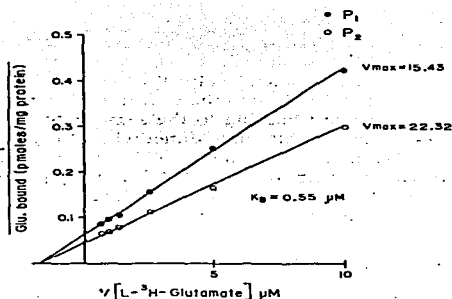

FIG. 5. Lineweaver–Burk plot of specific Na^+-independent [^{3}H]glutamate binding to retinal subcellular fractions. Experimental conditions and direct plot are shown in Figure 4. Each point is mean of 3–6 separate experiments performed in triplicate. ●, P_1 fraction; ○, P_2 fraction.

TABLE II
DISPLACEMENT OF L-[^{3}H]GLUTAMATE BY STRUCTURALLY RELATED COMPOUNDS.

Compound (1 mM)	% Displacement	
	P_1 Membranes	P_2 Membranes
L-Glutamate	100	100
L-Aspartate	89.0	98.9
LCysteic acid	95.8	84.3
L-Cysteine sulfinic acid	93.7	88.5
DL-α Amino adipic acid	60.0	73.4
D-Glutamate	45.5	95.0
D-Aspartate	47.5	70.5
N-Methyl-DL-glutamate	65.35	91.2
β-Methyl-DL-aspartate	62.2	73.9
L-Glutamate dimethyl ester	60.0	89.6
L-Glutamate-diethyl ester	77.0	85.2
Kainic acid	0	0
GABA	14.7	30.0
Glycine	20.0	11.0

Specific [^{3}H]glutamate binding was determined as described in the text at a final [^{3}H]glutamate concentration of 20 nM. Displacers were added to a final concentration of 1 mM. Results are expressed as means of 5–10 experiments which varied less than 15%.

bound glutamate in both P_1 and P_2 fractions. α-amino adipic acid and β-methyl-DL-aspartate produced in both fractions similar displacement (60–70%). Binding to the P_1 fraction showed some stereospecificity; D-glutamate and D-aspartate displaced only 50% bound glutamate at 1 mM concentration. In contrast, binding to the P_2 fraction proved less specific; both D-compounds displaced binding to the same extent as L-glutamate or L-aspartate (90–100%). L-Glutamate dimethyl and L-glutamate diethyl esters (GDEE), displaced glutamate about 70% in the P_1 fraction and 80% in the P_2 fraction. Other compounds, as GABA and glycine did not considerably displace binding. Kainic acid, considered as a glutamate analogue, did not displace binding to any extent.

DISCUSSION

One of the main criteria to be fulfilled by a neurotransmitter candidate, is its specific interaction with postsynaptic receptors. In the case of the amino acids GABA and glycine, which have been postulated as transmitters, binding studies have successfully correlated the physiological effects of the compounds with the distribution of postsynaptic receptors assayed through the radiolabeled ligand binding techniques (17–23). For these amino acids, specific modifications of the assay conditions have made it possible to distinguish between binding to uptake sites and to postsynaptic receptor sites in the membranes (20, 34). In these cases, the use of labeled agonists or antagonists of high specific radioactivity have been helpful in labeling postsynaptic receptors (42, 43). L-Glutamate binding sites have been characterized in different preparations of nervous tissue (24–29, 37, 38). In these studies, [^{3}H]glutamate was used as a ligand and the evidence for dealing with postsynaptic receptors comes mainly from pharmacological interactions and kinetic constants, although there is great divergence in reported data (24, 25, 27). In spite of the publication of specific reports on the methodology (37), the conditions in which these receptors should be assayed remains a matter of discussion, due to the lack of specific postsynaptic antagonists for glutamate. As glutamate itself is to be used as a ligand, difficulties arise in distinguishing binding to pre- or postsynaptic uptake sites, or to enzyme active sites for which glutamate is a substrate, and binding to postsynaptic receptors.

We have tried to characterize glutamate receptors by means of the method which has successfully been used for identifying other transmitter amino acid receptors (21). It has been accepted that binding measured at high temperature in the presence of sodium corresponds to an interaction with transport sites mainly, whereas, binding measured at low temperature and in the absence of sodium is mainly to postsynaptic sites (20).

Our results seem to agree with this criterion, for whereas, binding to fresh membranes in the presence of sodium was almost abolished in the absence of this ion, binding to frozen membranes at 4°C in a sodium-free medium was even slightly enhanced (Figure 1). These data closely agree with those obtained for GABA in cortical synaptic membranes (19, 20).

Endogenous inhibitors of glutamate (37) and GABA binding (36, 44) have been reported recently. These compounds are said to be removed from the membranes through repeated washing, preincubation at high temperature, or detergent treatment. Under our conditions, Triton treatment depressed binding to fresh membranes in the presence of sodium (uptake sites), while binding to frozen and thawed membranes in the absence of sodium was enhanced (Table I). These results are also in agreement with previous reports for GABA. Foster and Roberts (28) and Sharif and Roberts (37) have reported Triton treatment and freezing of the membranes to depress glutamate receptor binding in cerebellar and brain membranes, however, this discrepancy could be due to differences in the membrane preparations and the assay conditions used.

Binding of neurotransmitter amino acids to frozen membranes in the absence of sodium has been shown to concentrate in synaptic membranes as compared to whole homogenate membranes. Glutamate binding in our system did not concentrate in either subcellular fraction; this could be due to the existence of different synapses in the retina using glutamate as a transmitter.

Kinetics of glutamate binding revealed a single component of the binding system in whole retina, and consequently in both subcellular fractions (Figures 3, 4). The same K_B was obtained (0.555 μM) with a slightly higher number of binding sites in the P_2 fraction membranes. The constants obtained are in agreement with those reported by Enna and Snyder for the GABA receptor in rat brain (20), by Foster and Roberts (28) for glutamate receptors in rat cerebellum (0.7 μM), and more recently, by Head et al. (29) in cat cerebellar membranes (0.33 μM). The last authors find a second component of lower affinity (1.8 μM), which was not reported by the former authors, nor detected by us in the retina, probably because of the differences in the concentration range explored. The dimensions of the K_B obtained, makes it unprobable that binding is to an enzyme, for K_M for enzymes are much closer to the millimolar range. Although binding constants are not necessarily in agreement with the transport constants, they generally are in the same range. Thomas and Redburn have recently reported in the chick retina, uptake constants for glutamate and aspartate, which are 1–2 μM for synaptosomal fractions (13). The constant we report for binding is of higher affinity, which would be in agreement with postsynaptic receptor binding.

The pharmacological specificity of glutamate binding was also investigated in order to support glutamate interaction with physiologically relevant receptors, and if possible, to distinguish between glutamate receptors at the outer and the inner plexiform layers. Glutamate itself was the most potent displacer of [^{3}H]glutamate binding. Other acidic amino acids which have close structural relation to glutamate were almost, as potent as, aspartate, cysteic acid, and cysteine sulfinic acid, which displaced 90 to 100% bound glutamate from both subcellular fractions. Both aspartate (14) and glutamate (16) have been proposed as the excitatory neurotransmitter released from photoreceptor terminals. L-Aspartate as well as the aspartate antagonist α-aminoadipic acid and β-methyl-DL-aspartate which is a potent aspartate agonist in the spinal cord (46, 47), displaced significantly less glutamate from the P_1 than from the P_2 fraction, which could indicate a higher number of glutamate over aspartate receptors at the outer plexiform layer. These data would be in agreement with those of Yazulla and Kleinschmidt (15), who have proposed that aspartate could be the transmitter released by cones, whereas, glutamate could be released by rods.

L-Glutamate dimethyl ester and L-glutamate diethyl ester (GDEE), which act as glutamate postsynaptic antagonists in some preparations (48), displaced glutamate binding in both P_1 and P_2 fractions.

L-Glutamate binding to the P_1 fraction showed to be stereospecific; D-isomers of glutamate and aspartate displaced binding only 50% in this fraction, even at a 10^4 fold excess over the ligand concentration. Other neurotransmitter candidates, as GABA, or glycine had non-significant displacing effects.

Kainic acid, the restricted glutamate analogue, which has been considered to act via glutamate receptors (39, 40), had no displacing effect on glutamate binding to any of the fractions (Table II). These results agree with those of Simon et al. (52), Foster and Roberts (28), and Head et al. (29), whom in different preparations did not find displacement of bound glutamate by kainate, and suggest that in the retina, as has been shown in other CNS regions (49–51), kainic acid interacts with a different kind of receptors than glutamate.

