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Índice

Resumen.

Los receptores acoplados a proteínas G (GPCRs) conforman la superfamilia más grande de receptores de membrana. Sus ligandos varían en cuanto a su composición bioquímica entre péptidos, proteínas aminoácidos, iones, fotones ó lípidos. Un ejemplo particular es el ácido lisofosfatídico (LPA), un factor particularmente abundante en suero que cuenta con actividad biológica regulando diversas funciones celulares como la proliferación, sobrevida, división celular, entre otras. Se han identificado seis receptores del tipo GPCRs capaces de responder de forma específica y selectiva a este ligando, nombrados LPA_{1-6}. Los primeros tres pertenecen a la familia EDG (del inglés *Endothelial Differentiation Gene*), los otros pertenecen a la familia P2Y (receptores tipo purinérgicos). Este trabajo tuvo como objetivo estudiar las respuestas fisiológicas inmediatas a la estimulación del receptor 2 para ácido lisofosfatídico (LPA_2) y cómo éstas se ven afectadas durante su desensibilización. A partir de células C9 se obtuvo una línea celular que expresa de manera estable el LPA_2 fusionado a un tipo de proteína verde fluorescente y se evaluó su localización celular. Se cuantificó la variación en la concentración de calcio intracelular en esta línea en respuesta a distintas concentraciones del ligando (LPA). Se observó que el LPA_2 es sensible a la desensibilización mediada por PMA y se estudiaron las posibles proteínas que pudiesen estar mediando esta desensibilización. Encontramos también que el receptor es capaz de activar las vías de ERK, p38 y Akt/PKB.

Introducción

La unidad fundamental de la vida es la célula, una necesidad común a todos los tipos de células es la capacidad de "sentir" lo que pasa en su entorno para poder adaptarse a diversas situaciones y comunicarse entre ellas.

Los seres vivos de este planeta están expuestos a constantes cambios, cambian las temperaturas, las superficies, la intensidad de la luz, las fuentes de comida, etc. Una de las propiedades más distinguibles de cualquier ser vivo es la adaptación a dichos cambios; Una célula incapaz de responder a los cambios en su entorno moriría por la más mínima diferencia ambiental (osmolaridad, temperatura, etc.), ya que no podría realizar modificaciones en sí misma o en su entorno próximo como una respuesta que le permita contender con dichos cambios.

Debido a esta necesidad, una de las primeras maquinarias obligadas a desarrollar por los primeros seres vivos fueron aquellas encargadas de la comunicación celular: los receptores y sus sistemas de señalización. Esta característica se hizo más compleja y relevante al momento de formar organismos multicelulares, ya que esto significa la necesidad de coordinar todo un grupo de células para realizar las mismas funciones, delegar tareas y establecer una red de comunicación que permita mantener en óptimas condiciones cada célula como parte de un todo, con una apropiada cantidad de nutrientes y un mínimo requerimiento de esfuerzo.

Los organismos vivos tienen una gran variedad de receptores de membrana, los cuales son proteínas encargadas de percibir las señales del medio externo y la manera en que cambia transduciendo esta primera señal, la cual puede ser mecánica, química ó física, en un cambio que la célula pueda interpretar para desencadenar una respuesta apropiada. Estas respuestas pueden observarse como cambios en el metabolismo, en la expresión de genes, en la morfología celular, etcétera.

Existen cuatro tipos principales de receptores celulares: 1) los receptores nucleares, que generalmente son factores de transcripción que se activan o inactivan al entrar en contacto con su ligando, 2) receptores con actividad enzimática, 3) receptores tipo canal iónico y 4) receptores acoplados a proteínas G (GPCRs). A estos últimos serán a los que me refiera de aquí en adelante ya que son los de interés en esta tesis.

Receptores Acoplados a Proteínas G

Los receptores acoplados a proteínas G (GPCRs), también conocidos como receptores de siete dominios transmembranales (7TM), constituyen la familia más grande de receptores transmembranales. Se considera que surgen evolutivamente con el origen de la multicelularidad. Existen alrededor de 800 genes que codifican para GPCRs en el genoma humano, los cuales están involucrados con una gran gama de procesos incluyendo la vista, el olfato, el gusto, la vaso-relajación, el ritmo cardiaco, la neurotransmisión, el desarrollo embrionario, entre otros. (Mombaerts P 2004).

