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LOS RECEPTORES PARA AMINOACIDOS EXCITADORES Y SU MODULACION POR AMINOACIDOS INHIBIDORES EN CELULAS DEL EPITELIO PIGMENTARIO DE LA RETINA EN CULTIVO

TESIS QUE PARA OPTAR POR EL GRADO DE MAESTRO EN
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R E S U M E N

El ácido L-glutámico (L-glu) y algunos de sus análogos inducen la fagocitosis de los discos de los segmentos externos de los fotorreceptores por el epitelio pigmentario de la retina (EPR), probablemente a través de la interacción con un receptor. En esta tesis se caracterizaron receptores para L-glu en las membranas de las células del epitelio pigmentario de la retina de pollo en cultivo primario, en estado no confluente (16 DIV) y confluente (25 DIV). La unión específica de L-glu-H^3 es saturable, reversible e insensible a temperatura, pero parcialmente dependiente de Na^+.

Asimismo, se identificaron receptores con características similares en células del EPR humano en cultivo, procedentes de donadores *post-mortem* de diferentes edades (6 y 29 días, 4 meses, 15 y 33 años). Las características cinéticas fueron similares en todos los casos (K_D de 1181 a 1,482 nM; Bmax de 8.6 a 13.1 pmol/mg de proteína). El perfil farmacológico de la interacción sugiere la existencia de receptores tanto de tipo ionotrópico (NMDA), como metabotrópico (sensible a ACPD).

Tanto en el tejido intacto como en los cultivos celulares, el EPR recibe la influencia de otros aminoácidos, neurotransmisores potenciales, como la taurina, el GABA y la glicina. Se demostró que en todas las preparaciones, estos compuestos aumentan la unión del L-glu-H^3, efecto que disminuye inversamente a la diferenciación del tejido. En el EPR de pollo, la taurina aumenta el número de sitios específicos, mientras que en el EPR humano, la glicina en concentraciones micromolares, aumenta la afinidad de los receptores a L-glu. La estricnina no inhibe el efecto de la glicina, pero sí el 7 clorokinurenato, un antagonista del sitio alostérico de la glicina en el receptor de tipo NMDA. El estímulo producido por la glicina, se observa también en las membranas de las células en estado proliferativo, como fibroblastos de la esclerótica y la línea tumoral NB76, pero no en tejidos diferenciados, ni en células postmitóticas, como el cerebro y la retina adultos. Los resultados apoyan la participación del L-glu en la regulación de la fagocitosis y/o la diferenciación celular del EPR.

I N D I C E

I. ABREVIATURAS

aa - Aminoácidos
AAE - Aminoácidos excitadores
ACPD - Acido trans-1-amino-ciclopentil-1,3-dicarboxílico
AMPA - α-amino-3-hidroxi-5-metilizoxazol-4-propionato
AMPc - Adenosín monofosfato cíclico
AP3 - Acido L-fosfonopropiónico
APB - Acido 2-amino-4-fosfonobutírico
APV - Acido amino 7-fosfonovalérico
ATP - Adenosín trifosfato
CNQX - 6-ciano-7-nitroquinoxalín-2,3-diona
DG - Diacilglicerol
DGG - γ-D-glutamil-glicina
DIV - Días in vitro
DNA - Acido desoxirribonucleico
DNQX - 6,7-dinitroquinoxalín-2,3-diona
EC_{50} - Constante de estimulación
EDTA - Acido etilendiaminotetra-acético
EPR Epitelio pigmentario de la retina
GABA - Acido γ-aminobutírico
GDEE - Glutamato dietil ester
GTP - Guanosín trifosfato
IPs - Fosfatos de inositol
IP_3 - Trifosfato de inositol
JSTX - Toxina de la araña *Nephila clavata*
KA - Acido kaínico
L-Glu - Acido L-glutámico
L-Glu-H^3 - Acido L-glutámico tritiado
mGLUR 1-8 - Receptores metabotrópicos para ácido glutámico
NMDA - Acido N-metil-D-aspártico
PBS - Solución amortiguadora de fosfatos
PIP_2 - 4,5 difosfato-fosfatidil inositol
PKC - Proteína cinasa C
QA - Acido quisculálico
RCS - Rata Royal College of Surgeons con distrofia retinal hereditaria
RKB - Ringer Krebs bicarbonato
ROS - Segmentos externos de los bastones
RP - Retinitis pigmentosa
RPAD - Retinitis pigmentosa autosomica dominante
rpm - revoluciones por minuto
SNC - sistema nervioso central

I. INTRODUCCION

A. EPITELIO PIGMETARIO DE LA RETINA

1. MORFOLOGIA DEL EPR

El epitelio pigmentario de la retina (EPR) está formado por una capa simple de células cuboidales, situada entre la retina neural y la coroides, se halla en estrecha asociación con los fotorreceptores, que son las células de la retina sensibles a los fotones. De la superficie de la membrana apical se extienden procesos, llamados microvellosidades, hacia los segmentos externos de los fotorreceptores. La superficie basal, con sus numerosas invaginaciones, está funcionalmente relacionada con la membrana de Bruch de la coroides. Existen pequeñas diferencias en el tamaño de estas células entre las distintas especies de vertebrados, el grosor promedio de estas células es de 10 a 25 µm y la altura de 7 a 10 µm (Nguyen-Legros, 1978).

Las células del EPR están unidas entre sí por uniones estrechas que, junto con el endotelio de los coriocapilares, impiden muy eficientemente el intercambio de substancias potencialmente tóxicas entre la circulación coroidal y la retina neural, de tal forma que el EPR constituye una parte importante estructural y funcional de la barrera hemato-retiniana. Las células del EPR están polarizadas no sólo morfológica sino también bioquímicamente. Por ejemplo, la ATPasa Na^+-K^+ se localiza exclusivamente en la superficie apical, mientras que los receptores para la proteína del suero que une retinol se encuentra en las membranas plásmaticas basal y lateral en el EPR de rata y retina humana (Zinn y Marmor, 1979).

Las células del EPR contienen los organulos celulares característicos de toda célula eucariotica:

El núcleo se encuentra en la porción basal, contiene la doble membrana típica con numerosos poros. En la célula del organismo adulto es visible un solo gran núcleo, mientras que en la etapa proliferativa del estado de embriogénesis es frecuente la existencia de más de un núcleo (Hollenberg y Spira, 1972.) Hay sin embargo, en algunos

organismos como la salamandra, la rata y el bovino, un número significativo de células polinucleadas que aumenta con la edad (Berman y Schwell, 1974). Se ha considerado que estos núcleos poliploides pueden ser el resultado de un bloqueo espontáneo de la mitosis (Nguyen-Legros, 1978).

Contienen microperoxisomas, que son orgánulos de pequeño tamaño (0.15 a 0.25 µm de diámetro), típicos de células con gran actividad metabólica, de transporte y almacén de lípidos. Este factor va de acuerdo con la función del EPR como intermediario del metabolismo del retinol.

Las mitocondrias están localizadas principalmente en la zona basal de la célula. Son de un tamaño promedio entre 0.5 y 1.0 µm de longitud. Estos orgánulos tienen las enzimas necesarias para realizar el ciclo de Krebs y la cadena respiratoria para sintetizar el ATP.

Los melanosomas son orgánulos muy abundantes en el citoplasma de la células del EPR que le confieren a ésta el color obscuro característico.

El retículo endoplásmico rugoso esta presente en menor cantidad que el liso y este último se encuentra acumulado en la zona apical y en las microvellosidades de la célula (Clarck, 1986).

El citoesqueleto de las células del epitelio pigmentario contiene como componente más abundante a la actina, aunque existen tambien microtúbulos y filamentos intermedios hechos de vimentina (Docherty y col. 1987).

2. EMBRIOLOGIA DEL EPR

El EPR y la retina tienen un origen común, ambos derivan de la misma placa ectodérmica primitiva. La placa ectodérmica se invagina y forma un surco neural, después se origina el tubo neural y enseguida este se regionaliza en dos zonas, un segmento caudal y uno rostral que se convertirá en el encefalo. El extremo cefálico se divide en tres protuberancias conocidas como vesículas primarias, el proencéfalo, el mesencéfalo y el romboencéfalo. En el desarrollo posterior, el proencéfalo se subdivide en el telencéfalo y

el diencéfalo. A cada lado del diencéfalo se proyecta una evaginación que forma las vesículas ópticas, que se extienden y hacen contacto con la parte inferior del ectodermo externo, después de establecido este contacto la pared de las vesículas se invagina y forma la copa óptica, de doble pared. La pared externa de la copa óptica se pliega y da origen a dos capas: la más externa se diferencia en el EPR y la interna en la retina neural.

Las células del EPR se diferencian tempranamente en el desarrollo; durante esta etapa generalmente las células aumentan de tamaño y se lleva a cabo el proceso de melanogénesis. En el pollo la mayoría de las células dejan de dividirse del 4o. al 7o. día de incubación, el cual se completa al décimo cuarto día del desarrollo embrionario (Stroeva y Mitashow, 1983), aunque se ha reportado la presencia de melanina a las 42 horas de incubación (Zinn y Marmor, 1979). En la especie humana se completa la proliferación celular, a diferencia de otras especies, después del nacimiento.

3. CULTIVO DEL EPR

El estudio farmacológico de los receptores en los tejidos de humanos se facilita mediante el uso de cultivos celulares, ya que proporciona gran número de poblaciones puras de los tipos celulares de interés. Sin embargo, esto introduce factores de confusión desconocidos, como es que el colocar células en cultivo puede alterar muchas características estructurales y funcionales. Ha sido demostrado que las células del EPR humano en cultivo retienen muchas de las características que expresan in vivo y se han usado para estudiar gran variedad de las funciones del EPR (Das y Gouras, 1988; Mayerson y Hall, 1986; Hall y col, 1991; Gregory y col, 1994).

4. SISTEMAS DE TRANSDUCCION EN EL EPR

PRODUCCION DEL AMPc

Algunas investigaciones recientes no dejan duda de que el EPR es un tejido blanco para la acción de un gran número de hormonas y neurotransmisores. Sin embrago, la

naturaleza precisa de los sistemas de transducción estimulados por receptores membranales y los segundos mensajeros intracelulares generados por AAE no han sido aún identificados.

El EPR de primates contienen niveles bajos, pero detectables de AMPc (Newsome y col, 1974) y niveles endógenos similares están probablemente presentes en el EPR de otras especies (Friedman y col, 1987). Los niveles de AMPc en el EPR pueden modularse por una gran variedad de agonistas, lo que sugiere que estas células contienen un sistema para la adenilato ciclasa similar al encontrado en otros tipos celulares. La producción de AMPc intracelular en el EPR cultivado de embriones de pollo, se estimula por catecolaminas con el siguiente orden de potencia: L-isoproterenol > epinefrina ≥ norepinefrina > dopamina (Koh y Chader, 1984a). Esta respuesta es típica para los agentes β-adrenérgicos que interactúan con el receptor β-adrenérgico excitador. La señal se traduce a través de la interacción con una proteína G, en este caso Gs, lo cual tiene como resultado la activación de la adenilato ciclasa y la producción de AMPc. Las células del EPR de humano contienen una adenilato ciclasa estimulada por agonistas β-adrenérgicos (Koh y Chader, 1984b) y prostaglandinas (Friedman y col, 1987). Friedman y col en 1989 demuestran que los agonistas de la adenosina estimulan la acumulación de AMPc en las células intactas del EPR y la actividad de la adenilato ciclasa en preparaciones de membrana, lo que sugiere la presencia de receptores tipo A para la adenosina vinculados con la adenilato ciclasa en forma estimuladora.

HIDROLISIS DE FOSFOINOSITIDOS.

El mecanismo de hidrólisis de fosfoinositidos utiliza una combinación de segundos mensajeros: fosfatos de inositol y diacilglicerol, y en una etapa posterior iones Ca^{2+}, los cuales se generan a partir de la hidrólisis de 4,5-difosfato fosfatidil inositol de la membrana plasmática (figura 1b). Estas reacciones forman parte de un sistema de transduccion de señales que intervienen en otros tejidos para controlar una gran variedad

de procesos celulares que incluyen la secreción, el metabolismo, la fototransducción y la proliferación celular. El componente inicial, la molécula receptora de la superficie de la célula, transmite información a través de la membrana plasmática hacia el interior de la célula por medio de una proteína G: proteína de membrana que no puede activarse a menos que se una al GTP (Pfister y col, 1993). La proteína G, activa a la fosfolipasa C en la cara interna de la membrana; la fosfolipasa C a su vez corta el lípido de membrana fosfatidil inositol 4,5-difosfato en diacilglicerol y trifosfato de inositol. El IP_3 actúa predominanatemente liberando Ca^{2+} del retículo endoplásmico. El DG activa a una proteína cinasa unida a la membrana, a la que se le ha denominado cinasa C (PKC), la cual fosforila proteínas de la membrana plasmática (Berridge e Irvine, 1989; Osborne, 1988).

Osborne y col (1991) demostraron la presencia de receptores para acetilcolina de tipo muscarínico en el EPR de humano. Estos recepores detectados en cultivo están asociados con la producción de fosfatos de inositol. El carbacol a una concentración de 100 µM produce un aumento en la concentración de fosfatos de inositol de 4 veces sobre el nivel basal, y su EC_{50} es de 70 µM.

Se ha demostrado que diversas sustancias, entre estas los aminoácidos excitadores, estimulan la producción de fosfatos de inositol en diversas regiones del SNC, que incluyen la retina neural (Ghazi y Osborne, 1989). De esta forma, los AAE pueden actuar por el disparo de la cascada de segundos mensajeros además de activar canales iónicos.

5. FUNCIONES DEL EPR

El EPR realiza una amplia variedad de actividades, que incluyen la fagocitosis, captación y almacenamiento de retinoides, secreción de los componentes de la membrana basal y el transporte selectivo de substancias entre los fotorreceptores y los coriocapilares (Zinn y Marmor, 1979); ejerce influencia trófica sobre otros tejidos e induce la diferenciación de la coroides y de los fotorreceptores de la retina. Asimismo, el EPR

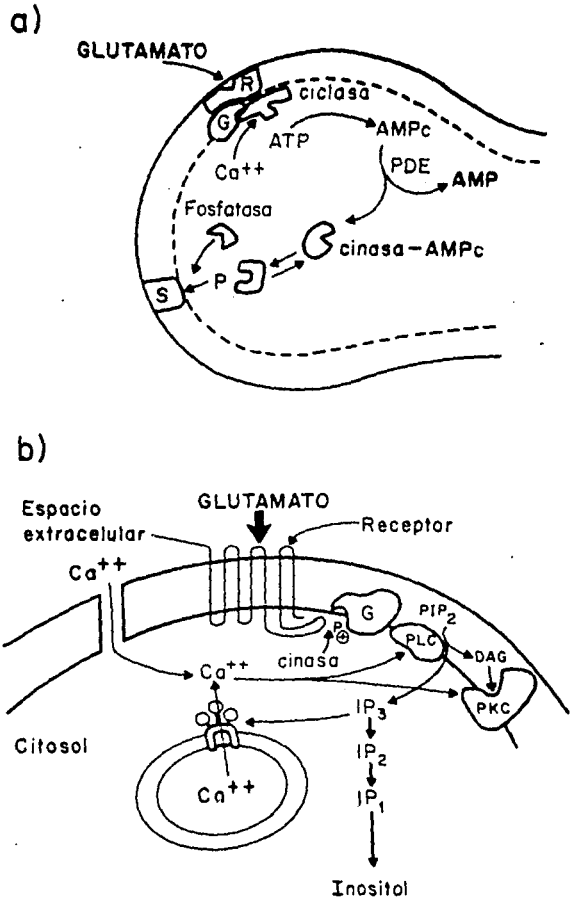

FIGURA 1. Mecanismos de transducción: **a**) cascada del AMPc; el ácido glutámico interactúa con su receptor (R) y activa a una proteína que une GTP (G), la cual en presencia de Ca^{2+} estimula a la ciclasa del AMP, generando AMPc a partir de ATP. El AMPc puede degradarse a AMP mediante la acción de una fosfodiesterasa (PDE), o bien participar en la activación de cinasas, las que a su vez fosforilan a diversas proteínas de la membrana(S). **b**) Activación de la hidrólisis de fosfoinosítidos descrita en el texto.

desempeña multiples actividades relacionadas con la fisiología de la retina, entre las que se encuentra el transporte de diferentes compuestos desde los coriocapilares hacia el espacio subretiniano y viceversa. Algunos estudios hechos en el EPR de rana indican que en la membrana apical se localiza una ATPasa Na^+-K^+, así como un transporte de cloro en dirección opuesta al flujo del sodio (retina a coroides), además de un transporte de calcio con una dirección de coroides a retina (Miller y Steinberg, 1977a). Y transportadores específicos para aminoácidos y bicarbonato (Miller y Steinberg, 1979). En 1977(b) Miller y Steinberg, demostraron que tanto la membrana apical como la basal tienen una alta conductancia para el potasio. La retina de algunos vertebrados como el pollo es avascular, en dicho caso el papel del EPR como una barrera selectiva hematorretiniana es aún más aparente. En el EPR el retinol constituyente del pigmento fotosensible de las células fotorreceptoras, se metaboliza, transporta y regenera por medio de la actividad de diversas deshidrogenasas y se almacena en forma esterificada principalmente como palmitato y estearato. Asimismo existen en el EPR varias proteínas que unen retinol y que lo transportan hacia la retina y fuera de ella, con lo que se impide su efecto tóxico (Zinn y Marmor, 1979).

FAGOCITOSIS

Una de las funciones más sobresaliente del EPR es su participación en el proceso de renovación de las células fotorreceptoras, actividad conocida como fagocitosis.