This study demonstrates the presence in the retina of high affinity glutamate receptors, which show certain degree of pharmacological specificity. Conditions in which binding was assayed have been shown to evidence mainly synaptic receptors, therefore, supporting the possibility for glutamate subserving a neurotransmitter role in this organ. Since it has been proposed to act at the photoreceptor level, an attempt was made to find some differences in glutamate receptors at the outer (P_1) and the inner (P_2) plexiform layers. Although membranes from the P_1 fractions,

which is enriched with photoreceptor terminals, and the P_2 fraction, which is enriched with nerve endings from the inner plexiform layer (30), both show high affinity glutamate binding; the P_1 fraction shows a slightly higher degree of pharmacological specificity. This might suggest that although glutamate could be used in more than one kind of synapses in the retina, as proposed by Murakami et al. (53), it could have a more specific receptor at the outer plexiform layer. However, as aspartate and its analogues are also efficient in displacing bound glutamate, we cannot rule out the possibility of aspartate being also a transmitter at the photoreceptor terminals, and experiments are now in progress to clarify this point.

ACKNOWLEDGMENT

The author would like to thank Miss Frida Somohano for her excellent assistance.

REFERENCES

1. Curtis, D. R., and Watkins, J. C. 1960. The excitation and depression of spinal neurones by structurally related amino acids. J. Neurochem 6:117–141.
2. Krnjević, K., and Phillis, J. W. 1963. Iontophoretic studies of neurones in the mammalian cerebral cortex. J. Physiol. (London) 165:274–304.
3. Coombs, J. S., Eccles, J. C., and Fatt, P. 1955. The specific ionic conductances and the ionic movements across the motoneuronal membrane that produce the inhibitory postsynaptic potential. J. Physiol. (London) 130:326–373.
4. Dowling, J. E., and Ripps, H. 1973. Effect of magnesium on horizontal cell activity in the skate retina. Nature, Lond. 242:101–103.
5. Trifonov, Y. A., 1968. Study of synaptic transmission between photoreceptors and horizontal cells by means of electrical stimulation of the retina. Biofizika 13:809–817.
6. Neal, M. J. 1976. Amino acid transmitter substances in the vertebrate retina. Gen. Pharmac. 7:321–332.
7. Murakami, M., Ohtsu, K., and Ohtsuka, T. 1972. Effects of chemicals on receptors and horizontal cells in the retina. J. Physiol. (London) 227:899–913.
8. Cervetto, L., and Piccolino, M. 1974. Synaptic transmission between photoreceptors and horizontal cells in the turtle retina. Science, 183:417–419.
9. Gershenfeld, H. M., and Piccolino, M. 1979. Pharmacology of the connections of cones and L-Horizontal cells in the vertebrate retina. Pages 213–226, in Schmitt, F. O. and Worden, F. G. (eds.). The Neurosciences: Fourth Study Program, MIT Press, Cambridge, Mass.
10. Kennedy, A. J., and Voaden, M. J. 1974. Distribution of free amino acids in the frog retina. Biochem. Soc. Trans. 2:1256–1258.
11. Neal, M. J., and White, R. D. 1971. Uptake of ^{14}C-L-glutamate by rat retina. Br. J. Pharm. 43:442–443.
12. White, R. D., and Neal, M. J. 1976. The uptake of L-glutamate by the retina. Brain Res. 111:79–83.

13. THOMAS, N. T., and REDBURN, D. A. 1978. Uptake of ^{14}C-aspartic acid and ^{14}C-glutamic acid by retinal synaptosomal fractions. J. Neurochem. 31:63–68.
14. NEAL, M. J., COLLINS, G. G., and MASSEY, S. C. 1979. Inhibition of aspartate release from the retina of the anaesthetised rabbit by stimulation with light flashes. Neurosci. Lett. 14:241–245.
15. YAZULLA, S., and KLEINSCHMIDT, J. 1980. The effects of intraocular injection of kainic acid on the synaptic organization of the goldfish retina. Brain Res. 182:287–301.
16. SLAUGHTER, M. M., and MILLER, R. F. 1981. 2-Amino-4-Phosphonobutyric acid: A new pharmacological tool for retina research. Science 211:182–185.
17. SANO, K., and ROBERTS, E. 1963. Binding of γ-aminobutyric acid by mouse brain preparations. Biochem. Pharmacol. 12:489–502.
18. PECK, E. J., SCHAEFFER, J. M., and CLARK, J. H. 1973. γ-Aminobutyric acid, bicuculline and postsynaptic binding sites. Biochem. Biophys. Res. Commun. 52:394–400.
19. ZUKIN, S. R., YOUNG, A. B., and SNYDER, S. H. 1974. Gamma-aminobutyric acid binding to receptor sites in the rat central nervous system. Proc. Natl. Acad. Sci. (USA) 71:4802–4807.
20. ENNA, S. J., and SNYDER, S. H. 1976. Properties of γ-aminobutyric acid (GABA) receptor binding in rat brain synaptic membrane fractions. Brain Res. 100:81–97.
21. ENNA, S. J., and SNYDER, S. H. 1976. Gamma-aminobutyric acid (GABA) receptor binding in mammalian retina. Brain Res. 115:174–179.
22. DE FEUDIS, F. U., ORENSANZ MUÑOZ, L. M., and FANDO, J. L. 1978. High-affinity glycine binding sites in rat CNS: regional variation and strychnine sensitivity. Gen. Pharmac. 9:171–176.
23. SNYDER, S. H. 1975. The glycine synaptic receptor in the mammalian central nervous system. Br. J. Pharmac. 53:473–484.
24. ROBERTS, P. J. 1974. Glutamate receptors in rat central nervous system. Nature (London) 252:399–401.
25. MICHAELIS, E. K., MICHAELIS, M. L., and BOYARSKY, L. L. 1974. High-affinity glutamate binding to brain synaptic membranes. Biochim. Biophys. Acta 367:338–348.
26. MICHAELIS, E. K. 1975. Partial purification and characterization of a glutamate-binding membrane glycoprotein from rat brain. Biochem. Biophys. Res. Commun. 65:1004–1012.
27. DE ROBERTIS, E., and FISZER DE PLAZAS, S. 1976. Isolation of hydrophobic proteins binding amino acids. Selectivity of the binding of L-[^{14}C]glutamate in cerebral cortex. J. Neurochem. 26:1237–1243.
28. FOSTER, A. C., and ROBERTS, P. J. 1978. High-affinity L-[^{3}H]glutamate binding to postsynaptic receptor sites on rat cerebellar membranes. J. Neurochem. 31:1467–1477.
29. HEAD, R. H., TUNNICLIFF, G., and MATHESON, G. K. 1980. Glutamate receptor binding to cat central nervous system membranes. Can. J. Biochem. 58:534–538.
30. NEAL, M. J., and ATTERWILL, C. K. 1974. Isolation of photoreceptor and conventional nerve terminals by subcellular fractionation of rabbit retina. Nature 251:331–333.
31. LÓPEZ-COLOMÉ,, A. M., SALCEDA, R., and PASANTES-MORALES, H. 1978. Potassium-stimulated release of GABA, glycine and taurine from the chick retina. Neurochem. Res. 3:431–441.
32. FRICKE, V. 1975. Tritosol: A New scintillation cocktail based on Triton-X-100. Anal. Biochem. 63:555–558.
33. LOWRY, O. H., ROSEBROUGH, W. J., FARR, A. L., and RANDALL, R. S. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
34. YOUNG, A. B., and SNYDER, S. H. 1973. Strychnine binding associated with glycine receptors of the central nervous system. Proc. Nat. Acad. Sci. (USA) 70:2832–2836.
35. BAUDRY, M., and LYNCH, G. 1979. Regulation of glutamate receptors by cations. Nature 282:748–750.