Los GPCRs están agrupados en seis clases desde la A hasta la F con base en su homología de secuencia; Receptores Tipo-Rodopsina (Clase A), Receptores de la familia Secretina (Clase B), Receptores de Glutamato Metabotrópico/Feromona (clase C), Receptores de Feromonas del Apareamiento de Hongos (clase D), Receptores de AMP cíclico (Clase E) y Receptores Frizzled/Smoothened (clase F) (Alexander SPH. et. al. 2015). La clase A comprende el mayor número de GPCRs. La clase B cuenta con alrededor de 50 miembros, activados por hormonas peptídicas y neuropéptidos. La clase C contiene a los receptores de Glutamato, los cuales son activados por aminoácidos e iones.

Las asas extracelulares de los GPCRs poseen dos motivos de cisteínas altamente conservadas que forman enlaces disulfuro que estabilizan la estructura del receptor (Cook J y Eidne K 1997). Los GPCRs carecen de actividad enzimática propia, en vez de esto funcionan acoplándose a proteínas G heterotriméricas, compuestas por las subunidades Gα, Gβ y Gγ. El ligando se une a un GPCR y estimula la disociación de las proteínas G en las subunidad Gα unido a GTP y el dímero Gβγ. Las subunidades de las proteínas G disociadas regulan la actividad de varias enzimas entre ellas proteínas cinasas, la fosfolipasa C y la adenilato ciclasa para generar segundos mensajeros (figura 1). Estos segundos mensajeros regulan diversas funciones celulares. La complejidad de la respuesta se aumenta con el hecho de que existen diversos tipos de subunidades de las proteínas G, al menos 20 Gα, 6 Gβ, y 11 Gγ reportados (Catteneo, F et al 2014).

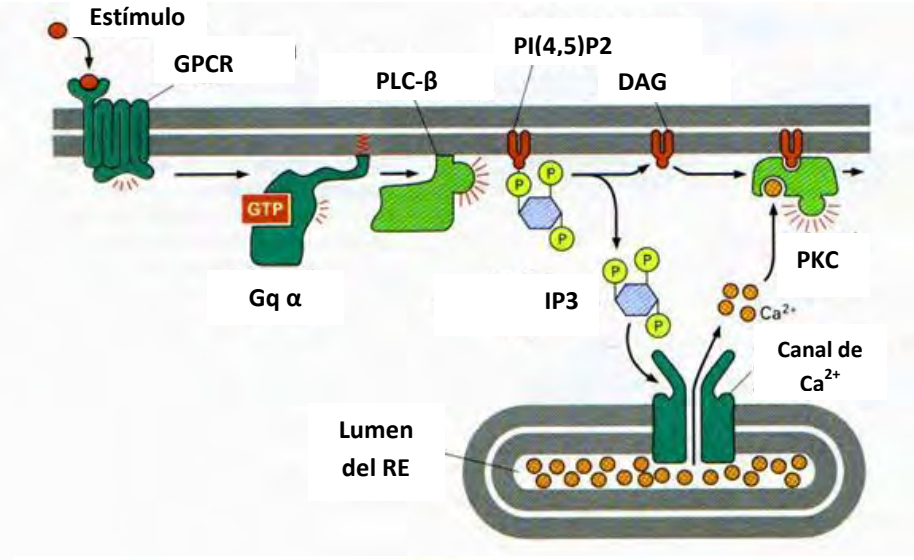
Las proteínas G, dependiendo de sus combinaciones y el contexto celular en el que se encuentren, desencadenan cascadas de señalización que inducen cambios celulares. Entre las principales familias de proteínas G encontramos las siguientes:

G_s que estimula a la adenilato ciclasa aumentando la concentración de cAMP dentro de la célula. G_i que inhibe la adenilato ciclasa disminuyendo la concentración de cAMP dentro de la célula. Así ambas regulan la actividad de diversas proteínas sensibles a cAMP. G_q estimula a la fosfolipasa C, la cual corta al fosfatidil inositol bifosfato (PIP2) en inositol 3 fosfato (IP3) y diacil-glicerol (DAG), el IP3 abre canales de calcio del retículo endoplásmico (RE) aumentando la concentración de calcio en citoplasma. El Ca^{2+} junto con el DAG activan a la proteína cinasa C (PKC). $G_{12/13}$ que estimula a la proteína RhoGEF un factor intercambiador de nucleótidos GTP.

