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CARACTERIZACIÓN DE LOS SISTEMAS DE SEGUNDOS MENSAJEROS ACOPLADOS A RECEPTORES PARA ÁCIDO GLUTÁMICO EN EL EPITELIO PIGMENTARIO DE LA RETINA.

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GABRIELA FRAGOSO CONTRERAS

DIRECTOR DE TESIS: DRA. ANA MARÍA LÓPEZ COLOMÉ.

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DISCONTINUA.

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G R A C I A S

RESUMEN

Los aminoácidos excitadores inducen el fenómeno de fagocitosis en las células del epitelio pigmentario de la retina (EPR), a través de su interacción con receptores específicos. Con el fin de determinar si el efecto de estos compuestos se debe a la activación de segundos mensajeros, se caracterizó la hidrólisis de fosfoinosítidos inducida por L-glutamato (L-glu) y algunos análogos en cultivos primarios de EPR de pollo. Los agonistas de los receptores ionotrópicos de L-glu estimulan con mayor eficiencia la generación de IPs-H^3: ácido N-metil-D-aspártico (NMDA) ≥ L-glu > ácido quiscuálico (QA) ≥ ácido kaínico (KA) > ácido (1S-3R)-1-aminociclopentano-1,3-dicarboxílico (ACPD), mientras que aquellos de los receptores metabotrópicos lo hacen con mayor potencia (ACPD ≥ L-glu > NMDA > QA > KA). El ácido L-2-amino-4-fosfonobutírico (L-AP4) también estimuló la acumulación de fosfatos de inositol. El efecto del NMDA se inhibió con los antagonistas ácido 2-amino-5-fosfonopentanoico (AP5), ácido 3,(RS)-(2-caboxipiperazin-4-il) propil-1-fosfónico (CPP) y maleato de dizocilpina (MK-801), mientras que el del KA se inhibió con los antagonistas de los receptores ácido α-amino-3-hidroxi-5-metilisoxazol-4-propiónico (AMPA)/KA, 6-ciano-7-nitroquinoxali-na-2,3-diona (CNQX) y 6-7-dinitroquinoxalina-2,3-diona (DNQX). El estímulo inducido por L-glu se inhibió parcialmente por ambos tipos de antagonistas. El antagonista metil-carboxi-fenilglicina (MCPG) inhibió el efecto de los agonistas trans-ACPD, dihidroxi-fenilglicina (DHPG) y L-AP4. La estimulación por NMDA requiere de Ca^{2+} extracelular y se inhibe por quelantes del mismo y por bloqueadores de los canales de Ca^{2+} sensibles al voltaje, así como por inhibidores de los movimientos intracelulares de Ca^{2+}. Ninguno de los agonistas probados modificó la concentración de adenosín monofosfato cíclico (AMPc) basal, ni la estimulada por forskolina o carbacol. Los resultados demuestran la presencia de receptores a aminoácidos excitadores en el EPR, acoplados a la hidrólisis de fosfoinosítidos, con características similares a aquellos de la retina neural, pero diferentes de los descritos en el sistema nervioso central; se discute la posibilidad de su participación en la fagocitosis.

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ABREVIATURAS

aa	aminoácidos
AAE	Aminoácidos excitadores
AC	Adenilato ciclasa
ACPD	Ácido trans-1-amino-ciclopentil-1,3-dicarboxílico
AMPA	Ácido α-amino-3-hidroxi-5-metilisoxazol-4-propiónico
AMPc	Adenosín monofosfato cíclico
AP3	Ácido 2-amino-3-fosfonopropiónico
AP4	Ácido 2-amino-4-fosfonobutírico
AP5	Ácido 2-amino-5-fosfonopentanoico
7ClK	Ácido 7 cloro-kinurénico
CNQX	6-ciano-7-nitroquinoxalina-2,3-diona
CPP	Ácido 3-(RS)-(2-carboxipiperazín-4-il)propil-1-fosfónico
DAG	Diacilglicerol
DHPG	3,5-dihidroxifenil-glicina
DNQX	6,7-dinitroquinoxalina-2,3-diona
EC_{50}	Constante de estimulación 50
EPR	Epitelio pigmentado de la retina
GABA	Ácido γ-amino butírico
IPs	Fosfatos de inositol
IP_3	Trifosfato de inositol
KA	Ácido kaínico
L-AP4	Ácido L-2-amino-4-fosfonobutírico
L-glu	Ácido glutámico
L-glu-H^3	Ácido glutámico tritiado
MCPG	(±)-L-metil-4-caboxifenil-glicina
MK-801	Maleato de dizocilpina
NMDA	Ácido N-metil-D-aspártico
PIP_2	4,5-difosfato-fosfatidil inositol
PKC	Proteína cinasa C
PLC	Fosfolipasa C
QA	Ácido quiscuálico
RKB	Ringer Krebs bicarbonato
SE	Segmentos externos de los bastones
SNC	Sistema nervioso central
t-ACPD	Ácido (1S,3R)-1-aminociclopentano-1,3-dicarboxílico

INTRODUCCIÓN

MORFOLOGÍA Y LOCALIZACIÓN DEL EPR

El epitelio pigmentado de la retina (EPR), es una capa celular importante del sistema visual, vital para el mantenimiento de la integridad de las células de la retina sensibles a la luz, los fotorreceptores.

El EPR está situado entre la coroides y la retina, íntimamente asociado a los fotorreceptores. Histológicamente el EPR consiste de una monocapa continua de células, que comienza en el borde inicial del nervio óptico y se extiende periféricamente hacia la ora serrata, zona en la cual termina la retina. En la región anterior a la ora serrata, el EPR se continúa como el epitelio pigmentado del cuerpo ciliar (Clark, 1986).

El EPR es una monocapa de células poligonales de forma hexagonal. Las células del EPR al igual que otras células epiteliales son asimétricas. Esta asimetría es esencial para muchas de las funciones llevadas a cabo por las células. La superficie apical del EPR orientada hacia la retina sensorial, está cubierta por procesos o microvellosidades, de 60 a 95 μm de largo que se asocian estrechamente a los fotorreceptores y se extienden hacia los segmentos internos (Nguyen-Legros, 1978).

Las células del EPR se relacionan entre ellas mediante uniones estrechas y desmosomas en banda. Este complejo de unión, también llamado de barra terminal, está localizado cerca de la región apical de la membrana plasmática e impide la difusión de moléculas de gran tamaño a través del espacio entre

células adyacentes. El sistema de barra terminal es el responsable de mantener la barrera hemato-retiniana a nivel del riego sanguíneo coroidal (Zinn y Marmor, 1979).

La membrana plasmática de las células fotorreceptoras y las del EPR no poseen puntos de contacto, y están separados por el espacio subretinal, constituido por una capa de matriz extracelular viscosa (Clark, 1986; Nguyen-Legros, 1978). Las células del EPR tienen un color pardo oscuro debido a la presencia de gránulos de melanina. Las observaciones histológicas muestran que las células del EPR son más largas y contienen más melanina en la zona macular que en la periferia. Esto está de acuerdo con la observación de que la mácula tiene una apariencia más oscura que otras regiones retinianas (Zinn y Marmor, 1979).

En la región basal del EPR, existe un complejo pentalaminar llamado membrana de Bruch. Los estudios con microscopía electrónica han demostrado que este complejo está constituido por la membrana basal del EPR, una capa de colágena interna, la capa elástica media, la zona de colágena externa y la membrana basal de los coriocapilares (Nguyen-Legros, 1978).

El núcleo de la célula pigmentada se localiza en la porción basal y mide aproximadamente de 8 a 12 μm de diámetro (Zinn y Marmor, 1979). Muchas de las células contienen varios núcleos, especialmente en la región de la ora serrata donde la relación de células mononucleadas y binucleadas es del

orden de 30:1 (Tsunematsu et al., 1981). En general el núcleo de la célula presenta un patrón de cromatina difuso y puede contener 1 ó 2 nucleolos. Los microtúbulos y filamentos de 10 nm de diámetro, que forman parte del citoesqueleto, parecen intervenir en el movimiento de organelos en estas células (Kline y Ali, 1981).

FUNCIONES DEL EPR

La estructura del EPR está íntimamente relacionada con muchas de sus funciones, que pueden clasificase como funciones físicas, ópticas, bioquímico-metabólicas y de transporte (Zinn y Marmor, 1979).

Las funciones físicas del EPR consisten en mantener una barrera de protección de la retina neural al paso de moléculas procedentes de la circulación coroidal, así como ofrecer una gran fuerza de adhesión retina-EPR a través del transporte de fluídos específicos y la síntesis de algunos mucopolisacáridos hacia el espacio subretinal (Miller y Steinberg, 1976; 1977a; Oakley et al., 1978). Las microvellosidades del EPR también podrían contribuir a esta adhesión por su estrecha relación estructural con los segmentos externos de los fotorreceptores (Matsumoto et al., 1987).

Las funciones ópticas que realiza el EPR consisten en disminuir la luz dispersa, lo que mejora la resolución de las imágenes, y absorber la energía luminosa por medio de los gránulos de melanina. Asimismo actúa como una barrera pigmentada para prevenir el daño que causaría un exceso de luz. (Rapp y Williams, 1980).

Por otra parte, el EPR metaboliza por esterificación los precursores de los pigmentos visuales como la vitamina A para la síntesis de rodopsina (Bridges et al., 1984), a la vez que lleva a cabo el almacenaje y transporte de los mismos. Asimismo, contiene enzimas como la tirosinasa que interviene en la síntesis y renovación de los gránulos de melanina (Zinn y Marmor, 1979).

Las funciones de transporte que realiza el EPR, son la de llevar numerosos metabolítos hacia y desde las células retinianas a la circulación coroidal, entre los que se encuentran aminoácidos como la taurina y la metionina y azúcares como la glucosa (Lake et al., 1977; Miller y Steinberg, 1976). Asimismo participa en el bombeo iónico, importante para mantener la homeostasis del espacio subretiniano (Miller y Steinberg, 1977a; 1977b; 1979).

Fagocitosis

Una función bioquimico-metabólica importante del EPR es la de fagocitar paquetes de discos de los segmentos externos de los fotorreceptores (Matsumoto et al., 1987; Tsunematsu et al., 1981). El desprendimiento de los discos, definido como la eliminación de los discos del extremo distal de los segmentos externos de conos y bastones, incluye la separación de grupos de discos, los cuales se fagocitan por las células adyacentes del EPR (Young, 1976). Se estima que cada célula del EPR podría fagocitar en promedio los segmentos externos de 30 a 45 fotorreceptores. A este respecto, los estudios

de Young (1971) en el mono *rhesus*, indicaron que cada bastón sintetiza de 80 a 90 nuevos discos del segmento externo cada 24 horas y el número de discos fagocitados por una célula de EPR diariamente es de 2,000 en la parafóvea, 3,500 en la perifóvea y aproximadamente 4,000 en la periferia. Esto se debe a que la relación entre las células del EPR y los fotorreceptores no siempre es 1:1 (Zinn y Marmor, 1979).

Young y Bok en 1969, usaron el término fagosomas para la inclusión de cuerpos con material de bastones y notaron que cada cuerpo estaba rodeado por dos membranas celulares separadas, la primera, o membrana interna, derivada del segmento externo del fotorreceptor, la segunda o membrana externa, derivada de la célula del EPR.

Por otra parte, las células del EPR contienen numerosas enzimas hidrolíticas en sus lisosomas, que intervienen en la degradación de los restos de las membranas procedentes de los segmentos externos de los fotorreceptores. Los fagosomas se unen a los lisosomas y forman estructuras especializadas llamadas fagolisosomas (Tamai y Chader, 1979).

La renovación de los segmentos externos (SE) requiere de la síntesis y el ensamble de nuevos discos en la región basal del SE y el desprendimiento intermitente de los discos más viejos de la región apical, los cuales se degradan en los fagolisosomas (Young y Bok, 1969). Esta función del EPR es de gran importancia, ya que en condiciones patológicas tales como la retinitis pigmentosa, los discos que se desprenden de los SE se acumulan en el

espacio subretiniano impidiendo el aporte de nutrientes a la retina.

La retinitis pigmentosa (RP) es un grupo de retinopatías hereditarias que afecta aproximadamente a 1 de cada 4,000 individuos, y se caracteriza por una deficiencia en la fagocitosis de los paquetes de discos de los SE de los fotorreceptores por parte del EPR, lo cual conduce a la ceguera progresiva e irrevesible del paciente. Se ha establecido una clasificación de éstas con base en las características genéticas en: dominante, recesiva y ligada al cromosoma X. El EPR es el sitio de lesión primaria en la distrofia retinal heretidaria de la rata Royal College of Surgeons, modelo animal de retinis pigmentosa (Bird, 1987).

RELACIÓN EPR-RETINA

Durante el desarrollo y la diferenciación

Durante el desarrollo embrionario, el diencéfalo, la segunda vesícula cerebral, da origen a las copas ópticas. La zona de la vesícula óptica opuesta a la superficie del ectodermo prolifera y forma una depresión que invagina la porción distal y media inferior lo que constituye la copa óptica, mientras que la superficie ectodérmica da origen a la vesícula del cristalino, que ocupa la concavidad de la copa óptica. La pared externa de la copa óptica permanece como una capa de células cuboidales que da origen al epitelio pigmentado de la retina, al cuerpo ciliar y al iris. La pared interna de la copa prolifera y da

origen a la retina, al epitelio ciliar no pigmentado y al epitelio posterior del iris (Barnstable, 1987; Zinn y Marmor, 1979).

En las etapas primarias del desarrollo embrionario, el fondo de la copa óptica que da lugar a la retina y la capa externa que da lugar al EPR son equipotenciales. El transplante del epitelio pigmentario o de retina en el estadio de copa óptica a distintas regiones del encéfalo embrionario, demuestran que tanto una capa como otra tienen la capacidad de generar tanto neuronas características de la retina como células del epitelio pigmentado (Clark, 1986; Zinn y Marmor, 1979).

La futura retina pierde la capacidad de transdiferenciarse en epitelio pigmentado en el estadio que precede a su estratificación. El epitelio pigmentado sin embargo, conserva por más tiempo la facultad de transformarse en neuronas de la retina, un fenómeno denominado también "metaplasia". La importancia de este fenómeno radica en su posibilidad de sustituir a células de la retina neural en el caso de daño durante el desarrollo embrionario.

En la retina adulta

La comunicación entre el epitelio pigmentado y la retina, es indispensable para el buen funcionamiento del proceso visual. En los bastones el proceso de fototransducción comienza con la incidencia de un fotón sobre la rodopsina, proteína integral de la membrana de los discos de los fotorreceptores, que es

sensible a la luz. El 11-cis retinal es el grupo prostético de la rodopsina; las células del EPR incorporan el trans-retinal del plasma por un proceso en el que se considera interviene un receptor de membrana (Das y Gouras, 1988). El trans-retinal se isomeriza a 11-cis retinal y posteriormente se esterifica a 11-cis ester. El 11-cis-ester puede ser hidrolizado y el alcohol formado es entonces oxidado por una deshidrogenasa estereoespecifica a 11-cis-retinaldehido. El 11-cis-retinaldehído es transferido a los segmentos externos de los fotorreceptores para posteriormente incorporarse a la opsina y formar la rodopsina (Bridges et al., 1984). El 11-cis retinal parece ser especifico del EPR ya que no se requiere en otra región del organismo.

Una de las actividades más importantes del EPR es su participación en el recambio de los segmentos externos de las células fotorreceptoras, al fagocitar paquetes de discos. En ratas con distrofia hereditaria de la retina, la actividad fagocítica se ve severamente reducida, lo que ocasiona la acumulación de membranas de los segmentos externos en el espacio subrretinal y por consiguiente, la degeneración de los fotorreceptores (Tamai y Chader, 1979; Weleber y Eisner, 1988).

También se pone de manifiesto la interacción EPR-retina en la respuesta eléctrica de la retina, puesto que la onda "a" del electrorretinograma se origina en la capa de los fotorreceptores, la onda "b" se genera principalmente por la actividad de las células bipolares ON y células (glia) de Müller y la onda "c" proviene principalmente de la despolarización de la membrana apical del EPR

(Zinn y Marmor, 1979).

RECEPTORES A AMINOÁCIDOS EXCITADORES

El ácido L-glutámico (L-glu) es un aminoácido no esencial, que posee un poderoso efecto excitador sobre la mayor parte de las neuronas del sistema nervioso central (SNC) y se sintetiza en el sistema nervioso principalmente a partir de la glutamina por acción de la glutaminasa (Gaetano Di Chiara y Gessa, 1981).