36. YONEDA, Y., and KURIYAMA, K. 1980. Presence of a low molecular weight endogenous inhibitor of [^{3}H]muscimol binding in synaptic membranes. Nature 285:670–673.
37. SHARIFF, N. A., and ROBERTS, P. J. 1980. Problems associated with the binding of L-glutamic acid to synaptic membranes: Methodological aspects. J. Neurochem. 34:779–784.
38. BIZIERE, K., THOMPSON, H., and COYLE, J. T. 1980. Characterization of specific, high-affinity binding sites for L-[^{3}H]glutamic acid in rat brain membranes. Brain Res. 183:421–433.
39. HERNDON, R. M., and COYDE, J. T. 1977. Selective destruction of neurons by a transmitter agonist. Science 198:71–72.
40. MCGEER, E. G., MCGEER, P. L., and SINGH, K. 1978. Kainate-induced degeneration of neostriatal neurons: dependency upon corticostriatal tract. Brain. Res. 139:381–383.
41. COYLE, J. T., BIZIERE, K., and SCHWARCZ, R. 1978. Neurotoxicity of excitatory amino acids in the neural retinal. Pages 177–188, in MCGEER, E. G. et al. (eds.), Kainic acid as a tool in neurobiology, Raven Press, New York.
42. LE FORT, D., HENKE, H., and CUÉNOD, M. 1978. Glycine specific [^{3}H]strychnine binding in the pigeon CNS. J. Neurochem 30:1287–1291.
43. WANG, Y. J., SALVATERRA, P., and ROBERTS, E. 1979. Characterization of [^{3}H]muscimol binding to mouse brain membranes. Biochem. Pharmacol. 28:1123–1128.
44. TOFFANO, G., GUIDOTTI, A., and COSTA, E. 1978. Purification of an endogenous protein inhibitor of the high affinity binding of γ-aminobutyric acid to synaptic membranes of rat brain. Proc. Natl. Acad. Sci. (USA) 75:4024–4028.
45. EHINGER, B., and FLACK, B. 1971. Autoradiography of some suspected neurotransmitter substances: GABA, glycine, glutamic acid, histamine, dopamine and L-DOPA. Brain Res. 33:157–172.
46. BERGEY, G. K., MARTIN, M. R., and HERMES, M. 1980. Effect of D-L-α aminoadipate on postsynaptic amino acids responses in cultured mouse spinal cord neurons. Brain Res. 193:199–207.
47. PADJEN, A. L., and SMITH, P. A. 1980. Specific effects of α-D,L-aminoadipic acid on synaptic transmission in frog spinal cord. Can. J. Physiol. Pharmacol. 58:692–698.
48. JOHNSTON, G. A. R. 1979. Central Nervous System Receptors for Glutamic acid. Pages 177–185, in FILER, L. J., JR. et al. (eds.) Glutamic Acid: Advances in Biochemistry and Physiology Raven Press, New York.
49. MICHAELIS, E. K., MICHAELIS, M. L., and GRUBBS, R. D. 1980. Distinguishing characteristics between glutamate and kainic acid binding sites in brain synaptic membranes. FEBS Letters 118:55–57.
50. SEIL, F. J., and WOODWARD, W. R. 1980. Kainic acid neurotoxicity in granuloprival cerebellar cultures. Brain Res. 197:285–289.
51. HALL, J. G., HICKS, T. P., and MCLENNAN, H. 1978. Kainic acid and the glutamate receptor. Neurosci. Lett. 8:171–175.
52. SIMON, J. R., CONTRERA, J. F., and KUHAR, M. J. 1976. Binding of [^{3}H]kainic acid, an analogue of L-glutamate, to brain membranes. J. Neurochem. 26:141–147.
53. MURAKAMI, M., OHTSUKA, T., and SHIMASAKI, H. 1975. Effects of aspartate and glutamate on the bipolar cells of the carp retina. Vision Res. 15:456–458.

Vision Res. Vol. 22, pp. 1495 to 1501, 1982
Printed in Great Britain

0042-6989/82/121495-07$03.00/0
Pergamon Press Ltd

CHARACTERIZATION OF [L-^{3}H]ASPARTATE BINDING TO CHICK RETINAL SUBCELLULAR FRACTIONS

A. M. López-Colomé* and F. Somohano

Centro de Investigaciones en Fisiologia Celular, Universidad Nacional Autónoma de México, Apartado Postal 70–600, 04510 México, D. F. México

Abstract—Binding of [L-^{3}H]aspartate to synaptic receptors was examined in membranes from whole chick retina and subcellular fractions enriched with photoreceptor terminals (P_1) or terminals from the inner plexiform layer (P_2). Na^+-independent, stereospecific, high affinity binding was concentrated in the P_1 fraction (K_b = 40 nM). P_2 fraction also showed a high affinity binding system (K_B = 11.8 nM) with lower capacity than in the P_1 fraction. Comparative studies with [L-H^3]-asparate, [L-H^3]-glutamate and [H^3]-kainate showed that L-aspartate and L-glutamate are the most potent inhibitors of the binding of the three ligands. Aspartate and glutamate binding were effectively displaced by *N*-methyl-DL-aspartate and α-amino adipate, whereas only [H^3]-glutamate binding was significantly inhibited by glutamate-diethyl-ester. Kainic acid exhibited negligible affinity for aspartate and glutamate binding sites. Results indicate the presence of different receptors for glutamate and aspartate in both plexiform layers of the retina.

INTRODUCTION

Considerable neurophysiologic evidence supports the concept that the neurotransmitter released in the dark from the photoreceptor cells in the vertebrate retina must be an excitatory compound. Light stimulation would produce the arrest of this neurotransmitter release through hyperpolarization of the photoreceptor cells (Trifonov, 1968). The chemical nature of this neurotransmitter is as yet unknown, although physiologic and biochemical studies have suggested that the excitatory amino acids glutamate (Glu) or aspartate (Asp) are released at the photoreceptor synaptic endings (Dowling and Ripps, 1973). Although electrophysiological experiments have shown the presence of Glu and/or Asp receptors at the external plexiform layer of the retina (Dowling and Ripps, 1973; Murakami *et al.*, 1972; Cervetto and Piccolino, 1974; Murakami *et al.*, 1975; Gershenfeld and Picollino, 1979), biochemical evidence on this point is more restricted. Both amino acids appear to be concentrated in the external layers of the retina (Neal, 1976; Kennedy and Voaden, 1974), and a high-affinity, Na^+-dependent uptake mechanism shared by glutamate and aspartate has been characterized in the rat retina (Neal and White, 1971; White and Neal, 1976) as well as in synaptosomal fractions from the rabbit retina (Thomas and Redburn, 1978). If a compound is to serve a neurotransmitter role, specific synaptic receptors should be present at the restricted areas in which the compound should act. Specific, high affinity receptors for [^{3}H]Glu have been found in both plexiform layers of the chick retina, which satisfy some of the criteria to be met by physiological synaptic receptors for this compound (López-Colomé, 1981). However, the possibility of Glu or Asp interacting with a common receptor or being both retinal neurotransmitters has not yet been discarded. In this study, we have demonstrated the presence of high affinity aspartate receptors in the retina, and we have tried to establish some differences with receptors for Glu and Kainic acid (Ka) from the distribution, the kinetic and the pharmacological point of view, which could be of help in determining the nature of the excitatory amino acid transmitters in the retina.

The distribution and characteristics of L-Asp receptors were studied in membranes from the whole chick retina, as well as from the P_1 fraction (enriched with synaptic endings from the outer plexiform layer) and the P_2 fraction enriched with conventional nerve endings, mainly from the inner plexiform layer (Neal and Atterwill, 1974), and compared to those previously reported for L-Glu receptors (López-Colomé, 1981). Specific interactions of Glu and Asp with physiological receptors have been examined in several central nervous system preparations following the radiolabelled ligand technique (Roberts, 1974; Michaelis *et al.*, 1974; Michaelis, 1975; De Robertis and Fiszer de Plazas, 1976; Foster and Roberts, 1978; Head *et al.*, 1980, Roberts *et al.*, 1980; Foster *et al.*, 1981; Sharif and Roberts, 1981). In order to eliminate the possibility of [^{3}H]Glu or [^{3}H]Asp interacting with uptake as well as synaptic receptor sites, we have studied binding in the conditions reported by Enna and Snyder (1976a, b) for the GABA (α-aminobutyric acid) receptor, and tested by us for retinal Glu receptors (López-Colomé, 1981).

MATERIALS AND METHODS

Membrane preparation

Retinas free from pigment epithelium were obtained from 4–6 week old chicks and were osmotically shocked by homogenization in 20 vol. (w/v) of

*To whom correspondence should be sent.

double distilled water in a glass-glass homogenizer. The homogenate was placed on ice for 15 min and membranes were pelleted by centrifugation at 45,000 *g* at 4°C for 20 min, washed twice with the same volume of water and recentrifuged. The final pellet was resuspended by homogenization in Krebs–Tris buffer pH 7.4, without glucose for binding assays. Primary subcellular fractions were isolated as previously described (López-Colomé *et al.*, 1978) from retinas homogenized in 0.32 M sucrose containing 10^{-4} M $MgSO_4$. The crude nuclear fraction (P_1) and the crude synaptosomal fraction (P_2) were osmotically disrupted by homogenization in 20 vol. of water, and membranes were obtained as described for the whole retina.