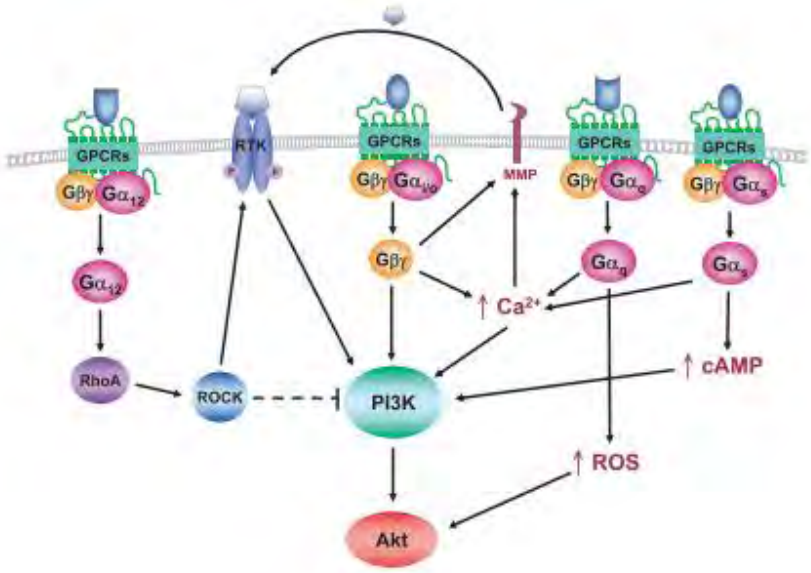
Lo anterior provoca cambios en la actividad de diversas proteínas sensibles a Ca^{2+}, cAMP o GTP influyen en la sobrevivencia, el metabolismo, la morfología e incluso el transcriptoma de la célula. Para realizar estas funciones finales se conocen proteínas clave del proceso. Entre ellas encontramos a las MAPKs (del inglés *Mitogen Activated Protein Kinase*) las más estudiadas de estas son ERK1 y ERK2 (del inglés *Extracellular-signal Regulator Kinase*) encargada de regular procesos como la proliferación, la diferenciación y la motilidad celular fosforilando diversos factores de transcripción (TFs), prácticamente la activación de cualquier proteína G lleva eventualmente a la activación de las ERK1/2. Otra es la vía de Akt/PKB que inhible la muerte celular y tiene diversos efectos en el metabolismo. Esta última es activada por la cinasa dependiente de fosfatidil inositol 3-fosfato (PIP3) llamada PDK (Liebmman, C. 2001).

Algo común a todos los receptores es que después de haber respondido al estímulo deben volver a un estado no-activado. Se le llama desensibilización al mecanismo de adaptación que se basa en disminuir la cantidad de receptores o llevarlos a un estado poco sensible mediante cambios postraduccionales. Este proceso puede ser causado por el contacto entre el receptor y su ligando (desensibilización homóloga) o por la activación de otras proteínas que no están directamente relacionadas con el proceso inicial de señalización como puede ser otro receptor o diversas cinasas (desensibilización heterológa).

A


Estímulo
GPCR
PLC-β
PI(4,5)P2
DAG
GTP
Gq α
IP3
PKC
Ca2+
Canal de Ca2+
Lumen del RE