Werman en 1966 estableció seis criterios básicos para que un compuesto pueda ser considerado como neurotransmisor: 1) su presencia en terminales sinápticas, 2) existencia de un mecanismo de sintesis, 3) un sistema de desactivación del compuesto, como puede ser un sistema de recaptura de alta afinidad dependiente de Na^+ y ATP, 4) un sistema de liberación del compuesto, 5) identidad farmacológica y 6) inducir el mismo resultado que el transmisor natural en la célula postsináptica.

El ácido glutámico reune la mayor parte de estos criterios en las neuronas del SNC de los vertebrados (López Colomé, 1986): El L-glu representa del 10 al 15 % de la poza de aminoácidos libres y su concentración promedio es de aproximadamente 4 µmol/g en la mayoría de las células de los vertebrados (Voaden et al., 1977).

Las enzimas que catalizan la síntesis del L-glu en la retina, están concentradas en los segmentos internos de los fotorreceptores, lo cual podría

indicar la participación de este compuesto como neurotransmisor (López Colomé, 1981). El L-glu se acumula en la retina por un sistema de captura de alta afinidad que depende de la presencia de sodio. Los estudios autorradiográficos en retinas de rata y conejo (Gaetano Di Chiara y Gessa, 1981) indican que el L-glu se acumula preferentemente en las células gliales. También se ha registrado su acumulación en fotorreceptores de varias especies (Watkins y Evans, 1981), por lo que Brandon y Lam en 1983 propusieron al ácido glutámico como candidato a neurotransmisor en los conos de la retina de rata. En cuanto a la liberación estimulada por despolarización, el L-glu se libera de los fotorreceptores aislados, en forma dependiente de Ca^{2+}(Johnston, 1979). Inicialmente se consideró que el L-glu sólo actuaba sobre receptores controlados por ligandos asociados a canales relacionados con la transmisión sináptica excitadora rápida. Recientemente se ha demostrado que el L-Glu interactúa con receptores acoplados a proteínas G (que unen GTP), las que a su vez activan o inactivan la síntesis de segundos mensajeros en las células (Nakanishi, 1992). La clasificación de los receptores para glutamato y compuestos químicos relacionados (aminoácidos excitadores, AAE) basada en estudios bioquímicos, farmacológicos y electrofisiológicos, señala la existencia de dos grandes grupos: Los receptores ionotrópicos y los receptores metabotrópicos. I. Los receptores ionotrópicos son proteínas heteroméricas en las que el ligando controla la apertura de un canal catiónico inespecífico; se clasifican a su vez de acuerdo con sus

propiedades farmacológicas y electrofisiológicas en los tipo NMDA (N-metil-D-aspartato; McBain y Mayer, 1994), AMPA (α-amino-3-hidroxi-5-metilisoxazol-4-propioanato) y KA (ácido kaínico) por ser estos los compuestos con mayor afinidad para activar a sus correspondientes receptores. II.- Los receptores metabotrópicos (mGluRs), son estructuras monoméricas acopladas a proteínas G (que unen GTP) que modulan la síntesis de segundos mensajeros mediante la regulación de la actividad de enzimas como la fosfolipasa C, la fosfolipasa D y la adenilato ciclasa, así como de canales de Ca^{2+} y de K^{+} (Pin y Duvoisin, 1995).

El KA y otros agonistas de los aminoácidos excitadores inducen la liberación del L-asp, L-glu y taurina de los fotorreceptores y del GABA y la Gly de la capa plexiforme interna de la retina. Con excepción del GABA, estos agentes en altas concentraciones, pueden causar el desprendimiento de los discos de los fotorreceptores en *Xenopus sp*, por un mecanismo dependiente de Ca^{2+} (Besharse y Dunis, 1983), lo que apoya la posibilidad de que el agente endógeno relacionado con el desprendimiento pudiera ser el propio glutamato.

Receptores ionotrópicos tipo NMDA

Los receptores de tipo NMDA son permeables al Ca^{2+} y al Na^{+}, y su activación causa un incremento de la concentración de Ca^{2+} intracelular. Además de su sitio de reconocimiento para el L-glu, este receptor tiene un sitio alostérico positivo para la glicina, actuando esta como coagonista; tiene un

sitio modulador negativo para el Zn^{2+}, y algunas poliaminas también regulan su actividad (Calderón y López Colomé, 1998), aumentando la unión tanto al sitio de reconocimiento como al de la glicina. Asimismo, en el canal iónico del receptor hay sitios inhibidores para el Mg^{2+} (dependiente de voltaje) y para anestésicos disociativos como la ketamina, la fenciclidina y el maleato de dizocilpina (MK-801; McBain y Mayer, 1994).

Experimentos electrofisiológicos y bioquímicos han demostrado que la activación del canal requiere al menos de dos eventos secuenciales: una despolarización que permita eliminar el bloqueo del canal por el Mg^{2+} (Nowak et al., 1984), y la unión del L-glu y de la glicina a sus respectivos sitios (Johnson y Ascher, 1987).

Los receptores a NMDA se distribuyen en prácticamente todo el sistema nervioso central, pero específicamente en áreas de la corteza cerebral y de los ganglios basales, así como en regiones relacionadas con los sistemas sensoriales. Estudios electrofisiológicos y con radioligandos indican que las características bioquímicas del receptor de NMDA varían en las diferentes regiones del SNC. En particular, las variaciones regionales de la unión específica de L-glu-H^3, de CPP-H^3 y de la modulación de la unión de MK-801-H^3 por los diversos agonistas y antagonistas del L-glu, han facilitado la descripción de cinco subtipos de receptores, entre los que se encuentran los que tienen preferencia por los agonistas, los que tienen preferencia por los antagonistas, los receptores del tálamo medio, los receptores del cerebelo y

los receptores presinápticos (Laurie y Seeburg, 1994; Wong y Thukral, 1996).

Las técnicas de biología molecular han permitido la identificación de una gran variedad de subunidades correspondientes a receptores de glutamato (Hollmann y Heinemann, 1994). Los ovocitos de *Xenopus*, las células renales HEK-293 y ciertas neuronas de mamíferos se han usado como sistemas de expresión para el estudio biofísico y farmacológico de distintas combinaciones de subunidades clonadas, y aunque se desconoce la estequiometría de las mismas en el receptor, se sabe que de las 5 subunidades identificadas para el receptor de NMDA (NR1, NR2A, NR2B, NR2C y NR2D) los ensambles de subunidades NR1 presentan las propiedades características de los receptores nativos, por ejemplo la unión del agonista y la unión del coagonista (Moriyoshi et al., 1991; Johnson y Ascher, 1987), alta permeabilidad al calcio (Mc Dermott et al., 1986) y bloqueo por magnesio dependiente del voltaje (Nowak et al., 1984). Asimismo, se ha demostrado que la amplitud de la corriente registrada en estos ensambles homoméricos no corresponde a la observada en receptores nativos postsinápticos, y que los ensambles que incluyen al menos una de las 4 subunidades NR2 conocidas presentan un patrón más frecuente (Ikeda et al., 1992). Recientemente se han identificado receptores de NMDA presinápticos en neuronas noradrenérgicas del *locus ceruleus*, y dopaminérgicas de la sustancia negra que regulan la liberación del neurotransmisor y que presentan una farmacología de receptores homoméricos (Fink et al., 1990; Wong y Thukral, 1996).

De manera general, el patrón de expresión de las subunidades mencionadas en el cerebro de la rata es el siguiente: la subunidad NR1 se expresa prácticamente en todas las células del sistema nervioso. Se han encontrado 8 variantes debidas al corte alternativo del RNAm que la codifica y que podría indicar diferencias regionales importantes durante el desarrollo y en el estado maduro (Ishii et al., 1993; Monyer et al., 1992). Las subunidades NR2 presentan un patrón de expresión y de localización más específico, que se superpone en ocasiones. En términos generales se sabe que la subunidad NR2A se expresa tan ampliamente como la subunidad NR1 lo cual indica la existencia de un receptor de NMDA con una composición heteromérica común en varias neuronas de diversas estructuras del SNC; la subunidad NR2B se expresa predominantemente en el cerebro anterior y se colocaliza con la subunidad NR2A en la corteza cerebral, en el hipocampo y en el cuerpo estriado, mientras que la subunidad NR2C se encuentra en el cerebelo, en el tálamo y en el bulbo olfatorio. Con respecto a la subunidad NR2D, se ha localizado predominantemente en estructuras del diencéfalo, del cerebro medio y del tallo cerebral; en estas dos últimas estructuras se colocaliza con la subunidad NR2A. En el tálamo y en el bulbo olfatorio se expresan y colocalizan las 4 subunidades NR2 (Wenzel et al., 1995).

La heterogeneidad funcional y farmacológica del receptor de NMDA depende de la composición heteromérica del mismo; las afinidades de los distintos sitios farmacológicos pueden modificarse al coexpresar cualquiera de

las subunidades NR2 en receptores recombinantes que expresan la subunidad NR1 (Williams, 1993; Laurie y Seeburg, 1994). Sobre esta base, se ha postulado que los receptores que contienen las subunidades NR1-NR2A corresponden al tipo de receptores que unen preferentemente a los agonistas, los ensambles NR1-NR2B corresponden a los receptores con alta afinidad por los antagonistas, los heterómeros NR1-NR2C corresponden a los receptores cerebelares y los NR1-NR2D, a los receptores característicos del tálamo medio (Laurie y Seeburg, 1994). Por otra parte, se ha demostrado que la coexpresión de más de dos tipos de subunidades NR2 predomina sobre la expresión de heterómeros con tan solo un tipo de esta subunidad (Wafford et al., 1993), dificultando así la clasificación de los receptores basada en su estequiometría, la cual hasta la fecha se desconoce.

En la retina se han hecho estudios de hibridación *in situ* para localizar la expresión de los mRNA que codifican para cada una de las subunidades del receptor de NMDA así como de otros receptores ionotrópicos glutamatérgicos (Brandstätter et al., 1994). El patrón de expresión de los RNAm de las subunidades NR1 y NR2A-C es muy parecido al observado en el SNC, con excepción del mRNA para la subunidad NR2D que no fué detectado, ya que la subunidad NR1 se expresa en prácticamente todas las capas celulares de la retina, mientras que las subunidades NR2 presentan un patrón de distribución restringido a la retina interna (células glanglionares, células amacrinas y células bipolares). Existe evidencia de que en cultivos de células de retina de

pollo de embriones de 8 días enriquecidos con células amacrinas hay una baja sensibilidad al bloqueo por Mg^{2+} y que aparentemente no hay potenciación de la actividad del canal al agregar 10 μM de glicina en condiciones saturantes de NMDA (Duarte et al., 1996). Para explicar lo anterior se ha planteado que exista cierto nivel basal de despolarización que mantenga una liberación constante del cotransmisor endógeno (glicina o su análogo) que se encuentre modulando a los receptores de NMDA (Duarte et al., 1996), aunque también existe la explicación alternativa de que los receptores de NMDA que se expresan por las células amacrinas no exhiben potenciación por la glicina debido a la composición heteromérica que presentan.

En condiciones de isquemia en la retina de la rata, se ha observado la participación importante de receptores ionotrópicos del tipo NMDA y no NMDA, ya que la aplicación de antagonistas competitivos y no competitivos del glutamato en estos receptores, reduce considerablemente la muerte de neuronas colinérgicas y gabaérgicas a concentraciones muy bajas (50 nM) (Lombardi y Moroni, 1994). Coincidentemente, el ácido 7-cloro-kinurénico (7ClK), un antagonista altamente selectivo para el sitio de glicina en el receptor de NMDA (Moroni et al., 1992) no reduce la muerte neuronal a concentraciones de hasta 200 nM.

Con respecto a la toxicidad por aplicación excesiva de NMDA en la retina, la aplicación de ACPC o de glicina disminuye la muerte celular, probablemente a través de una disminución de la actividad del receptor de NMDA, y por lo

tanto, contrario a lo que se podría esperar para un receptor de NMDA que potencia su actividad a concentraciones cada vez mayores del coagonista (Boje et al., 1992). Sin embargo no hay evidencia directa de que dicho efecto suceda a nivel del receptor.

Receptoes ionotrópicos tipo AMPA/KA

Los receptores AMPA/KA son preferentemente permeables a cationes monovalentes y se les relaciona con el componente rápido de los potenciales postsinápticos excitadores que precede a la activación dependiente de voltaje de los receptores de NMDA (Gasic y Hollmann, 1992). Los receptores tipo AMPA son canales activados por ligandos constituidos por 5 subunidades y se codifican por 4 genes denominados GluR1 (GluR-A) a GluR4 (GluR-D). La expresión de la subunidad GluR2 forma receptores heteroméricos impermeables a Ca^{2+}. Ahora se sabe que los receptores tipo AMPA que no expresan la subunidad GluR2 son permeables a cationes divalentes incluyendo al Ca^{2+} (Carvalho et al., 1998; Taschenberger y Grantyn, 1998)

La estructura secundaria propuesta para este receptor incluye: 1) un gran dominio amino terminal extracelular, 2) tres dominios transmembranales (TM1, TM3 y TM4), 3) un cuarto segmento transmembranal hidrofóbico que forma una asa similar a la región que forman las secuencias peptídicas de los canales de K^+ (Nakanishi, 1992), 4) un sitio de unión para agonistas formado por las regiones S1 y S2 extracelulares, 5) un extremo carboxilo terminal

intracelular (Domenico et al., 1997).

Las subunidades del receptor tipo AMPA se expresan en todas las neuronas registradas del cerebro, sin embargo cada célula puede diferir en el número y tipo de subunidades expresadas. La mayoría de las células sintetizan receptores heteroméricos compuestos por al menos 2 diferentes subunidades, pero también se pueden expresar como homómeros. No existen anticuerpos específicos para cada subunidad del receptor para KA, aun así los estudios inmunocitoquímicos que se han hecho usando un anticuerpo que reconoce las subunidades GluR5, GluR6 y GluR7 para el receptor de KA, revelan su presencia de manera abundante en neuronas de hipocampo. Además de las neuronas, estos receptores se presentan en células gliales astrocíticas (Betther y Mulle, 1995).

Desde el descubrimiento de que los receptores para AMPA son sensibles también al ácido kaínico, para diferenciar los efectos del AMPA y KA recientemente Tarnawa et al. (1993), sintetizaron una serie de 2,3-benzodiazepinas que actúan como antagonistas no competitivos en la respuesta mediada por AMPA. Uno de estos, el GYK153655 es selectivo para AMPA, su uso permite la observación de la corriente de iones inducida por KA en neuronas cultivadas.

Receptores metabotrópicos

En 1985 (Sladeczek, et al.) se demostró que el L-glu estimula a la

fosfolipasa C (PLC) en neuronas de estriado en cultivo, a través de la estimulación de un receptor que no pertenece a las familias de receptores de tipo NMDA, AMPA o KA; esto mismo se observó en rebanadas de hipocampo, células granulares del cerebelo y astrocitos en cultivo. La existencia de estos receptores se confirmó usando ovocitos de *Xenopus* al expresar en ellos los RNA mensajeros correspondientes y el NMDA, AMPA y KA no activan directamente a los mGluRs clonados. Consecuentemente, los antagonistas de los receptores de tipo NMDA (MK-801, AP5) y los de los receptores de tipo AMPA/KA (CNQX, NBQX, GAMS), tampoco afectan la actividad de los mGluRs de manera directa.

Se han sintetizado ya varios ligandos selectivos para los diferentes tipos de mGluRs, que permiten distinguirlos farmacológicamente. Antes de 1993 el ácido L-2-amino-3-fosfonopropiónico (L-AP3; Irving et al., 1992; Schoepp et al., 1990) y el β-hidroxamato del L-aspártico eran los antagonistas más usados; actualmente se consideran poco selectivos y de baja potencia. El agonista general más empleado es el trans ACPD ((±)-1-aminociclopentano-tans-1,3-dicarboxilato), mezcla equimolecular de (1S,3R) y (1R,3S)-ACPD, que interactúa de manera diferencial con todos los mGluRs y cuyo isómero más activo es el 1S,3R-ACPD, el AP4 (2-amino-fosfonobutirato) que activa a un tipo de receptores acoplados a la inhibición de la adenilato ciclasa, los agonistas derivados de la CCG-1, así como una serie de agonistas y antagonistas derivados de la fenilglicina la mayor parte de los cuales actúa

sobre mGluRs presinápticos (Roberts, 1995).