Para la renovación de las membranas de los segmentos externos de los bastones (ROS), en los fotorreceptores de los vertebrados se sintetizan y ensamblan nuevos discos en el extremo basal del segmento e intermitentemente se desprenden los discos apicales viejos (Young y Bok, 1969; Young, 1976). El desprendimiento de los discos incluye la separación de grupos de ellos, los cuales son fagocitados por el EPR adyacente (Young y Bok, 1969) y degradados en fagolisosomas (Ishikawa y Yamada, 1970; Marshall y Ansell, 1971). La fagocitosis de los discos de los ROS se considera generalmente como un

proceso de pasos multiples, que comprenden la unión de los discos al EPR, la ingestión de éstos y las fases de degradación (Young y Bok, 1969; Clarck, 1986). Mucho del trabajo reciente se ha enfocado al estudio del control del desprendimiento de los discos por estímulos fisiológicos (revisión de Besharse, 1982). Por ejemplo, el desprendimiento y fagocitosis ocurren normalmente como una respuesta diurna que tanto in vivo como in vitro está regulada por el patrón de luz-oscuridad, así como por influencias circádicas (LaVail, 1976; Basinger y col, 1976; Flannery y Fisher, 1979; Besharse y col, 1980). Sin embargo, el tratamiento de las copas ópticas de *Xenopus* en cultivo, con los candidatos a neurotransmisores L-aspartato y L-glutamato produce un incremento en el desprendimiento de los discos, independientemente de la luz (Greenberger y Besharse, 1985) y un aumento en la adhesividad retina-EPR (Defoe y col, 1989). Ciertos aminoácidos no excitadores, tales como la taurina y la glicina, también aumentan el desprendimiento de los discos y la consecuente fagocitosis de éstos por parte del EPR. Producen además adhesividad aparente (Sweatt y Besharse, 1988) sin causar neurotoxicidad significativa, como la que si causan los AAE.

Se ha observado que el desprendimiento de los discos provocado por luz en las copas ópticas, requiere de Ca^{++} extracelular (Greenberger y Besharse, 1983) y se bloquea por antagonistas de los canales de Ca^{++} (Besharse y col, 1986).

6. PATOLOGIA DEL EPR

RETINITIS PIGMENTOSA

El EPR es el sitio de lesión primaria en la distrofia retinal hereditaria de la rata Royal College of Surgeons (RCS), modelo animal de retinitis pigmentosa. La RP se caracteriza por una deficiencia en la fagocitosis de los paquetes de discos de los segmentos externos de los fotorreceptores por parte del EPR.

La retinitis pigmentosa en un grupo de retinopatías hereditarias que afecta aproximadamente 1 de cada 4,000 individuos. Se ha establecido una clasificación con

base en las características genéticas: dominante, recesivo y formas relacionadas con el cromosoma X. Los genes de la retinitis pigmentosa autosómica dominante están localizados en el cromosoma 3q que codifica para una proteína de los fotorreceptores, La Rodopsina; el cromosoma 6q, con una deleción en el codón 118/119 del gen que codifica a la Periferina, está asociado con la retinitis pigmentosa. No se conoce el efecto directo del cromosoma 8, que también participa en esta familia de alteraciones. La RPAD progresa lentamente en las primeras décadas de vida, pero causa daños visuales severos hasta alcanzar la ceguera completa.

Más de 40 mutaciones genéticas en la opsina de humano están relacionadas con algunas formas de esta enfermedad tan heterogénea. Los fotorreceptores de los pacientes con RPAD y mutaciones en el gen de la opsina sintetizan la rodopsina normal junto con la rodopsina mutada y esta causa, por un mecanismo aún desconocido, la degeneración lenta de las células fotorreceptoras.

Los ratones heterocigóticos con el gen mutado de la opsina generan fotorreceptores casi normales durante el desarrollo postnatal temprano. Estos tienen segmentos externos de menor longitud que la normal. Conforme avanza la edad, tanto los conos como los bastones se reducen en número y hay un decremento en la respuesta del electrorretinograma a los estímulos fóticos. Estos ratones con mutaciones visuales son utilizados como modelos para correlacionar esta enfermedad en humanos.

Se ha sugerido que el EPR de las ratas distróficas es deficiente en la producción de factores tróficos importantes para la sobrevivencia de los fotorreceptores. De acuerdo al trabajo de Malecaze y col. (1993), existe en el EPR de ratas distróficas sólo el 20% del número de receptores para el factor de crecimiento fibroblástico, comparado con las células de ratas normales.

B. LOS AMINOACIDOS EXCITADORES

Los aminoácidos excitadores (AAE) L-glutamato en particular, pero también L-aspartato y homocisteato, parecen ser los neurotransmisores excitadores más abundantes en el SNC de los vertebrados (revisiones de Watkins y Evans, 1981; Fagg y col, 1983; Fonnum y col, 1981; Cotman y col, 1987). Sus acciones se realizan por medio de por lo menos cinco clases de receptores, de los cuales el receptor de N-metil-D-aspartato es el mejor caracterizado (Monaghan y col, 1989; Wroblewski y Danysz, 1989).

1. RECEPTORES PARA AAE DE TIPO NMDA

El estudio farmacológico de los receptores de tipo N-metil-D-aspartato (NMDA) es el más desarrollado actualmente. Existen por lo menos 5 sitios farmacológicamente distintos por medio de los cuales diversos compuestos pueden alterar la actividad de este receptor (McDonald y Johnson, 1990; Monaghan y col, 1989).

Estos incluyen:

a) un sitio de unión para el transmisor al cual se une el L-glutamato,

b) un sitio alostérico positivo al cual se une la glicina,

c) un sitio en el canal que une fenciclidina y otros anestésicos disociativos

d) un sitio de unión en el canal para el Mg^{2+}, dependiente de voltaje

e) un sitio para cationes divalentes con acción inhibitoria al cual puede unirse el Zn^{++}, localizado fuera del canal y

f) un sitio de regulación al cual se unen las poliaminas.

Aunado a esto, el análisis de unión de ligandos indica que hay 2 sitios de unión distintos (o estados distintos) asociados con el sitio de reconocimiento del transmisor, uno que preferentemente une agonistas y otro que une antagonistas. El canal de este receptor es permeable al Ca^{2+}, Na^{+} y K^{+} (Monaghan y col, 1989).

Johnson y Ascher (1987) descubrieron que la glicina aumenta considerablemente los efectos de los agonistas del NMDA, pero que esta no tiene efecto por sí misma. En

concentraciones tan bajas como 0.1 µM, la glicina incrementa considerablemente la respuesta del receptor de NMDA sin ser bloqueada por estricnina. Los análisis de canales unitarios por medio del "patch-clamp" indican que la glicina aumenta la frecuencia de apertura del canal, pero no la amplitud de la corriente y disminuye el período de desensibilización (figura 2).

2. RECEPTORES PARA AAE DE TIPO NO-NMDA

Los ácidos kaínico y quisculálico fueron inicialmente aislados durante la purificación de la actividad antihelmíntica encontrada en el alga Diosgenea simplex (kainato) y en semillas de la planta Quiscualis fructus (quiscualato) (Barnes y Henley, 1992).

El GDEE inhibe la respuesta inducida por quiscualato, pero no afecta la respuesta del kaínato (Haldeman y McLennan, 1972; McLennan y Liu, 1982). La despolarización inducida por el agonista selectivo del QA, el AMPA, son también bloqueados selectivamente por GDEE (Krogsgaard-Larsen y col., 1980).

Los compuestos que inhiben el efecto del KA, pero no el del QA son: el 2-amino-4-fosfonobutirato (APB) (Davies y Watkins, 1979), γ-D-glutamil-glicina (DGG) (Teichberg y col, 1989; Davies y Watkins, 1981) y el ácido kinurénico (Ganong y col, 1983). Los antagonistas más comunes de los receptores de KA y QA son los derivados de la quinoxalina: la 6-ciano-7-nitroquinoxalín-2,3-diona (CNQX) y la 6-7-dinitroquinaxalín-2,3-diona (DNQX), reportados por Honoré y Drejer (1988). Las concentraciones micromolares bajas de estos compuestos bloquean las respuestas del KA y QA, pero también tienen efecto sobre las respuestas inducidas por NMDA (Honoré y Drejer, 1988; Blake y col, 1988; Drejer y Honoré, 1988). En altas concentraciones la DNQX antagoniza la respuesta evocada por el NMDA en una forma no competitiva (Birch y col, 1988). El antagonista ácido 6,7-dicloro-3-hidroxi-2-quinoxalinocarboxílico ha sido reportado como bloqueador selectivo del receptor KA con respuesta relativa sobre QA (Frey y col, 1988).

La estructura pentamérica de los receptores de tipo no-NMDA sería consistente con las estructuras reportadas para otros canales iónicos controlados por ligandos, incluyendo el del ácido γ-aminobutírico tipo A ($GABA_A$), la glicina y los receptores para acetilcolina de tipo nicotínico.

3. RECEPTORES PARA AAE DE TIPO METABOTROPICO

Además de sus efectos en receptores ionotrópicos, el glutamato ejerce acciones por medio de receptores metabotrópicos (figura 2). En el año 1991, Nakanishi y col. clonaron un DNA complemetario para un receptor de glutamato metabotrópico (mGluR1) y recientemente aislaron clonas de DNA complemetanrio para 4 mGluRs de rata adicionales (mGluR2 a mGluR6) (Tanabe y col, 1992). Mientras que el mGluR1 y el mGluR5 estimulan la hidrólisis de fosfolípidos de inositol, los receptores metabotrópicos restantes parecen estar acoplados a sistemas de transducción diferentes. Por ejemplo, con el mGluR2 y el mGluR3 expresados en ovocitos de hamster chino se encontró que inhiben la estimulación de la adenilato ciclasa por forskolina y por consiguiente la acumulación de AMPc; y el mGluR4 parece corresponder a un receptor para APB, localizado en la presinápsis, que regula la liberación del ácido glutámico (Schoepp y Conn, 1993). Nakajima y col (1993), caracterizaron un nuevo receptor, el mGluR6, con una alta afinidad por el APB y acoplado inhibitoriamente con la adenilato ciclasa. Asimismo Saugstad y col. reportaron en 1994 al mGluR7 y Duvoisin y col (1995) al mGluR8, homólogos al receptor mGluR4.

El receptor metabotrópico es activado por L-glutamato, tbotenato, quisqualato y ACPD y no es bloqueado por antagonistas de KA/QA como la CNQX o la toxina de la araña Nephila clavata (JSTX).

Si bien se desconoce la función del receptor activado por el ACPD, que resulta en la formación de fosfatos de inositol, vía una proteína G sensible a la toxina pertusis

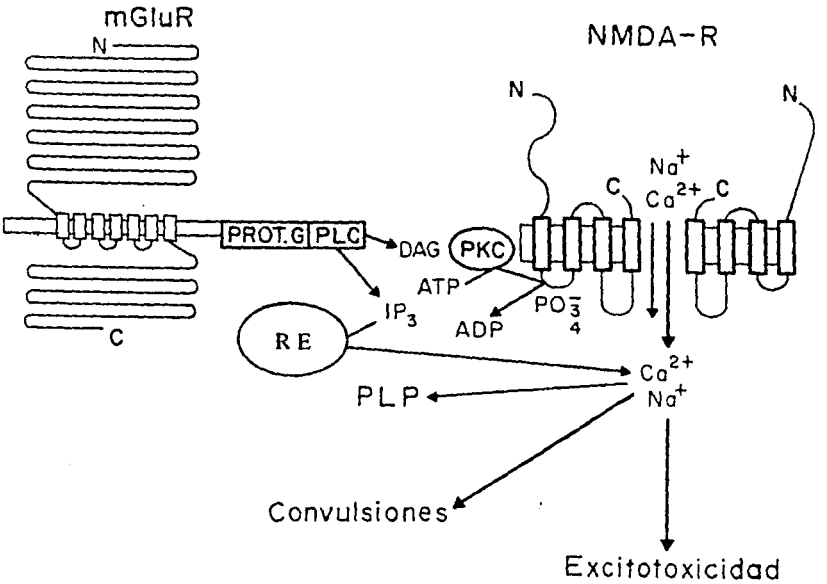

FIGURA 2. Representación esquemática de un receptor ionotrópico (NMDAR) y uno metabotrópico (mGluR) para L-Glu, así como su posible interrelación. N, extremo amino terminal de la proteína; C, extremo carboxilo terminal; Prot. G, proteína que une GTP; PLC, fosfolipasa C; DAG, diacilglicerol; IP, trifosfato de inositol; RE, retículo endoplásmico; PKC, proteína cinasa C; PLP, potenciación a largo plazo. La estimulación del mGluR activa la hidrólisis de fosfoinosítidos y subsecuentemente a la PKC. Esta enzima fosforila al NMDAR, cuya estimulación produce la elevación del calcio citosólico. La elevación del Ca^{++} se relaciona con numerosos procesos normales y patológicos. Modificado de Schoepp y Conn, 1993.

(Monaghan y col, 1989), hay razón para especular que este receptor juega un papel importante en la estabilización sináptica. En común con muchos factores de crecimiento, este receptor regula la formación de IPs. Además, la actividad de este receptor disminuye rapidamente después de la sinaptogénesis (Nicoletti y col, 1986) y reaparece después de la desdiferenciación (Nicoletti y col, 1987).

La transcripción de cada uno de los mGluRs muestra distintos patrones de expresión en el cerebro.

La transmisión excitadora parece incluir acciones mediadas por uno o más combinaciones de estos receptores, ionotrópicos y metabotrópicos, hasta en las sinapsis simples.

4. RELACION DE LOS AAE CON LA FAGOCITOSIS

Los AAE estimulan el desprendimiento de discos de los fotorreceptores (Greenberger y Besharse, 1985) en forma independiente del Ca^{++} (Besharse y col, 1986) lo que sugiere la posibilidad de que los AAE puedan mimetizar los efectos de un agente endógeno.

Defoe y col (1989), mostraron que el desprendimiento de discos inducido por L-glutamato va acompañado de un incremento aparente en la adhesividad EPR-fotorreceptor. El lugar del efecto del L-glutamato, así como de otros aa, sobre el incremento de la adhesividad o el desprendimiento de los discos no se conoce, pero se considera que puede intervenir un receptor membranal porque el efecto de este aminoácido se bloquea por el antagonista específico kinurenato (Besharse y Spratt, 1988).

5. LOS AAE EN EL DESARROLLO DEL SNC

Los estudios recientes han revelado que las diversas formas moleculares de los receptores para AAE muestran especificidad celular y patrones temporales de expresión (Somohano y col, 1988). Así, los canales de receptores para AMPA expresados

tempranamente en el desarrollo difieren en estructura y propiedades funcionales de los canales expresados en el cerebro adulto (Sommer y Seeburg, 1992).

Los receptores para aminoácidos excitadores parecen no solo intervenir en la transmisión sináptica normal en las vías excitadoras, sino también participar en la modificación de las conexiones sinápticas durante el desarrollo (Wroblewski y Danysz, 1989) y los cambios en la eficiencia de la transmisión sináptica a lo largo de la vida (Watanabe y col, 1992; Riuzicha y Jhamandas, 1993; Palmer y col, 1990). Irónicamente, la sobreactivación de receptores selectivos también pueden causar la degeneración neuronal y hasta la muerte celular (Watkins y Evans, 1981; Schoepp y Conn, 1993).

Muchos de los trabajos sobre la regulación de los receptores para AAE durante el desarrollo, se ha basado en los experimentos de unión de ligandos específicos y tales estudios han proporcionado evidencias de los cambios en la cantidad de receptores de glutamato en las primeras semanas de vida postnatal (Slater y col, 1992). Recientemente varios estudios de hibridización in situ han proporcionado nueva información sobre como se regula la expresión del gen del receptor AMPA-kainato durante el desarrollo (Sommer y Seeburg, 1992).

C. TAURINA

La taurina (ácido β-amino-etanosulfónico) es un aminoácido ampliamente distribuido en el organismo (Kendler, 1989), con las concentraciones más altas en el músculo estriado y cardiaco (Huxtable, 1986; 1989), glándulas pineal y pituitaria (Vellan y col, 1970), plaquetas (Ahtee y col, 1974), células fibroblastoides (Wright, 1986), hígado (Jacobsen y Smith, 1968) y la retina (Pasantes-Morales y col, 1972). Se ha caracterizado como un nutriente esencial condicionado (Chipponi y col, 1982), y participa en una gran cantidad de funciones que incluyen la osmorregulación (Thurston y col, 1980), proliferación celular (Gabrielian y col, 1992; Gaull y col, 1983), modulación del flujo de calcio (Sebring y Huxtable, 1985), estimulación de la glicólisis y gluconeogénesis (Kulakowski y Maturo,

1984), modulación de la excitabilidad neuronal (Bernardj, 1985), destoxificación (Emudianughe y col, 1983) y estabilización membranal (Pasantes-Morales y col, 1985).

La taurina es el aminoácido más abundante en la retina, su concentración es de 10 a 40 mM en la retina de los vertebrados (Pasantes-Morales y col, 1972; Cohen y col, 1973; Starr y Voaden, 1972), y se concentra en los fotorreceptores más que en las capas internas de la retina (Cohen y col, 1973). Además de los fotorreceptores y las células gliales, el EPR y algunas células amacrinas acumulan taurina (Ehinger, 1973; Lake y col, 1977; Lake y col, 1978; Pourcho, 1981), la captación de la cual es por procesos de alta y baja afinidad (Kennedy y Voaden, 1976; Salceda y col, 1979; Dawson y Neal, 1984).

Existe un sistema de tansporte para taurina en el EPR (Salceda y Saldaña, 1993). Sivakami y col (1992), aislaron vesículas de membranas apicales de células del EPR de bovino y encontraron dos sistemas de transporte: el transportador específico para taurina y el transportador para taurina y GABA. Este último sistema de transporte está presente en las células normales del EPR, pero no en las células de ratas distróficas. El sistema de transporte para taurina es dependiente de los iones Cl^- y Na^+, que son necesarios para su óptima función, que se realiza en contra del gradiente de concentración del aminoácido, los β-aminoácidos y el GABA pueden interactuar con este sistema, pero no los α-aminoácidos; tiene una alta afinidad por taurina e hipotaurina y es un sistema simple, saturable y según los estudios realizados utilizando vesículas aisladas de membranas apicales de EPR de bovino, este sistema tiene una Vmax = 1,068 pmol/min/mg de proteína y una K_t = 96 µM.