B


GPCRs
RTK
MMP
Gβγ
Gα12
Gαi/o
Gαq
Gαs
↑ Ca2+
RhoA
ROCK
PI3K
↑ cAMP
↑ ROS
Akt

C

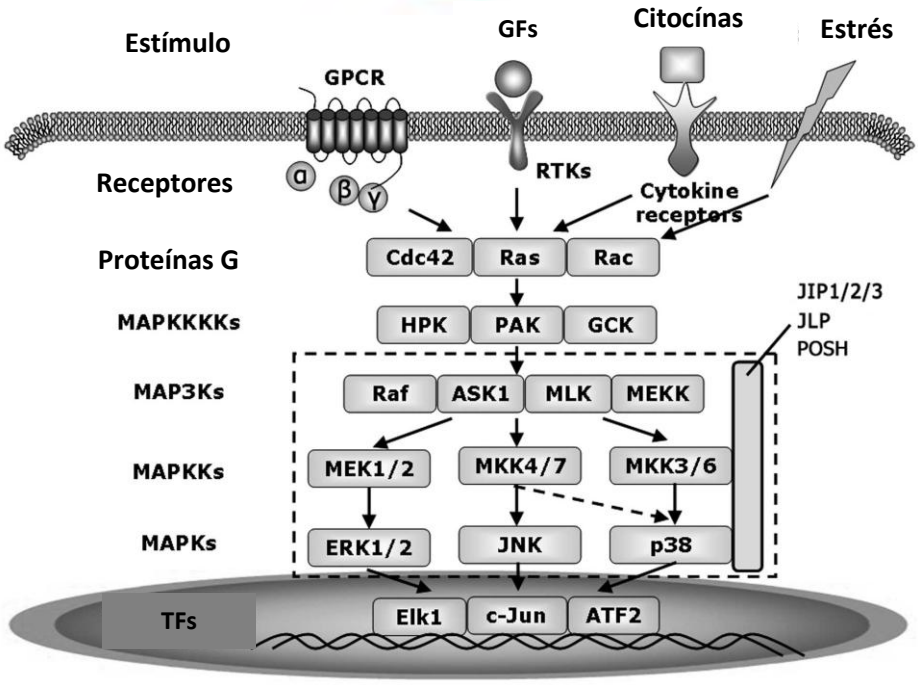

Estímulo
GFs
Citocínas
Estrés
GPCR
RTKs
Cytokine receptors
Receptores
Proteínas G
Cdc42
Ras
Rac
MAPKKKKs
HPK
PAK
GCK
JIP1/2/3
JLP
POSH
MAP3Ks
Raf
ASK1
MLK
MEKK
MAPKKs
MEK1/2
MKK4/7
MKK3/6
MAPKs
ERK1/2
JNK
p38
TFs
Elk1
c-Jun
ATF2

Figura 1. Vías principales de señalización de los GPCRs. A) Vía de Diacil glicerol/calcio B) Activación de Akt. C) Vía de activación de Erk. (Figuras modificadas de *Alberts, Bruce et al.* 2008, *New, David C. et al.* 2007, *Zhao, Hua-Fu et al.* 2015).

Actualmente se conocen a *grosso modo* tres mecanismos principales para este proceso: (1) El secuestro del receptor: que al ser internalizado a través de pequeñas vesículas intracelulares, llamadas endosomas, restringiendo su acceso al ligando. (2) La regulación por disminución (*down-regulation)* del contenido total del receptor: envía estas vesículas a lisosomas para destruir a los receptores y quedarse con una cantidad menor de éstos. (3) Inactivación del receptor por medio de modificaciones postraduccionales, como la fosforilación de ciertos reciduos, que inducen cambios conformacionales que le impidan interaccionar con las proteínas G.

Son dos las principales familias de proteínas que se encargan de regular la actividad de los GPCRs en la desensibilización homóloga: Las cinasas de los GPCR (GRKs) y las β-arrestinas. Las GRKs regulan la actividad mediante la fosforilación de los dominios intracelulares seguido de la liberación de las proteínas G acopladas, esto permite la interacción de las β-arrestinas con los GPCRs lo que previene la re-asociación con las subunidades de las proteínas G, aunque existen reportes de que algunos GPCRs son capaces de seguir interaccionando con las proteínas G al mismo tiempo que están asociados a las β-arrestinas (Thomsen, A et. al. 2016).

Recientemente se ha observado que la β-arrestina puede funcionar como una proteína de andamiaje y activadora de otras proteínas de señalización río abajo del receptor. Así, la interacción con las β-arrestinas le permite a los GPCRs desencadenar cascadas de señalización independientes de las proteínas G (figura 1). Además de la regulación por GRKs existen otros grupos de cinasas que fosforilan a los GPCRs afectando su actividad, como son la proteína cinasa dependiente de AMP cíclico (PKA) y las proteínas cinasas C (PKCs) que originalmente se describió como dependiente de calcio pero ahora se sabe que no todas las isoformas poseen esa característica.