Binding assay

Essentially, binding assays were carried out following the procedure described by Enna and Snyder (1976a) except that Krebs–Tris buffer was used instead of Krebs–Citrate buffer. Na^+-dependent binding was measured in freshly prepared membranes; the membrane pellet for these assays was resuspended in Krebs–Tris buffer (118 mM NaCl; 1.2 mM KH_2PO_4; 4.7 mM KCl; 2.5 mM $CaCl_2$; 1.17 mM $MgSO_4$; 26 mM Tris–HCl) at pH 7.4 without glucose. Assays for sodium-independent binding were carried out in membranes frozen for a period between one and two weeks. These membranes were incubated 30 min at 37°C in 50 vol. (w/v) of Krebs–Tris buffer pH 7.4 without NaCl in the presence of 0.05% Triton-X-100 (final concentration) prior to the binding assay. Membranes were recovered by centrifugation and resuspended in Krebs–Tris buffer from which NaCl had been omitted or substituted by sucrose.

For the binding assay, 1 ml of the membrane suspension (0.5 mg protein, approx.) was incubated on ice for 10 min in the presence of 20 nM [L-^{3}H]glutamate, the same concentration of [L-^{3}H]aspartate or 40 nM [^{3}H]kainic acid in the absence (control) or the presence of the unlabelled compound at the indicated excess concentration. The reaction was stopped by centrifugation at 45,000 *g* at 4°C for 20 min. After decanting the supernatant, the membrane pellets containing bound radioactivity were superficially rinsed twice with 5 ml of double distilled water and solubilized in 0.1 ml of NCS (Tissue solubilizer, Amersham). Bound radioactivity was determined after the addition of 10 ml of Tritosol (Fricke, 1975) followed by liquid scintillation spectrometry. All samples were kept 12 hr in the dark before counting. Radioactivity was corrected for quenching, background, and counting efficiency. Calculation of the specific binding was made by subtraction of the non-specific binding component which persisted in the absence of the unlabelled ligand. Protein was measured by the method of Lowry *et al.* (1951).

Materials

[*l*-^{3}H]glutamate (43–46 Ci/m mol), [L-^{3}H]aspartate (10–17.3 Ci/m mol), and [^{3}H]kainic acid (7.8 Ci/m mol) were obtained from New England Nuclear Corp., Boston, Massachusetts. NCS (Tissue Solubilizer) was from Amersham-Searle Corp., Arlington Hills, IL. All common analytical agents and drugs were from Sigma Chemical Co., St Louis, MO.

RESULTS

Na^+-Dependence of glutamate and aspartate binding

The presence of sodium-dependent and sodium-independent binding sites was assayed in membranes obtained from the whole retina (Table 1). Binding of aspartate to freshly prepared membranes was higher than for glutamate and in both cases, was considerably reduced in the absence of sodium. In contrast, binding of asparate and glutamate to frozen and thawed membranes, did not vary in the presence or absence of sodium, although a slight tendency to increase was observed in the absence of this ion. Although aspartate specific binding to fresh membranes was greater than glutamate binding in the presence of sodium, binding of Glu to membranes that had been frozen was 3 times greater than Asp binding (Fig. 1). As shown previously for Glu binding (López-Colomé, 1981), Triton treatment slightly

Table 1. Na^+-dependence of specific [^{3}H]glutamate and [^{3}H]aspartate binding to membranes from whole retina

Membrane preparation	[L-^{3}H]glutamate binding (pmol/mg protein)		[L-^{3}H]aspartate binding (pmol/mg protein)	
	Fresh	Frozen	Fresh	Frozen
+118 mM NaCl	0.75 ± 0.120 (4)	0.292 ± 0.009 (3)	1.33 ± 0.182 (3)	0.088 ± 0.010 (3)
−NaCl	0.059 ± 0.008 (3)	0.32 ± 0.035 (7)	0.160 ± 0.041 (3)	0.095 ± 0.007 (4)

Membranes were prepared as described in experimental procedure. [^{3}H]glutamate and [^{3}H]aspartate (20 nM) were displaced with 1 mM cold glutamate or aspartate. Results are the means ± SEM of the number of experiments indicated in parentheses.

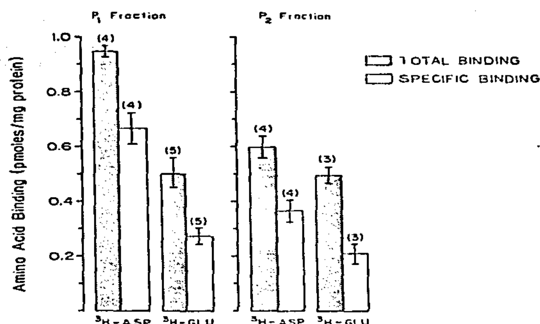

Fig. 1. Subcellular distribution of Na^+-independent binding of [L-^{3}H]aspartate and [L-^{3}H]glutamate. Subcellular fractions and membranes from these fractions were obtained as described in the Methods section. Frozen-thawed membranes preincubated with Triton were used, and the binding assay was conducted as described in the text. Results are the means ± SEM of the number of experiments included in parentheses. All experiments were performed in duplicate.

enhanced [^{3}H]Asp binding to membranes that had been frozen.

Subcellular distribution of aspartate and glutamate Na^+-independent binding

The concentrations of specific sodium-independent Asp and Glu binding sites in membranes from the P_1 fraction (enriched with synaptic terminals from the outer plexiform layer) and the P_2 fraction (enriched with synaptic terminals from the inner plexiform layer) were compared (Fig. 1). Whereas binding of Glu did not concentrate in any of the fractions as related to whole retinal membranes, Asp binding did concentrate 7 times in the P_1 fraction and 4 times in the P_2 fraction compared with the membranes from whole retina (Table 1 and Fig. 1). Glu-specific binding in this condition was 54% of total binding (0.452 ± 0.05 pmol/mg protein) in P_1 and 42% of total (0.448 ± 0.025 pmol/mg protein) in the P_2 fraction. Aspartate-specific binding was 71% of total (0.94 pmol/protein) in the P_1 and 60% of total (0.60 pmol/mg protein) in the P_2 fraction.

Kainic acid binding

Specific binding of [^{3}H]kainic acid to whole retinal membranes and membranes from subcellular fractions was compared in the presence and absence of a physiological sodium concentration (118 mM). Results in Table 2, show that although total binding was slightly increased in the presence of sodium, specific binding was not significantly changed in the presence of this ion. Ka binding did not concentrate (in the presence or absence of sodium) in any of the subcellular fractions.

Kinetics of aspartate binding to membranes from subcellular fractions

Binding of Asp was examined through a concentration range from 1.0 to 30 nM, in frozen-thawed membranes from P_1 and P_2 fractions (Fig. 2). Binding

Table 2. Subcellular distribution of [^{3}H]kainic acid binding to retinal membranes

Membrane preparation	[^{3}H]kainic acid binding (p mol/mg protein) Fresh membranes + NaCl		Frozen membranes − NaCl	
	Total	Specific	Total	Specific
Whole retina	0.209 ± 0.008	0.054 ± 0.007	0.160 ± 0.011	0.048 ± 0.011
P_1	0.175 ± 0.024	0.062 ± 0.012	0.127 ± 0.036	0.041 ± 0.004
P_2	0.129 ± 0.040	0.040 ± 0.004	0.116 ± 0.040	0.058 ± 0.020

Membranes were prepared as described in Experimental Procedures. Kainic acid binding (40 nM) was measured in the presence or absence of 118 mM NaCl. Binding was displaced by 1 mM cold kainic acid. Results are the mean ± SEM of 4–6 experiments.