Mediante el uso de técnicas de Biología Molecular se han clonado 8 genes que codifican para mGluRs, algunos de los cuales generan variantes postrancripcionales ya sea por empalme alternativo o bien por un mecanismo de edición del RNA mensajero (Pin y Duvoisin, 1995). Dos laboratorios aislaron clonas de DNAc que codifican para el receptor metabotrópico ahora designado como mGluR1a cuya secuencia se ha empleado como sonda para conformar bibliotecas de DNAc mediante hibridación y reacción en cadena de la polimerasa.

Con base en la homología de su secuencia de aminoácidos, así como en su mecanismo de transducción, los 8 mGluRs se clasifican en 3 grupos. Los mGluRs del mismo grupo tienen aporximadamente 70% de similitud en su secuencia, mientras entre los diferentes grupos este porcentaje se reduce a 45%. El grupo I comprende al mGluR1 y mGluR5, el grupo II al mGluR2 y mGluR3 y el grupo III a los 4 restantes. Sus mecanismos de transducción se han estudiado al inducir su expresión en ovocitos de *Xenopus*, células de ovario de hamster chino (CHO), células de riñón de hamster neonato o en células de riñón embrionario humano (BHK; Thomsen, 1996).

Los mGluRs del grupo I están acoplados principalmente a la hidrólisis del fosfolípido de membrana fosfatidilinositol-4,5-difosfato (PIP_2), pero entre estos, la variante "a" (anteriormente denominada α) del mGluRI, se relaciona con la activación de la adenilato ciclasa (Sortino et al., 1996), mientras que aquellos

de los grupos II y III, están acoplados a la inhibición de la adenilato ciclasa.

a) Grupo I de receptores metabotrópicos a glutamato (mGluRI)

A este grupo pertenecen los receptores mGluR1 con cinco isoformas (a, b, c, d, e) y mGluR5 con dos isoformas (a y b). En los sistemas de expresión examinados, el grupo I de receptores al interactuar con su ligando, en su mayoría, estimulan a la fosfolipasa C lo que se traduce en un aumento en el recambio de fosfoinosítidos, y la liberación de Ca^{2+} de compartimientos intracelulares (Bardsley y Roberts, 1983). El componente inicial, la molécula receptora en la superficie de la célula, transmite información a través de la membrana plasmática hacia el interior de la misma, por medio de una proteína G, que se activa mediante la unión de GTP. La proteína G, activa la la PLC en la cara interna de la membrana; la PLC a su vez hidroliza al fosfolípido PIP_2 en DAG e IP_3. El IP_3 libera Ca^{2+} del retículo endoplásmico mediante su interacción con receptores específicos en este organelo (Fig. 1). El DAG en forma sinérgica con el Ca^{2+}, activa a una proteína cinasa unida a la membrana, a la que se le ha denominado PKC, que a su vez fosforila a proteínas de la membrana plasmática (Abe et al., 1992).

Por otra parte, la variante postranscripcional mGluR1a también induce el aumento intracelular de AMPc, lo que implica la activación de la enzima responsable de su síntesis, denominada adenilato ciclasa (Sortino et al., 1996). Esta glicoproteína está anclada a la membrana plasmática y tiene un

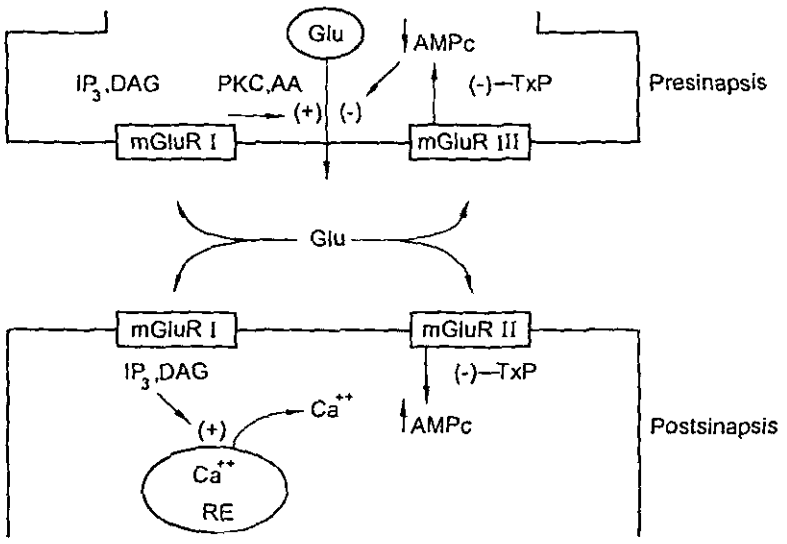

Figura 1. El diagrama muestra la comunicación sináptica glutamatérgica entre dos neuronas, la localización de los diferentes tipos de receptores metabotrópicos para el ácido glutámico (mGluR), y las reacciones inducidas por la activación de éstos en la terminal presináptica y la zona postsináptica. Abreviaturas: AA, ácido araquidónico; AMPc, adenosín monofosfato cíclico; Ca^{++}; ión calcio; DAG, diacilglicerol; IP_3, trifosfato de inositol; Glu; ácido glutámico; PKC, proteína cinasa C; TxP, toxina pertusis; (+), activación; (-), inhibición.

peso molecular aproximado de 150,000. Su sitio catalítico se localiza en la superficie interna de la membrana, transforma al MgATP en AMPc y su actividad está regulada por proteínas G. El AMPc es importante en la fisiología celular por su acción como segundo mensajero, que activa cinasas de proteína, especificamente la PKA (dependiente de AMPc), la cual está formada por dos subunidades reguladoras y causa su disociación de las subunidades catalíticas; las subunidades catalíticas transfieren el γ-fosfato del MgATP a los residuos de aminoácidos de diversas enzimas, cuya función se regula por fosforilación. La síntesis de AMPc y la activación de la PKA permite la amplificación considerable de la señal inicial. Una herramienta farmacológica útil en el estudio de los receptores que estimulan a la PKA es la forskolina, fármaco que es permeable a la membrana celular y que activa a la AC directamente, un diterpeno aislado de la raíz de la planta medicinal hindú, *Coleus forskohlii* (Shoepp et al., 1990; Fig. 2).

La cinética de la liberación de Ca^{2+} inducida por los mGluRI a, b y c expresados en sistemas heterólogos como ovocitos de *Xenopus* y células de mamífero transfectadas presenta algunas diferencias. La liberación inducida por los mGluR1b y c es más lenta y de más larga duración que la inducida por el mGluR1a. La estimulación de la PLC por activación de mGluR1a puede ser inhibida parcialmente con la toxina pertussis, lo que sugiere la participación de una proteína G de la familia Gi-Go. En este grupo, el mGluR1a activa a la AC cuando se expresa en ovocitos de hamster chino, lo que aumenta la

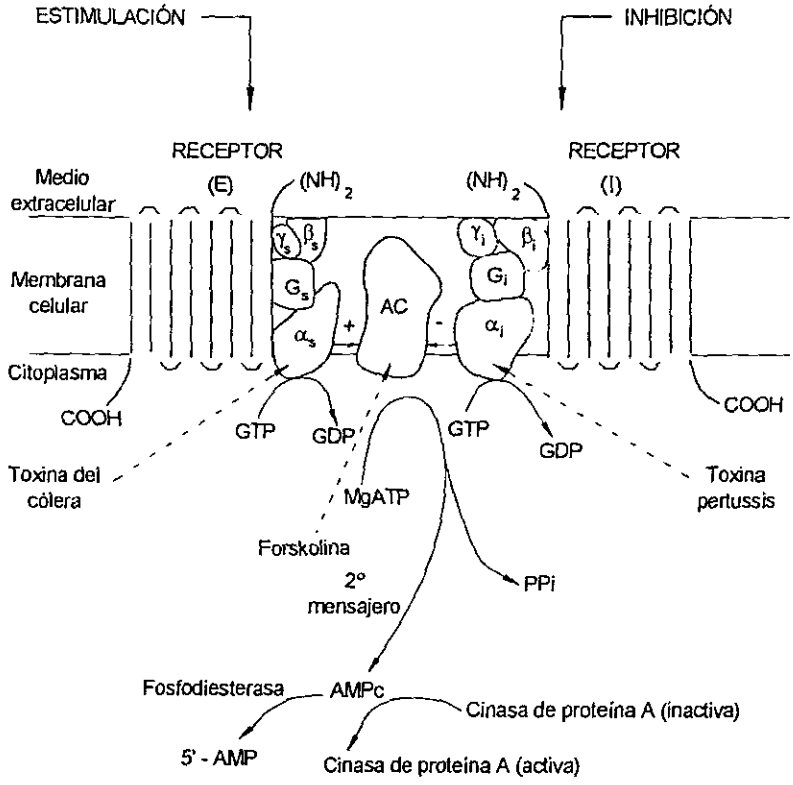

Figura 2. Diagrama que muestra el control bifásico de la adenilato ciclasa (AC). Los agonistas fisiológicos, transmisores y drogas, se unen a receptores específicos de la membrana plasmática, acoplados a proteínas G (Gs o Gi), representadas por las subunidades αs o αi, βs o βi, γs o γi. La transducción de esta señal al sitio catalítico de la AC, localizada en la superficie interna de la membrana, produce su activación o inhibición. Ciertos agentes, como la toxina del cólera o la toxina pertussis, causan la ribosilación de la subunidad de la proteína G y estimulan o inhiben respectivamente su actividad. La forskolina activa directamente a la AC, sin la intervención de la proteína G reguladora (Gs) y aumenta la concentración de AMPc intracelular.

concentración intracelular de AMPc.

Entre los AAE que activan a los receptores metabotrópicos, así como a los ionotrópicos, están el QA, el IBO y el L-Glu. El QA es agonista más potente en los receptores metabotrópicos que en los ionotrópicos AMPA/KA. El análisis farmacológico de los receptores mGluR1a transfectados en líneas celulares de mamífero y ovocitos de *Xenopus*, muestra que los agonistas tienen el siguiente orden de potencia: QA > L-Glu > IBO > L-CCG >1S,3R-ACPD; el QA tiene efecto a concentraciones micromolares bajas, mientras que el ACPD a concentraciones micromolaes altas o milimolares. Cuando se elimina una corta secuencia por empalme alternativo del extremo carboxilo de mGluR1a, esta remoción afecta su potencia pero no el orden de sensibilidad a los agonistas. Aunque es difícil distinguir farmacológicamente entre los receptores mGluR1 y 5, el mGluR5 es más sensible al ACPD que el mGluR1. Por otra parte, la mayoría de las fenilglicinas son antagonistas para el mGluR1a, siendo las más potentes la 4C3HPG y la 4CPG (Roberts, 1995; Jane et al., 1995).

Se ha demostrado por hibridación con el RNAm correspondiente, que la distribución de estos receptores en el SNC de la rata es heterogénea, aunque no es posible definir esto en términos cuantitativos; las regiones con las más altas concentraciones son: la capa de células de Purkinje, así como las células granulares del cerebelo, aunque no se ha definido su función en el cerebelo. En el area CA3 y el giro dentado del hipocampo se observa un nivel mayor de expresión que en la región CA1 y la substancia nigra, lo que junto con otras

evidencias ha permitido asociarlos con las funciones de memoria y aprendizaje. El tálamo, el bulbo olfatorio, el septum, el hipotálamo y los núcleos subtalámicos tienen un nivel moderado de expresión; la corteza cerebral, el estriado y la capa molecular del cerebelo tienen una concentración baja de estos receptores (Conn y Patel, 1994). Estos receptores no solo movilizan el Ca^{2+} intracelular, sino que al activar cinasas de proteína, pueden modular receptores para AAE de tipo ionotrópico alterando su estado de fosforilación, por lo que podrían intervenir en la modulación de la transmisión glutamatérgica rápida (Doherty et al., 1997).

b) Grupo II de receptores metabotrópicos a glutamato (mGluRII)

Los receptores del grupo II (mGluR2 y mGluR3) se han sobreexpresado por transfección en células CHO y BHK; en estas lineas derivadas de fibroblastos, la activación de estos receptores inhibe la estimulación de la AC producida por forskolina (Tanabe et al., 1993). Esta trasducción se inhibe totalmente con la toxina pertussis, sugiriendo que la proteína G involucrada en el acoplamiento pertenece a la familia Gi. En contraposición a los receptores del grupo I, el 1S,3R-ACPD es un potente agonista en este grupo, mientras que el QA es un agonista débil, aunque cabe aclarar que la sensibilidad al QA varía entre los miembros de este grupo, siendo la EC_{50} del QA para el mGluR2 de 1mM y para el mGluR3 de 40 μM, lo que permite diferenciarlos. El agonista DHPG, considerado más potente que el ACPD, es un compuesto específico para este

grupo. La L-CCG-I es aproximadamente 10 veces más potente que el 1S,3R-ACPD en los mGluR2, y para este último la MCPG es el único antagonista, pero su efecto es débil y también tiene efecto antagonista sobre el mGluR1a. El perfil farmacológico general de la potencia de los agonistas es: DCG-IV ≥ L-CCG-I > L-Glu ≥ DHPG ≥ (1S,3R)-ACPD > IBO > QA.

b) Grupo III de receptores metabotrópicos a glutamato (mGluRIII)

La DCG-IV es el más potente entre los derivados de la CCG, teniendo poca o nula actividad sobre los receptores de los grupos I y III. Paradójicamente, derivados de la fenilglicina como la 4CPG, 4C3HPG y 3C4HPG actúan como agonistas en el receptor mGluR2 y antagonistas en los mGluRI (Conn y Patel, 1994).

En este grupo, que incluye a los receptores mGluR4, 6, 7 y 8; el agonista más potente es el L-AP4 (Thomsen et al., 1992), seguido por el L-Glu. El 1S,3R-ACPD y el QA son menos activos o carecen de efecto. El orden de potencia de los agonistas es: L-AP4 > L-Glu ≥ L-CCG-I ≥ L-SOP. El mGluR4 se identifica por su alta sensibilidad al IBO, mientras que el mGluR6 es más sensible a la L-SOP que al L-Glu y su presencia se ha demostrado hasta ahora, exclusivamente en la capa interna de la retina. Estos receptores están acoplados a la inhibición de la AC, por consiguiente, su activación disminuye los niveles intracelulares de AMPc; se considera que estos receptores corresponden a receptores presinápticos cuya función es regular la liberación

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activadas por la vía de los fosfoinositidos. Esta cadena de reacciones se activa por 1S,3R-ACPD así como por QA, IBO y L-CCG-I, pero no por agonistas ionotrópicos como el NMDA o el KA. Puesto que este efecto no se bloquea por antagonistas de los receptores ionotrópicos, se postula que se debe a la activación de mGluRs.

d) Características estructurales de los mGluR

La secuencia de aa de los mGluRs es homóloga de la de otros receptores acoplados a proteínas G. Cada uno tiene un péptido señal, el dominio amino terminal del receptor propuesto como extracelular, 7 segmentos hidrofóbicos asociados muy estrechamente y que atraviesan la membrana, definidos como dominios transmembranales. El dominio carboxilo terminal, propuesto como intracelular es variable en longitud y poco conservado entre los miembros de esta familia de receptores. La secuencia más conservada corresponde a una región hidrofóbica localizada en el dominio extracelular, propuesta como el sitio de unión del ligando. Existen 21 residuos de cisteína que se conservan en todos los mGluRs; 9 de estos residuos están localizados en la porción carboxilo terminal. Los receptores poseen numerosos sitios de fosforilación en secuencias intracelulares y sitios de glicosilación extracelulares, propuestos como zonas de modulación (Pin y Duvoisin, 1995; Fig. 3).