Los GPCRs son activados por una molécula (primer mensajero) cuya naturaleza es variable (lípidos, péptidos, proteínas, aminoácidos, iones o fotones). El descubrimiento de que los lípidos son capaces de fungir como moléculas

señalizadoras que evocan respuestas celulares a través de los GPCRs fue un descubrimiento relativamente tardío y a éstos se les llamó lípidos bioactivos. Un caso particular de éstos es el ácido lisofosfatídico (LPA).

Ácido Lisofosfatídico (LPA)

El ácido lisofosfatídico (LPA) es un lisofosfolípido sumamente sencillo compuesto por un glicerol unido a un grupo fosfato por medio de un enlace fosfoéster en el hidroxilo del carbono uno y en el hidroxilo del carbono dos ó del tres unido por un enlace éster presenta un ácido graso de tamaño variable que, en la naturaleza, generalmente oscila entre 16 y 20 carbonos y puede estar saturado o insaturado (figura 2).

Figura 2. Estructura del ácido lisofosfatídico, un lípido bioactivo. (Editada de Shinichi, O 2010)

Originalmente se consideraba que el LPA era solamente un intermediario del metabolismo de lípidos sin mayor relevancia fisiológica, pero posteriormente se encontró que éste es un lípido bioactivo con efectos pleiotrópicos sobre las células de los mamíferos como la contracción muscular, proliferación, agregación de plaquetas, invasión de células tumorales, entre otros. Los receptores encargados de mediar las funciones fisiológicas del LPA son principalmente del tipo GPCRs, más adelante se detallará este punto.

El LPA es abundante en suero, fluido folicular, saliva y fluido seminal. Es secretado de forma constitutiva por plaquetas y su secreción por otras células puede darse en respuesta a estrés osmótico, factores de crecimiento, hormonas, entre otros estímulos. Algunas células tumorales secretan LPA de forma constitutiva.

Existen dos formas de sintetizar LPA (figura 3). La primera de ellas, se lleva acabo principalmente en el interior de la célula por la fosfolipasa A1 o la fosfolipasa A2 las cuales remueven una de las cadenas alifáticas del ácido fosfatídico (PA) (Akoi, J 2008). También puede sintetizarse LPA a partir de un lisofosfolípido que, por acción de una fosfolipasa D como la autotaxina (ATX), pierde su cabeza polar (colina, serina, etanolamina, etc.) La lisofosfatidilcolina (LPC) es el principal sustrato de la ATX (Tokumura et al 1986) (Shinichi et al. 2010). Ésta segunda vía es la más importante debido a que es la principal fuente de LPA extracelular capaz de activar a los receptores para LPA de la superficie celular. Existen muchas enzimas que participan en el camino que lleva a un fosfolípido a convertirse en LPA (figura 3), pero desde el punto de vista fisiológico la que tiene mayor relevancia es la autotaxina (ATX). Evidencia de ello puede verse en que un ratón mutante heterócigo de $ATX^{-/+}$ produce aproximadamente la mitad del LPA en comparación a lo que produce un ratón silvestre (Tanaka 2006). Además se ha observado que los ratones mutantes de ATX presentan los mismos fenotipos que los mutantes de los receptores para LPA.