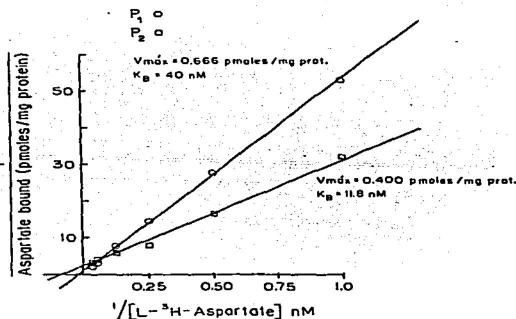

Fig. 2. Lineweaver–Burk plot of specific, Na^+-independent L-aspartate binding to membranes from subcellular fractions. Membranes were prepared as described in the text. Frozen membranes preincubated with Triton were resuspended in Krebs-Tris medium without NaCl. Membranes were incubated in the presence of increasing concentrations of [^{3}H]aspartate. Each point is the mean of 3–8 experiments performed in triplicate, O, P_1 fraction; □, P_2 fraction.

to both fractions (in the absence of sodium) was found to be saturable. Scatchard analysis (not shown) as well as Lineweaver–Burk plots showed a $K_B = 40$ nM and a $B_{max} = 0.666$ pmol/mg protein for the P_1 fraction and a $K_B = 11.8$ nM and $B_{max} = 0.4$ pmol/mg protein for the P_2 fraction. Further analysis of binding up to 1000 nM Asp, revealed the existence in the P_1 fraction of a second component of the binding system with $K_b = 291$ nM and $B_{max} = 4.8$ pmol/mg protein. A second component of lower affinity which was not saturated at 1 μM concentration was also observed in the P_2 fraction. In contrast, binding of Glu, showed a single component in both fractions, through a concentration range 1–2000 nM, with a $K_B = 555$ nM, and B_{max} value slightly higher in the P_2 than in the P_1 fraction (López-Colomé, 1981).

Displacement [^{3}H]Asp specific binding by some structurally related compounds

Some pharmacological characteristics of Asp binding to membranes from subcellular fractions were examined in order to assess an interaction with physiologically relevant receptors. Displacement of [^{3}H]Ka specific binding was also investigated and characteristics compared with Asp and Glu binding. Initially, the effect of a single concentration (100 μM) of displacing compounds was tested (Table 3), followed by the determination of IC_{50} for active compounds at [^{3}H]Asp and [^{3}H]Glu concentrations of 20 nM and 40 nM[^{3}H]Ka (Table 4). Table 3 shows that in both fractions, L-Asp is preferentially displaced by its correspondent structural analogues. While [^{3}H]Asp is more effectively displaced by DL- α-aminoadipate (α-AA) and N-methyl-DL-asparate (NMDLA) [^{3}H]Glu is preferentially displaced by glutamate diethyl ester (GDEE), although α-aminoadipate and NMDLA also showed significant displacing effects. Noticeably, although [^{3}H]Ka is 100% displaced by both, Glu and Asp, cold kainate up to 1.0 mM concentration did not displace Glu, whereas it did displace bound Asp by about 50% at a 100 μM concentration. Binding of all three ligands to P_1 fraction membranes showed only 50% displacement by the D-isomers of Glu and Asp.

DISCUSSION

Glutamate (Glu) and aspartate (Asp) have been proposed as photoreceptor transmitters in the vertebrate retina (Neal, 1976). Evidence in support of this assumption derives mainly from electrophysiological studies which have demonstrated that either of these amino acids mimics the effects of the natural transmitter released from the photoreceptor cell (Dowling and Ripps, 1973; Cervetto and Piccolino, 1974; Gershenfeld and Piccolino, 1979). Further studies (Wu and Dowling, 1978) support Asp as the cone-transmitter in the carp retina, and Slaughter and Miller (1981) have proposed Glu as the transmitter of the On pathways in the mudpuppy retina. Biochemical studies have demonstrated that Glu and Asp share a high-affinity uptake mechanism (Neal and White, 1971 and 1976; Thomas and Redburn, 1978) and are mainly concentrated in the outer layers of the retina (Neal, 1976). Studies in an eye-cup preparation have demonstrated a decrease of Asp release following light stimulation of the rabbit retina (Neal *et al.*, 1979) and electron microscopy studies following kainic acid lesions in the goldfish retina, suggest that while Asp could be the cone-transmitter, Glu could function as

Table 3. Displacement of specific [L-^{3}H]aspartate binding by some related compounds at 100 μM concentration

Compound	% Displacement P_1 fraction membranes	P_2 fraction membranes
L-aspartate	100	100
L-glutamate	100	100
D-aspartate	47	45
D-glutamate	48	40
L-glutamate-diethyl ester	48	50
DL-α-amino-adipate	71	71
β-methyl-DL-aspartate	79	63
2-amino-4-phosphonobutyrate	40	27
Kainate	48	33

Results are expressed as % of maximal displacement of [^{3}H]ASP by 1 mM cold Asp. Final [^{3}H]Asp concentration was 20 nM, and displacers were added to 100 μM final concentration. Data are the mean of 6–10 experiments performed in duplicate, which varied less than 15%.

the rod-transmitter (Yazulla and Kleinschmidt, 1980). Glutamate and aspartate receptor binding to central nervous system membranes has been extensively studied, and the correlation of synaptic pharmacology and binding data support the suggestion that Glu and Asp are involved in synaptic transmission in the central nervous system (Watkins, 1978; Johnston, 1979; Foster *et al.*, 1981; Foster and Roberts, 1978; Roberts *et al.*, 1980; Sharif and Roberts, 1981; Fabb *et al.*, 1981). In the retina, binding of [^{3}H]Glu has been characterized in both plexiform layers, in conditions believed to expose synaptic receptors López-Colomé (1981), and Mitchell *et al.* (1981) have reported the presence of high affinity receptors for [^{3}H]Glu (K_B = 12 nM, 72 nM and 800 nM) and for Asp assayed through [^{3}H]NMDA binding K_B = 8.4 nM and 82 nM) at the inner plexiform layer of the bovine retina.

In the present work we have demonstrated the presence of high-affinity, saturable binding sites for [^{3}H]asparate at the outer and inner plexiform layers of the chick retina with K_B = 40 nM and 12 nM respectively, together with a lower affinity system with a K_B in the μM range (Fig. 2). These values are of higher affinity than those reported by us for Glu binding site (555 nM) in the same preparation; however they are in the same range as the kinetic constants reported for [^{3}H]Glu and [^{3}H]NMDA binding in the bovine retina (Mitchell *et al.*, 1981). The binding constants for [^{3}H]Asp are also in close correspondence with those reported by Enna and Snyder (1976b) for the GABA receptor in the bovine retina.

The conditions in which [^{3}H]Asp binding was conducted were the same as we used for studying [^{3}H]Glu receptors (López-Colomé, 1981). Because no specific antagonist of the synaptic action of Glu and Asp has been demonstrated under conditions where physiological actions and effects on binding are parallel, the use of the same compound as a ligand raises the possibility of an interaction with uptake sites, or enzymes for which the compound is a substrate (Foster and Roberts, 1978; Roberts, 1981; Sharif and Roberts, 1981). In order to eliminate these

Table 4. Inhibition of the specific binding of [^{3}H]Glu, [^{3}H]Asp and [^{3}H]Ka in membranes from whole retina

Compound	IC$_{50}$ (μM) [L-^{3}H]aspartate	[L-^{3}H]glutamate	[^{3}H]kainate
L-aspartate	1.5	10.0	6.0
L-glutamate	0.6	0.35	0.15
NMDLA	0.8	6.0	>100
GDEE	>100	300.0	>100
2-APB	>100	>100	>100
DL-α AA	7.0	5.0	—

NMDLA—*N*-methyl-DL-aspartate; GDEE—glutamate diethyl ester; 2-APB—2-amino-4-phosphonobutyric acid; α-AA—α-amino adipic acid. Binding was performed as described in methods. Final Glu and Asp concentration was 20 nM; final Ka concentration was 40 nM. Compounds were added over a concentration range 0.01–100 μM, and 5 different concentrations were tested, except for GDEE, in which a range of 0.01–1000 μM was tested. IC$_{50}$ were determined by log dose/percentage plots of 3 sets of data (duplicate determinations) which varied by less than 20%.

possibilities, we have conducted our assays under conditions known to diminish these interactions by at least 80% (Schwarcz, 1981). Sharif and Roberts (1980) have reported the loss of Glu and Asp specific binding upon freezing and a deleterious effect of Triton treatment in cerebellar and brain membranes, and have chosen to use fresh preparations for their assays. However, in our hands, freezing for more than a week does not significantly affect Na^+-independent binding of either ligand, although it greatly diminishes Na^+-dependent one. Similar to Glu, Triton slightly increased Na^+-independent Asp binding.