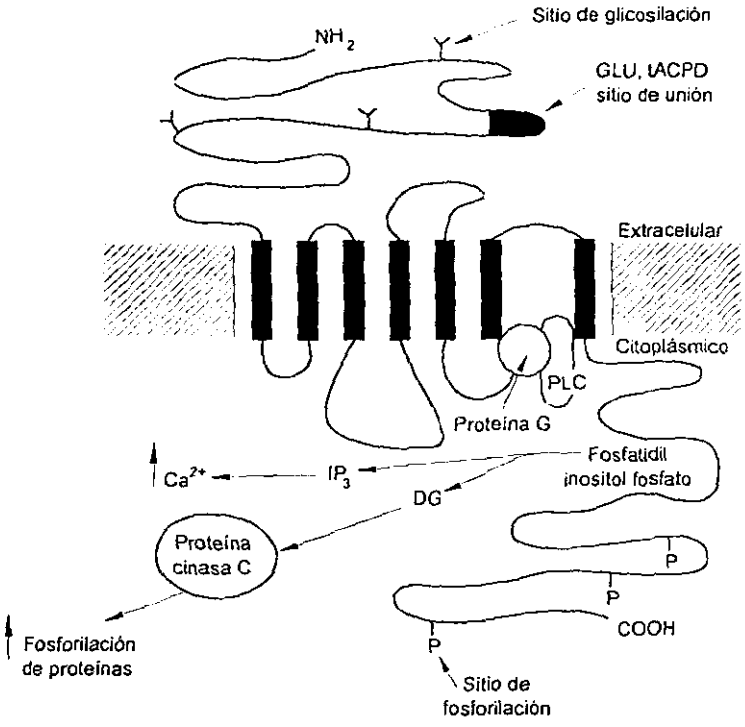

Figura 3. Diagrama de la estructura general de los receptores metabotrópicos para AAE. Abreviaturas: Ca^{2+}, ión calcio; COOH, extremo carboxilo; DG, diacilglicerol; IP_3, trifosfato de inositol; GLU, ácido glutámico; NH_2, extremo amino; PLC, fosfolipasa C; tACPD, (±)-1-aminociclopentano-trans-1,3-dicarboxilato. Estos receptores también se relacionan con la neurotoxicidad inducida por L-Glu en desórdenes neuroconvulsivos y neurodegenerativos como la Corea de Huntington y la enfermedad de Alzheimer. Asimismo participan en la regulación de la liberación del propio L-Glu.

e) Relación entre los mGluRs y la fisiología neuronal

Se ha demostrado que los mGluRs participan en muchas funciones cerebrales como fenómenos de plasticidad sináptica, desarrollo del sistema nervioso, la potenciación y la depresión a largo plazo que son la base fisiológica del aprendizaje y la memoria (Conn y Pin, 1997).

Papel de los mGluRs en el desarrollo.- La hidrólisis de fosfoinositidos inducida por la activación de mGluRs (específicamente mGluR5a), es particularmente elevada en el SNC durante el período inmediatamente posterior al nacimiento, y esta función disminuye progresivamente durante el resto del desarrollo postnatal. El acoplamiento entre los mGluRs y la fosfolipasa C durante los estados tempranos del desarrollo sugiere que estos receptores pueden participar en el desarrollo postnatal al proporcionar una señal trófica, promover la sobrevivencia y/o conferir neuroprotección contra la muerte celular apoptótica. Bruno et al.(1994) demostraron que en células granulares del cerebelo, el t-ACPD protege de la muerte celular apoptótica inducida por despolarización.

En la neurotoxicidad y neuroprotección.- El L-Glu es el principal agente neurotóxico, y responsable del daño que se observa en el cerebro en los cuadros de isquemia, anoxia e hipoglicemia. Se entiende por neurotoxicidad a la sobreactivación descontrolada de estos receptores, que desencadena

respuestas intracelulares tales como el aumento excesivo de Ca^{2+}. El Ca^{2+} es considerado un segundo mensajero que activa enzimas que en exceso, llegan a causar la muerte celular. La evidencia bibliografica indica que el receptor tipo NMDA es el principal involucrado por su alta permeabilidad al Ca^{2+}, sin embargo, los mGluRs también están involucrados, ya que el QA ha mostrado ser un potente neurotóxico en cultivos corticales; el grado de esta neurotoxicidad depende de la dosis y del tiempo de exposición, siendo las EC_{50} = 250 μM (5 minutos) y 1 μM (20-24 horas). La neurotoxicidad aguda del QA es independiente del Ca^{2+} extracelular e insensible a los antagonistas para los receptores ionotrópicos tipo NMDA y No-NMDA. Sin embargo, los mGluRs parecen también ejercer el papel contrario, porque el ACPD actúa como neuroprotector contra la toxicidad producida por NMDA en cultivos corticales de células granulares del cerebelo y de la retina (Koh et al., 1986). Esta neuroprotección se revierte con inhibidores de cinasas de proteína, como el H7 y el HA-1004. En este efecto protector participa probablemente una proteína G, ya que se revierte pretratando al tejido con toxina pertussis. Asimismo la L-CCG y la L-SOP que son agonistas de los mGluRs de los grupos acoplados negativamente a la AC (grupos II y III) pueden atenuar la neurotoxicidad del NMDA, indicando que en el efecto protector interviene la inhibición de esta enzima. Una posibilidad alternativa podría ser que la neuroprotección fuera el resultado de la inhibición de los canales de Ca^{2+} sensibles al voltaje debido a que generalmente, durante el daño agudo

cerebral, como trauma, hipoglicemia y epilepsia, la sobreestimulación del receptor de NMDA despolariza la membrana e induce la entrada excesiva de Ca^{2+} a las neuronas a través de estos canales. Por su ubicación en terminales presinápticas, se considera que los receptores mGluRI participan en la neurotoxicidad al estimular la liberación del AAE endógeno y por localizarse en la periferia del botón presináptico, sólo se activarían cuando la concentración del neurotransmisor fuera excesiva. Mientras que los receptores acoplados de manera inhibidora a la AC como los mGluRII y III se consideran neuroprotectores porque ubicados en la presinapsis bloquean la liberación del L-Glu.

En la potenciación a largo plazo.- La LTP que consiste en el aumento sostenido de la eficiencia sináptica, considerada como la facilitación neuronal para responder a las señales provenientes de otras células que induce una estimulación eléctrica de alta frecuencia en vías cerebrales específicas de los mamíferos, es hasta ahora el modelo más aceptado para el aprendizaje y la memoria. La LTP requiere un aumento en la concentración de Ca^{2+} libre en el citoplasma a través de canales membranales. Esta entrada de Ca^{2+} puede ocurrir a través de 2 canales distintos: a) los canales del receptor tipo NMDA, b) a través de canales de Ca^{2+} dependientes de voltaje; el aumento intracelular de Ca^{2+} se consigue también a través de su liberación de almacenes intracelulares por medio de receptores para IP_3, que se produce por la

activación de la PLC acoplada a mGluRs. Existe evidencia que apoya la participación de los mGluRs en el hipocampo, entre la que se incluye el que la LTP se bloquee por inyección intracerebro-ventricular de toxina pertussis, y la proteína G sensible a esta parece localizarse en la presinapsis de las fibras musgosas de la region CA3 del hipocampo. El D,L-AP3 (1mM) reduce la potenciación a largo plazo en las mismas fibras musgosas; las neuronas piramidales del area CA3 del hipocampo y células granulares del núcleo dentado tienen niveles altos de RNAm para los mGluR1 y 5 (Monaghan et al., 1996).

La técnica para la medición de receptores postsinápticos in situ, que emplea un radioligando, fue desarrollada originalmente para el estudio de interacciones hormona-receptor y aplicada a los aa por Snyder y Bennett, y ha sido usada frecuentemente para diversos neurotransmisores. Esta técnica consiste en cuantificar la interacción del mismo neurotransmisor propuesto o de un agonista o antagonista especifico, de alta actividad especifica, con las membranas. Para receptores de aa, cuando el radioligando es el mismo aminoácido, es necesario discriminar entre los sitios de unión postsinápticos y los sitios de recaptura del compuesto. Es posible hacer esta diferenciación por medio de manipulaciones técnicas al medir los sitios de unión, así como por la caracterización farmacológica de la interacción. Así la unión del ligando a los sitios de recaptura requiere de Na^+, depende de la temperatura, y los sitios se inactivan con la congelación, mientras que la unión a los receptores sinápticos

no se afecta por el Na^+, no depende de la temperatura, y en ocasiones se hace más evidente al congelar y descongelar las membranas.

Receptores a AAE en el EPR

Con respecto a la relación que existe entre la fisiología del EPR y los receptores para AAE, se ha demostrado que el L-glutamato estimula el desprendimiento de los discos de los fotorreceptores (Greenberger y Besharse, 1985), y se ha sugerido que los AAE actúan vía un receptor del tipo del ácido kaínico para el inducir el desprendimiento de los mismos (Besharse et al., 1986).

López Colomé y colaboradores (1993, Artículo I;), demostramos que los receptores para aminoácidos excitadores del epitelio pigmentado de la retina del pollo son similares, pero no idénticos a los estudiados en el SNC (Monaghan et al., 1989). La unión de L-glu-H^3 en cultivos de epitelio pigmentado de retinas de pollo es farmacológicamente sensible a los agonistas y antagonistas de los receptores de NMDA, así como al agonista de los receptores metabotrópicos, t-ACPD. Por otra parte, la glicina incrementa considerablemente la unión del L-glu-H^3 a través de un mecanismo que depende de la edad de cultivo y cuyos agonistas pueden ser los aminoácidos taurina y con mucho menor potencia el GABA (López.-Colomé y Fragoso, 1995, Artículo II).

Utilizando la técnica de unión del ligando radiactivo L-glu-H^3, también

demostramos que existen receptores específicos para este neurotransmisor en células cultivadas de EPR humano (López-Colomé et al., 1994, Artículo III). Estos receptores, así como en el EPR de pollo, se modulan por los aminoácidos glicina, taurina y GABA, aumentando la unión del ligando de manera inversamente proporcional a la edad del donador. Las características farmacológicas de unión indican que exiten receptores ionotrópicos de tipo NMDA, y receptores metabotrópicos sensibles a t-ACPD en este sistema celular.

A continuación se presentan los artículos correspondientes a estos estudios:

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Specific Interaction of Glutamate With Membranes From Cultured Retinal Pigment Epithelium

A.M. López-Colomé, R. Salceda, and G. Fragoso
Instituto de Fisiología Celular, U.N.A.M. México, D.F. México

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 $K_D = 333$nM 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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Address reprint requests to Dr. A.M. López-Colomé, Instituto de Fisiología Celular, U.N.A.M, Apartado Postal 70-600, 04510 México D.F., (49) Mexico.

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; Sweatt 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 (López-Colomé 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 (López-Colomé et al., 1991). Eyes were enucleated, freed from vitreous 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 (López-Colomé 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-Colomé, 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]L-Glutamate (spec.act. 35–59 Ci/mmol) was obtained from New England Nuclear (Boston, MA).

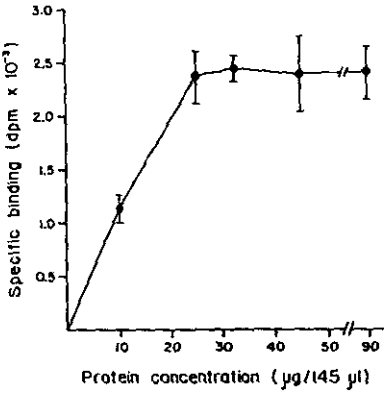

Fig. 1. Protein-dependence of [^{3}H]L-glutamate binding. Frozen/thawed membranes from RPE cultures of 16 DIV were used. Assay was performed as described in Materials and Methods, at 37°C. [^{3}H]L-glutamate was 50 nM. Data are the mean ± S.D. of four experiments performed in triplicate.

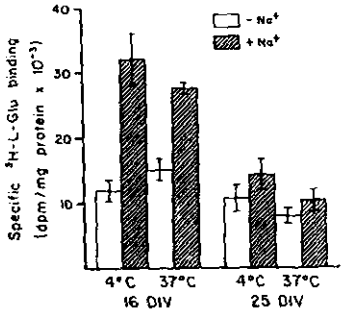

Fig. 2. Effect of temperature and sodium on [^{3}H]L-glutamate binding. Membranes were isolated from cultures of 16 and 25 DIV and binding was measured as described in Materials and Methods. [^{3}H]Glutamate concentration was 50 nM. Results are expressed as the mean ± S.D. of seven independent experiments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

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, 1981) 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).

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^{++}	0.335 ± 0.020	74
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 ± 0.009 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

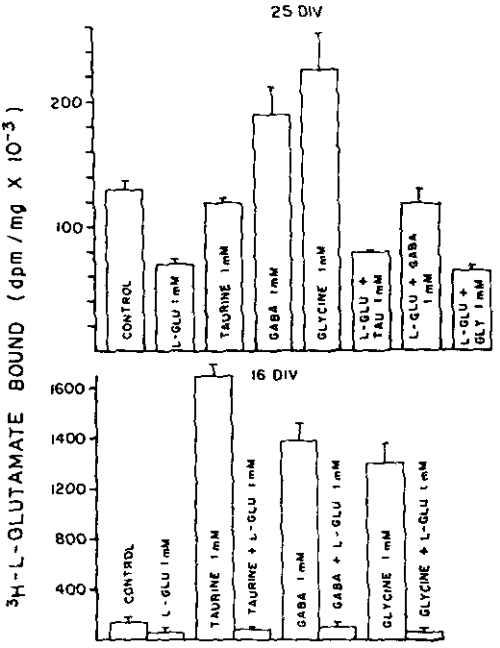

Fig. 3. Enhancement of glutamate binding by inhibitory amino acids. Experiments were performed in Na^+-free buffer, in the presence of 50 nM [^{3}H]glutamate. The ordinate axis represents total binding. Data are the mean ± S.D. of 10 experiments performed in triplicate. In all cases, specific binding 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, 16.9 ± 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 a B_{max} = 3.2 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)	1.705 ± 0.045
D-Serine (1mM)	0.929 ± 0.020
HA-966 (100 μM)	1.088 ± 0.030
7-Chlorokynurenate (100 μM)	0.095 ± 0.015
Glycine (1mM) + strychnine (100 μM)	1.599 ± 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-hydroxy3-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 (López-Colomé, 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 site 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

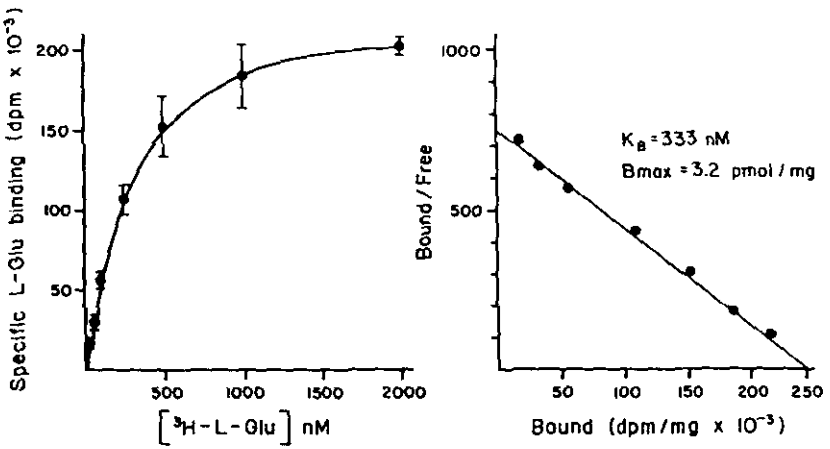

Fig. 4. Saturation curve and Scatchard analysis of [^{3}H]glutamate binding. Specific binding was measured in frozen/thawed membranes from 16 DIV cultures at 37°C, in the absence of sodium. Data were analyzed using the INPLOT (Version 3.1) program from GraphPAD Software, San Diego, CA. Hill coefficient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

presence of magnesium, as has been shown for 3-[(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonate (CPP) (López-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 (López-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.

ACKNOWLEDGMENTS

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ARTÍCULO II

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Glycine Stimulation of Glutamate Binding to Chick Retinal Pigment Epithelium

A.M. López-Colomé[1] and G. Fragoso[1]

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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_{50} = 200 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 (mGluRs), 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, and decreased with increasing 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 participation in several aspects of RPE physiology, including phagocytosis and cell division.

KEY WORDS: Glutamate receptors; retina; cell culture; glycine; taurine.

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, addition-

[1] Instituto de Fisiología Celular, U.N.A.M. Apartado Postal 70-253, 04510, México, D.F., México.

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.] cycloheptan-5, 10-imine; PIP_2, phosphatidyl inositol bisphosphate. ED = embryonic day; DIV = days in vitro; RPE, retinal pigment epithelium; EAA, excitatory amino acids.