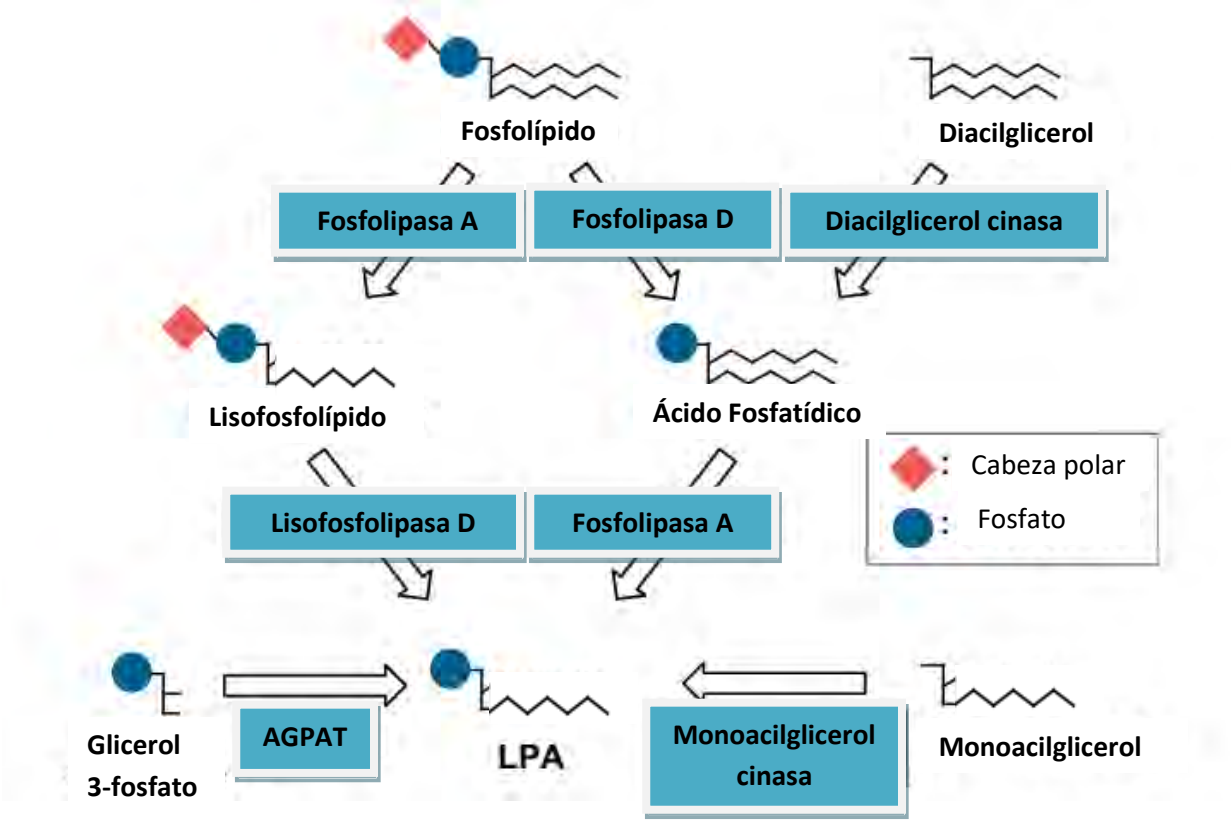


Figura 3. Síntesis de ácido lisofosfatídico. (Modificada de Shinichi 2010)

La autotaxina es una fosfolipasa D secretada, descubierta en 1986 por Tokumura, codificada por el gen ENPP2 (Miembro 2 de la familia fosfodiesterasa/pirofosfatasa exonucleótido), clasificada en esta familia por su homología estructural. Sin embargo, a pesar de presentar actividad de exonucleasa, ésta es muy baja comparada con su actividad como lipasa (con una Km=250 µM para la LPC vs una Km=14 mM para los ácidos nucleícos) (Tokumura et al 2002) (Umezu-Goto et al 2002). Además su localización extracelular rara vez la pone en contacto con ácidos nucléicos.

La autotaxina se identificó primero en el sobrenadante de células de melanoma humano en 1992 (Stracke et al 1992) (Orosa B. et al 20015). Diez años después se purificó la fosfolipasa D del suero fetal bovino y comprobaron que se trataba de la misma enzima, la autotaxina (ATX) (Tokumora et al 2002). Ahora se sabe que se encuentra principalmente en suero; es secretada por células plasmáticas activadas y células de algunos tipos de cáncer. Entre sus efectos, se ha reportado que promueve la motilidad de manera autocrina, es decir que produce su acción sobre la misma célula que la secreta.

A pesar de que ATX es clasificada como una lisoPLD es distinta a las PLDs clásicas debido a que carece de homología con ellas y es selectiva para los lisofosfolípidos. Posee 3 isoformas: ATX-t abundante en plasma, ATX-m de melanoma y la ATX-γ principalmente expresada en cerebro. Contiene un péptido-señal de secreción de 19 aminoácidos en el amino terminal.

El LPA, producto principal de la ATX, se ha mencionado como el factor de crecimiento más abundante en suero, con una concentración aproximada de 5µM, debido a que sus funciones mejor conocidas son la proliferación, crecimiento, sobrevida y diferenciación de las células. También participa en la regulación del metabolismo de lípidos y puede producir un cambio en el patrón de expresión de genes. Estas funciones las lleva a cabo por medio de sus receptores.