El transporte de taurina se ha estudiado utilizando cultivos primarios de diversos tipos celulares y líneas celulares. Edwards (1981) estudió las propiedades de la captación de taurina en cultivos primarios del EPR de rata y mostró que las células acumulan activamente taurina y este es un proceso dependiente de Na^+, temperatura y sensible a venenos metabólicos. El sistema es simple con una K_t de 16 µM.

La pérdida de la captación de taurina por el sistema de alta afinidad en la retina de ratas RCS está relacionada con la degeneración de los fotorreceptores (Schmidt y Berson, 1978).

La taurina es sintetizada en el hígado y el EPR la transporta desde el flujo sanguíneo coroidal hacia la retina, debido a que la síntesis de este aminoácido en la retina no cubre la concentración milimolar que existe en este tejido. Los niveles de taurina en los fotorreceptores podrían mantenerse por el transporte desde los coriocapilares vía el EPR, mientras la retina interna puede sintetizar taurina de novo (Salceda y col, 1979). Se ha sugerido que la fagocitosis de los segmentos externos de los fotorreceptores por el EPR es regulada por la relación melatonina-taurina en el espacio del interfotorreceptor (Ogino y col, 1983).

D. ACIDO γ-AMINO BUTIRICO

El ácido gamma-aminobutírico (GABA) está virtualmente presente en cada area del cerebro (Vellan y col, 1970) y es activo en aproximadamente del 20 al 40% de las sinapsis del mismo (Bloom e Iverson, 1971). El GABA se une a dos subtipos diferentes de receptores y participa en una gran variedad de respuestas fisiológicas.

A pesar de que ambos subtipos de receptores participan en la inhibición neuronal, son diferentes en su perfil farmacológico, distribución e interacción con iones y nucleótidos. Estos sitios se especifican como $GABA_A$ y $GABA_B$.

El receptor $GABA_A$ es el receptor clásico de GABA. Es un complejo proteico con multiples sitios de reconocimiento. El receptor GABA A contiene por lo menos, un sitio de unión al GABA acoplado al canal de Cl^-. Este receptor incluye sitios de unión para benzodiazepinas y barbituratos (Bowery y col, 1987). Los dos antagonistas más comunes en el complejo del receptor GABA, bicuculina y picrotoxina, disminuyen la permeabilidad de la membrana para el Cl^- (Silvilotti y Nistri, 1991).

Los receptores del subtipo $GABA_B$ tienen un perfil farmacológico distinto al receptor $GABA_A$. Los receptores $GABA_B$ son insensibles a bicuculina, barbituratos y benzodiazepinas. Estos receptores son estereoespecíficamente activados por baclofen y no están asociado con un ionóforo de Cl^- más bien parecen estar acoplados a canales de CA^{2+} y K^+. El baclofen actúa sobre este receptor disminuyendo la amplitud de la corriente de Ca^{2+} e incrementando la conductancia al K^+.

E. GLICINA

Varios estudios han demostrado la presencia de un receptor para el aminoácido glicina en el cerebro anterior de los mamíferos que es distinto del receptor de glicina clásico encontrado en médula espinal y tallo cerebral (Kishimoto y col, 1981; Johnson y Ascher, 1987). El receptor de glicina en el cerebro anterior es insensible a la estricnina y muestra una selectividad diferente para los aminoácidos, es relativamente insensible a la β-alanina y taurina y más sensible a la D-serina (Kishimoto y col, 1981). Este efecto lo ejerce la glicina por medio de su interacción con un sitio modulador del receptor de tipo NMDA de los AAE.

Con el descubrimiento de que la glicina aumenta considerablemente la activación del receptor de NMDA, el papel potencial de la glicina en la transmisión de mensajes en el SNC se ha expandido enormemente, se considera que actua como modulador de la excitabilidad en el SNC por medio de su acción como potenciador sobre el receptor de NMDA (Johnson y Ascher, 1987). Mientras que la acción de la glicina como un neurotransmisor inhibidor se restringe a las regiones cerebrales inferiores (Kemp y Leeson, 1993).

II. OBJETIVO

Una de las funciones más importantes del EPR es la de fagocitar paquetes de discos de los segmentos externos de los fotorreceptores. Para llevar a cabo esta función es necesario que exista un mecanismo de comunicación y se propusó la hipótesis de que esta comunicación pudiera llevarse a cabo por medio de mensajeros químicos como el ácido glutámico y/o la taurina.

Ya se ha demostrato la presencia de receptores para taurina en membranas de células cultivadas del EPR de embriones de pollo (López-Colomé y col, 1991), los cuales tienen características similares a las que presentan los receptores de tipo postsináptico en otras regiones del SNC (Kontro y Oja, 1989).

Por otra parte se conoce la influencia que ejercen ciertos aminoácidos como la glicina (Reynolds y col, 1987; Snell y col, 1987) y el GABA (Walden y col, 1989) en potenciar la respuesta de los AAE, el ácido glutámico y sus análogos.

Se consideró factible la posibilidad de que tanto la taurina, como el GABA y la glicina aumentaran la unión de ácido glutámico a las membranas aisladas del EPR. El objetivo fue caracterizar la influencia de estos aminoácidos sobre la unión de glu-H^3 a dos edades de cultivo de las células del EPR de pollo: en un estado no diferenciado (16 DIV) y en un estado diferenciado (25 DIV). Para comprobar que la condición de cultivo no altera el sistema de receptores a estudiar, se probó el aislamiento de membranas de embriones de pollo a diferentes edades para comparar con lo que se encontró en cultivo.

Asimismo se busco caracterizar este sistema de receptores para ácido glutamico en las membranas de células cultivadas de EPR de humano, se consideró la posibilidad de que estos receptores estuvieran preservados filogenéticamente. El estudio de los receptores de humanos se realizó con células cultivadas de donadores post-mortem de diferentes edades.

III. MATERIALES Y METODOS

A. CULTIVO DE EPR DE POLLO

Se utilizaron embriones de pollo de 7 días de desarrollo a los cuales se les extrajó la copa óptica en condiciones estériles, a ésta se le hizo un corte ecuatorial, se desechó la parte anterior y de la posterior se extrajo el EPR. Se lavó el tejido con RKB, compuesto por: NaCl 118 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.17 mM, glucosa 1.6 mM y NaHCO 35 mM; se centrifugó 5 minutos a 500 rpm a temperatura ambiente, la pastilla que se obtuvo se resuspendió en solución Ringer fresca y se centrifugó en las mismas condiciones. Se repitió la operación 3 veces más. La disociación de las células se hizo por incubación con tripsina 0.13% en PBS durante 5 minutos a temperatura ambiente. Las células se sembraron en cajas Falcon de 20 cm^2 con medio de cultivo Difco TC-199 con 10% de suero de bovino fetal inactivado, 2 mM de glutamina, 100 UI/ml de penicilina y 100 µg/ml de estreptomicina, a una densidad de 25,000 células por cm^2 y se mantuvieron a una temperatura constante de 37 °C durante 16 o 25 días. El cambio de medio se realizó a intervalos de 5 días y el aspecto de estos cultivos a los 16 DIV es fibroblástica y a los 25 DIV epitelial.

B. CULTIVO DE EPR DE HUMANO

Los ojos de los donadores post-mortem se obtuvieron de The National Disease Research Interchange, Philadelphia, Pennsylvania USA. Los cultivos de células del epitelio pigmentario de la retina obtenidos provinieron de donadores de diferentes edades: 9 y 29 días, 4 meses, 15 y 33 años. Ninguno de los donadores presentaba enfermedades crónicas, ni se había sometido a una medicación prolongada; la muerte se debió a un trauma severo. Los ojos fueron extraídos y se mantuvieron en medio estéril durante 4 horas, y enviados en hielo al laboratorio en un lapso de 36 horas. Las células del EPR se aislaron usando una modificación de Stramm y col. (1983). Las células se disociaron con una solución de pancreatina con 0.1% de EDTA, se centrifugaron a 40 g durante 10

minutos y se lavaron dos veces con medio RPMI 1640 complementado con 16% de suero de bovino fetal, glutamina (5 mM), 100 U/ml de penicilina y 100 µg/ml de estreptomicina. Las células se sembraron a una densidad de 5 X 10^4 células por ml en placas de cultivo de 30 mm insertadas en filtros millicell-Ha (Millipore, Bedford, Ma). Dspués de alcanzar la confluencia, las células se transfirieron a frascos de plástico de 75 cm^2 (Becton Dickinson Co, Oxnard, Ca USA). Las células en cultivos mantuvieron su morfología cuboidal sin apariencia fibroblástica hasta los pasajes 5 o 6 en los cuales perdieron la pigmentación.

C. OBTENCION DE MEMBANAS

Las células del EPR de pollo se cosecharon a los días de cultivo indicados para cada experimento (16 y 25 DIV). Después de eliminar el medio de cultivo y lavar con RKB, las células se desprendieron con una espátula y se resuspendieron en 5 ml de RKB. La resuspenstón se centrifugó a 500 rpm durante 5 minutos, se desechó el sobrenadante y la pastilla de células se homogenizó a 4°C, en 20 volúmenes (p/v) de agua bidestilada; la suspensión se mantuvo 15 minutos en hielo con objeto de inducir la ruptura total de las células por choque osmótico. Se centrifugó a 45,000 xg durante 20 minutos para sedimentar las membranas y se lavó 2 veces resuspendiendo por homogenización. Finalmente, las membranas se congelaron durante un mínimo de 48 horas a -70°C.

Las células de EPR de humano recibieron un tratamiento de lavado para la obtención de membranas idéntico al hecho en células de pollo.

D. CUANTIFICACION DE RECEPTORES POR LA TECNICA DE UNION DE UN LIGANDO RADIACTIVO

Los receptores se midieron por la unión especifica de un radioligando tritiado (L-glutamato-H^3) a las membranas de las celulas cultivadas de las diferentes edades. Los ensayos se realizaron por el método de filtración con filtros de fibra de vidrio Whatman (tipo GF/B). Las membranas se homogenizaron en amortiguador Tris-HCl 0.1 M a un

pH de 7.4 con o sin NaCl 118 mM y a temperatura de 37 ó 4°C. El aminoácido marcado se añadió a una concentración de 50 nM con variaciones según el experimento. La unión inespecífica se tomó como la radiactividad que permanece unida a las membranas en presencia de 1 mM de los compuestos fríos, agonistas o antagonistas.

Los filtros se disolvieron en 10 ml de titosol (Fricke, 1975) y la radiactividad se midió en un contador de centelleo líquido.

E. CUANTIFICACION DE PROTEINA

En todos los experimentos se determinó la cantidad de proteína por el método de Lowry y col (1951), utilizando albúmina sérica de bovino (Sigma) como standard.

F. REACTIVOS

El ácido L-glutámico-H^3 (Actividad específica, 45-59 Ci/mmol) se obtuvo de Amersham International PLC (Amersham, UK). Los filtros de fibra de vidrio tipo GF/B se obtuvieron de Whatman. El medio de cultivo TC-199 y el suero de bovino fetal se adquirieron de Difco (Detroit, Michiga, USA). Los agonistas del ácido glutámico se obtuvieron de Tocris Neuramin Charlotte St, Bristo, BS1 5PP, England). Los demás reactivos fueron de Sigma Chemical Co (St Louis, Mo, USA).

IV. RESULTADOS

A. PRIMERA PARTE (ARTICULOS 1 y 2).

DATOS CORRESPONDIENTES AL EPR DE POLLO

Por ser el objetivo de esta tesis el estudio de los receptores para ácido glutámico y su interacción con otros aminoácidos considerados inhibidores (taurina, GABA y glicina), el artículo número 1 (López Colomé y col, 1993) muestra que estos tres aminoácidos aumentan la unión específica del ácido glutámico en más de 10 veces en cultivos de 16 DIV, se observó una disminución considerable del efecto en cultivos de 25 DIV (figura 3). Este efecto parece ser específico para el EPR, ya que no se presenta en la retina de pollo de un día post-eclosión ni en la de 7 días de desarrollo embrionario, tampoco existe en la corteza cerebral de la rata adulta como lo muestra el artículo número 2 (figura 3) (López-Colomé y col en prensa). En las membranas aisladas del EPR de pollo de un día de edad (post-eclosión), se muestra un efecto equivalente al encontrado en el ensayo hecho con cultivos de 25 DIV (artículo 2, figura 1).

Con el objeto de eliminar la posibilidad de que este efecto se produjera como consecuencia de las condiciones de cultivo, se realizaron experimentos equivalentes en EPR de embriones de pollo en diferentes estados de desarrollo embrionario. En la figura 1- artículo 2, se muestra que hay un aumento en la unión de glutámico inducido por taurina, GABA y glicina, la taurina es el compuesto más potente en producir el efecto. El aumento es más evidente durante los primeros días del desarrollo embrionario y se pierde paulatinamente hacia el tiempo de eclosión.

La figura 2-artículo 2 muestra el análisis cinético del sistema estudiado en ausencia y presencia de taurina y glicina a una concentración de 1 mM. Las curvas de saturación y análisis de Scatchard comparativos muestran que existe un aumento en los sitios de unión para ácido glutámico, sin cambio en la afinidad del neurotransmisor por su receptor cuando el ensayo se realiza en presencia de taurina, y aparece un sitio de menor afinidad y aumenta el número de sitios cuando el ensayo se hace en presencia de glicina.

Se estudió además las características farmacológicas del sistema por medio del uso de agonistas y antagonistas específicos de los receptores para L-glutamato sobre la unión específica de glutamato-H^3: L y D-glutamato, L y D-aspartato, como agonistas generales, el dietil ester del glutamato (GDEE), antagonista de los receptores tipo quiscualato; el quiscualato (QA), agonista de los receptores tipo QA; el N-metil-D-aspartato (NMDA), agonsita de los receptores tipo NMDA, el kainato (KA), agonista del receptor tipo kainato y el L-glutamato-4-monohidroxamato, así como el DL-aspartato-β-hidroxamato como inhibidores de la captación del glutamato. El estudio farmacológico se hizo con cultivos de 16 DIV (tabla 1-artículo 1) y 25 DIV (tabla 2-artículo2), y los resultados muestran que a la primera edad probablemente existan más de un tipo de receptor para L-glutamato, y al comparar con los datos de la segunda edad se observó un cambio en las propiedades farmacológicas, aparición de nuevos sitios (KA) y desaparición de otros (L-AP3).

Con objeto de probar que se requiere la presencia de taurina para que se produzca el efecto, las membranas se preincubaron con este compuesto durante 10 y 20 minutos. Posteriormente se eliminó el medio de incubación, al centrifugar las membranas, y con la pastilla se procedió a realizar el ensayo. La preincubación con taurina (1 mM) no afectó la unión de glu-H^3, lo que comprobó que efectivamente se requiere para que realice su efecto.

B. SEGUNDA PARTE (ARTICULO 3).

DATOS CORRESPONDIENTES AL EPR DE HUMANO.

El artículo 3 (López-Colomé y col. 1994) muestra que con el objeto de estandarizar el tiempo de reacción del sistema de receptores para AAE, se realizó el experimento probando diferentes tiempos de incubación con el agonista marcado radiactivamente. De los resultados obtenidos en la curva de tiempo se consideró en lo sucesivo realizar los ensayos con un tiempo de incubación de 10 minutos debido a que en este lapso de alcanzaba prácticamente la unión máxima.

Se muestra la presencia en membranas de células cultivadas de EPR de humano de sitios de unión para el ácido glutámico, la interacción es reversible y de alta afinidad. En las figuras 4 y 5 se muestran las curvas de saturación en las membranas de diferentes donadores, la concentración del ligando radiactivo varía en los niveles de 25 nM a 2 µM y en presencia de glicina para el caso del donador más joven. El análisis de Scatchard (figuras 4 y 5) mostró una sola población de receptores en cada uno de las gráficas realizadas. En la cinética comparativa en las membranas del donador de 29 días se muestra que en presencia de glicina hay un aumento en la afinidad de los receptores para L-glu.

Se estudió el efecto de varios agonistas y antagonistas específicos de los receptores de L-glutamato: L y D-glutamato, L y D-aspartato, GDEE, QA, NMDA, APV, KA, L-glutamato-4-monohidroxamato y LD-aspartato-β-hidroxamato a una concentración del agonista marcado radiactivamente (ácido glutámico-H^3) de 50 nM. El orden en la potencia desplazamiento de estos compuestos utilizados a una concentración de 1 mM tuvo algunas variaciones en relación a la edad del donador (tabla I).

En las figura 1 se muestra que la estimulación con glicina, GABA y taurina presenta una relación inversa respecto tanto a la edad del donador como al número de pasajes in vitro en los cultivos procedentes del mismo tejido. El efecto de la glicina se detecta en concentraciones micromolares de este compuesto (figura 3) que provoca un aumento en la unión específica del L-glu por un cambio en la afinidad de los sitios de unión (figura 4). Este efecto no se inhibe en presencia de estricnina.

Se estudió la unión del ligando en presencia de taurina, GABA y glicina en otros cultivos celulares obtenidos de donadores humanos post-mortem: linfoblastos provenientes de una línea celular y células de esclerótica, provenientes del donador de 29 días. Como lo indican las gráficas correspondientes (tablas III y IV), existe un efecto positivo de los AAE sobre el aumento en la unión de ácido glutámico.

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Specific Interaction of Glutamate With Membranes From Cultured Retinal Pigment Epithelium

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Excitatory amino acids (EAA) have been shown to induce phagocytosis in retinal pigment epithelial (RPE) cells. In order to explore if this action is receptor-mediated, we have identified and characterized receptors for L-glutamate through the binding of [^{3}H]L-glutamate to membranes from chick RPE cells in primary culture. Specific binding was found saturable, with $K_D = 333nM$ and $B_{max} = 3.2$ pmol/mg protein in frozen/thawed membranes. Na^+-independent binding was present in cultures of 16 and 25 days in vitro, and was not affected by temperature. Pharmacological profile of analogues of EAA at different receptor types suggests the presence of a metabotropic type receptor (L-glutamate > S-2-amino-3-phosphonopropionate > 2-amino-4-phosphonobutyrate = trans-(1S,3R)-1-aminocyclopentane-1,3-dicarboxylate > quisqualate). Excitatory amino acid analogues acting at the NMDA-receptor also displaced bound L-glutamate, and a noticeable stimulation of specific binding of this ligand by glycine was shown; this effect was mimicked by D-serine and 1-hydroxy-3-aminopyrrolidone-2 (HA-966) but not by 7-chlorokynurenate, and was not inhibited by strychnine. Since taurine and GABA also increased specific binding, it is likely that modulation of EAA receptors in RPE differs from that in neurons.