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ally, Tau promotes the proliferation of RPE cells (9). 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 function, the chemical communication between these tissues is still poorly understood. Glutamate (L-Glu) and other excitatory amino acids (EAA) have been shown to induce phagocytosis in RPE (10,11), 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_2) 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 $[Ca^{2+}]$ 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 those described for CNS cells (18,21). Pharmacologically, these receptors are sensitive mainly to agonists and antagonists of NMDA receptors and to the mGluR agonist trans-ACPD (19,20). Glycine, which has been identified as an NMDA coagonist (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 0.13% trypsin pH 7.4, RPE cells were dissociated and seeded at a density of 10^5 cells per Falcon Flask of 25 cm^2 growth area. Cultures were maintained in TC-199 medium supplemented with 10% heat-inactivated fetal bovine serum, 2.0 mM glutamine, 100 Units/ml of penicillin and 100 mg/ml streptomycin. Cells reached confluency at 16 days in vitro (DIV) 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 45,000 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 30–50 µg protein per assay (175 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 unlabeled glutamate. Agonists and antagonists, when tested, were added instead of glutamate. Reaction was terminated by dilution and further filtration on glass fiber filters (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. [^{3}H]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 [^{3}H]L-Glu Binding to Membranes from Intact RPE: Effect of Gly, Tau, and GABA. Binding of [^{3}H]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

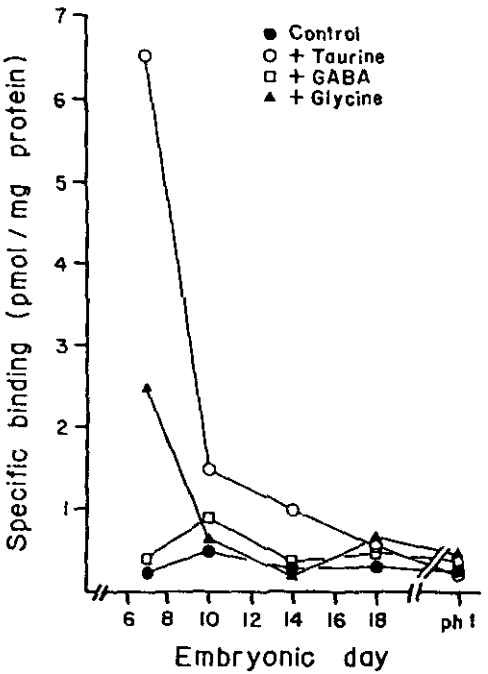

Fig. 1. Effect of Gly, Tau and GABA on [³H]L-Glu binding to embryonic RPE. RPE was obtained from chick embryos at the indicated age, and membranes were prepared as described in Methods. [³H] Glu binding (50 nM) was determined in the absence (control) or presence of 1 mM Gly, Tau or GABA. 1 mM unlabeled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

Table I. Effect of Taurine, GABA, and Glycine on [³H]L-Glu Binding to Membranes from Brain and Retina

	Specific [³H]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. [³H]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 ± SEM of 4 experiments performed in triplicate. ED, embryonic day.

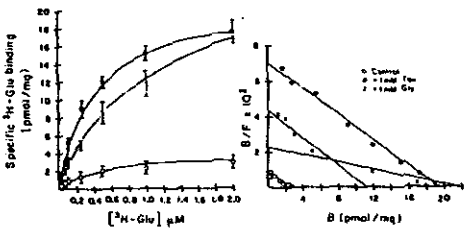

Fig. 2. Effect of Gly and Tau on the kinetic parameters of Glu-binding. Experiments were performed as described in Methods. Saturation curve and Scatchard analysis were studied in membranes from 16 DIV cultured RPE cells. [³H]L-Glu concentration was increased from 25 to 2000 nM; 1 mM unlabeled L-Glu was used for defining non-specific binding. Results are expressed as the mean ± SEM of 4 experiments performed in triplicate. Data were analyzed using the INPLOT (version 3.1) program from Graph PAD software, San Diego, CA. Hill coefficients were >0.80 in all cases.

post-hatching. As can be seen in Fig. 1, with the exception of ED 10 in which specific binding was significantly higher (0.477 ± 0.008 pmol/mg protein), values remained similar to those in the mature eye (0.268 ± 0.009 pmol/mg protein).

1 mM Gly or Tau significantly increased specific binding in early developmental stages; only Gly showed an effect in the mature tissue (Fig. 1). Although GABA also stimulated binding, this 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 [³H]L-Glu Binding to Membranes from Retina and Brain. 1 mM concentration of Gly, Tau, or GABA did not affect [³H]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 [³H]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 of 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 19.8 pmol/mg protein, as compared to B_{max} in the absence of the amino acid which was 3.2 pmol/mg protein; no significant change in affinity was seen: K_D = 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

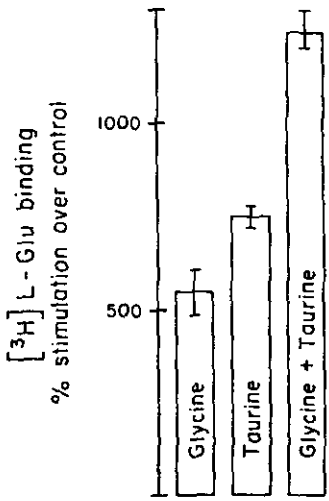

Fig. 3. Additivity of the effect of taurine and glycine on [^{3}H]L-Glu binding. Membranes from 16 DIV cultures were prepared as described in the methods section, the same as the binding assays. [^{3}H]L-Glu concentration was 50 nM, and glycine, taurine or both, were added at 1 mM concentration. Data 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.

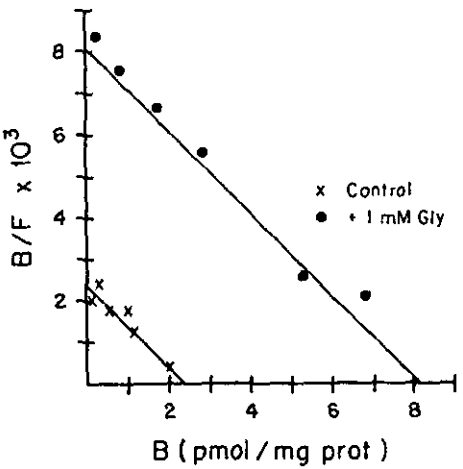

Fig. 4. Scatchard analysis of Glu binding to membranes from cultures of 25 DIV. Saturation curve, Scatchard analysis and statistics were as described for Fig. 2. Data are the mean ± SEM of 3 experiments in triplicate.

the K_d (259 nM). However, in the presence of Gly a second, lower affinity binding site for Glu was apparent, with K_d = 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} = 2.3 to B_{max} = 8.1 pmol/mg protein.

Effect of Gly and Tau on the Pharmacological Properties of [^{3}H]L-Glu Binding. Membranes obtained from 16 DIV cultures were preincubated for varying periods of time with 1 mM Gly or 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 16 DIV cultures, age in which cells posses two binding sites for L-Glu. The efficacy of the different agonists and antagonists of EAA-receptors (25 DIV) for displacing Glu at 1 mM concentration was L-Glu > QA= KA= NMDA= AP5> 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≥ CNXQ≥ 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

Table II. Displacement of [³H]L-Glu Binding by Related Compounds in Membranes from 16 and 25 DIV Cultures

Displacer		Specific [³H]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)	0.032 ± 0.009	0.363 ± 0.077
NMDA	(1 mM)	0.289 ± 0.009	0.117 ± 0.065
CPP	(200 μM)	0.129 ± 0.010	0.225 ± 0.052
MK-801	(5 μM)	0.269 ± 0.006	0.187 ± 0.006
D-AP5	(1 mM)	0.261 ± 0.006	0.232 ± 0.010
L-AP4	(1 mM)	0.180 ± 0.008	0.400 ± 0.063
L-AP3	(1 mM)	0	0.428 ± 0.024

Membranes were prepared as described in Methods. The concentration of [³H]L-Glu was 50 nM; total binding was 0.529 ± 0.060 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-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.] cyclohepten-5,10-imine; D-AP5, D-2-amino-5-phosphonopentanoate; L-AP4, L-2-amino-4-phosphonobutyrate; L-AP3, L-2-amino-3-phosphonopropionate. Results are the mean ± SEM of 3 independent experiments performed in triplicate. *Some of these compounds were previously tested (12), but a new series of experiments was performed in order to compare with new compounds.

Table III. Kinetic Constants of [³H]L-Glu Binding to Cultured RPE Membranes

Age in culture	[³H]Glu	[³H]Glu + Tau	[³H]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 = 958 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.

to ionotropic receptors is increased by these agents. Additionally, 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} ≈ 200 μM in membranes from cultures of

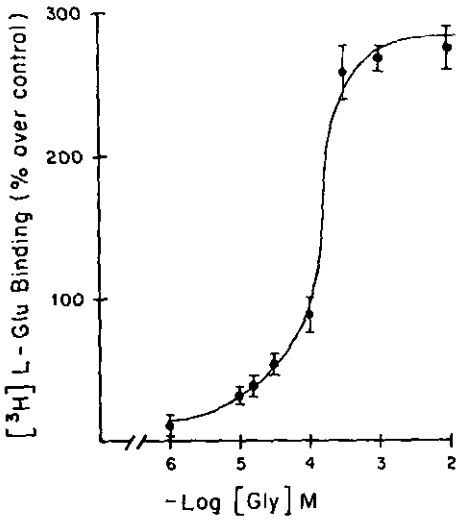

Fig. 5. Concentration dependence of Gly-induced increase in [³H]L-Glu binding. Glu binding to membranes from 25 DIV cultures was measured in the presence of concentrations of Gly from 1 μM to 10 mM. The concentration of [³H]L-Glu was 50 nM; 1 mM unlabeled Glu was used as displacer. Results are the mean of 3 experiments in triplicate ± SEM.

Table IV. Effect of 7-Chlorokynurenate and Strychnine on Stimulated Glutamate Binding

Additions	Total binding (pmol/mg protein) 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
Glycine + Strychnine	0.694 ± 0.050	0.680 ± 0.024

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-chorokynurenate. All compounds were added simultaneously at 100 μM concentration. [³H]L-Glu concentration was 50 nM.

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 receptor sites for [³H]L-Glu in chick RPE. Bind-

ing to these sites 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 25 DIV are compared, in younger tissue compounds known to interact with mGluRs 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 (16 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 25 DIV, the high-affinity Glu-binding site is not present, and Gly but not Tau promotes binding of L-Glu to the low affinity site (Fig 4, Table III); the pharmacology of this site is closer to an ionotropic type 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 agonist 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 (18). 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. 3): one specific for Gly, and another at which Tau can also act. The latest is present only at immature, actively dividing stages of the culture, whereas the first remains up to the differentiated non-dividing stage. The general decrease in 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 from 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 (13). 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 is known regarding the functions of EAA in RPE. We have suggested a role for these compounds in phagocytosis and/or cell division (12,13), since calcium entry is an underlying requisite for both. The mGluRs could be involved in cell differentiation, probably through the activation of PIP_2 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 on the biochemical characteristics of RPE EAA receptors will be of help in the understanding of some retinal pathologies involving retina - RPE communication.

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Current Eye Research

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

A. M. López-Colomé, G. Fragoso, C. E. Wright[1] and J. A. Sturman[1]

Instituto de Fisiología Celular, UNAM, Apartado Postal 70-253, 04510, México, D. F. México and [1]Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314, USA

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_D) 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

Correspondence: Dr John A. Sturman, Department of Developmental Biochemistry, Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314, USA

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×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 I. 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.

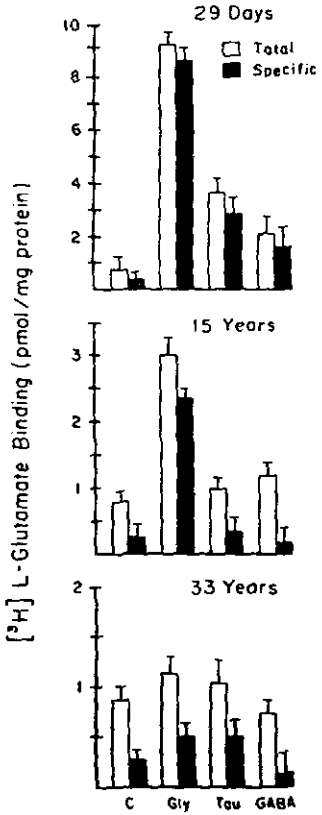

Figure 1. Effect of glycine, taurine and GABA on [^{3}H]L-Glu binding to RPE membranes from donors of different age. Experiments were performed as described in methods, in the presence of 1 mM glycine, taurine or GABA. For control values, experiments were performed without additions. 1 mM L-Glu was added 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.

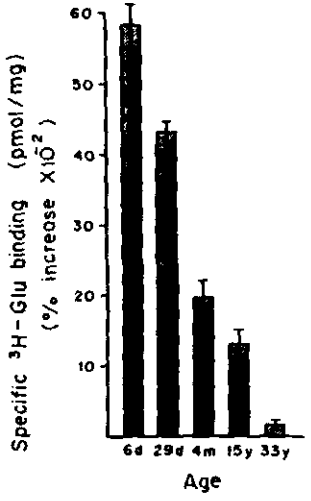

Figure 2. Decrease in glycine-stimulation of [^{3}H]L-Glu binding with the age of donor. The concentration of [^{3}H]L-Glu was 50 nM; 1 mM L-Glu was added for defining non-specific binding. Results are the mean ± S.E.M. of the means of three experiments each performed in triplicate.

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

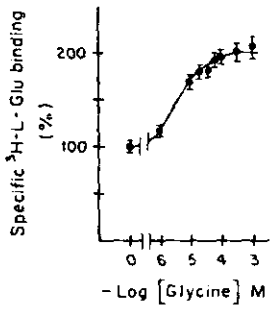

Figure 3. Dose-dependence of glycine stimulation of [^{3}H]L-Glu was determined as described in Methods. Membranes were from a 33 year-old donor. [^{3}H]L-Glu concentration was 50 nM. Results are expressed in pmol/mg protein and represent the mean ± S.D. of the means of 3 independent experiments each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

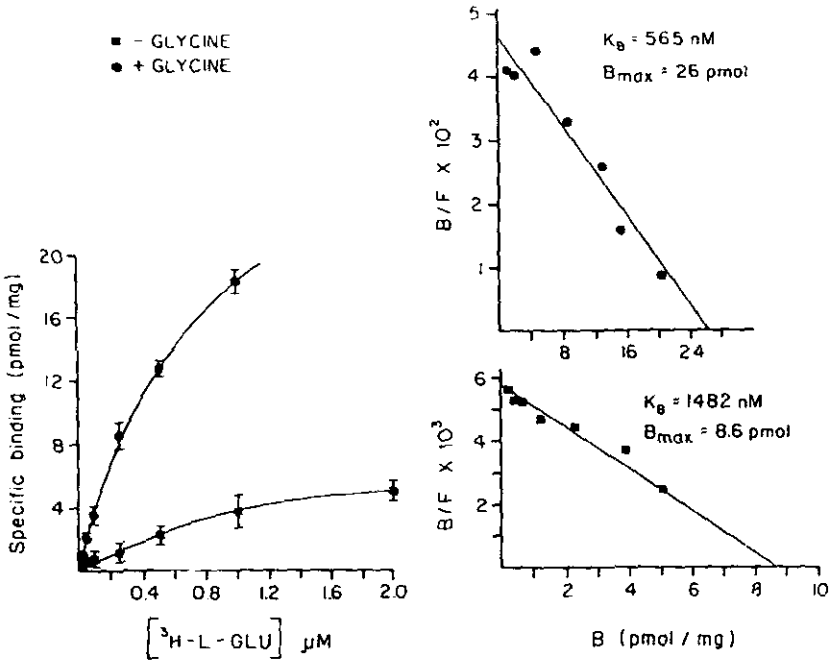

Figure 4. Saturation curve and Scatchard analysis of Glu binding to RPE membranes from a 29 day-old donor. Experiments were performed as described in Methods, using [³H]L-Glu within a concentration range of 50–2000 nM, in the absence or presence of 1 mM Glycine. Results are the mean ± S.D. of the means 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 [³H]L-Glu binding to RPE membranes

Saturation curves using [³H]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. [³H]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

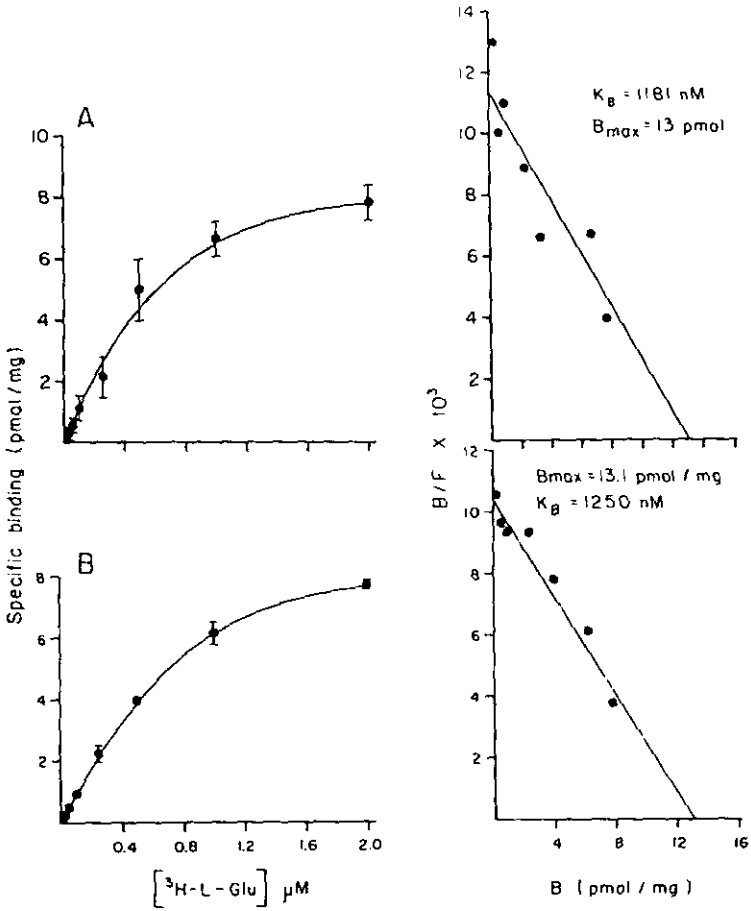

Figure 5. Saturation curve and Scatchard analysis of Glu binding to RPE membranes from a 15 year old (A) and a 33 year-old (B) donors. Experiments were as described in Methods. [^{3}H]L-Glu concentration was increased from 50 to 2000 nM, and non-specific binding was defined with 1 mM cold Glu. No glycine 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.