Receptores para ácido lisofosfatídico

El LPA extracelular se une al menos a seis receptores de superficie específicos (LPA_{1-6}), pertenecientes a la clase A de la superfamilia de los GPCRs. Los primeros tres pertenecen a la familia EDG (gen de diferenciación endotelial) y sus secuencias de aminoácidos son homólogas entre 50 y 60%. Los otros tres están relacionados a la familia de receptores P2Y (receptores tipo purinérgicos) (Kihara Y. et al 2008). Por otro lado existe evidencia de que el LPA puede señalizar mediante el receptor nuclear PPARγ (del inglés *Peroxisome Proliferator-Activator Receptor gamma*) el cual participa en la diferenciación a adipocitos (McIntyre et al 2003).

Los receptores para LPA tienen la capacidad de acoplarse a diferentes proteínas G (figura 4), lo que podría explicar la gran diversidad de efectos de este ligando, aunado a la combinación de receptores y de proteínas G que expresa cada tipo celular.

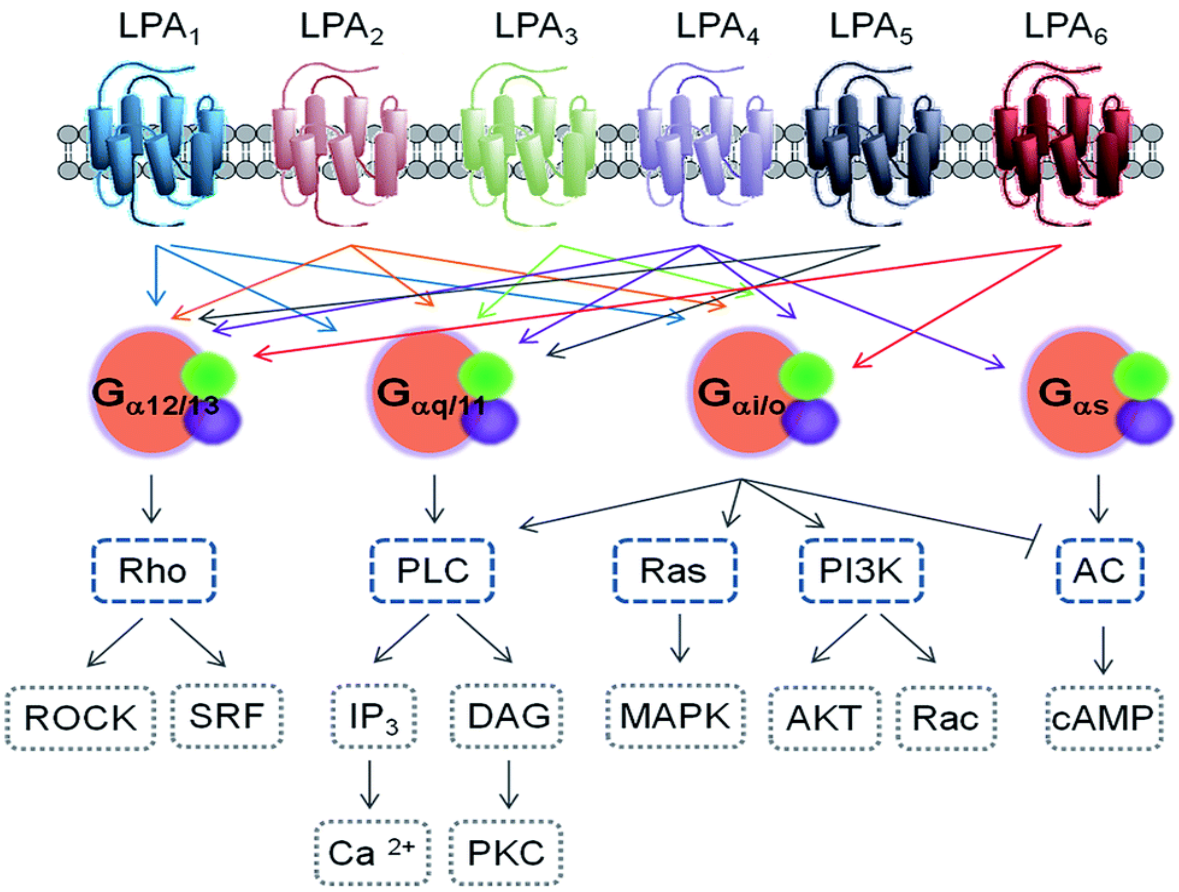


Figura 4. Receptores para LPA y su asociación a diversas proteínas G. (Tomada de Sheng X et al. 2015)