Key words: excitatory amino acids, metabotropic receptors, phagocytosis, NMDA receptors

INTRODUCTION

A close relationship exists between the retina and the retinal pigment epithelium (RPE) from the anatomical and functional point of view, since this cell monolayer shares the same embryological origin with the retina in vertebrate species (Mund and Rodrigues, 1979) and is capable of transdifferentiation in early developmental stages (Pittack et al., 1991), as well as in some cases of retinal damage (Detwiler and van Dyke, 1953; Coulombre and Coulombre, 1965). Additionally, the presence of RPE is required for the correct stratification of retinal cells during development (Vollmer et al., 1984; Wolburg et al., 1991). Among the functions subserved by RPE, which are related to retinal physiology, are the transport of molecules from blood vessels to the retina, (Steinberg and Miller, 1979; Lake et al., 1975), the regulation of ionic concentrations in the subretinal space (Immel and Steinberg, 1986), as well as the phagocytosis of the disks during photoreceptor outer segment shedding (Young and Bok, 1969). Regarding this last function, it has been proven that some glutamate related excitatory amino acids (EAA) such as kainate (KA), induce degeneration of retinal cells when administered systemically to newborn rats (Salceda et al., 1979) or alternatively, when applied intravitreally to mature animals (López-Colomé and Somohano, 1982; 1986) or to neurons in primary tissue culture (Zambrano and Hyndman, 1983). These findings suggest that the concentration of glutamate (L-glu) in the subretinal space should be low and finely regulated in order to avoid retinal toxicity (Olney, 1982).

On the other hand, regarding ROS shedding, it has been proposed that the signal from the retina to the RPE for activating phagocytosis could be a diffusible substance released by the retina in conditions in which shedding is increased, i.e., light stimulation (Hollyfield et al., 1976; Young, 1977).

L-Glu, L-aspartate (L-asp), KA, and to a minor extent quisqualate (QA), have been shown to induce phagocytosis in a light- (Greenberger and Besharse, 1985) and Ca^{++}-independent fashion (Besharse and Spratt, 1988; Besharse et al., 1986). This induction, as well as light-triggered phagocytosis, which is Ca^{++} dependent (Greenberger and Besharse, 1983), is blocked

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by the specific EAA receptor antagonists kynurenate and D-O-phosphoserine (Besharse et al., 1988) which suggests that EAA-induced shedding is a receptor-mediated process.

Receptors for EAA have been extensively studied in nervous tissue (Monaghan et al., 1989; Cotman and Monaghan, 1987). Ionotropic EAA receptors have been classified as N-Methyl-D-aspartate (NMDA) and non-NMDA types, the latest including the KA, QA, and possibly the 2-amino-4-phosphonobutyrate (AP4) type (Foster and Fagg, 1984; Watkins and Evans, 1981). More recently, a metabotropic class of EAA receptor which is QA-sensitive but α-amino-5-methylisoxazole-4-propionate (AMPA)-insensitive has been characterized in neurons (Sugiyama et al., 1987; Sladeczek et al., 1985; Schoepp and Johnson, 1988) and glial cells (Milani et al., 1989), which mediate the activation of the inositol phosphate cascade. However, not much is known regarding the properties of EAA receptors or their participation in the physiology of RPE.

Several findings support the possibility of taurine participation in the triggering of phagocytosis. Taurine and glycine have been shown to induce shedding (Greenberger and Besharse, 1985; Stovall and Besharse, 1988), and taurine is also capable of reversing the inhibition of phagocytosis induced by cyclic AMP and melatonin (Ogino et al., 1983). Taurine is readily available to RPE, since it is highly concentrated in retinal ROS (Orr et al., 1976; Pasantes-Morales et al., 1972) and is released from these structures by light (Salceda et al., 1977) which is also the natural trigger for phagocytosis (LaVail, 1976). Additionally, we have previously demonstrated the presence of specific high-affinity, Na^+-independent binding sites for this compound, which could mediate its action in RPE (Lopez-Colome et al., 1991).

Since it is conceivable that a glutamate-related excitatory amino acid could participate in the induction of phagocytosis, we have now investigated the presence of specific receptors for this compound, using [^{3}H]L-glu binding to membranes from chick RPE cells in primary culture. We have characterized these sites from the biochemical and pharmacological point of view, and we here demonstrate that taurine and glycine greatly stimulate glutamate specific binding, suggesting the possibility of a joint action of these compounds in the regulation of phagocytosis.

MATERIALS AND METHODS

Cell Culture

Cultures were set using RPE from seven-day-old chick embryos from a local strain, as described (Lopez-Colome et al., 1991). Eyes were enucleated, freed from sclera and retina, placed in isotonic Krebs-Ringer bicarbonate buffer (KRB) containing (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.17; KH_2PO_4, 2.0; $NaHCO_3$, 25; glucose, 5.6; pH 7.4, and rinsed four times with the same ringer. RPE was dissociated in TC-199 medium after incubating for 5 minutes with 0.13% trypsin in phosphate buffered saline, pH 7.4. Cells were seeded at a density of 10^5 cells per Falcon Flask of 25 cm^2 growth area, and incubated at 37°C in TC-199 medium supplemented with 10% heat-inactivated fetal calf serum, 2.0 mM glutamine, 100 units/ml of penicillin and 100 mg/ml streptomycin. Cultures form a confluent monolayer at 16 days in vitro (DIV); at this time, cells acquire typical cuboidal shape and initiate melanine synthesis. The purity of the culture is indicated by the epithelioid form and presence of pigment in every cell (Salceda et al. 1992).

Membrane Preparation

After 16 DIV, the medium was removed and cells were washed once with KRB. Cells were detached from the dish using a rubber policeman, pooled in KRB and sedimented by centrifugation at 500 rpm for 5 min. The supernatant was removed, and the cell-pellet was homogenized in 20 vol (w/v) of distilled water on ice, in order to osmotically shock the cells. Membranes were then obtained by centrifugation at 45000 × g for 20 min at 4°C. After three washes, membranes were frozen for 2 to 7 days prior to the binding assay. The frozen pellets were thawed and washed once more with buffer prior to the assay (Lopez-Colome and Somohano, 1987). Protein was measured by the method of Lowry et al. (1951).

Binding Assay

Membrane pellets were resuspended in TRIS-HCl buffer 0.05 M, pH 7.4 with or without 118 mM NaCl as indicated. Binding assay was performed as previously described (López-Colome, 1981). Briefly, membrane suspension (30–50 μg protein per assay) was incubated in the presence of 50 nM [^{3}H]L-glu in a final volume of 175 μl for the indicated period of time. 1 mM cold L-glu was used for defining non-specific binding. In pharmacological experiments, specific agonists and antagonists were added instead of cold glutamate at the concentration indicated in each case. The reaction was stopped by dilution with 3 ml of cold buffer, and membranes were recovered by filtration in glass fibre filters (GF/B) and washed twice with 5 ml of cold buffer. Filters were added 10 ml of Tritosol (Fricke, 1975) and counted for radioactivity in a Beckman liquid scintillation counter. Corrections were made for quenching and counting efficiency.

Materials

[^{3}H]-Glutamate (spec. act. 35–59 Ci/mmol) was obtained from New England Nuclear (Boston, MA).

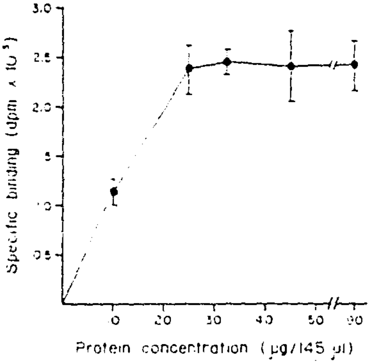

Fig. 1. Protein-dependence of [^{3}H]L-glutamate binding. Frozen thawed membranes from RPE cultures of 16 DIV were used. Assay was performed as described in Materials and Methods, at 37°C. [^{3}H]L-glutamate was 50 nM. Data are the mean ± S.D. of four experiments performed in triplicate.

Glass microfibre filters (GF/B) were from Whatman. TC-199 medium and fetal calf serum were from Difco (Detroit, MI). Excitatory amino acid analogues were from Tocris-Neuramin, Bristol, England. Fertilized eggs were from "Armour Hatchery". All other reagents and chemicals were from Sigma (St. Louis, MO).

RESULTS

Characteristics of [^{3}H]L-Glutamate Binding

Protein concentration was increased from 10 to 50 µg per assay (Fig. 1). Results showed that an optimum specific binding was obtained from 20 µg; hence, a working concentration of 30–40 µg per assay was chosen. Specifically bound L-glutamate was 50–60% of total binding in these conditions.

In order to determine equilibrium time, the binding reaction was stopped at incubation times from 5 to 30 min. Specific binding increased up to 10 min remaining constant thereafter. All other experiments were performed at 10 min incubation.

Effect of Sodium and Temperature on Binding

Binding of glutamate to synaptic receptors in the CNS (for rev. see Monaghan et al., 1989) and the retina (López-Colomé, 1986) has been shown to be Na⁺- and temperature-independent. Frozen-thawed membranes were used in order to eliminate the possible binding to uptake sites (Schwarcz, 1981). Results in Fig. 2 show that temperature does not affect binding in cultures of two ages, 16 DIV (confluent) and 25 DIV (old). The presence of physiological NaCl concentrations (118 mM) in contrast, increases specific binding by two-fold in young (16 DIV) but not in old cultures. All following experiments were performed in young confluent cultures (16 DIV), unless stated otherwise.

Additionally, binding was also performed in freshly obtained membranes from 16 and 25 DIV cultures as a control and in this preparation, binding to uptake sites absolutely Na⁺-dependent, was observed (results not shown).

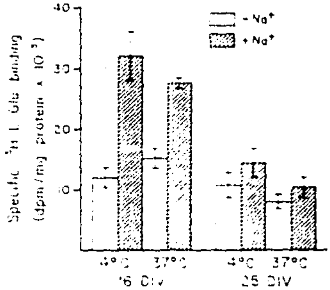

Fig. 2. Effect of temperature and sodium on [^{3}H]L-glutamate binding. Membranes were isolated from cultures of 16 and 25 DIV and binding was measured as described in Materials and Methods. [^{3}H]Glutamate concentration was 50 nM. Results are expressed as the mean ± S.D. of seven independent experiments performed in triplicate.

Effect of EAA Analogues on [^{3}H]L-Glutamate Binding

Some compounds which have been shown to interact with the different excitatory amino acid receptor subtypes were tried for determining their efficiency as competitors at the L-glutamate binding sites at 1 mM concentration. As can be seen in Table I, the most potent displacer was L-glu followed by (s)-2-amino-3-phosphonopropionate (S-AP3), AP4 and (1S,3R)-1-aminocyclopentane-1,3-dicarboxylate (trans-ACPD), compounds proposed to interact with the metabotropic glutamate receptor (Schoepp and Johnson, 1988); the inactive analogue R-AP3 showed a much lower effect. D-Isomers of glutamate and aspartate were weaker than their L-isomers, indicating stereospecificity of this interaction. Among ionotropic receptor analogues, QA and AMPA were less potent whereas no displacement of bound glu by KA was seen. Although NMDA displaced to some extent, the antagonist 2-amino-5-phosphonopentanoate (AP5) had no effect. However, upon addition of 1mM $MgCl_2$, the potency of AP5 increased considerably whereas that of NMDA remained similar. The uptake

TABLE I. Displacement of [^{3}H]L-Glutamate by Analogues

Analogue	Specific displacement (pmol mg protein)	%
L-Glutamate	0.450 ± 0.050	100
D-Glutamate	0.245 ± 0.021	54
L-Aspartate	0.360 ± 0.032	80
D-Aspartate	0.240 ± 0.036	53
Quisqualate	0.204 ± 0.051	45
AMPA	0.108 ± 0.060	24
Kainate	0	
trans ACPD	0.400 ± 0.015	89
S-2-Amino-3-phosphonopropionate	0.428 ± 0.024	95
R-2-Amino-3 phosphonopropionate	0.117 ± 0.029	26
RS-2-Amino-4-phosphonobutyrate	0.400 ± 0.063	89
N-Methyl-D-aspartate	0.117 ± 0.065	26
N-Methyl-D-aspartate Mg^{++}	0.200 ± 0.060	44
RS-2-Amino-5-phosphonopentanoate	0.060 ± 0.020	13
RS-2-Amino-5-phosphonopentanoate Mg^{++}	0.338 ± 0.020	75
L-Glutamate-γ-monohydroxamate	0.050 ± 0.008	11
DL-Aspartate-β-hydroxamate	0.050 ± 0.015	11
L-Alanine	0	
β-Alanine	0	

Assays were performed in the absence of Na^+. [^{3}H]Glutamate concentration was 50 nM. Analogues were added at 1 mM concentration. Mg^{++} when present, was 1 mM $MgCl_2$. Results are expressed as the mean ± S.D. of 3 to 15 experiments performed in triplicate. Total binding was 0.851 ± 0.089 pmol/mg protein.

inhibitors L-glutamate-γ-monohydroxamate and DL-aspartate-β-hydroxamate (Roberts and Watkins, 1975) did not displace glutamate, and the same was true for L-alanine and β-alanine.

The inhibitory amino acids taurine, glycine and GABA, when added at 1 mM concentration, increased glutamate binding by 5 to 10 times in cultures of 16 DIV (Fig. 3). This increase was restricted to specifically bound L-glu, since it was displaceable by 1 mM cold L-glu, while non-specific binding remained unchanged; taurine was the most potent stimulator of binding at 16 DIV. When the same experiment was performed with old cultures (25 DIV), although these amino acids continued to enhance specific binding, the effect was much lower than in young confluent cultures; the order of efficiency in the latter case was glycine > GABA > taurine (Fig. 3). As can be seen in Table II, the enhancement of specific binding by glycine, was not inhibited by strychnine up to 100 μM concentration. Similar enhancement was obtained in the presence of D-serine, an agonist at the glycine modulatory site of the NMDA receptor, and by HA-966, partial agonist at this same site (reviewed in Thomson, 1990). 7-chlorokynurenate, considered as an antagonist at this site (Kleckner and Dingledine, 1989), did not enhance glutamate binding.

The dose-dependence of taurine effect was determined using 100 nM [^{3}H]L-glutamate as a ligand in 16 DIV cultures. Specific binding was increased from 0.768

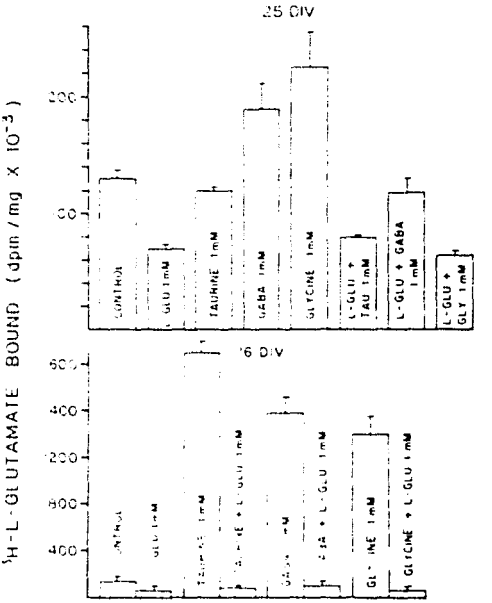

Fig. 3. Enhancement of glutamate binding by inhibitory amino acids. Experiments were performed in Na^+ free buffer, in the presence of 50 nM [^{3}H]glutamate. The ordinate axis represents total binding. Data are the mean ± S.D. of 10 experiments performed in triplicate. In all cases, specific binding in the presence of glycine or GABA was significantly different between them ($P < 0.01$) and from control ($P < 0.001$) at 16 DIV; $P < 0.005$ at 25 DIV). Taurine effect was significant only at 16 DIV ($P < 0.001$). Student's "t" test was applied.

pmol/mg protein without taurine, to 15.7 ± 0.10, 15.9 ± 0.05, 18.9 ± 0.2 and 21.6 ± 0.53 in the presence of 1, 10, 100 and 500 μM taurine respectively, the latest value being equivalent in percent increase, to that shown in Fig. 3 (16 DIV) in the presence of 1 mM taurine.

Saturation Curve of [^{3}H]L-Glutamate Binding

Varying [^{3}H]L-glu concentration within the range of 25 to 2000 nM generated a saturation curve with a single slope by Scatchard analysis (Fig. 4). This suggested a single population of high affinity receptors with K_D = 333 nM and B_{max} = 2 pmol/mg protein.

DISCUSSION

The phagocytosis of ROS disks by retinal pigment epithelium is essential for the maintenance of visual

TABLE II. Effect of Glycine on [^{3}H]L-Glutamate Binding

Compound	Glutamate displaceable binding (pmol/mg protein)
Control	0.450 ± 0.050
Glycine (1mM)	[illegible].708 ± 0.045
D-Serine (1mM)	[illegible].629 ± [illegible]
HA-966 (100 μM)	1.088 ± 0.030
7-Chlorokynurenate (100 μM)	0.098 ± 0.015
Glycine (1mM) + strychnine (100 μM)	1.500 ± 0.009

Binding was measured in the absence of Na$^+$. [^{3}H]Glutamate concentration was 50 nM. Specific binding in control was total binding minus binding in the presence of 1 mM cold glutamate. For glycine related compounds, bound [^{3}H]glutamate in the presence of the compound was displaced by 1 mM glutamate. Data are the mean ± S.D. of 9 experiments performed in triplicate. HA-966: 1-hydroxy-3-aminopyrrolidone-2.

function in vertebrates. The failure of this process induces the degeneration of the retina, probably due to diffusional problems caused by the accumulation of debris in the subretinal space (Dowling and Sidman, 1962; Herron et al., 1969). Phagocytosis is considered a multistep process encompassing a) recognition binding, b) ingestion, and c) digestion (Bok and Young, 1979; Clark, 1986). Regarding recognition, although the molecular mechanism remains unknown, the surface of both, RPE cells and the particle to be engulfed are involved; this process has been shown to be selective and does not require energy. As for ingestion, it has been proposed that a signal is required for triggering this stage, which is energy-dependent (Bok and Young, 1979). This signal could be a messenger from the retina interacting with specific receptors on the RPE cells, and activating subsequently a second messenger intracellular system (Clark, 1986). Different mechanisms for ingestion are present in RPE which could be related to the nature of the phagocytized particle (Clark, 1986). The excitatory amino acids L-glu, L-asp, KA and QA seem to be involved in both, binding and ingestion, since they increase adhesiveness between RPE and retinal surface (Matsumoto et al., 1987), and induce hyper-shedding and phagocytosis (Greenberger and Besharse, 1985), by two causally unrelated mechanisms (Defoe et al., 1989).