Acknowledgements

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OBJETIVO

El estudio farmacológico de los receptores se facilita mediante el uso de cultivos celulares, ya que constituyen una población homogénea de los tipos celulares de interés. Se ha demostrado que las células del EPR en cultivo retienen la mayor parte de las características que expresan *in vivo*, por lo que gran parte de las funciones del mismo se han estudiado en este sistema. Previamente reportamos la presencia de receptores específicos para el L-glu en células del EPR de pollo (López-Colomé et al., 1993; 1995) así como el EPR humano (López-Colomé et al., 1994) en cultivo primario. Las características bioquímicas y farmacológicas de estos receptores son similares en ambos casos, y sugieren la participación de los AAE tanto en la inducción de la fagocitosis como en el control de la proliferación de las células del EPR. Con el fin de definir la participación de los AAE como posibles mediadores en la inducción de la fagocitosis en el EPR, se estudiaron los mecanismos de transducción activados por dichos compuestos en cultivos primarios de EPR de pollo. Se caracterizó la hidrólisis de fosfatidil-inositol bifosfato y la síntesis de AMPc como productos de la activación de los receptores a AAE. Los resultados obtenidos acerca del mecanismo normal que inducen los AAE en el EPR nos permitirá en el futuro determinar su relación con la fagocitosis y las condiciones patológicas en las que el EPR se altera.

MÉTODOS

Cultivo. Se utilizaron embriones de pollo de 7 días de desarrollo de los cuales se extrajo la copa óptica en condiciones estériles; a ésta se le hizo un corte ecuatorial, se desechó la parte anterior y de la posterior se aisló el EPR . Se lavó el tejido con Krebs Ringer Bicarbonato (KRB), 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 5.6 mM y $NaHCO_3$ 35 mM y se centrifugó 5 minutos a 500 rpm a temperatura ambiente; la pastilla que se obtuvo se resuspendió en solución KRB fresca y se centrifugó en la mismas condiciones. Se repitió la operación 3 veces. La disociación de las células se hizo por incubación con tripsina 0.13% en amortiguador de fosfatos durante 5 minutos a temperatura ambiente. Las células se sembraron en placas de 12 multipozos 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 pozo y se mantuvieron en un incubador Lab-Line a una temperatura constante de 37 °C en ambiente húmedo y gaseado con 95:5 de CO_2:O_2 durante 21-25 días. La identidad de las células y la pureza del cultivo se establecieron por la morfología epiteloide y la presencia de melanina en todas las células (Salceda et al., 1992).

Cuantificación de fosfatos de inositol. Para cuantificar la producción de fosfatos de inositol en las células del EPR, cada pozo de cultivo se incubó con

mio-inositol-H^3 (2 μCi/pozo) durante 20-24 horas. Posteriormente se lavó 3 veces cada pozo con KRB para eliminar el compuesto radiactivo no incorporado y se mantuvo durante 10 minutos en KRB conteniendo 1mM de LiCl. Los agonistas de AAE se añadieron a los pozos y se incubaron a diferentes tiempos a 37 °C según se indica en la sección de resultados. La reacción se detuvo añadiendo a cada muestra 1 ml de cloroformo/metanol (1:2), 0.75 ml de H_20 y 0.5 ml de cloroformo. Las muestras así obtenidas, se centrifugaron para separar la fase acuosa, que contiene los fosfatos marcados radiactivamente con tritio de la fase lipídica. Los fosfatos de inositol se separaron por cromatografía de intercambio aniónico en una columna DOWEX AG1-X8 (100-200 mesh, forma formiato), mediante una modificación de la técnica de Berridge (1989). Las muestras se colectaron en viales y las desintegraciones por minuto (DPM) se obtuvieron en un contador de centello líquido.

Cuantifiación de proteína. En todos los experimenos se determinó la concentración de proteína por el método de Lowry et al (1951), utilizando albúmina sérica de bovino (Sigma) como estándar.

Reactivos. El mio inositol-H^3 (actividad específica: 20.0-23.45 Ci/mmol) fué de DuPont NEN Research Products. El medio de cultivo TC-199 y el suero de bovino fetal se adquirieron de Difco(Detroit, Michigan, USA). Los agonistas y

antagonistas del L-Glu se obtuvieron de Tocris Cookson Inc. (St. Louis Mo 63146, U.S.A.), con excepción del L-Glu, el KA y la nifedipina que fueron de Sigma y el MK-801 obtenido de Research Biochemicals International. Los demás reactivos fueron de Sigma Chemical Co. (St Louis, Mo, USA).

RESULTADOS

Eficiencia de agonistas generales del L-Glu para inducir la acumulación de IPs-H^3

La fig. 4 muestra la estimulación inducida por los agonistas generales para los diferentes tipos de receptores para AAE tanto ionotrópicos como metabotrópicos. El orden de eficiencia de los agonistas a la concentración de 1 mM fué: NMDA ≥ L-glu > QA ≥ KA > ACPD. El NMDA y el L-glu son los agonistas más eficientes al aumentar la hidrólisis de fosfoinosítidos 220% y 215% respectivamente, mientras que el ACPD es el menos eficiente (120%).

La curva de tiempo en la figura 5 indica que desde un minuto de estimulación hay una respuesta evidente, y el estímulo máximo se alcanza a partir de los 15 minutos de estimulación. En experimentos subsiguientes, el tiempo de estimulación fué de 15 minutos.

Potencia de los AAE en la inducción de la hidrólisis de PIP_2

Se elaboraron curvas dosis-respuesta de la acumulación de IPs-H_3 inducida por agonistas de los diferentes subtipos de receptores de AAE, en un intervalo de concentraciones entre 10 μM y 1.0 mM. Las EC_{50} se calcularon a partir de las gráficas correspondientes. En la Fig. 6 se observa que el orden de potencia de los agonistas probados fué: ACPD (EC_{50} = 45 μM) > L-glu (EC_{50} = 72 μM) > NMDA (EC_{50} = 135 μM) > QA (EC_{50} = 185 μM) > KA (EC_{50} = 255 μM). El compuesto más potente y menos eficiente fue el agonista metabotrópico

ACPD, mientras que los más eficientes fueron el agonista ionotrópico NMDA y el agonista general L-glu.

Farmacología del efecto ionotrópico de los AAE sobre la acumulación de IPs-H^3

La especificidad de la inducción de la síntesis de IPs-H^3 por los agonistas de los diferentes subtipos de receptores a L-Glu se determinó mediante el uso de antagonistas específicos (Tabla I). El efecto del agonista general L-glu se inhibió por el antagonista del NMDA , AP5 (45%), así como por la DNQX, antagonista de los receptores AMPA/KA (53%). La estimulación por KA se inhibió 22% por la DNQX y 31% por CNQX, mientras que la estimulación inducida por el agonista metabotrópico L-AP4, no se inhibe por las quinoxalinas, pero sí por el antagonista metabotrópico MCPG (21%). Asimismo se estudió el efecto de antagonistas especificos de NMDA, el agonista ionotrópico más eficiente para inducir la hidrólisis de PIP_2. Los antagonistas competitivos del sitio de reconocimiento del receptor, AP5 y CPP inhibieron la estimulación producida por NMDA 200 μM, siendo más potente el CPP. Asimismo, el MK-801, bloqueador de canal abierto del receptor, inhibió eficientemente el efecto del NMDA (Tabla I).

Efecto de agonistas y antagonistas de los mGluR sobre la acumulación de IPs-H^3

La Fig. 7 muestra el estudio farmacológico de la respuesta inducida por el ACPD, y la DHPG, agonistas generales de los receptores metabotrópicos para los AAE (Pin y Duvoisin, 1995). La DHPG estimula la síntesis de IPs con mayor eficiencia que el ACPD. La MCPG, antagonista general de los receptores metabotrópicos (Roberts, 1995), bloqueó la estimulación inducida por ACPD a la concentración de 1 mM. Por otra parte, la respuesta inducida por concetraciones máximas de la DHPG no se modifica por la adición simultánea de dosis máximas de ACPD, lo que demuestra un mismo sitio de acción de estas drogas.

Dependencia de Ca^{2+} externo en el efecto de los AAE

La estimulación por NMDA requiere la presencia de Ca^{2+} externo (Fig. 8), puesto que se inhibe notablemente en presencia de 1mM de EGTA. La inhibición es más notable en el caso de los agonistas ionotrópicos QA, NMDA y KA; tanto el agonista general L-Glu como el agonista metabotrópico L-AP4 presentan una estimulación menor en ausencia de Ca^{2+} externo (Tabla 1). Con el objeto de determinar la vía de entrada del Ca^{2+} externo, se estudió el efecto de los bloqueadores de los canales de Ca^{2+} sensibles al voltaje nifedipina (tipo L) y verapamil (tipo T), así como los inhibidores de transportadores intracelulares de Ca^{2+} dantroleno y tapsigargina sobre la estimulación por

NMDA 200 μM. Como se observa en la Fig. 8, todos estos compuestos disminuyeron parcialmente la estimulación.

Efecto de los AAE sobre la concentración de AMPc

Se ha demostrado que los AAE activan la síntesis de AMPc en algunas preparaciones a través de su interacción con los mGluRs I (Schoepp y Conn, 1993). Con el fin de explorar esta posibilidad, se estudió el efecto de los agonistas L-Glu, L-Asp y NMDA a concentración saturante (1 mM), sobre la concentración de AMPc, sin observarse ningún efecto (Tabla 2). La concentración de AMPc no se modificó por la presencia de AAE en el medio. Ninguno de los agonistas metabotrópicos o ionotrópicos estimuló o inhibió la síntesis de este segundo mensajero. La forskolina, activador directo de la AC, aumentó la síntesis de AMPc, sin embargo este efecto no se modificó en presencia de L-glu ó L-asp (1 mM), agonistas generales de los receptores para AAE, añadidos minutos antes que la FKS. Por otra parte, el aumento en la síntesis de AMPc producido por la estimulación de los receptores colinérgicos (Onali et al., 1994) tampoco se modificó por estimulación con L-glu 1 mM (Tabla 2).

Artículo IV

EXCITATORY AMINO ACID-INDUCED INOSITOL PHOSPHATE FORMATION IN CULTURED RETINAL PIGMENT EPITHELIUM

GABRIELA FRAGOSO[1] AND ANA MARIA LOPEZ-COLOME [1,2]

[1]Instituto de Fisiología Celular, U.N.A.M. [2]Department of Biochemistry, Faculty of Medicine, U.N.A.M. Apartado Postal 70-253, 04510, México, D.F., México

Aceptado en Visual Neuroscience

Correspondence:
Dr. Ana Maria López-Colomé
Instituto de Fisiología Celular, U.N.A.M.
Apartado Postal 70-253
04510, México, D. F., México
acolome@ifcsun1.ifisiol.unam.mx
Tel. (525)622-56-17
Fax: (525)622-56-07

Abstract

Excitatory amino acid (EAA)-induced production of inositolphosphates (IPs) was studied in primary cultures of chick retinal pigment epithelium (RPE) following *in vitro* incorporation of [^{3}H] myo-inositol. Glutamic acid (L-glu) significantly increased [^{3}H]-IPs accumulation (215 %). L-glu agonists stimulated [^{3}H]IPs accumulation in the following order of efficiency: N-methyl-D-aspartate (NMDA) ≥ L-glu > quisqualate ≥ kainate > (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD). Stimulation was dependent on external Ca^{2+}. The NMDA-induced response was blocked by (+)-5-methyl-10,11-dihydro-5H-dibenzo-cyclohepten-5,10-imine maleate (MK-801) and 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) and was decreased by the L-Ca^{2+}-channel blockers verapamil and nifedipine as well as by dantrolene. The metabotropic glutamate receptor (mGluR) antagonist (+)-α-methyl-4-carboxyphenylglycine inhibited 3,5-dihydroxyphenylglycine and ACPD-induced stimulation, which demonstrates the presence in RPE of mGluRs 1 and/or 5, as well as NMDA receptors, coupled directly, or through the influx of external Ca^{2+}, to phospholipase C-γ activation. L-glu agonists showed no effect either on basal level of intracellular cyclic adenosine monophosphate, nor on forskolin- or carbachol-induced stimulation of adenylyl cyclase. Since L-glu is released from the retina upon illumination, and receptors for this compound are present in RPE, the activation of the inositide pathway could be involved in the regulation of retina-RPE interaction, which is essential for the visual process.

Keywords: Glutamate receptors; Retinal pigment epithelium; Inositol phosphates.

Abbreviations

L-glu, L-glutamate; QA, quisqualate; KA, kainate; NMDA, N-methyl-D-aspartate; ACPD, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid; L-AP4, L-2-amino-4-phosphonobutyric acid; CNQX, 6-cyano-7-nitroquinoxaline-1,3-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-cyclohepten-5,10-imine maleate; PIP_2, phosphatidyl-inositol bisphosphate; DIV, days *in vitro*; RPE, retinal pigment epithelium; EAA, excitatory amino acids.

Introduction

The retinal pigment epithelium (RPE), plays a pivotal role in the maintenance of the structural and functional integrity of the neural retina, since it participates in functions such as transepithelial transport of nutrients and minerals (Steinberg & Miller, 1973), light and dark adaptation, storage and conversion of vitamin A esters, synthesis of the acid-mucopolysaccharide complex, ensheathing of the outer segments of photoreceptors (Zinn & Benjamine-Henkind, 1979), and also undertakes the phagocytic function, essential for the turnover of the rod outer segment discs (Hogan et al., 1974; Young & Bok, 1969).

L-Glutamate (L-glu) is considered as a primary neurotransmitter in excitatory synaptic pathways within the central nervous system (for review, see Mayer & Miller, 1990; Monaghan et al., 1989; Nakanishi, 1992), however, excessive glutamatergic stimulation is also involved in the etiology of stroke, epilepsy and neurodegenerative disorders (Choi, 1988). Receptors for L-glu can be classified into two distinct groups based on their signal transduction mechanism: 1) ionotropic glutamate receptors, which include N-methy-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), or kainic acid (KA; MacDermott et al., 1986; Murphy et al., 1987), and 2) metabotropic glutamate receptors (mGluRs), coupled to G-proteins (Mayer & Miller, 1990; Monaghan et al., 1989, Nahorski & Potter, 1989; Schoepp and Conn, 1993), which include at least 8 subtypes and a few additional variants

(Pin & Duvoisin, 1995). These, in turn, are classified into three different groups (Pin & Duvoisin, 1995). The phospholipase C (PLC)-coupled receptors (mGluR1, mGluR5 and their variants) constitute group I and are highly sensitive to quisqualate (QA; Aramori & Nakanishi, 1992), in contrast to the second group, negatively coupled to adenylyl cyclase (mGluR2 and mGluR3), at which (2S,3S,4S)-α-(carboxycyclopropyl)glycine (L-CCG-I, Hayashi et al., 1992) is the most selective agonist. L-2-amino-4-phosphonobutyric (L-AP4) acid and L-serine-O-phosphate (L-SOP) act as potent agonists of the third group, composed of mGluR4, mGluR6, mGluR7 and mGluR8, also coupled to the inhibition of the cyclase (Thomsen et al., 1992; Tanabe et al., 1992). (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) is a non selective agonist for all mGluRs (Palmer et al., 1989; Shoepp et al., 1992).