Although binding sites for both Glu and Asp were found in membranes from both plexiform layers of the retina, important differences were determined which suggest different specific sites for these two compounds. Asp receptors show higher affinity than Glu receptors. Glu receptors do not concentrate in any of the fractions, whereas Asp binding concentrates seven times in the P_1 fraction and four times in the P_2 fraction, compared with the membranes from whole retina (Table 1, Fig. 1). As in the case of brain membranes, (Foster *et al.*, 1981), the number of Glu receptors exceeded that of Asp receptors. Pharmacologically, the binding of [^{3}H]Asp exhibited higher specificity than [^{3}H]Glu binding. L-Glu and L-Asp were the most potent inhibitors of binding, perhaps due to the fact that L-Glu is in a flexible conformation and might interact with Asp receptors as well as Glu receptors. α-Amino-adipate and NMDLA effectively displaced Asp binding, which would be in agreement with their physiological actions as Asp antagonist and agonist respectively in other nervous tissue preparations (Padjen and Smith, 1980; McCullock, Johnston *et al.*, 1974), whereas GDEE, considered as a Glu antagonist in some central nervous system areas, is a very weak displacer of Asp binding. D-isomers of Asp and Glu at 100 μM concentration displaced less than 50% Asp binding, indicating the stereospecificity of Asp receptor. D-Asp is also considered as an uptake inhibitor, and this result would suggest that we are not dealing with uptake receptors. Although electrophysiologically D- and L- isomers of aspartate show similar potencies, the fact that they have different effects on binding is interesting, and has also been reported for cerebellar membranes (Sharif and Roberts, 1981). Similarly to L-Glu binding, L-Asp binding is not significantly affected by kainate nor by the Glu analogue 2-amino-4-phosphonobutyric acid (Tables 3 and 4).

Although kinetic constants for [^{3}H]Asp binding are close to those reported for [^{3}H]kainate binding in the same tissue (Biziere and Coyle, 1979), the fact that kainate receptors do not concentrate in the inner plexiform layer and its lack of effect as a Asp or Glu displacer, suggests that, as reported in other preparations (Hall *et al.*, 1978; Michaelis *et al.*, 1980; Slevin and Coyle, 1981) the receptors for this compound are different from those for Glu or Asp.

In conclusion, we have demonstrated the presence of high-affinity, specific binding sites for [^{3}H]Asp in both plexiform layers of the retina. However, a more profound characterization of Glu and Asp receptors is needed in order to establish definitely their role as transmitters at precise synapses in this organ.

Acknowledgements—This work was supported in part by a grant from the Consejo Nacional de Ciencia y Tecnologia (Project PCCBNAL-800800).

REFERENCES

Biziere K. and Coyle J. T. (1979) Localization of receptors for Kainic acid on neurons in the innernuclear layer of retina. *Neuropharmacology* 18, 409–413.

Cervetto L. and Piccolino M. (1974) Synaptic transmission between photoreceptors and horizontal cells in the turtle retina. *Sciences* 183, 417–419.

De Robertis E. and Fiszer de Plazas S. (1976) Isolation of hydrophobic proteins binding amino acids. Selectivity of the binding of L-^{14}C glutamate in cerebral cortex. *J. Neurochem.* 26, 1237–1243.

Dowling J. E. and Ripps H. (1973) Effect of magnesium on horizontal cell activity in the skate retina. *Nature, Lond.* 242, 101–103.

Enna S. J. and Snyder S. H. (1976a) Properties of γ-aminobutyric acid (GABA), receptor binding in rat brain synaptic membrane fractions. *Brain Res.* 100, 81–97.

Enna S. J. and Snyder S. H. (1976b) Gamma-aminobutyric acid (GABA) receptor binding in mammalian retina. *Brain Res.* 115, 174–179.

Fagg G. E., Foster A. C., Mena E. E., Koerner J. F. and Cotman C. W. (1981) Calcium ions and the pharmacology of acidic amino acid receptor sites. *Trans. Am. Soc. Neurochem.* 12, 122.

Foster A. C. and Roberts P. J. (1978) High-affinity L-^{3}H-glutamate binding to post-synaptic receptor sites on rat cerebellar membranes. *J. Neurochem.* 31, 1457–1477.

Foster A. C., Mena E. E., Fagg G. E. and Cotman C. W. (1981) Glumate and asparate binding sites are enriched in synaptic junctions isolated from rat brain. *J. Neurosci.* 1, 620–625.

Fricke V. (1975) Tritosol: A new scintillation cocktail based on Triton-X-100. *Analyt. Biochem.* 63, 555–558.

Gershenfeld H. M. and Piccolino M. (1979) Pharmacology of the connections of cones and L-horizontal cells in the vertebrate retina. In *The Neurosciences: Fourth Study Program* (Edited by Schmitt F. O. and Worden F. G.), pp. 213–226. MIT Press, Cambridge, MA.

Hall J. G., Hicks T. P. and McLennan H. (1978) Kainic acid and the glutamate receptor. *Neurosci. Lett.* 8, 171–175.

Head R. H., Tunnicliff G. and Matheson G. K. (1980) Glutamate receptor binding to cat central nervous system membranes. *Can. J. Biochem.* 58, 534–538.

Johnston G. A. R. (1979) Central Nervous System Receptors for Glutamic Acid. In *Glutamic Acid: Advances in Biochemistry and Physiology* (Edited by Filer L. J. Jr *et al.*). pp. 177–185. Raven Press, New York.

Kennedy A. J. and Voaden M. J. (1974) Distribution of free amino acids in the frog retina. *Biochem. Soc. Trans.* 2, 1256–1258.

López-Colomé A. M., Salceda R. and Pasantes-Morales H. (1978) Potassium-stimulated release of GABA, glycine and taurine from the chick retina. *Neurochem. Res.* 3, 431–441.

López-Colomé A. M. (1981) High-affinity binding of L-glutamate to chick retinal membranes. *Neurochem. Res.* 6, 1019–1033.

Lowry O. H., Rosebrough W. J., Farr A. L. and Randall R. S. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* 193, 265–275.

ESTA TESIS NO DEBE SALIR DE LA BIBLIOTECA

Michaelis E. K., Michaelis M. L. and Boyarsky L. L. (1974) High-affinity glutamate binding to brain synaptic membranes. *Biochim. biophys. Acta* 367, 338–348.

Michaelis E. K. (1975) Partial purification and characterization of a glutamate-binding membrane glycoprotein from rat brain. *Biochem. biophys. Res. Commun.* 65, 1004–1012.

Michelis E. K., Michaelis M. L. and Grubbs R. D. (1980) Distinguishing characteristics between glumate and kainic acid binding sites in brain synaptic membranes. *FEBS Lett.* 118, 55–57.

Mitchell C., Hampton C. and Redburn D. (1981) Localization of receptor and transport sites for glutamate and aspartate in innerplexiform layer of bovine retina. *Invest. Ophthal. visual Sci.* 20, 215.

Murakami M., Ohtsu K. and Ohtsuka T. (1972) Effects of chemicals on receptors and horizontal cells in the retina. *J. Physiol., Lond.* 227, 899–913.

Murakami M., Ohtsuka T. and Shimasaki H. (1975) Effects of aspartate and glutamate on the bipolar cells of the carp retina. *Vision Res.* 15, 456–458.

Neal M. J. and White R. D. (1971) Uptake of ^{14}C-L-glutamate by rat retina. *Br. J. Pharm.* 43, 442–443.

Neal M. J. and Atterwill C. K. (1974) Isolation of photoreceptor and conventional nerve terminals by subcellular fractionation of rabbit retina. *Nature* 251, 331–333.

Neal M. J. (1976) Amino acid transmitter substances in the vertebrate retina. *Gen. Pharmac.* 7, 321–332.

Neal M. J., Collins G. G. and Massey S. C. (1979) Inhibition of aspartate release from the retina of the anaesthetised rabbit by stimulation with light flashes. *Neurosci. Lett.* 14, 241–245.

Padjen A. L. and Smith P. A. (1980) Specific effects of α-D,L-aminoadipic acid on synaptic transmission in frog spinal cord. *Can. J. Physiol. Pharmac.* 58, 692–698.

Roberts P. J. (1974) Glutamate receptors in rat central nervous system. *Nature, Lond.* 252, 399–401.

Roberts P. J., Sharif N. A. and Swait J. C. (1980) Characteristics of L-^{3}H-aspartate binding [illegible] synaptic membranes. *Br. J. Pharmacol.* 70, 146P.

Roberts P. J. (1981) Binding studies for the investigation of receptors for L-glutamate and other excitatory amino acids. In *Glutamate: Transmitter in the Central Nervous System* (Edited by Roberts P. J., Storm-Mathisen J. and Johnston G.A.R.), pp. 35–53. Wiley, New York.

Schwarcz R. (1981) Effects of tissue storage and freezing on brain glutamate uptake. *Life Sci.* 28, 1147–1154.

Sharif N. A. and Roberts P. J. (1980) Problems associated with the binding of L-glutamic acid to synaptic membranes: Methodological aspects. *J. Neurochem.* 34, 779–784.