We have demonstrated that binding sites for L-glutamate are present in membranes from chick RPE cells in culture. These sites show the properties of receptor molecules since a finite number of these are present as can be concluded from data in Fig. 1 as well as from the saturable kinetics shown in Fig. 4. Binding was found specific, reversible and Na$^+$-independent in 25 DIV cultures, whereas 16 DIV cultures showed in addition, Na$^+$-dependent binding sites (Fig. 2). In order to exclude the possibility of binding to transport sites, the pharmacological and biochemical characterization of this interaction was performed using frozen membranes in the absence of Na$^+$, condition known to decrease by 80% the number of uptake sites (Schwarcz, 1981). The glu uptake inhibitors glu- and asp-hydroxamates were without effect, further supporting the assumption that we are not dealing with uptake sites (Table I).

Another characteristic of binding to receptors, the high affinity of the interaction, was also demonstrated since the K_B is in the nanomolar range (333 nM), and in the same range reported for [^{3}H]L-glu binding to synaptic receptors in nerve tissue (Foster and Fagg, 1984) and the retina (Lopez-Colome, 1981). Pharmacology using agonists and antagonists at the different subtypes of EAA receptors, indicated that the most potent compound for displacing bound L-glu was L-glu itself, followed by the metabotropic receptor agonist trans-ACPD and antagonists S-AP3 and APB, whereas R-AP3, L-asp, D-glu, and D-asp were less potent, suggesting a certain degree of stereospecificity. QA and its antagonist glutamate-diethyl-ester (GDEE) were also moderately potent. NMDA was weak, and its antagonist AP5 was without effect in the absence of Mg^{++}, however in the presence of Mg^{++}, the potency of NMDA and most importantly of AP5 was greatly increased (Table I). KA and AMPA showed no effect on [^{3}H]glu binding, discarding the presence of KA receptors.

These results agree with those in which the effect of glu and its analogues on shedding were tested (Greenberger and Besharse, 1985), with the exception of KA, which potently stimulates shedding but does not displace L-glu in our system. This result however, suggests that in intact cells, KA could be inducing the release of L-glu which in turn would act as the messenger from the retina (Besharse and Spratt, 1988), since KA has been shown to release GABA, glycine, taurine, glu and asp from postreceptoral neurons in this tissue (Campochiaro et al., 1985; Yazulla, 1983; Yazulla et al., 1985).

An interesting finding regarding L-glu binding properties was the noticeable enhancement of specific binding induced by taurine, glycine and GABA, which decreased with the age of the culture (Fig. 3). If these changes are related to maturational modifications in the specificity of the phagocytic function of RPE, remains to be established.

Taken together, the pharmacological characteristics of glutamate binding to EPR membranes suggest the presence of either one receptor type for excitatory amino acids showing different properties from those described in the CNS, or alternatively, two populations of receptors with equal affinity: one of the metabotropic type, sensitive to trans-ACPD, S-AP3 and AP4 (and QA) and another, possibly of the NMDA type, since the effect of the competitive antagonist AP5 is greatly enhanced in the

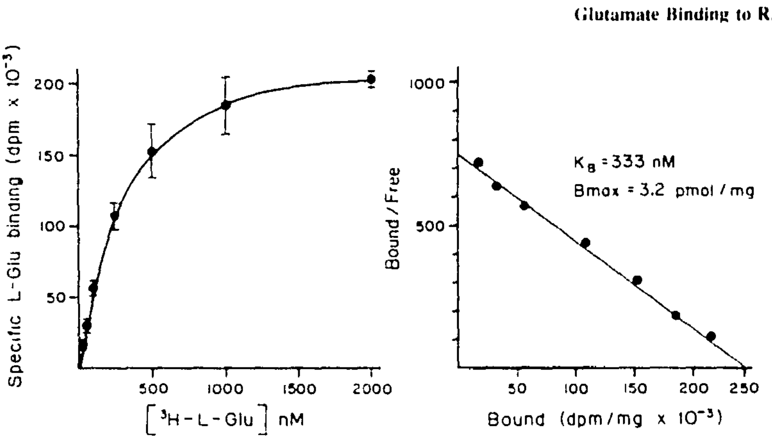

Fig. 4. Saturation curve and Scatchard analysis of [^{3}H]glutamate binding. Specific binding was measured in frozen-thawed membranes from 16 DIV cultures at 37°C, in the absence of sodium. Data were analyzed using the INPLOT (Version 3.1) program from GraphPAD Software, San Diego, CA. Hill coefficient = 0.86. Data are the mean ± S.E.M. of five independent experiments performed in triplicate.

presence of magnesium, as has been shown for [(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonate (CPP) (Lopez-Colomé and Somohano, 1992). Additionally glycine, which acts as an allosteric modulator at the NMDA receptor complex (Johnson and Ascher, 1977), highly increases specific glu (agonist) binding and this increase is mimicked by the glycine agonist D-serine and the partial agonist HA-966, but not by its antagonist 7-chlorokynurenate, as has been seen in nervous tissue (for review see Thomson, 1990). The site through which glycine exerts its effect is not blocked by strychnine (Table II), suggesting that it is not an inhibitory glycine receptor. On the other hand, we cannot discard the possibility of an allosteric interaction through taurine receptors since these sites show high affinity for glycine (Lopez-Colomé et al. 1991).

Regarding the possible function of these receptors, two of the main problems for proposing glu as the messenger inducing shedding and phagocytosis have been: 1) the high concentration of glu (mM) required for this induction would generate retinal toxicity, and 2) the apparent lack of specificity of the effect, since taurine, glycine and glutamine can also induce shedding. On the basis of the data obtained in this study, it is tempting to suggest the possibility that glu coming from neural retina in very low concentrations, could diffuse tonically to the subretinal space. Upon light stimulation, taurine released from the photoreceptor outer segments would increase glu binding to its receptors in RPE (present results), thus activating phagocytosis. Since an active high affinity uptake system for taurine has been demonstrated in RPE (Lake et al., 1975), the removal of the amino acid in the absence of light would allow a return to basal conditions. Experiments in order to further test this hypothesis are now under way.

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ARTICULO 2

Glycine Stimulation of Glutamate Binding to Chick Retinal Pigment Epithelium

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ACEPTADO EN NEUROCHEMICAL RESEARCH

ABSTRACT

The effect of glycine (Gly) and taurine (Tau) on the biochemical and pharmacological properties of [^{3}H]L-glutamate ([^{3}H]-Glu) binding to membranes from primary cultures of chick retinal pigment epithelium (RPE), as well as from intact tissue during development was studied. Gly and Tau increase B_{max} of [^{3}H]-Glu binding to a high affinity site (K_d ~ 300 nM) in membranes from 16 days *in vitro* (immature) cultures; additionally, Gly discloses a low affinity Glu-binding site (K_d ~ 970 nM) at this stage. In membranes from 25 days *in vitro* (mature) cultures, the high affinity site is no longer present and Tau has no effect on Glu-binding; Gly still stimulates binding to the low affinity site by four fold, with an EC = 300 µM. Pharmacological profile using specific excitatory amino acid (EAA) receptor agonists and antagonists suggests that at 16 days *in vitro* Glu binds preferentially to metabotropic Glu receptors similar, and at 25 days *in vitro* to ionotropic receptors different from neuronal ones. The stimulatory effect of Gly and Tau was also observed in intact RPE, at increasing with successive embryonic age. Glu binding was also stimulated in membranes from chick retina, but not in those from rat brain. Results support the possibility of EAA [illegible] physiology, including phagocytosis and cell division.

Key words: [illegible]; glutamate; glycine; taurine.

Abbreviations: L-Glu, L-glutamate; QA, quisqualate; KA, kainate; NMDA, N-methyl-D-aspartate; trans-ACPD, (±) 1-aminocyclopentane-trans-1,3-dicarboxylic acid; D-AP5, D-2-amino-5-phosphonopentanoic acid; L-AP4, L-2-amino-4-phosphonobutyric acid; L-AP3, L-2-amino-3-phosphonopropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; (+)MCPG, (+)α-methyl-4-carboxyphenyl-glycine; DHPG, (RS)3,5-dihydroxyphenyl-glycine; CPP, 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; MK-801, (+)-5-methyl-10, 11-dihydro-5H-dibenzo [a,d] cycloheptene-5, 10-imine; PIP$_2$, phosphatidyl inositol biphosphate. ED= embryonic day; DIV= days in vitro; RPE, retinal pigment epithelium; EAA, excitatory amino acids.

INTRODUCTION

Retinal pigment epithelium (RPE) shares the same embryological origin with the retina (1) and is in close anatomical and physiological relation with this tissue (2). In addition to protecting the retina from photic damage, RPE is involved in several functions as the regulation of ionic environment in the subretinal space, retinoid metabolism, nutrient transport from the blood to the retina, and the formation of the blood retinal barrier (3). Additionally in some species, RPE can transdifferentiate into retinal neurons under specific circumstances (4). One of the most important functions of RPE is its participation in retinal photoreceptor renewal; disruption of retina-RPE communication at this level could be involved in retinopathies such as retinitis pigmentosa or proliferative vitreoretinopathy (5).

Light stimulates phagocytosis, and also releases Tau and Gly from photoreceptor outer segments (6) and from post receptoral neurons (7). Both compounds have been shown to stimulate phagocytosis (8) and, additionally, Tau promotes the proliferation of RPE cells [illegible]. These amino acids could reach RPE from the retina and participate in phagocytosis and/or cell division.

Although the normal activity of RPE is a requisite for retinal [illegible] is still poorly understood. Glutamate (L-Glu) and other excitatory amino acids (EAA) [illegible], possibly through the activation of specific receptors. L-Glu

receptors have been characterized in chick (12) and human (13) RPE, hence it is likely that EAA could participate in the *in vitro* induction of this phenomenon. On the other hand, RPE undergoes phenotypic and metabolic changes related to de-differentiation and subsequent return to active proliferation in culture (14) and also *in vivo*, during the regeneration of damaged sensory retina (15). Since N-methyl-D-aspartate (NMDA) receptors are involved in proliferation (16) as well as differentiation (17), we have proposed the participation of EAA in phagocytosis and/or proliferation, possibly through an increase in internal calcium concentration (13).

Several types of EAA receptors have now been described and classified in two main categories: ionotropic receptors of the NMDA, kainate (KA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) types (18), and metabotropic receptors (mGluRs) linked to the activation of phosphoinositide (PIP) hydrolysis, the activation or inhibition of adenylyl cyclase, and the generation of arachidonic acid (19). The stimulation of most of the EAA receptors generates an increase in internal [Ca^{++}] through different mechanisms (20), followed by the activation of several enzyme systems. We have previously determined that EAA receptors present in chick and human RPE (12, 13), are similar but not identical to [illegible]. Briefly, these receptors are sensitive mainly to agonists and antagonists of NMDA receptors and to [illegible], which has been identified as an NMDA agonist (22), greatly

increases [^{3}H]L-Glu binding; this effect is age-related and mimicked by Tau and to a smaller extent by GABA. In an attempt to contribute information regarding the role of these receptors in RPE physiology, we have further characterized kinetically and pharmacologically the effect of Gly and Tau on [^{3}H]L-Glu binding at young and mature ages of RPE in culture as well as in the intact tissue. We here demonstrate that Glu-binding sites as well as their modulation by Gly and Tau undergo changes during differentiation, which could be related to changes in the functions subserved by EAA during development.

EXPERIMENTAL PROCEDURE

Cell Culture

Chick RPE cells were cultured as described previously (12). Eyes were enucleated from seven day-old embryos (local strain); the vitreous and the retina were removed, and the eyecups were rinsed four times with Krebs-Ringer Bicarbonate buffer (KRB) containing (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.17; KH_2PO_4, 2.0; $NaHCO_3$, 25; glucose, 5.6; pH 7.4. After a 5 min incubation in phosphate buffered saline containing 0.15 % trypsin pH 7.4, RPE cells were dissociated and seeded at a density of 10^6 cells per Falcon Flask of 25 cm^2 growth area. Cultures were mantained in TC-[illegible] bovine serum, 2.0 mM glutamine, 100 Units/ml of penicillin and 100 mg/ml [illegible] exhibiting fibroblast - like morphology; cuboidal shape and

melanine synthesis are achieved at 25 DIV (mature cells). The purity of the culture is indicated by the epithelioid form and presence of pigment in every cell (23).

Membrane Preparation

RPE cells were harvested at 16 or 25 DIV, pooled in KRB and pelleted by centrifugation at 500 rpm for 5 min. The supernatant was removed, and the cells were homogenized in 20 vol (w/v) of distilled water on ice, in order to induce complete osmotic shock. Membranes were then obtained by centrifugation at 45000 x g for 20 min at 4°C, washed 3 times and frozen from 2 to 7 days; the frozen pellets were thawed and washed once more with buffer prior to the assay (24). Protein was determined by the method of Lowry et al. (25).

Membranes from intact RPE, adult and embryonic chick retina, as well as from rat cerebral cortex were obtained following the same procedure as for those from RPE cultures.

Binding Assay

[^{3}H]L-Glu binding was measured as previously described (24). Membranes were resuspended in TRIS-HCl buffer 0.05 M, pH 7.4 to a protein concentration of 0.2 mg protein per assay (250 µl final volume). Unless stated otherwise, [^{3}H]L-Glu concentration was 50 nM and non-specific binding was defined in the presence of 1 mM quisqualate. Agonists or antagonists, when tested, were added instead of glutamate. Reaction was terminated by dilution and further filtration through glass fiber filters (Whatman GF/B) followed by two washes with cold buffer. Filters were counted for radioactivity

after the addition of 10 ml Tritosol (26) in a Beckman liquid scintillation counter. Corrections were made for quenching and counting efficiency. Data were analyzed using the INPLOT (version 3.1) program from Graph PAD software, San Diego, Ca.

Materials

[3H]L-Glutamate (Sp.Act. 35-59 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Microfibre glass filters (GF/B) were from Whatman. Fetal bovine serum (FBS) and TC-199 medium were from Difco (Detroit, MI). All excitatory amino acids and related compounds were from TOCRIS-Cookson, Bristol, England. Chick embryos and fertilized eggs were from the University Animal House. All common reagents and chemicals were from Sigma (St. Louis, MO).

RESULTS

Developmental profile of [3H]L-Glu binding to membranes from intact RPE: Effect of Gly, Tau, and GABA.

Binding of [3H]L-Glu to frozen thawed membranes from chick RPE was measured in the absence of sodium at embryonic days 7,10,14,18 and one day post-hatching. As can be seen in Fig. 1, with the exception of E[illegible] in which specific binding was significantly higher (0.477 ± 0.068 pmol/mg protein), values remained similar to those in the mature eye (0.268 ± 0.009 pmol/mg protein).

[illegible] binding in early developmental stages; only Gly showed an effect in the mature [illegible] stimulation follows a different developmental pattern from that of

Gly and Tau, becoming uneffective by day 14 ED for which its action was not followed further. Non-specific binding did not vary significantly in any of these conditions (0.515 ± 0.015 pmol/mg protein).

Effect of Gly, Tau, and GABA on [^{3}H]L-Glu binding to membranes from retina and brain.

1 mM concentration of Gly, Tau, or GABA did not affect [^{3}H]L-Glu binding to membranes obtained from rat cerebral cortex (Table I). In the same condition, Tau (15%) and Gly (7%) showed a small but consistent stimulating effect on binding to membranes from chick mature retina, which was more evident in embryonic (ED7) tissue (Table I).

Kinetics of [^{3}H]L-Glu binding

Saturation curves for Glu binding were performed in the absence (control) and presence of 1 mM Gly or Tau in order to determine if the increased binding to membranes from 16 DIV cultures (12) was due to a change in the affinity at receptors or rather in the number of binding sites.

As can be seen in Fig. 2, in the presence of Tau, the B_{max} was increased to 13.4 pmol/mg protein, as compared to B_{max} in the absence of the amino acid which was 5.2 pmol/mg protein; no significant change in affinity was seen: K_m= 320 nM and 283 nM in the absence and presence of Tau, respectively. Gly 1 mM also increased B_{max} to 11.4 pmol/mg protein, without changing the K_m (259 nM). However, in the presence of Gly a high affinity binding site for Glu was apparent, with K_m= 960 nM and B_{max} = 22

pmol/mg protein. When Gly and Tau were added simultaneously at 1 mM concentration, their effect was additive (Fig.3).

Since we had previously demonstrated that the stimulatory effect of Tau was no longer present in membranes from 25 DIV cultures (12), saturation curves were performed in this preparation in the absence and presence of 1 mM Gly. Fig. 4 shows that a single, low affinity site (K_d = 958-987 nM) was present, binding to which was stimulated by Gly from B_{max} = 2.2 to B_{max} = 3.1 pmol/mg protein.

Effect of Gly and Tau on the pharmacological properties of [^{3}H]L-Glu binding.

Membranes obtained from 15 DIV cultures were preincubated for varying periods of time with 1 mM Gly + Tau, pelleted, resuspended, and binding of Glu measured. In this condition, no increase in binding was observed when compared to controls preincubated in the absence of the amino acids (not shown). This result indicated that a permanent modification of membrane structure (i.e. activation of cytoskeleton degrading enzymes) by Gly or Tau can be discarded.