The inositol phosphate/diacyl glycerol (IP/DAG) pathway is triggered by receptor-mediated activation of PLC (Berridge, 1987), generating inostiol trisphosphate (IP_3) and diacylglycerol (DAG). Stimulation of this pathway increases intracellular Ca^{2+} and protein phosphorylation, leading to the physiological responses of the cells (Berridge & Irvine, 1989).

We have previously identified and characterized specific L-glu receptors in chick (López-Colomé et al., 1993; López-Colomé & Fragoso, 1995) and human RPE cells (López-Colomé et al., 1994) in culture. Although still controversial, previous studies have proposed the involvement of IP_3 (Heth et al., 1995) and cyclic AMP (Hall et al., 1993), in the regulation of phagocytosis. In the present

work we have studied the effects of EAA on IPs and cAMP formation in RPE cells, in order to explore the possibility of EAA participation in the induction of this phenomenon.

Materials and methods

Cell culture

Cultures were set using RPE cells isolated from seven-day-old chick embryos as previously described (López-Colomé et al., 1991). Eyes were enucleated, freed from vitreous and neural retina, placed in isotonic Krebs-Ringer bicabonate (KRB) containing (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.17; KH_2PO_4, 2.0; $NaHCO_3$, 35; 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 (1.2×10^5) onto 3.5 cm diameter multiwell dishes and incubated at 37°C in TC-199 medium supplemented with 20% heat-inactivated fetal bovine serum, 2.0 mM glutamine, 100 units/ml of penicillin and 100 μg/ml streptomycin. Cultures form a confluent monolayer at 7 days *in vitro* (DIV); at this time, cells acquire typical cuboidal shape and initiate melanine synthesis. The purity of the culture was assessed as described (Salceda et al., 1992).

Measurement of inositol phosphate accumulation

Confluent RPE cell cultures were incubated in the presence of 2 μCi of myo-[2-^{3}H(N)]-inositol per well for 20-24 h. Cells were then washed three times with 1 ml prewarmed KRB containing 10 mM LiCl. In most experiments Mg^{2+} was omitted from the incubation buffer. Cells were pre-incubated in 1 ml of KRB for 10 min at 37°C under gentle shaking, after which agonists were added,

at the indicated concentrations, for varying periods of time. Antagonists, when used, were added after the pre-incubation period. At the end of the incubation, the medium was removed and 1 ml of chloroform/methanol (1:2) added to each culture well. After 20 min, the extract was collected and the wells washed with 0.75 ml of water and 0.5 ml of chloroform. The pooled extracts were mixed and centrifuged at 1,000 xg for 5 min in order to partition the phases. One milliliter of the upper aqueous phase was used for separating inositol phosphates. Extract was applied to a column containing 0.5 g Dowex AG1-X8 (100-200 mesh, formate form), and subsequently eluted with 10 ml of 5 mM myo-inositol and 10 ml of 60 mM sodium formate/5 mM sodium tetraborate. Inositol monophosphate (IP_1), bisphosphate (IP_2), and trisphosphate (IP_3) were extracted in "batch" by eluting with 2 ml of 1.0 M ammonium formate/0.1 M formic acid (Berridge et al., 1983). Radioactivity was determined, after the addition of 5 ml Tritosol (Fricke, 1975), in a liquid scintillation counter. Sister wells from each experiment, were used for determining cell number in a Coulter counter, and for protein determination by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Adenylyl cyclase assay

Adenylyl cyclase activity was measured as the concentration of cAMP. Briefly, cultured RPE cells grown to confluence, were washed three times with KRB, followed by the addition of 1 ml of KRB containing 1 mM 3-isobutyl-1-

methylxanthine (IBMX), and incubated at 37°C for 10 min. EAA or carbachol were added to a final concentration of 1 mM; forskolin (2.5 or 30 μM, final concentration) was added after a 5 min preincubation. After a 30 min incubation with each drug, the medium was removed and cell monolayers were resuspended in 0.5 ml of $HClO_4$ (0.4 M), homogenized, and centrifuged for 2 min (12,000 rpm) in a Beckman microfuge. The supernatants were neutralized to pH 7.0 with K_2CO_3/KOH (0.62M/1.4N) and centrifuged for 2 min at 12,000 rpm. The upper phase was removed to fresh tubes and stored at -20°C for subsequent analysis. The concentration of cyclic AMP in the extract was determined using a radioimmunoassay kit (Amersham Life Science).

Statistics

Dose-response curves were fitted by non-linar regression using the Prims program (Graphpad Software, San Diego, CA.).

Materials

Myo-[2-^{3}H(N)]-inositol (sp.act. 20 to 45 Ci/mmol) was from New England Nuclear. Dowex anion-exchange resin AGI-X 8 (200-400 mesh, formate form) was from BioRad (Waltford, Herts., UK). TC-199 medium and fetal bovine serum were from Difco (Detroit, MI). EAA analogues were from Tocris-Cookson (Bristol, England). MK-801 was from Research Biochemicals International. Fertilized eggs were from "Alpes" (Puebla, México). All other reagents and

chemicals were from Sigma (St. Louis, MO).

Results

Effect of L-glu agonists on [^{3}H]IPs accumulation

Confluent primary cultures of chick RPE were used for every experiment. The effect of 1 mM L-glu as well as ionotropic and metabotropic L-glu receptor agonists was tried. [^{3}H]IPs formation was increased by EAA (Fig. 4) in the following rank of efficiency: NMDA ≥ L-glu > QA ≥ KA > (1S,3R)-ACPD. The stimulatory effect of these compounds increased with time; equilibrium was reached by 15 min, remaining unchanged up to 30 min in the presence of the agonist (Fig. 5).

Pharmacology of the L-glu-induced increase on IPs concentration

L-glu agonists increased the levels of [^{3}H]IPs in RPE cells in a concentration-dependent manner (Fig. 6). The potency of EAA analogues for stimulating [^{3}H]IPs formation was determined within a concentration range from 10 μM to 1 mM. The metabotropic L-glu agonist ACPD was the most potent agent (EC_{50}=45 μM), followed by L-glu (EC_{50}=72 μM) > NMDA (EC_{50}=135 μM) > QA (EC_{50}=185 μM) > KA (EC_{50}=255 μM). In terms of efficiency, however, L-glu and NMDA increased IPs by 215%, QA and KA by 167%, and ACPD was the least efficient compound (120%).

The action of specific antagonists for the different subtypes of ionotropic L-glu receptors, on basal and agonist-stimulated increase in IPs, was also tested (Table 1). Stimulation by L-glu was inhibited by the AMPA/KA receptor

antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 50 μM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX; 50 μM) as well as by the recognition site antagonists of NMDA receptor, CPP (200 μM) and 2-amino-5-phosphonopentanoate (AP5; 200 μM), and by the channel blocker MK-801 (5 μM). NMDA stimulation was inhibited by 200 μM CPP or AP5, as well as by 5 μM MK-801. CNQX and DNQX decreased the KA- as well as QA-induced reponses which were, also, slightly inhibited by 200 μM of the partial agonist 2-amino-3-phosphonopropanoate (AP3), and 1mM of the metabotropic antagonist MCPG.

The effect of ACPD (1 mM) was potently antagonized by 1mM MCPG (Fig. 7) and by 200 μM AP3. Accumulation of [^{3}H]IPs induced by DHPG (metabotropic agonist for group I receptors) was more pronounced than the effect of the general metabotropic agonist ACPD. The simultaneous addition of both compounds showed no additive effect, suggesting an interaction with a single site.

Effect of calcium channel blockers on EAA-induced increase in [^{3}H]IPs

In order to test the participation of extracellular Ca^{2+} on IPs increase, experiments were performed in the presence of 1 mM EGTA; no agonist-triggered accumulation of [^{3}H]IPs was seen in this condition, which demonstrates the requirement of Ca^{2+} influx for the activation of PLC-γ. Stimulation of cultures in the presence of verapamil (10 μM) or nifedipine (10

μM), inhibitors of voltage sensitive L-type Ca^{2+} channels, partially prevented NMDA-stimulation of [^{3}H]IPs accumulation. Thapsigargin and dantrolene, known to interfere with internal Ca^{2+} movements (Simpson et al., 1993), also decreased IPs accumulation induced by NMDA (Fig. 8).

Effect of EAA on cAMP synthesis

cAMP participation in the regulation of phagocytosis (Ogino et al., 1983; Gregory et al., 1994), and other RPE functions as fluid transport (Miller et al., 1982) and retinomotor movements (Burnside et al., 1982) has been proposed. Since mGluRs 2,3,4,6, 7 and 8 are linked to the inhibition, and mGluR 1a to the activation of adenylyl cyclase, the effect of EAA on cAMP accumulation was also tested. Our results show that L-glu has no effect either on basal cyclase activity nor on stimulation by forskolin (30μM) or carbachol (1 mM; Table 2). Stimulation by 2.5 μM fosrkolin was not affected either by 1 mM L-glu. In pmoles of cAMP/mg protein: control, 1.4 ± 0.2; forskolin, 3.73 ± 0.35; L-glu, 1.6 ± 0.2; forskolin + L-glu, 3.8 ± 0.3.

Discussion

Dysfunctional RPE cells cause secondary changes in the neurosensory retina, which can lead to permanent loss of vision, as in the case of proliferative vitreoretinopathy (PV) or retinitis pigmentosa (RP; Bird, 1987). An increasing amount of evidence suggests that induction of phagocytosis of shed tips of rod and cone outer segments, is mediated by specific cell surface receptors on RPE (Mayerson & Hall, 1986). The disc-shedding process, balances the continuous renewal of photosensitive membrane. Rod disc-shedding is normally induced by light (Basinger et al., 1976); however, excitatory amino acids such as L-glu or KA have been shown to stimulate this process independently of a dark-light transition (Besharse et al.,1986; Greenberger & Besharse, 1985).

L-glu mediates a number of distinct intracellular events through receptor-mediated activation of second messenger pathways. Specific receptors for L-glu have been characterized in chick (López-Colomé, et al., 1993) and human RPE in culture (López-Colomé et al., 1994), and shown here to stimulate the IPs cascade. Although the physiological meaning of these findings is still uncertain, IPs modulate diverse cell processes as growth, neurotoxicity, and could possibly relate to phagocytosis (Heth et al., 1995; Pacheco & Jope, 1996).

Two contradictory lines of evidence exist regarding the role of IP_3 in phagocytosis. Heth et al. (1995) have shown an increase in rod outer segment

phagocytosis by RCS-RPE, induced by IP_3; however, Hall et al. (1996) failed to observe this effect. Our data demonstrating that L-glu and specific analogues increase IP_3 concentration through an interaction with specific receptors, are in line with results by Heth et al. (1995), since L-glu analogues have been shown to induce shedding (Besharse et al., 1986). However, the possibility of these two phenomena being unrelated cannot be reled out.

L-Glu analogues increase [^{3}H]IPs accumulation through activation of specific receptors

Previous studies have demonstrated the presence of specific binding sites for ^{3}H-L-glu, which pharmacologically correspond to ionotropic (NMDA and AMPA/KA), as well as metabotropic receptors (López-Colomé et al., 1993; 1994; López-Colomé & Fragoso, 1995), in chick and human RPE. We show here, that ionotropic L-glu receptor agonists stimulate IPs accumulation, NMDA being more potent and efficient than QA or KA (Figs. 4 and 6). The increase in [^{3}H]IPs induced by NMDA was inhibited by the competitive antagonists AP5 and CPP as well as by the receptor-channel blocker MK-801. Correspondently, the action of KA and QA was blocked by the quinoxalines CNQX and DNQX. This pharmacological profile (Table 1) parallels that established for these receptors in the CNS and the retina (Choe et al., 1996).

Our results also demostrate the activation of mGluRs, which correspond either to mGluR1 and/or to mGluR5, since ACPD and DHPG showed potent

agonist action, which was antagonized by MCPG (Fig. 7).

cAMP concentration is not altered by L-glu

Since the variation in cAMP concentration has been related to phagocytosis in RPE (Edwards & Bakshian, 1980), we explored the possibility of L-glu having an effect through mGluRs coupled either to the inhibition of AMP cyclase (groups II and III) or (mGluRs 1a) to the stimulation of this enzyme (Pin & Duvoisin, 1995). L-Glu did not alter the basal level of cAMP nor the stimulation of the cyclase induced by 30 μM forskolin (FSK). L-Glu has been shown, in brain tissue, to inhibit the increase of cAMP induced by carbachol (Pintor et al., 1994), however, no change could be observed in RPE using the same concentration seen to increase IP_3 (Table 2).

Possible mechanism for EAA-induced increase in IPs concentration

The mechanism through which the activation of ionotropic L-glu receptors increases IPs in RPE could relate to a raise in intracellular Ca^{2+} via the receptor channel itself or, alternatively, to the entry of this ion through voltage-dependent channels. Ca^{2+} has been implicated in phagocytosis (Hall et al., 1991), since the total Ca^{2+} concentration in RPE cells is very high (Hess, 1975). It is possible that the accessibility of bound Ca^{2+} to the cytoplasm, where it would affect cellular functions, is tightly controlled, and L-glu receptors could participate in such control, through the activation of PLC. On this matter,

although Heth et al., (1995) have observed an increase in phagocytosis by IP_3 in RCS rats, which could be induced by Ca^{2+}, Hall et al., (1991) showed that elevation of Ca^{2+} concentration, down-regulates this phenomenon; hence, the link between Ca^{2+} an IP_3 concentration as well as their possible effects on phagocytosis remain unclear. In our system the effect of EGTA, verapamil and nifedipine, suggests the participation of external Ca^{2+} entry through L-type Ca^{2+} channels (Fig. 8), in the increase of IPs concentration induced by EAA. In addition, intracellular Ca^{2+} release also seems to play a role in the regulation of IPs in RPE. Dantrolene, which inhibits Ca^{2+}-induced Ca^{2+} release without affecting IP_3-mediated Ca^{2+} release in other excitable cells (Ohta et al., 1990; Frandsen & Schousboe, 1992), decreased stimulation by NMDA (Fig. 8). Thapsigargin , a specific inhibitor of endoplasmic reticulum Ca^{2+}-ATPases which has been shown to release Ca^{2+} from the IP_3-sensitive pool (Lytton et al., 1991; Charles et al., 1993), also generated a decrease in EAA-induced stimulation of IPs production (Fig. 5). These results suggest that elevation of internal Ca^{2+} both, through plasma membrane channels (including receptors) or internal reservoirs, is required for the stimulation of the inositol pathway by L-glu.

The relationship of L-glu-stimulated IPs formation with induction of phagocytosis, though speculative at present, would have important implications regarding normal retinal function and some pathological states as RP and PV. Experiments are now on line in order to demostrate such link.

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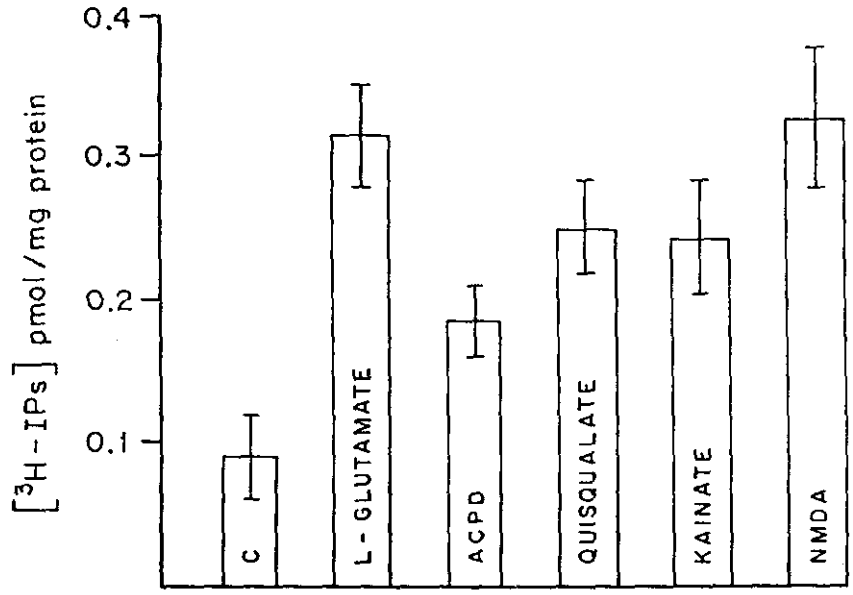

Fig. 4 EAA-stimulated accumulation of [^{3}H]IPs. EPR cultures were incubated for 15 min in the presence of 1 mM concentration of EAA agonists. The order of efficiency was: N-methyl-D-aspartate (NMDA) ≥ L-glutamate (L-Glu) > quisqualate (QA) ≥ kainate (KA) > (1S,3R)-1-aminocyclopentane-3,3-dicarboxylate (ACPD). Results are expressed as the mean ± SEM of five independent experiments performed in triplicate. c, control.