Sharif N. A. and Roberts P. J. (1981) L-Aspartate binding sites in rat cerebellum. A comparison of the binding of L-^{3}H-aspartate and L-^{3}H-glutamate to synaptic membranes. *Brain Res.* 211, 293–303.

Slevin J. T. and Coyle J. T. (1981) Ontogeny of receptor binding sites for ^{3}H-glutamic acid and ^{3}H-kainic acid in the rat cerebellum. *J. Neurochem.* 37, 531–533.

Slaughter M. M. and Miller R. F. (1981) 2-amino-4-phosphonobutyric acid; A new pharmacological tool for retina research. *Science* 211, 182–185.

Thomas N. T. and Redburn D. A. (1978) Uptake of ^{14}C-aspartic acid and ^{14}C-glutamic acid by retinal synaptosomal fractions. *J. Neurochem.* 31, 63–68.

Trifonov Y. A. (1968) Study of synaptic transmission between photoreceptors and horizontal cells by means of electrical stimulation of the retina. *Biofizika* 13, 809–817.

Watkins J. C. (1978) Excitatory amino acids. In *Kainic Acid as a Tool in Neurobiology* (Edited by McGeer E. G., Olney J. W. and McGeer P. L.), pp. 37–69. Raven Press, New York.

White R. D. and Neal M. J. (1976) The uptake of L-glutamate by the retina. *Brain Res.* 111, 79–83.

Yazulla S. and Kleinschmidt J. (1980) The effects of intraocular injection of kainic acid on the synaptic organization of the goldfish retina. *Brain Res.* 182, 287–301.

DISCUSION

Existen dos problemas principales involucrados en el estudio de los receptores a glutamato y a aspartato que aquí se presenta:

1) ¿Existen receptores postsinápticos para estos compuestos en la retina? ¿Pueden distinguirse los receptores de glutamato de los de aspartato?

2) A qué células corresponden estos receptores y por lo tanto, qué células podrían usar estos compuestos como neurotransmisores?.

Respecto al primer punto, el mayor problema para la identificación de estos receptores consiste en que no existen antagonistas específicos de la acción postsináptica de los aminoácidos excitadores, lo cual hace difícil diferenciar los receptores sinápticos a estos compuestos de otros tipos de receptores para los mismos como serían los receptores presinápticos de captación de alta afinidad. Como consecuencia, los receptores deben medirse empleando como radioligandos al ^{3}H-glutamato o al ^{3}H-aspartato; dado que estos compuestos se encuentran en concentración elevada en las células nerviosas en las que desempeñan diversas funciones, es necesario eliminar de la preparación membranal todo rastro del compuesto endógeno. Asimismo, se hace necesario el tratamiento de las membranas con un detergente ya que se ha descrito la existencia de inhibidores endógenos de la unión de estos compuestos con el receptor, los cuales deben ser extraídos cuando se desea medir los receptores sinápticos y determinar sus características cinéticas

y farmacológicas.

Otro problema derivado del uso del glutamato o el aspartato como ligandos del receptor consiste en poder distinguir la unión con los receptores sinápticos, de la unión con los receptores presinápticos de captación o con enzimas que pudieran usar estos compuestos como sustrato. Para obviar este problema, las determinaciones se llevan a cabo a bajas temperaturas (0-4°C) y en ausencia de sodio y de glucosa en el medio de incubación, ya que estas condiciones impiden la realización de procesos que, como la unión al receptor de captación, requieren tanto de la presencia de sodio como de una fuente de energía. Una forma alternativa de impedir la participación de los receptores de captación dejando intactos a los receptores sinápticos es la congelación de las membranas durante un mínimo de 24 horas antes de medir en ellas los receptores (36). Como puede observarse en los artículos presentados, las condiciones experimentales empleadas permiten afirmar que los receptores estudiados son únicamente receptores sinápticos.

Para que la interacción de un compuesto con su receptor tenga significación fisiológica, debe presentar una cinética de saturación con una constante de disociación en el rango de nM. Los receptores a glutamato y a aspartato en la retina presentan respectivamente una K_B de 550 nM y 40 nM, lo cual nos permite afirmar por este criterio también, que se trata de receptores sinápticos.

Desde el punto de vista farmacológico, la interacción de un transmisor con su receptor postsináptico debe presentar estereoespecificidad y modificarse en presencia de agonistas y antagonistas de la acción

fisiológica del compuesto. En el caso de los receptores a glutamato y aspartato en la retina, la potencia relativa de algunos análogos estructurales para inhibir la unión con el receptor (IC_{50}) parece ser paralela a la acción activadora o inhibidora que estos compuestos presentan sobre la acción de los aminoácidos excitadores en diversas preparaciones del sistena nervioso central. Así, encontramos que en la retina el dietil-éster del ácido glutámico (GDEE) que es un antagonista de la acción fisiológica del glutamato (32), es un bloqueador más potente de la unión del glutamato que del aspartato, mientras que el β-metil-DL_aspartato que es un potente agonista del aspartato en la médula espinal (46), desplaza preferentemente al aspartato de su receptor. Dado que los D-isómeros de ambos compuestos inhiben la unión con el receptor en un 50% mientras que los L-isómeros inhiben totalmente la interacción a las mismas concentraciones, podemos concluir que se trata de receptores que desde el punto de vista farmacológico, llenan los requisitos para ser considerados como receptores sinápticos.

En conclusión, se han identificado y caracterizado receptores a glutamato y a aspartato en la retina de pollo, los cuales por la metodología empleada, por sus constantes cinéticas y por sus propiedades farmacológicas, parecen ser receptores sinápticos.

Respecto a la localización de las células de la retina que poseen estos receptores, se ha postulado que la transmisión entre los fotorreceptores y las células bipolares (43), así como entre las células bipolares y las ganglionares (28) está mediada por un transmisor de tipo excitador que podría ser el ácido glutámico y/o el ácido aspártico.

La estructura laminar de la retina permite el aislamiento de fracciones enriquecidas en terminales sinápticas de los fotorreceptores o bien de la capa plexiforme interna conteniendo entre otras, las terminales de las células bipolares (30); la presencia de receptores a glutamato o a aspartato en las membranas de una u otra capa, o en forma alternativa su mayor concentración en una de ellas permitiría sugerir el tipo de célula que posee estos receptores. Los dos tipos de receptores, a glutamato y a aspartato, se localizaron en ambas capas sinápticas de la retina, lo cual apoya la posibilidad de que la transmisión entre las células bipolares y las ganglionares podría estar mediada por los mismos transmisores que la del fotorreceptor a las células bipolares, y que éste podría ser cualquiera o ambos aminoácidos excitadores.

Recientemente se ha descrito una técnica (27) que permite degenerar selectivamente los diferentes tipos de neuronas en la retina mediante la inyección intraocular de dosis crecientes de ácido kaínico, cuya toxicidad en la retina está bien demostrada (37). Dado que las neuronas de la retina poseen diferente sensibilidad a este compuesto (amacrinas > horizontales > bipolares > ganglionares y fotorreceptores), podemos eliminar de la retina uno o varios de estos tipos celulares. Esperamos poder determinar mediante esta técnica a qué células pertenecen los receptores a glutamato y a aspartato que hemos caracterizado, y consecuentemente a qué nivel de la retina podrían actuar como neurotransmisores.

Referencias

1 Bradford, H.F. y Richards, C.D. (1976) Specific release of endogenous glutamate from piriform cortex stimulated in vitro. Brain Res. 105, 168–172.

2 Bradford, H.F., Ward, H.K. y Thomas, A.J. (1978) Glutamine – A major substrate for nerve endings. J. Neurochem. 30: 1453–1459.

3 Cotman, C.W. Hamberger, A. (1978). Glutamate as a CNS neurotransmitter. Properties of release inactivation and biosynthesis. En: Amino acids as Neurotransmitters (ed. F. Fonnum) pp. 379–412. Raven, New York.

4 Cotman, C.W., Foster, A. y Lanthorn, T. (1980). An overview of glutamate as a neurotransmitter. En: Glutamate as a neurotransmitter, ed. Di Chiara, G. y Gessa, G.L., Raven Press, New York.

5 Crawford, A.C. y McBurney, R.N. (1976). The time course of action of L-glutamate at the excitatory neuromuscular junction in Maia squinado. J. Physiol. (London) 254, 47–48 P

6 Curtis, D.R. y Watkins, J.C. (1960) The excitation and depression of spinal neurons by structurally related amino acids. J. Neurochem. 6, 117–141.

7 Curtis, D.R. y Watkins, J.C. (1965) The pharmacology of amino acids related to gamma-aminobutyric acid. Pharmacol. Rev. 17, 347–391.