The pharmacological profile of Glu-binding was determined at 25 DIV, age in which a single receptor population is present, and compared with that in membranes from 15 DIV cultures, age in which [illegible]. The affinity of the different agonists and antagonists of EAA-receptors (25 DIV) for [illegible] (CNQX > AMPA > ACPD > CPP > AP4 > MCPG≤ DHPG, with AP3 having no effect

(Table II). This order of efficacy differs from the pharmacological properties at 16 DIV (12), age in which ACPD, AP3 and AP4 are highly efficient, whereas KA has no effect (L-Glu> AP3> ACPD≤ MCPG≤ AP4≥ DHPG> DNQX> CPP> QA> AMPA≥ CNQX≥ NMDA).

In order to define the pharmacological properties of the two Glu receptor populations present at 16 DIV, the same experiments were performed in the presence of 1 mM Gly or Tau. The order of efficacy did not change with Gly compared to control; however, in the presence of Tau, mGluR-interacting drugs became less potent, similar to the profile obtained at 25 DIV without Tau (L-Glu> QA> AP5> AP3> KA> NMDA> ACPD> AP4). Comparison of this result with control profile at 16 DIV in the absence of Tau, suggests that Glu binding to ionotropic receptors is increased by these agents. Additionaly, Glu binds with high affinity to another population of receptors, possibly of the metabotropic type, which are sensitive to stimulation by Gly and Tau, and are absent in membranes from older cultures (Table III). The stimulating effect of Gly showed to be dose-dependent in a concentration range from 1 µM to 10 mM, with an EC_{50} = [illegible]00 µM in membranes from cultures of 25 DIV (Fig.5), and was insensitive to 100 µM strychnine and 7-Cl-Kyn at all ages (Table IV).

DISCUSSION

We have previously demonstrated the presence of specific [illegible] which was stimulated by Gly in young undifferentiated cultures, and in

mature epithelioid ones. This effect was mimicked by Tau, only in the young cultures (12).

In this study we have further characterized this effect, and we have demonstrated the presence of two distinct Glu-binding sites in RPE showing high and low affinity, with different pharmacological properties and age-dependent expression.

In membranes from 16 DIV cultures, Gly and Tau both increase Glu-binding due to a large increase in B_{max} (Fig. 2). Since this effect is additive, Tau and Gly probably promote binding through an action at different sites (Fig.3); Gly also induces Glu binding to a second, lower affinity site. When the pharmacological profile at 16 and 35 DIV are compared, in younger tissue compounds known to interact with [illegible] as MCPG (competitive antagonist of PI hydrolysis and AP4 presynaptic receptors) and DHPG (agonist of t-ACPD), as well as AP3 and AP4 are the most efficient displacers, whereas they become the weakest in older cultures. Pharmacology of Glu interaction in the presence of Tau or Gly in DIV membranes), shows that compounds which interact with both, ionotropic and mGluRs (18, 19) displace bound L-Glu, however the effect of ionotropic-interacting drugs becomes more potent, suggesting that these are the sites stimulated by Tau and Gly (see Results). Our present data also show that in cultures of 35 DIV, the high-affinity [illegible] are not present, and Gly but not Tau promotes binding of L-Glu to the low affinity site (Fig 4, Table [illegible]), thus pointing to a [illegible] receptor (18), since AP3 does not displace binding, and ACPD and

AP4 are the less potent competing drugs. Also, MCPG (AP4) and DHPG (ACPD) do not displace Glu at this age, whereas they are active in 16 DIV membranes.

No thorough characterization of EAA receptors in RPE has been performed, although based on the common embryonic origin of RPE, retina and neural tissue, some similarity would be expected. Gly interacts with strychnine-insensitive sites which could be allosterically linked to the NMDA receptor (27); if such was the case, NMDA receptors in RPE bear properties different from those in CNS neurons (28), since Gly effect is not inhibited by 7-Cl-Kyn. Taurine seems to act on a different site from Gly, transiently producing the same effect.

In rat brain, Gly potentiates [illegible] binding and reduces antagonist binding to NMDA receptors possibly through a conversion from an antagonist-preferring conformation, relatively unresponsive to Glu, to an agonist-preferring conformation, highly responsive to Glu (29). This could also be the case in RPE, since the efficacy of L-Glu and NMDA is increased, although the one of AP5 remains unchanged.

According to our data, Glu receptor binding can be modulated by the inhibitory amino acids Tau and Gly acting at different receptors (Fig.1): one specific for Gly, and another at which Tau can also act. The latter is present only at immature, actively dividing stages of the culture, whereas the first remains up to the [illegible]. The potency of Gly effect agrees with the observation that NMDA receptors in the CNS

become less sensitive to Gly with age (29), possibly due to variations in the composition of heteromeric NMDA receptors during development (30). The clarification of this point must await to the establishment of the Glu-receptor subunits which are expressed by RPE.

The possibility of a mGluR sensitive to ACPD with very close affinity to the ionotropic ones exists. In several areas of the CNS mGluRs are mainly expressed during differentiation and early postnatal ages, or following nerve injury (31). This is also true for cortical neurons in culture which are protected from NMDA toxicity by ACPD, before but not after 18 DIV (32).

In order to discard culture-condition as the cause for Gly and Tau effects, studies were performed in membranes of intact embryonic RPE, in which an age-related decrease in Glu binding stimulation was also observed (Fig.1). We have also demonstrated Gly and Tau effect in human RPE cultures, which decreases inversely to the age of the donor (33). A developmental correlation is also supported by the lack of effect of Gly and Tau in membranes from adult brain and retina whereas in membranes from ED7 retina, these compounds show a clear stimulatory action on Glu-binding (Table I).

Regarding the physiological meaning of these results, no direct correlation can be established at this point, since little [illegible] AAA in RPE. We have suggested a role for these compounds in phagocytosis and/or cell division [illegible]. The mGluRs could be involved in cell differentiation, probably

through the activation of PIP hydrolysis, as well as in phagocytosis, through the inhibition of adenylyl cyclase (19), since cAMP has been shown to also inhibit phagocytosis (33). On the other hand, ionotropic Glu receptors have been shown to induce phosphoinositide hydrolysis through the entry of calcium in some cells (34).

Binding of Glu to the low affinity receptor in turn, could be related to the movement of calcium required for support functions such as enzyme activation, and ion channel regulation. EAA receptors in RPE, are distinct from those described in neurons (18) and glia (21), and their activity could be regulated by the concentration of Gly and Tau in the extracellular compartment. This is in keeping with recent findings showing colocalization of Gly transporter and NMDA receptors in rat brain (35), and also with the fact that Gly and Tau are released by the retina upon light stimulation (6), which in turn, triggers phagocytosis *in vivo*. Further studies in the biochemical characterization of RPE EAA receptors will be of help in the understanding of some retinal pathologies involving retina - RPE communication.

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Legends to the Figures.

Fig 1.- Effect of Gly, Tau and GABA on [^{3}H]L-Glu binding to embryonic RPE.

RPE was obtained from chick embryos at the indicated age, and membranes were prepared as described in Methods. [^{3}H] Glu binding (50 nM) was determined in the absence (control) or presence of 1 mM Gly, Tau or GABA. 1 mM unlabeled Glu was used for defining non-specific binding. Results are the mean of 3 independent experiments which varied less than 10%.

Fig 2.- Effect of Gly and Tau on the kinetic parameters of Glu-binding.

Experiments were performed as described [illegible]. Saturation curve and Scatchard analysis were studied in membranes from 16 DIV cultured RPE cells. [^{3}H]L-Glu concentration was increased from 25 to 2000 nM; 1 mM [illegible] was used for defining non-specific binding. Results are expressed as the mean ± S.E.M. of 4 experiments performed in triplicate. Data were analyzed using the [illegible] program from Graph PAD Software, San Diego, CA. Hill coefficients were ~0.90 in all cases.

[illegible] Effect of [illegible] on [^{3}H]L-Glu binding.

Membranes from 16 DIV [illegible] were [illegible] as described in the methods section, the same as the binding assays.

[³H]L-Glu concentration was 50 nM, and glycine, taurine or both, were added at 1 mM concentration. Data are % over total control binding in the absence of taurine or glycine in pmol/mg protein. Results are expressed as the mean ± SEM of five experiments from different cultures, performed in triplicate.

Fig 4.- Scatchard analysis of Glu binding to membranes from cultures of 25 DIV.

Saturation curve, Scatchard analysis and statistics were as described for Fig 2. Data are the mean ± S.E.M. of 3 experiments in triplicate.

Fig 5.- Concentration dependence of Gly-induced increase in [³H]L-Glu binding.

Glu binding to membranes from 25 DIV cultures was measured in the presence of concentrations of Gly from 1 µM to 10 mM. The concentration of [³H]L-Glu was 50 nM; 1 mM unlabeled Glu was used to displace. Results are the mean of 3 experiments in triplicate ± S.E.M.

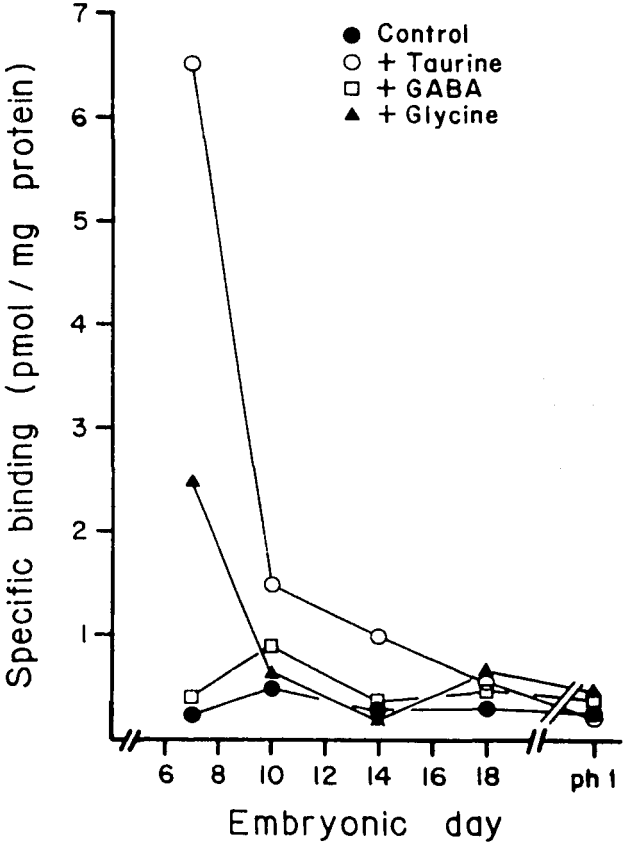
Control
+ Taurine
+ GABA
+ Glycine
Specific binding (pmol / mg protein)
7
6
5
4
3
2
1
6
8
10
12
14
16
18
ph 1
Embryonic day

FIGURA 1.

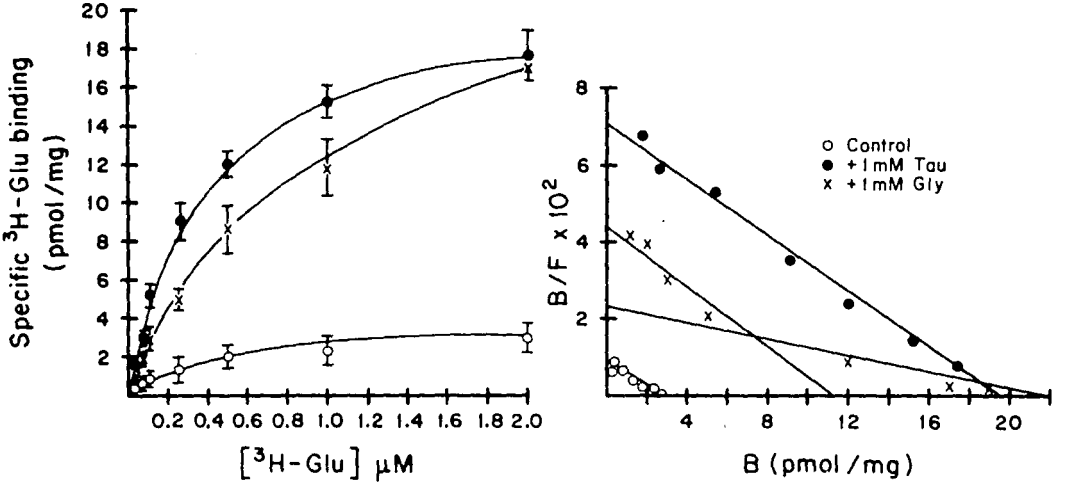
Specific ^{3}H-Glu binding
(pmol/mg)
20
18
16
14
12
10
8
6
4
2
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
[^{3}H-Glu] μM
B/F x 10^2
8
6
4
2
4 8 12 16 20
B (pmol/mg)
o Control
● +1mM Tau
x +1mM Gly

FIGURA 2.

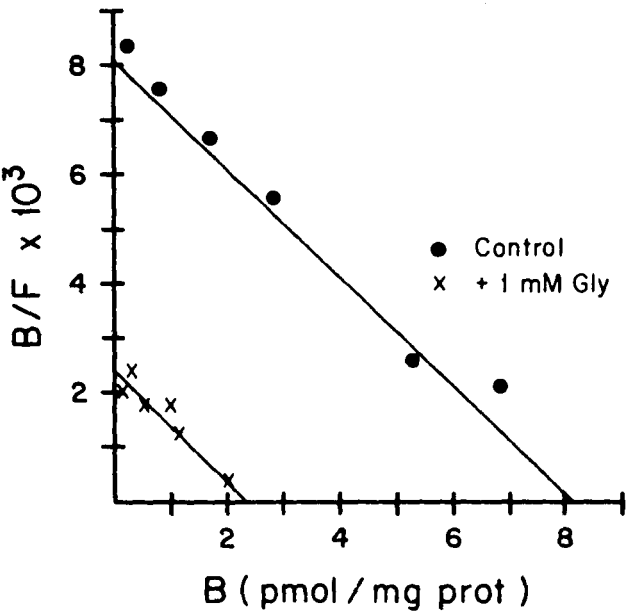

FIGURA 4.

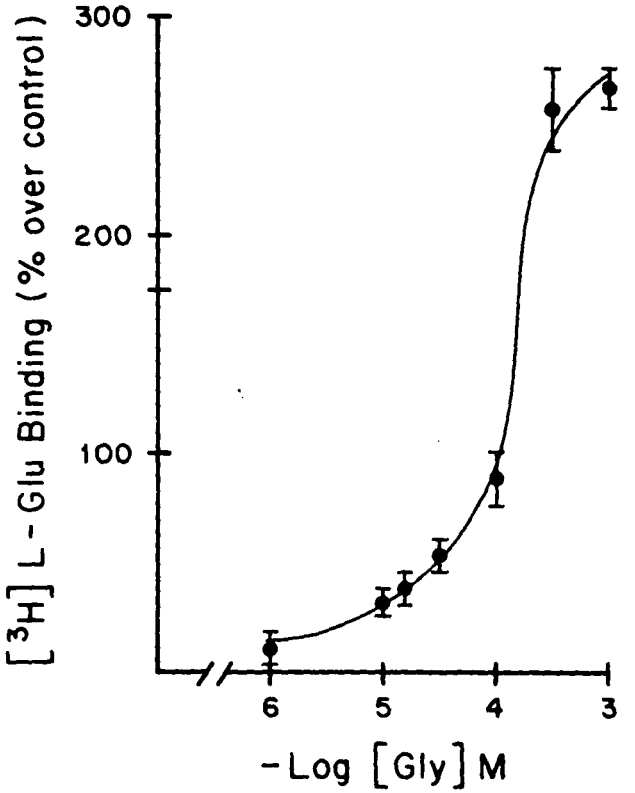

FIGURA 5.

Table I. Effect of Taurine, GABA and Glycine on [^{3}H]L-Glu binding to membranes from brain and retina.

	Specific [^{3}H]L-Glu binding (pmol/mg protein)		
	Rat Cortex	Chick Retina (adult)	Chick Retina (ED-7)
Control	0.149 ± 0.026	0.488 ± 0.061	0.268 ± 0.053
+ Taurine	0.130 ± 0.021	0.564 ± 0.090	0.684 ± 0.012
+ Glycine	0.198 ± 0.034	0.526 ± 0.025	0.626 ± 0.063
+ GABA	0.115 ± 0.023	0.480 ± 0.049	0.450 ± 0.100

Experiments were performed as described in Methods. [^{3}H]L-Glu concentration was 50 nM. Non-specific binding was defined by 1 mM unlabeled L-Glu in the absence (control) or presence of added compounds. Taurine, GABA and Glycine were 1 mM. Results are the mean ± S.E.M. of 4 experiments performed in triplicate. ED, embryonic day.

Table II. Displacement of [^{3}H]L-Glu binding by related compounds in membranes from 16 and 25 DIV cultures.

Displacer		Specific [^{3}H]L-Glu displaced (pmol/mg protein)	
		25 DIV	16 DIV
Glutamate	(1 mM)	0.328 ± 0.002	0.450 ± 0.05
Quisqualate	(1 mM)	0.307 ± 0.011	0.204 ± 0.05
AMPA	(1 mM)	0.230 ± 0.006	0.108 ± 0.06
CNQX	(50 μM)	0.244 ± 0.010	0.107 ± 0.09
Kainate	(1 mM)	0.302 ± 0.010	0
Trans-ACPD	(1 mM)	0.211 ± 0.015	0.400 ± 0.015
(+) MCPG	(250 μM)	0.040 ± 0.010	0.410 ± 0.084
DHPG	(250 μM)	0.0[illegible] ± [illegible]	[illegible] ± 0.077
NMDA	(1 mM)	0.[illegible]89 ± [illegible]	[illegible]17 ± 0.065
CPP	(200 μM)	0.129 ± 0.010	0.235 ± 0.052
MK-801	(5 μM)	0.269 ± 0.006	0.187 ± 0.006
D-AP5	(1 mM)	0.[illegible]61 ± [illegible]	[illegible]52 ± 0.010
L-AP4	(1 mM)	0.180 ± 0.008	0.400 ± 0.063
L-AP3	(1 mM)	0	0.[illegible]45 ± 0.024

Membranes were prepared as described in Methods. The concentration of [^{3}H]L-Glu was 0.1 mM; total binding was 0.[illegible] ± 0.0[illegible] pmol/mg protein at 25 DIV, and 0.800 ± 0.005 at 16 DIV. NMDA, N-methyl-D-Aspartate; trans-ACPD, (±) 1-aminocyclopentane-trans-1,3-dicarboxylic acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; (+) MCPG, (+)α-methyl-4-carboxyphenylglycine; DHPG, [illegible]; CPP, [illegible]-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo (a,d) cycloheptene-5,10-imine; D-AP5, D-2-amino-5-phosphonopentanoate; L-AP4, L-2-amino-4-phosphonobutyrate; L-AP3, [illegible]. A [illegible] experimental [illegible] these compounds were previously tested (12), four [illegible] new series of experiments was performed in order to compare with new compounds.
Table II. Displacement of [^{3}H]L-Glu binding by related compounds in membranes from 25 DIV cultures.