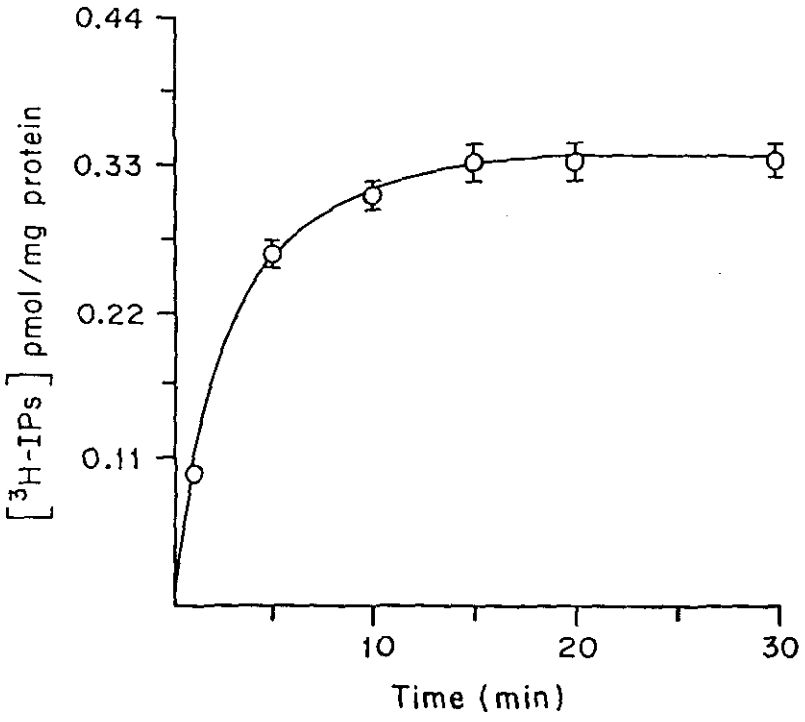

Fig. 5 Time course of L-glu-induced [^{3}H]IPs accumulation in RPE cells. Cells were incubated by various periods of time in the presence of 1 mM L-glu. The data are the mean ± SEM of six independent experiments performed in triplicate.

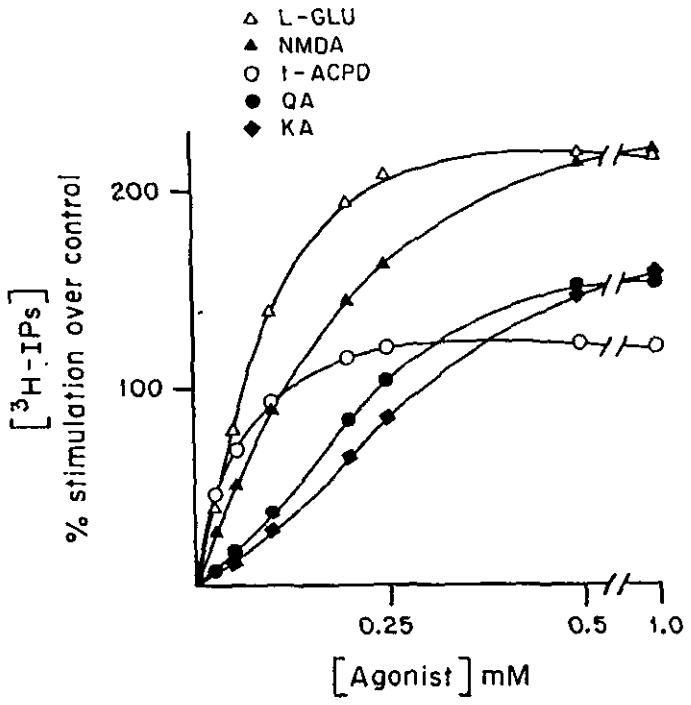

Fig. 6 Concentration-dependence of agonist-induced [^{3}H]IPs accumulation. Confluent cultures were incubated for 15 min in the presence of the indicated concentration of agonists; each concentration series was performed in parallel using sister cultures. Data are the mean of four experiments which varied less than 10%, performed in triplicate.

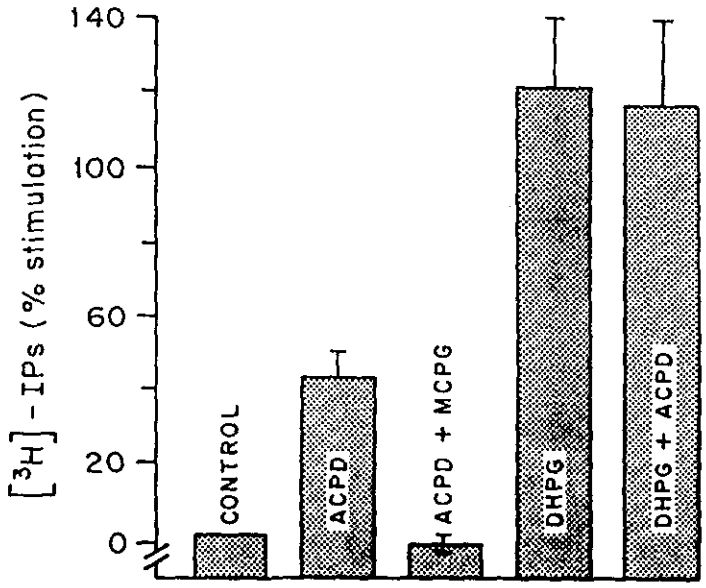

Fig. 7 Effect of metabotropic receptor agonists and antagonists on [^{3}H]-IPs formation. The antagonist MCPG and the agonist DHPG were added to the incubation buffer 5 min before ACPD. Values are the mean ± SEM (n = 3).

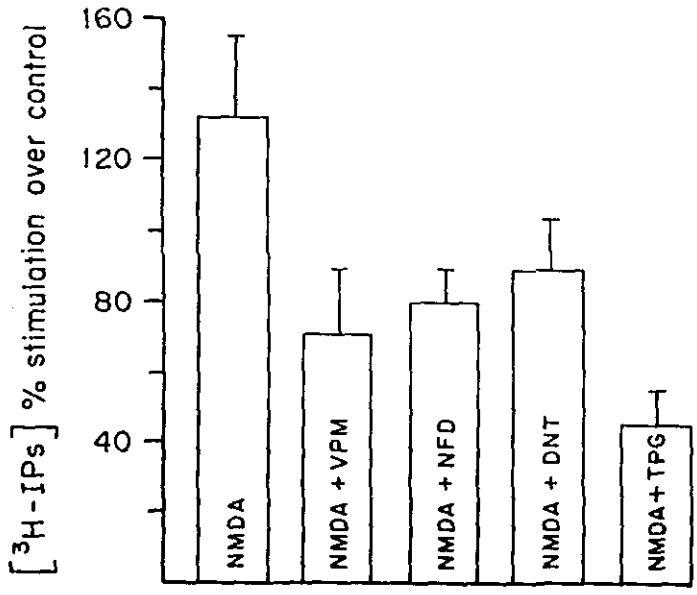

Fig. 8 Effect of calcium channel blockers on the NMDA-induced stimulation of [^{3}H]-IPs accumulation in RPE cells. The concentration of the drugs was: N-methyl-D-aspartate (NMDA, 200 μM), Verapamil (VPM, 10 μM), Nifedipine (NFD, 10 μM), Dantrolene (DNT, 30 μM) and Thapsigargin (TPG, 10 μM). Results are the mean ± SEM of three separate experiments carried out in triplicate.

Table 1. Effect of Antagonists on EEA-Induced IPs Accumulation.

Antagonists	Agonists				
	% Stimulation over control				
	L-Glu (200 μM)	NMDA (200 μM)	KA (500 μm)	QA (500 μM)	AP4 (1 mM)
None	204 ± 9	149 ± 7	57 ± 2	80 ± 2	61 ± 2
CNQX (50 μM)	64 ± 3	n.d.	9 ± 0.5	60 ± 2	61 ± 2
DNQX (50 μM)	44 ± 2	n.d.	20 ± 0.5	65 ± 3	72 ± 3
CPP (200 μM)	111 ± 4	14 ± 0.5	n.d.	83 ± 3	80 ± 3
AP5 (200 μM)	68 ± 3	38 ± 1	n.d.	n.d.	67 ± 3
MK-801 (5 μM)	133 ± 5	12 ± 0.5	n.d.	79± 3	n.d.
AP3 (200 μM)	200 ± 9	n.d.	n.d.	70 ± 3	15 ± 0.5
MCPG (1mM)	200 ± 9	n.d.	n.d.	20 ± 1	26 ± 1
-Ca^{2+} + EGTA (1mM)	62 ± 4	5 ± 0.5	5 ± 0.7	35 ± 3	28 ± 3

Selective antagonists for the different subtypes of glutamate receptors were tested in each case. Data are referred to the non-stimulated value, which was 0.093 ± 0.011 pmol/mg protein (100 %). Antagonists were added 5 min before agonists. Data are the mean ± SEM of 3-6 experiments carried out in triplicate.

Tabla 2. Effect of EAA on cAMP concentration

pmoles of cAMP/well			
Additions	Control	Forskolin (30μM)	Carbachol (1mM)
None	1.9 ± 0.20	8.1 ± 0.40	5.95 ± 0.55
L-Glu (1mM)	2.0 ± 0.12	7.8 ± 0.30	6.02 ± 0.4
L-Asp (1mM)	1.9 ± 0.11	8.1 ± 0.55	n.d.
NMDA (1mM)	1.9 ± 0.11	n.d.	n.d.

Cyclic AMP was measured by radioimmunoassay, as described in Methods. Experiments were performed in sister cultures. Results are the mean ± S.E.M. of three independent experiments performed in triplicate.

DISCUSIÓN Y CONCLUSIONES

Los resultados de este trabajo demuestran que agonistas del L-Glu que interactúan tanto con los receptores ionotrópicos del tipo NMDA y AMPA/KA, como con receptores metabotrópicos sensibles al ACPD, inducen la hidrólisis de PIP_2 en cultivos primarios de células del EPR. Los agonistas ionotrópicos son los más eficientes, con un perfil farmacológico NMDA ≥ L-Glu > QA ≥ KA > ACPD, mientras que los metabotrópicos son los más potentes en el orden ACPD ≥ L-Glu > NMDA > QA > KA.

Nuestros estudios previos han demostrado la presencia de receptores de L-Glu tanto ionotrópicos como metabotrópicos en membranas de células cultivadas del EPR de pollo (López-Colomé et al., 1993, Artículo I; 1995, Artículo II) y EPR humano (López-Colomé et al., 1995, Artículo III). En el presente trabajo, se comprueba que dichos receptores están acoplados a la síntesis de fosfoinosítidos, y no a la modulación, positiva ó negativa, de la adenilato ciclasa (Tabla 2). Las características farmacológicas de los receptores ionotrópicos involucrados coinciden con las descritas para los mismos en el SNC y la retina, ya que la acumulación de IPs-H^3 inducida por NMDA, se inhibe tanto por antagonistas del sitio de reconocimiento del receptor (AP5 y CPP), como por el bloqueador del canal, MK-801. La estimulación por KA en cambio, se inhibe por las quinoxalinas CNQX y DNQX, y consecuentemente, la del L-Glu, agonista general, se inhibe parcialmente

por ambos tipos de antagonista (Tabla I).

En otros tipos celulares, como son los de la retina (López-Colomé et al., 1986), se ha demostrado la activación de la cadena de fosfoinositidos por estimulación de receptores ionotrópicos. Dado que la retina y el EPR tienen un mismo origen embrionario, y las células del EPR tienen la capacidad de transdiferenciarse a neuronas retinianas en ciertas circunstancias (Pittack et al., 1991), podrían tener mecanismos y estructuras comunes de los receptores de AAE. Tanto los receptores de NMDA como los de KA generan entrada de Ca^{2+} a las células, el cual podría activar a la PLC y generar así un aumento en la concentración de IPs. El requerimiento casi absoluto de Ca^{2+} externo para la estimulación mediada por el NMDA (Fig 8) apoya esta idea. Por otra parte, la inhibición del efecto por los bloqueadores de canales sensibles a voltaje (VPM y NFD), sugiere la activación de la PKC como consecuencia tanto de la entrada inicial de Ca^{2+}, a través de los canales del receptor, como por la acción del IP_3 sobre el retículo endoplásmico; esta enzima a su vez podría activar dichos canales de fosforilación. Esta hipótesis también es congruente con el hecho de que el dantroleno y la tapsigargina, inhibidores de la liberación de Ca^{2+} de pozas intracelulares (Frandsen y Schousboe, 1992; Charles et al., 1993), inhiban la estimulación por NMDA, dato que no elimina la participación de Ca^{2+} endógeno. El perfil de potencia de los agonistas, sin embargo, difiere del descrito para otros tejidos, en los cuales el QA es el agonista más potente y el ACPD el menos potente (Roberts, 1995). Esto podría indicar un ensamble

diferente de las subunidades que integran a los receptores para AAE en el EPR.

En cuanto a los receptores metabotrópicos, estos podrían pertenecer al grupo I (mGluRs 1 y 5), ya que se acoplan a la hidrólisis de PIP_2. Tanto el ACPD como su agonista DHPG estimulan con alta potencia, interactuando con el mismo receptor (Fig 7). El efecto de ambos compuestos, así como el del L-AP4, agonista de los mGluRs del grupo III (4,6,7,8) se inhibe por la MCPG, antagonista del ACPD. Se ha demostrado (Roberts, 1995), que el mGluR5 es relativamente insensible a la MCPG, por lo que podemos suponer que el receptor del EPR es del tipo mGluR1. Sin embargo, nuestros resultados sugieren la presencia en el EPR de al menos otro subtipo de mGluR, diferente de los ya descritos, puesto que el L-AP4 está alcoplado a la cadena de fosfatidil inositol, y no a la del AMPc, como sucede en el SNC (Okamoto et al., 1994).

Considerando que los AAE inducen a través de su interacción con receptores específicos la fagocitosis de discos de los SE de los fotorreceptores por parte del EPR, tanto en copas aisladas de *Xenopus* (Greenberger y Besharse, 1985), como en cultivos celulares de EPR de mamífero, la producción de fosfatos de inositol a través de la activación de cinasas y aumento de Ca^{2+} intracelular, podrían participar en la inducción de este fenómeno. Si como se reporta (Strauss et al., 1998), los fosfatos de inositol intervienen en la activación de la fagocitosis y el AMPc en la modulación de la

misma (Nash y Osborne, 1995), suponemos que los receptores para AAE en estas células intervienen al inicio del proceso permitiendo la entrada de Ca^{2+} y la generación de de IPs.

Se postula que en el modelo animal de retinitis pigmentosa en rata, la causa sea una alteración de la función de proteínas en la ruta de segundos mensajeros. No se ha trabajado todavía en la búsqueda de los receptores para ácido glutámico en este modelo, por lo que sería un proyecto a seguir encontrar defectos en la expresión de estos receptores o una alteracón en la secuencia de eventos inducida por estos en el EPR. A este respecto cabe mencionar que tampoco se conoce que subunidades, formando los diferentes receptores a glutamato, estan presentes en el organismo con función fagocítica normal.

El presente reporte propone una nueva perspectiva para abordar el problema de alteraciones visuales, tales como la retinitis pigmentosa, ya que considerando el efecto fisiológico que tienen estos receptores al inducir la fagocitosis de discos y la importancia que tienen el ensamble correcto de subunidades que forman a los receptores de tipo ionotrópico, una alteración genética podría involucrar cambios en el ensamble de las subunidades de estos receptores que podrían alterar su fucionamiento correcto y como consecuencia, ser ineficientes para inducir la función fagocítica.

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