8 Curtis, D.R. y Johnston, G.A.R. (1974). Amino acid transmitters in the mammalian central nervous system. Ergebn. Physiol. 69: 97–188.

9 Divac, I., Fonnum, F. y Storm-Mathisen, J. (1977). High affinity uptake of glutamate in terminals of cortico striatal axons. Nature 266: 377–378.

10 Dowling, J.E. (1970) Organization of vertebrate retinas. Invest. Ophtalmol. 9:655–680.

11 Enna, S.J. y Snyder, S.H. (1976). Properties of γ-aminobutyric acid (GABA) receptor binding in rat brain synaptic membrane fractions. Brain Res. 100: 81–97.

12 Fonnum, F., Malthe-Sorenssen, D., Kvale, I., Soreide, A., Skrede, K.K. y Walaas, I. Glutamergic Neurons: Localization and Release of the Transmitter. En: Regulatory Mechanisms of Synaptic Transmission. eds. Tapia, R. y Cotman, C.W., pp. 59–70. Plenum Press, New York.

13 Foster, A.C. y Roberts, P.J. (1978). High-affinity L-^{3}H-glutamate binding to postsynaptic receptor sites on rat cerebellar membranes. J. Neurochem. 31: 1467–1477.

14 Foster, A.C., Mena, E.E., Fagg, G.E. y Cotman, C.W. (1981) Glutamate and aspartate binding sites are enriched in synaptic junctions isolated from rat brain. J. Neurosci 1: 620-625.

15 Gershenfeld, H.M. y Piccolino, M. (1979). Pharmacology of the connections of cones and L-horizontal cells in the vertebrate retina. En: The Neurosciences: Fourth Study Program, ed. Schmitt, F.O. y Worden, F.G. pp. 213-226. MIT Press, Cambridge, Mass.

16 Henke, H., Schenker, T.M. y Cuenod, M. (1976). Effects of retinal ablation on uptake of glutamate, glycine, GABA, proline and choline in pigeon optic tectum. J. Neurochem. 26: 131-134.

17 Honoré, T., Krogsgaard-Larsen, P., Hansen, J.J. y Lauridsen, J. (1981) Glutamate and aspartate agonists structurally related to ibotenic acid. Molec. and Cell. Biochem. 38: 123-128.

18 Jasper, H.H. y Koyama, I. (1969). Rate of release of amino acids from the cerebral cortex of the cat as affected by brain stem and thalamic stimulation. Can. J. Physiol. Pharmacol., 47: 889-902.

19 Johnston, G.A.R. (1976). Glutamate and aspartate as transmitters in the spinal cord. Adv. Biochem. Psychofarmacol. 15: 175-184.

20 Johnston, G.A.R. (1979). Central Nervous system receptors for glutamic acid. En: Glutamic Acid: Advances in Biochemistry and Physiology. ed. Filer, Jr., L.J. y col. pp. 177-185. Raven Press, New York.

21 Kondo, H. and Toyoda, J.I. (1980). Dual effect of glutamate and aspartate on the on-center bipolar cell in the carp retina, Brain Research 199; 240-243.

22 Krnjević, K., y Phillis, J.W. (1963). Iontophoretic studies of neurones in the mammalian cerebral cortex. J. Physiol. 165, 274-304.

23 Levi, G. y Raiteri, M. (1974) Exchange of neurotransmitter amino acid at nerve endings can stimulate high affinity uptake. Nature (London) 250: 735-737.

24 Logan, W.J., y Snyder, S.H. (1972). High affinity uptake systems for glycine, glutamic and aspartic acids in synaptosomes of rat central nervous tissue. Brain Res. 42: 413-431.

25 Marc, R.E., and Lam., D.M.K. (1981). Uptake of aspartic and glutamic acid by photoreceptors in goldfish retina. Proc. nat. Acad. Sci. USA 7:7185-7189.

26 McGeer, P.L., McGeer, E.G., Schener, U. y Singh, K. (1977). A Glutamatergic cortico-strial path? Brain Res. 128: 367-373.

27 Morgan, I.G. and Ingham. C.A. (1981), Kainic acid affects both plexiform layers of chick retina, Neurosci. Lett., 21; 275-280.

28 Naka, K.I. Neuronal circuitry in the catfish retina, Invest. Ophthalmol. 15; 926-934.

29 Neal, M.J. (1976). Amino acid transmitter substances in the vertebrate retina. Gen. Pharmac. 7: 321-332.

30 Neal, M.J. and Atterwill, C.K. (1974) Isolation of photoreceptor and conventional nerve terminals by subcellular fractionation of rabbit retina. Nature 251: 331-333.

31 Neal, M.J., Collins, G.G. and Massey, S.C. (1979), Inhibition of aspartate release from the retina of the anaesthetized rabbit by stimulation with light flashes, Neurosci. Lett., 14; 241-245.

32 Nistri, A. y Constanti, A. (1979). Pharmacological characterization of different types of GABA and glutamate receptors in vertebrates and invertebrates. En: Progress in Neurobiology, Vol. 13, pp. 177-235, Pergamon Press.

33 Perry, T.L., Berry, K., Diamond, S., and Mok, C. (1971). Regional distribution of amino acids in human brain obtained of autopsy. J. Neurochem. 18, 513-519.

34 Redburn, D.A. (1981) GABA and Glutamate as Retinal Neurotransmitters in Rabbit Retina. In G. Di Chiara and G.L. Gessa (Eds.), Glutamate as a Neurotransmitter, Raven Press, New York, pp. 79-89.

35 Rowe, J.S. and Ruddok, K.H. (1982) Depolarization of retinal horizontal cells by excitatory amino acid neurotransmitter agonists, Neurosci. Lett. 30; 257-262.

36 Schwarcz, R. (1981). Effects of tissue storage and freizing on brain glutamate uptake. Life Sci. 28, 1147-1154.

37 Schwarcz, R. and Coyle, J.T. (1977). Kainic acid: neurotoxic effects after intraocular injection, Invest. Ophthalmol., 16; 141-148.

38 Slaughter, M.M. and Miller, R.F. (1981) 2-amino-4-phosphono-butyric acid; A new pharmacological tool for retina research, Science. 221: 182-185.

39 Snyder, S.H., Young, A.B., Oster-Granite, M.L. y Herndon, R.M. (1975). Glutamic acid as a putative neurotransmitter of cerebellar granule cells: evidence from the virally induced granulo prival cerebellum. En: Metabolic compartmentation and neurotransmission. ed. Berl, S., Clarke, D.D. y Schneider, D. pp. 1-11, Plenum Press., New York.

40 Spencer, H.J. (1976). Antagonism of cortical excitation of striatal neurons by glutamic acid diethyl ester: Evidence for glutamic acid as an excitatory transmitter in the rat striatum. Brain Res. 102: 91-101.

41 Storm-Mathisen, J. (1977). Glutamic acid and excitatory nerve endings: Reduction of glutamic acid uptake after axotomy. Brain Res. 120, 379-386.

42 Takeuchi, A. y Takeuchi, N. (1964). The effect on crayfish muscle of iontophoretically applied glutamate, J. Physiol. (London) 170: 296-317.

43 Trifonov, Y.A. (1968). Study of synaptic transmission between photoreceptors and horizontal cells by means of electrical stimulation of the retina, Biofizika 13: 809-817.

44 Van den Berg, C.J. (1970). Glutamate and Glutamine. En: Handbook of Neurochemistry, Vol. III (ed. A. Lajtha), pp. 355-379. Plenum Press, New York, London.

45 Van den Berg, C.J. Matheson, D.F., Ronda, G., Reijnierse, G.L.A., Blokhius, G.G.D., Kroon, M.C., Clarke, D.D. y Gerfinke, D. (1975). A model of glutamate metabolism in brain: A biochemical analysis of heterogeneous structure. En: Metabolic compartmentation and Neurotransmission ed. Berl, S., Clarke, D.D. y Schneider, D., pp. 515-543. Plenum Press, New York.

46 Watkins, J.C. (1978). Excitatory Amino Acis. En: Kainic Acid as a Tool in Neurobiology, Ed. McGeer, E.G. y col. pp. 37-69. Raven Press, New York.

47 Wu, S.M. and Dowling, J.E., (1978), L-Aspartate: evidence for a role in cone photoreceptor synaptic transmission in the carp retina, Proc. nat. Acad. Sci. (Wash.), 75: 5205-5209.

48 Yazulla, S. and Kleinschmidt, J. (1980), The effects of intraocular injection of kainic acid on the synaptic organization of the goldfish retina, Brain Research, 182; 287-301.