Table III. Kinetic constants of [^{3}H]L-Glu binding to cultured RPE membranes.

Age in culture	[^{3}H]Glu	[^{3}H]Glu + Tau	[^{3}H]Glu + Gly
16 DIV	K_D= 320 nM Bmax= 3.2	K_D= 283 nM Bmax= 19.8	K_{D1}= 259 nM Bmax= 11.4 K_{D2}= 960 nM Bmax= 22
25 DIV	K_D= 358 nM Bmax= 2.3		K_D= 987 nM Bmax= 8.1

Data were calculated from Figs. 2 and 3. Bmax is expressed in pmol/mg protein. The concentration of Tau and Gly was 1 mM

Table IV. Effect of 7-chlorokynurenate and strychnine on stimulated glutamate binding.

	Total binding (pmol/mg protein)	
Additions	16 DIV	25 DIV
None	0.375 ± 0.021	0.420 ± 0.030
Glycine	0.741 ± 0.006	0.669 ± 0.012
7-ClKyn	0.350 ± 0.009	0.412 ± 0.015
Glycine + 7ClKyn	0.735 ± 0.008	0.700 ± 0.042
Taurine	1.100 ± 0.017	___
Taurine + 7 ClKyn	1.075 ± 0.020	___
Strychnine	0.400 ± 0.031	0.417 ± 0.012
[illegible]	[illegible]	[illegible]

Experiments were performed as described in Methods. Results are expressed as the mean ± SEM of three experiments, from different cultures, performed in triplicate. Taurine was not tried at 25 DIV, since it does not stimulate binding at this age. 7-ClKyn, 7-Chlorokynurenate. All compounds were added simultaneously at 100 μM concentration. [^{3}H]L-Glu concentration was 50 nM.

Excitatory amino acid receptors in membranes from cultured human retinal pigment epithelium

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Abstract

The presence of specific, saturable receptor sites for excitatory amino acids (EAA) in membranes from cultured human retinal pigment epithelium (RPE) was established through the binding of [^{3}H]L-glutamate (L-Glu). The age of the donors ranged from 6 days to 33 years.

The affinity of the binding (K_B) sites was between 1.2 and 1.5 μM, and did not change with the age of the donor, whereas the B_{max} was slightly increased (8.6 to 13.0 pmol/mg) in membranes from the 33 year-old compared to the 29 day-old donor. The efficacy profile of agonists and antagonists acting at EAA receptors for displacing [^{3}H]L-Glu was L-Glu = L-Aspartate > 2-amino-4-phosphonovalerate (AP5) > N-methyl-D-Aspartate (NMDA) > 1-aminocyclopentane-1,3 dicarboxylate (trans-ACPD) > 2 -amino-3-phosphonopropionate (AP3). These data suggest the presence of either an NMDA-receptor sensitive to the metabotropic agonist trans-ACPD or alternatively, the presence of two different populations of receptors with similar affinity for the agonist: NMDA and metabotropic.

Glycine highly stimulated Glu-binding; this effect was inversely related to the age of the donor. Taurine and to a lesser extent GABA, mimicked this effect. Stimulation by glycine was dose-dependent, insensitive to strychnine and 80% inhibited by 7-chlorokynurenate. This effect was also present in human RPE-derived fibroblasts, human scleral fibroblasts and the human lymphoblastoid cell line NB76, all continuously dividing cells. The results further support the possibility of the participation of EAA receptors in the regulation of phagocytosis in RPE. Curr. Eye Res. 13: 553–560, 1994.

Key words: excitatory amino acid receptors; cell culture; human retinal pigment epithelium

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Introduction

The normal function of RPE is a necessary requisite for visual function, since, among other things, in addition to its participation in the metabolism of visual pigment (1), this cell layer is involved in the renewal of photoreceptor outer segments in the retina (2).

Although it has been known for some time that phagocytosis of the photoreceptor outer segment disks from the retina is a light-triggered phenomenon, the nature of the message between the retina and the RPE is still unknown. Previous work has suggested the involvement of excitatory amino acids (EAA) in the induction of this process (3–6).

According to their transduction mechanism, EAA receptors have been classified as ionotropic and metabotropic. The first class includes N-methyl-D- aspartate (NMDA), α-amino-5-methylisoxazole-4-propionate/quisqualate (AMPA/QA), kainate (KA), and possibly the presynaptic 2-amino-4-phosphonobutyric acid (AP4) subtypes; several subunits of these receptors have been cloned and expressed (7, 8). As for metabotropic glutamate (Glu) receptors, six different subunits have been cloned: R_1 and R_5 have been found linked to the stimulation of phosphoinositide (PI) hydrolysis, whereas the other receptor subunits are negatively coupled to adenylate cyclase (9).

L-Glu, L-aspartate (L-Asp) and some other EAA analogues, as well as taurine and glutamine have been shown to induce phagocytosis of the retinal outer segments by the RPE in a light-independent fashion (3, 5), and also to increase retina–RPE adhesiveness in an isolated system (10).

Phagocytosis induced by EAA could be receptor-mediated, since it is blocked by general antagonists of EAA receptors (4), and also, specific receptors for L-Glu have been characterized in membranes from cultured chick RPE. Binding to these sites was found to be sensitive to analogues which interact with both ionotropic NMDA receptors and metabotropic receptors sensitive to trans-ACPD (11). If EAA are involved in shedding and phagocytosis, the presence of EAA receptors in human RPE could be meaningful for the maintenance of a normal relationship of RPE with the retina. In turn, a disruption of communication at

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this level may be involved in disease processes such as retinitis pigmentosa (12).

The aim of this study was to demonstrate, using [^{3}H]L-Glu as a ligand, the presence of specific binding sites for EAA in membranes from cultured human RPE, and to establish their characteristics.

Materials and methods

Cell culture

Post mortem posterior poles of eyes with the anterior segments surgically removed were obtained from the National Disease Research Interchange, Philadelphia, Pennsylvania. These poles were the result of corneal transplantation. Eyes were enucleated in a sterile field within four hours, and shipped to the laboratory within 36 hours on wet ice. Individuals had no history of chronic disease, were not on any prolonged medication and died rapidly of severe trauma in vehicular accidents. None received any life support. Retinal pigment epithelial (RPE) cell cultures were established from donors of different ages from 6 days to 33 years, as indicated in each case. The RPE cells were isolated using a modification of a previously described technique (13). After careful removal of the vitreous and neural retina, the eyecup was rinsed with serum-free RPMI 1640 medium, and filled with a 10% (v/v) solution of pancreatin in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% EDTA. After 10 min at 25°C, RPE cells were removed and the eyecup was washed three times with medium containing 16% fetal bovine serum (FBS). Cells were pelleted by centrifugation at $40\times g$ for 10 min and washed twice with medium containing 16% FBS, 5 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin, and subsequently seeded at a density of 5×10^4 per ml onto 30 mm Millicell-HA culture plate inserts (Millipore, Bedford, MA) and incubated at 37°C in a humidified incubator, in an atmosphere of air/CO_2 95:5%.

Table 1. Displacement of [^{3}H]L-glutamate by amino acid analogues

	Specific binding (pmol/mg protein)	
	Age of donor	
Analogue	29 days	33 years
L-Asp	0.450 ± 0.0	0.325 ± 0.05
L-Glu	0.421 ± 0.07	0.393 ± 0.05
NMDA	0.198 ± 0.07	0.283 ± 0.05
AP5	0.028 ± 0.01	0.234 ± 0.09
Trans-ACDP	n.d	0.227 ± 0.07
AP3	n.d.	0.218 ± 0.05
D-Glu	No displacement	0.121 ± 0.0005
D-Asp	0.081 ± 0.03	0.122 ± 0.05

Experiments were performed at 37°C in sodium-free buffer, using 50 nM [^{3}H]L-Glu as a ligand. All analogues were added at 1 mM concentration. Data are expressed as the mean ± S.E.M. of the means of 3 experiments each performed in triplicate. n.d. = not determined.

At confluency cells were removed from the filter using pancreatin + 0.1% EDTA and transferred to 75 cm^2 plastic flasks. Cells were passed at confluency at a ratio of 1:4. Cultures maintained cuboidal morphology, but started losing pigmentation by passage 6; at later passages these cells acquired a fibroblastic appearance, as described previously (11–14). Except where noted (experiments reported in Table III) all membranes were prepared from RPE cells with cuboidal morphology.

Human lymphoblastoid cell line NB76 (obtained from Dr. Nicholas Beratis) was cultured as previously described (15) in RPMI 1640–16% FBS medium and subcultured 1:3 when cell numbers reached 1×10^6 viable cells per ml.

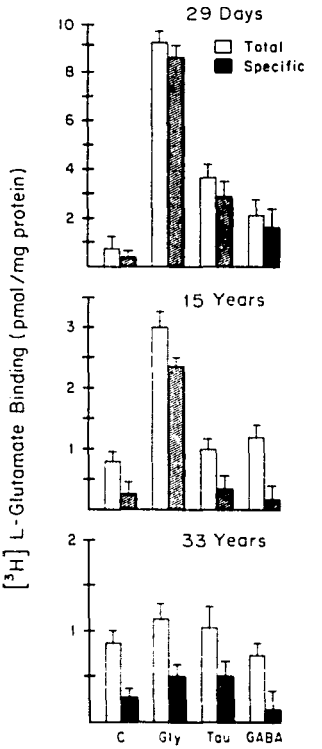

Figure 1. Effect of glycine, taurine and GABA on [^{3}H]L-Glu binding to RPE membranes from donors of different age. Experiments were performed as described in methods, in the presence of 1 mM glycine, taurine or GABA. For control values, experiments were performed without additions. 1 mM L-Glu was added for defining non-specific binding. Results are the mean of 3 experiments, each performed in triplicate. ± S.E.M. of the 3 means. Membranes from cells in passage 5 were used.

Membrane preparation

Membrane fractions were prepared from cultured RPE cells in passage number 5; some experiments were performed on cells from passages 8 to 14. Medium was removed and the cultures were washed once with Krebs Ringer-bicarbonate and then harvested in this same buffer. After pelleting at $1000 \times g$ for 5 min cells were homogenized in 20 ml water and placed on ice for 10 min for complete osmotic shock. Membranes were pelleted, washed twice at $45,000 \times g$ for 20 min and frozen until used for the binding assay.

The frozen pellets were thawed and washed once more with buffer prior to the assay (16). Protein was determined by the method of Lowry *et al.* (17).

Binding assay

Membrane pellets were resuspended in TRIS−HCl buffer 0.05 M, pH 7.4 and binding was measured as previously described (16). Membrane protein (30−50 μg per assay) was incubated in the presence of 50 nM [^{3}H]L-Glu in a final volume of 175 μl for the indicated periods of time. Non-specific binding was defined by the addition of 1 mM L-Glu. The reaction was stopped by dilution with 3 ml of cold buffer followed by filtration on glass microfiber filters and washed twice with the same buffer. After the addition of 10 ml of scintillation mixture (18), filters were counted for radioactivity. Corrections were made for quenching and counting efficiency.

Materials

[^{3}H]L-glutamate (spec. act. 45−59 Ci/mmol) was obtained from Dupont-NEN (Boston, MA). Culture media were from GIBCO/BRL, Grand Island, NY; glass microfiber filters (GF/B) were from Whatman International Ltd., Maidstone, England, and Millicell-HA Culture Plate Inserts from Millipore, Bedford, MA. Excitatory amino acid analogues were from Tocris-Neuramin, Bristol, England. All other reagents and chemicals were from Sigma, St Louis, MO.

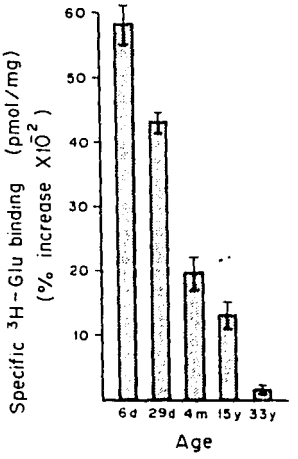

Figure 2. Decrease in glycine-stimulation of [^{3}H]L-Glu binding with the age of donor. The concentration of [^{3}H]L-Glu was 50 nM; 1 mM L-Glu was added for defining non-specific binding. Results are the mean ± S.E.M. of the means of three experiments each performed in triplicate.

Results

Binding of 50 nM [^{3}H]L-Glu to RPE membranes was measured at different protein concentrations from 10 μg to 100 μg per assay. As previously determined in the chick (11), 30−50 μg was found to be optimal. The time for the reaction to reach equilibrium was determined to be 10 ± 3 min. Using these assay conditions, specific binding was between 30 and 50% of total binding in most

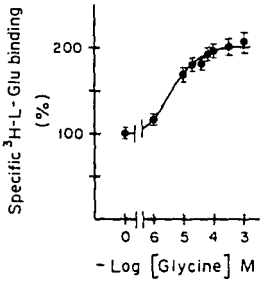

Figure 3. Dose-dependence of glycine stimulation of [^{3}H]L-Glu was determined as described in Methods. Membranes were from a 33 year-old donor. [^{3}H]L-Glu concentration was 50 nM. Results are expressed in pmol/mg protein and represent the mean ± S.D. of the means of 3 independent experiments each performed in triplicate. Binding in the absence of Gly was taken as 100%.

Table II. Effect of glycine and related compounds on [^{3}H]L-glutamate binding

Compound	Glutamate displaceable binding (pmol/mg protein)
Control	0.299 ± 0.06
Glycine (1mM)	5.420 ± 0.34
β-Alanine (1mM)	2.970 ± 0.22
Strychnine (1mM)	No displacement
7-Chlorokynurenate (10μM)	No displacement
Glycine (1mM) + Strychnine (1mM)	5.250 ± 0.31
Glycine (1mM) + 7-Chlorokynurenate (10μM)	0.512 ± 0.07

Experiments were performed as described in Methods. Membranes were from a donor 4 months old. [^{3}H]L-Glu concentration was 50 nM. In controls, 1mM L-Glu only was used as displacer. Results are the mean ± S.E.M. of the means of 5 experiments each performed in triplicate.

Table III. Specific [^{3}H]L-glutamate binding to membranes from RPE-derived fibroblastic cells (pmol/mg protein)

	Age of donor			
	4 Months	9 Months	17 Months	5 Years
Control	0.170 ± 0.09	0.245 ± 0.02	0.270 ± 0.06	0.231 ± 0.02
Taurine (1 mM)	4.916 ± 0.08	4.790 ± 0.06	5.120 ± 0.23	4.610 ± 0.09
Glycine (1 mM)	2.551 ± 0.10	2.422 ± 0.30	1.780 ± 0.42	1.722 ± 0.24
GABA (1 mM)	3.738 ± 0.07	4.152 ± 0.17	3.748 ± 0.22	3.769 ± 0.11

Membranes were obtained from RPE cultures which had become fibroblastic, lost pigment and were actively dividing. In control experiments, 50 nM [^{3}H]L-Glu was displaced by 1 mM L-Glu. In experiments in the presence of taurine, glycine or GABA, non-specific binding was also defined with 1 mM L-Glu. Results are the mean ± S.E.M. of the means of four experiments each performed in triplicate.

cases. Binding to these sites was sodium-independent, unlike binding to uptake sites which requires the presence of sodium.

Pharmacological properties of [^{3}H]L-Glu binding to RPE membranes

[^{3}H]L-Glu binding was measured in membranes of cultures from donors of different ages (29 days, 15 and 33 years). Displacement of [^{3}H]L-Glu by agonists and antagonists acting at EAA receptor subtypes at 1 mM concentration was measured in an attempt to establish a correlation with those previously characterized in nerve cells (19).

Results in Table I show mainly quantitative changes in the pharmacological profile of binding to membranes from donors of two different ages. The general agonists L-Glu and L-Asp were the most efficient displacers. NMDA and its competitive antagonist, 2-amino-5-phosphonovalerate (AP5), were also active. Displacement by these compounds was higher in membranes of cultures from the older donor.

The metabotropic Glu-receptor agonist trans-ACPD and the antagonists 2-amino-3-phosphonopropionate (AP3) and AP4 were also potent displacers of bound [^{3}H]L-Glu. Agonists at the non-NMDA ionotropic receptors were also tried: KA was without effect, and AMPA showed negligible activity (data not shown). D-Isomers of Glu and Asp showed much lower activity than the L-isomers, suggesting a stereospecific interaction. The hydroxamates of L-Glu and L-Asp, competitive inhibitors of Glu uptake (20) were entirely without effect.

Effect of glycine, GABA and taurine on [^{3}H]L-Glu binding

a) Membranes from RPE

In membranes from a 29 day-old donor, glycine, taurine and GABA at 1 mM concentration, stimulated the glutamate displaceable (specific) binding of [^{3}H]L-Glu, without altering the non-specific binding (Figure 1); the effect of glycine decreased with the age of the donor (Figure 2), whereas taurine and GABA were effective only in membranes of cultures from the younger (29 days old) donor (Figure 1). The dose-dependence of the glycine effect in a range of 1–100 μM was tested (membranes from the 33 year-old donor). Figure 3 shows that glycine reaches maximum stimulating effect at about 100 μM concentration, with an approximate EC_{50} of 5 μM. The pharmacological characteristics of the glycine-induced effect were studied, since this was the most powerful compound in increasing Glu binding. As can be seen in Table II, the effect of glycine is mimicked by β-alanine, a structural analogue of glycine, and by taurine at younger ages (Figure 1); the stimulatory effect was not affected by concentrations of strychnine up to 1 mM, but was 80% inhibited by 10 μM 7-chlorokynurenate, an antagonist of the glycine modulatory site at the NMDA neuronal receptor (21).

Table IV. Effect of taurine, GABA and glycine on [^{3}H]L-glutamate binding to membranes from proliferating cells

	Specific displacement (pmol/mg protein)	
	Scleral fibroblasts	Cell line NB76
Control	0.521 ± 0.01	0.391 ± 0.04
Taurine	3.608 ± 0.03	3.153 ± 0.01
GABA	0.614 ± 0.01	0.609 ± 0.03
Glycine	3.613 ± 0.04	6.232 ± 0.02

The concentration of Tau, GABA and Gly was 1 mM. In all cases, non specific binding was defined in the presence of 1 mM cold L-Glu. Cell line NB76 is a lymphoblastoid line (Tallan *et al.*, 1983). Results are expressed as the mean ± S.E.M. of the means of 3 experiments each performed in triplicate in membranes from the same batch of cells.

b) Membranes from cells different from RPE

As a control for cell-type specificity, [^{3}H]L-Glu binding to membranes from cultures of other cells of human origin was measured.

Fibroblastic cells derived from RPE cultures which have lost pigment also showed an increase in [^{3}H]L-Glu binding by glycine (Gly), GABA and taurine (Tau) (Table III). The order of potency in these cells was Tau > GABA > Gly, and as a difference with RPE, stimulation did not vary with the age of the donor.

In membranes from human scleral fibroblasts and in the human lymphoblastoid cell line NB76, stimulation by Gly and Tau was observed, but that of GABA was much lower than in RPE and RPE-derived fibroblasts (Table IV).

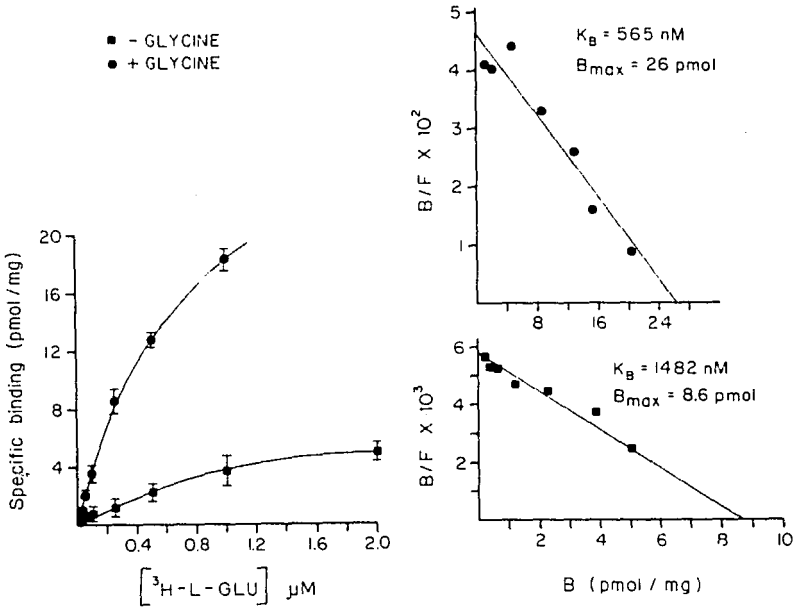

Figure 4. Saturation curve and Scatchard analysis of Glu binding to RPE membranes from a 29 day-old donor. Experiments were performed as described in Methods, using [^{3}H]L-Glu within a concentration range of 50–2000 nM, in the absence or presence of 1 mM Glycine. Results are the mean ± S.D. of the means of three experiments each performed in triplicate. Data were analyzed using the INPLOT (version 3.1) program from Graph PAD software, San Diego, CA. Hill coefficients (n_H) were > 0.8 for both curves.

Kinetics of [^{3}H]L-Glu binding to RPE membranes

Saturation curves using [^{3}H]L-Glu concentrations from 50 nM to 2.0 µM in membranes of cultures from 29 day-old, 15 and 33 year-old donors showed that binding is saturable and reversible (Figures 4 and 5). A slight, but significant, increase in the number of binding sites was observed in membranes from 15 year-old compared to those from the 29 day-old (8.6 to 13.0 pmol/mg protein, respectively) and remained unchanged in those from 33 year-old (13.1 pmol/mg protein). The K_B (1.2–1.5 µM) remained constant at all ages studied (Figures 4 and 5). Figure 4 shows that in the presence of 1 mM Gly, the K_B was decreased threefold, to 565 nM, and the binding sites doubled, to 26 pmol/mg protein (membranes from 29-day-old donor).

Discussion

In this study, we demonstrate the presence of specific Glu receptors in membranes from cultured human RPE, showing partial pharmacological resemblance with those previously described in diverse areas of the CNS (19) and the retina (22), although the affinity of the RPE receptors (K_B = 1.2–1.5 µM) is much lower (Figures 4 and 5) than that reported in excitable tissues (200–500 nM). The fact that binding was not inhibited by the hydroxamates of Glu and Asp, and did not require the presence of sodium, rules out the possibility of an interaction with transport sites for the amino acid (20).

The pharmacological profile obtained using agonists and antagonists at the main subtypes of EAA receptors suggests an NMDA-receptor, since after the general agonists L-Glu and L-Asp, NMDA and its specific antagonist, AP5, were the most efficient displacers of bound Glu. The metabotropic receptor agonist trans-ACPD and its antagonists, AP3 (9) and AP4 (8), were also potent displacers, raising the possibility of the presence of two different receptor subtypes in RPE (Table I). Since kinetic analysis of binding showed a single population of receptors, we cannot discriminate at this level between the possibility of a single population of receptor sites, with different pharmacological properties and/or subunit composition from those described up to now in the CNS (7, 8, 19), and the presence of two separate binding sites with very similar affinity for the agonist. [^{3}H]Antagonist binding will be of help in solving this problem in the future. Our results also show that KA and AMPA, specific agonists at non-NMDA receptors, displayed none or very low potency for displacing L-Glu, which supports the suggestion of an NMDA receptor (data not shown).

Due to the difficulty in obtaining material from young donors, especially, it was not possible to repeat experiments on material from the same age. Experiments were performed with membranes of cultures from donors of 6 and 29 days, 4 months, 15 and 33 years of age. Specific Glu-binding was slightly higher in cultures from older donors, although the affinity of the receptor was the

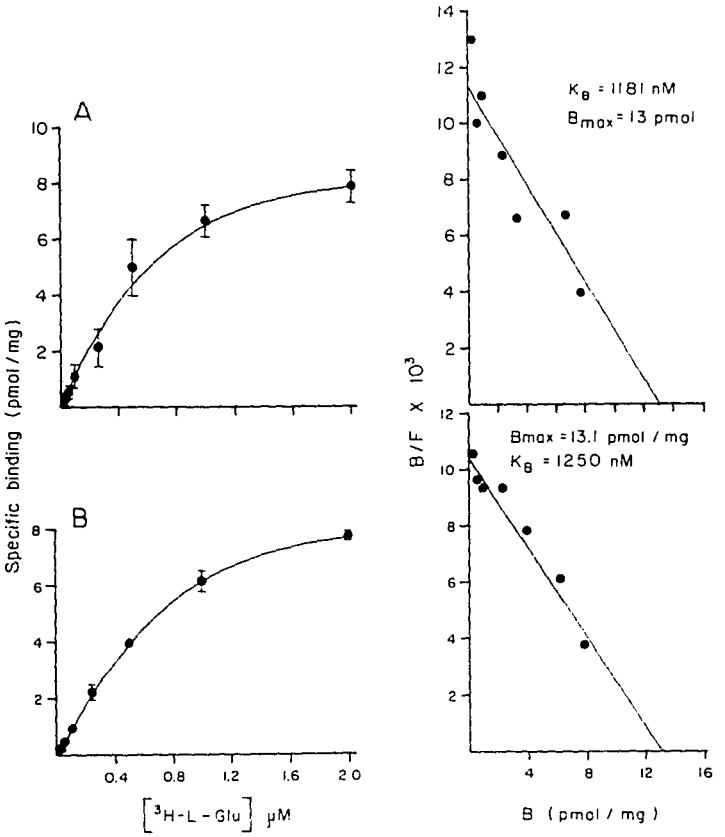

Figure 5. Saturation curve and Scatchard analysis of Glu binding to RPE membranes from a 15 year old (A) and a 33 year-old (B) donors. Experiments were as described in Methods. [^{3}H]L-Glu concentration was increased from 50 to 2000 nM, and non-specific binding was defined with 1 mM cold Glu. No glycine was added. Results are the mean ± S.D. of the means of three experiments each performed in triplicate. Hill coefficients (n_H) were: A = 0.82; B = 0.89.

same in all preparations (Figures 4 and 5); in spite of this fact, significant pharmacological differences were observed. Pharmacologically however, the capacity of NMDA and its competitive antagonist AP5 for displacing bound L-Glu was 2 and 8 times higher, respectively, in membranes from the 33 year-old cultures compared to those from the 29 day-old. Similar changes in the properties of binding to NMDA sites have been reported in the chick retina during maturation (23). Such coincidence is not unexpected since both tissues share the same embryological origin (24) and RPE cells can transdifferentiate into retinal cells under specific conditions (25).

As previously shown in RPE from the chick (11), glycine, taurine and GABA noticeably increase Glu-specific binding, glycine being the most potent (Figure 1). The effect of glycine on Glu binding was dose-dependent with an EC_{50} of 5 μM, which is in the range of saturation of the allosteric glycine site of the neuronal NMDA receptor (21). The fact that the glycine coagonist-site antagonist, 7-chlorokynurenate, inhibited the effect of glycine, whereas strychnine, a blocker of inhibitory receptors for the amino acid did not (Table II), also suggests an action of this compound at an NMDA-type receptor which shares properties with those in neurons, but also shows differences, since neither taurine nor GABA can substitute for glycine at NMDA-receptors described in the CNS (21).

The stimulatory effect of inhibitory amino acids on binding was decreased (glycine) or completely lost (taurine and GABA) with increasing age of the donor (Figures 1 and 2); differences do not derive from culture conditions or number of passages,

since these were kept constant (see materials and methods), and also since the same effect has been observed in intact RPE from the chick (López-Colomé and Fragoso, submitted).

In some species, including humans, RPE cells in culture undergo a de-differentiation following a number of passages, becoming fibroblastic and regaining accelerated cell division (14). The biochemical mechanisms responsible for these transformations are poorly understood, but could be related to the reception and transduction of signals and hence, to the receptors involved. Binding to membranes from cultures of RPE-derived fibroblastic cells (Table III), scleral fibroblasts and human lymphoblastoid line NB76 (Table IV), all actively dividing cells, was also increased by glycine, taurine and GABA. Differences in pharmacological specificity between RPE-derived fibroblasts (Table III) and fibroblasts derived from sclera (Table IV) were observed, which support the different origin of these cells.

NMDA receptors seem to be involved in both differentiation (26) and proliferation (27), and also can modulate the activity of metabotropic receptors (mGluRs) through the entry of Ca^{++} and the subsequent activation of several enzyme systems and ion channels (9). On this basis it seems possible that Ca^{++} entry through NMDA receptors is required for cell division in RPE. This possibility is suggested by the fact that dividing cells, such as lymphoblasts, fibroblasts and young RPE show positive modulation at this receptor, whereas mature RPE does not. Transient variations in the properties of L-Glu receptor modulation, such as those observed here in RPE, have been reported for NMDA receptors in the cerebellum (28), and could be explained by differential expression of the receptor subunits with age (7).

As for the involvement of EAA receptors in phagocytosis, previous studies have shown that L-Glu, but not NMDA, is capable of inducing this phenomenon (4, 6). This is in favor of two populations of Glu receptors in RPE, one of them of the metabotropic type. Activation of specific subclasses of mGluRs results in adenylate cyclase inhibition (9). Since cAMP has been shown to exert an inhibitory effect on phagocytosis, which is antagonized by taurine (29), the possibility exists that the stimulation of mGluRs sensitive to trans-ACPD in RPE could trigger phagocytosis by lowering the level of this compound.

Although very little is known about the molecular mechanisms which regulate the function of RPE and its relationship with the retina, the present results suggest that EAA could be involved in proliferation and phagocytosis in this tissue through the activation of two different glutamate receptors: an NMDA-gated receptor channel and a subtype of mGluR. Since impairment of these processes is related to several retinopathies, further understanding of the function of EAA receptors in RPE could be of clinical relevance.

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V. DISCUSION Y CONCLUSIONES

Los resultados obtenidos demuestran que existen receptores específicos de unión para el ácido glutámico-H^3 en membranas de células cultivadas de EPR de humano y de pollo. La unión a estos sitios es saturable, reversible, de alta afinidad e independiente de Na^+. En el EPR de pollo se presenta una disminución de la cantidad de receptores en la etapa de células diferenciadas (25 DIV) con respecto a la etapa no diferencaida (16 DIV) y en ambos sistemas estos receptores cambian durante el desarrollo.

Los ácidos glutámico, aspártico y kaínico estimulan el desprendimiento de los discos de los segmentos externos de los fotorreceptores y la subsecuente fagocitosis de estos por el EPR, independientemente de la condición de luz-oscuridad (Greenberger y Besharse, 1985). Se ha propuesto que estos compuestos actúen vía un receptor, ya que por medio de antagonistas específicos se bloquea el efecto de estos aminoácidos (Besharse y Spratt, 1988). A este respecto los autores antes mencionados utilizaron las copas ópticas de *Rana pipiens* para sus ensayos y encontraron que el kinurenato, D-O-fosfoserina y el ácido cis-2,3-piperidín dicarboxílico bloquean tanto el efecto neurotóxico y desprendimiendo de discos inducido por kainato. En este trabajo se utilizaron antagonistas para el ácido kaínico y el resto de los agonistas para aminoácidos excitadores sin embargo, no encontramos receptores para kaínico en etapas iniciales del desarrollo, sino hasta el tiempo de eclosión en el caso del tejido aislado y en cultivos diferenciados (25 DIV). Por lo tanto, podemos concluir que posiblemente los receptores que no involucran al ácido kaínico tienen una función diferente al de la fagocitosis en etapa inicial de desarrollo, que en anfibios la fagocitosis presenta un mecanismo de inducción diferente, o que in vivo, el ácido kaínico pueda inducir liberación de otros mensajeros que directamente intervengan en el proceso fagocítico. A este respecto, es necesario un estudio exhaustivo de la inducción de fagocitosis por el EPR inducida por los AAE en células de pollo y de humano.

Otros aminoácidos como la L-glutamina, L-glicina y taurina estimulan el desprendimiento de los discos sin dañar la capa interna de la retina como lo hacen los aminoácidos excitadores (Greenberger y Besharse, 1985). En relación a esto, la unión específica de L-glu-H^3 a sitios de la membrana en las células cultivadas y aisladas de EPR de pollo y en membranas aisladas de células cultivadas de EPR de humano reciben la influencia de otros aminoácidos no excitadores (taurina, GABA y glicina). Estos aminoácidos, a una concentración de 1 mM, aumentan la unión específica de L-glu-H^3 en membranas de células cultivadas de EPR de humano y de pollo y en membranas aisladas de EPR de pollo. Este aumento en la unión es específica para el EPR, porque este fenómeno no se presenta en membranas de otros tipos celulares, como por ejemplo la retina de pollo o las membranas aisladas de la corteza de rata. Existe además un efecto aditivo parcial taurina-glicina que hace suponer que estos aminoácidos actúan sitios de reconocimiento diferentes, aunque puedieran interactuar entre sí.

La taurina aumenta la unión del ácido glutámico e incrementa los sitios de unión y la afinidad de los receptores por su mensajero. Existe la posibilidad de que pudiese realizar una función in vivo al interactuar con los receptores del glutámico, porque se ha demostrado que la fagocitosis se inhibe por melatonina y AMPc y este efecto se revierte muy eficientemente por taurina (Ogino y col, 1983). Aunque la función de la taurina en la retina se desconoce, se sospecha que se trata de una función importante, ya que su deficiencia causa la degeneración de los fotorreceptores en el gato (Hayes y col, 1975; Schmidt y col, 1976). La taurina podría actuar modulando un receptor metabotrópico del glutámico acoplado a la inhibición de la adenilato ciclasa, de la que disminuye la actividad y concomitantemente la del AMPc, ésto permite la actividad de fagocitosis. Este aminoácido pudiera regular la fagocitosis al presentarse en el espacio subretiniano después de liberarse de los fotorreceptores cuando se estimulan con luz (Salceda y col, 1977) e interactuando después con receptores específicos en el EPR (López-Colomé y col, 1991). La glicina por otra parte, pudiera actuar sobre el sitio modulador alostérico positivo del

receptor para NMDA, reportado en el SNC (Kemp y Leeson, 1993), estimulando así su actividad y disparando un efecto similar al de la taurina.

Se conoce que los AAE pueden modular los sistema GABAérgicos en el SNC, modulando, por ejemplo, la liberación del GABA, pero poco se sabe de la influencia que ejerce el GABA sobre sitios para AAE, aunque existe evidencia de que el GABA aumenta los potenciales de campo corticales inducidas por NMDA, QA y ácido glutámico (Walden y col, 1989). Sería necesario caracterizar sitios de unión específicos para GABA en estas células con el propósito de dilucidar el mecanismo por el cual modula estos receptores para AAE.

Sería importante continuar investigando el papel que juegan estos receptores para aminoácidos excitadores en las funciones fisiológicas del EPR, probablemente la fagocitosis de los segmentos externos de los fotorreceptores, el control de la división célular de estas células, diferenciación celular, transdiferenciación del EPR a neuronas retinales, etc. Que a su vez serviría para explicar por comparación la causa de alteraciones patológicas como lo es la retinitis pigmentosa. Esta tesis por tanto hace una pequeña aportación para explicar el mecanismo de comunicación retina-EPR a través de receptores para AAE.

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