Receptor 2 para ácido lisofosfatídico (LPA_2)

En el laboratorio se estudian los tres receptores para LPA de la familia EDG. Éste trabajo se centró en caracterizar las propiedades del receptor LPA_2 el cual es uno de los menos estudiados debido, entre otras cosas, a su falta de fenotipo evidente en las cepas de ratón que no expresan este gen, y a que se ha considerado como redundante con respecto al LPA_1. Sin embargo, existen claras diferencias entre estos dos subtipos (difieren en secuencia, posibles sitios de fosforilación, localización tisular y proteínas asociables) por lo que posiblemente tengan distintos papeles fisiológicos. A continuación se resume de la literatura previa la información conocida sobre este receptor.

El LPA_2 es una proteína de 351 aminoácidos que fue descubierta en una búsqueda de secuencias homólogas al LPA_1 (Songzhu An et al 1998). Se acopla, en respuesta a su ligando, el LPA, a las proteínas $G\alpha_{i/o}$, $G\alpha_{q/11}$ y $G\alpha_{12/13}$. Para llevar a cabo sus diversas funciones activa las vías de las MAPK (del inglés *Mitogen-Activated Protein Kinase*) y Akt/PKB. Se ha postulado que el LPA_2 es redundante con el LPA_1 debido a que el doble *knockout* (mutante que ha perdido dicho gen) no muestra diferencias con respecto al mutante que sólo tiene deletado al LPA_1 (Contos J et al 2002). La deleción del LPA_2 no tiene efecto sobre el desarrollo embrionario (Fang-Tsyr et al 2008). Aún es incierto cómo estas vías le permiten al LPA2 realizar sus funciones y cómo se regula la activación de éstas.

Además se sabe que el LPA_2 posee al menos dos importantes dominios de interacción proteína-proteína. El dominio PDZ, un dominio estructural de 80 a 90 aminoácidos que necesita cuatro aminoácidos para su función (DSTL, Apartato-Serina-Treonina-Leucina). Curiosamente estos cuatro aminoácidos no estaban presentes en el primer cDNA que se obtuvo de LPA_2 ya que provenía de una biblioteca de cDNA de un tumor de ovario, por lo que esta característica se mantuvo oculta por un tiempo (Songzhu An et al 1998). Por otro lado, contiene sitios de reconocimiento para dedos de Zinc. (Fang-Tsyr et al 2008)

Mediante su dominio PDZ el LPA_2 (y no el LPA_1) es capaz de asociarse a la proteína PDZ2 (también llamada NHERF2) lo que potencia la activación de ERK mediada por LPA y la inducción de COX2 (ciclooxigenasa 2) (Oh YS et al 2004). Este dominio

también puede facilitar el reclutamiento de Rho GEFs (del inglés *Guanine nucleotide Exchange Factor*), acercándolos a la proteína $G\alpha_{12/13}$, esto aumenta la capacidad GEF de RhoA.

En cuanto a las interacciones proteína-proteína mediadas por dominios de dedos de Zinc, el LPA2 se une al receptor TRIP6 (del inglés *Thyroid-hormone Receptor Interaction Protein 6*), con ayuda de LIM1. Ésta asociación en el carboxilo terminal (Lin F et al 2007) es mediada por un motivo tipo dedo de Zinc. Por otra parte Siva-1, una proteína proapoptótica que se activa por p53 y E2F1 durante la respuesta al daño en el DNA, lleva al receptor a las placas de adhesión focal donde no hay ligando. TRIP6 (ZRP-1) es una molécula presente en las adhesiones focales que se sabe que regula la motilidad celular (Xu J et al 2004).

TRIP6 conduce al LPA_2 a asociarse con los complejos focales (complejos de proteínas situados en las placas de adhesión focal), ahí activa otras proteínas como c-Src, ésta interacción tiene un efecto positivo en la activación de ERK mediada por LPA ya que se ha encontrado a c-Src como intermediario de ésta vía.

Otros estudios en el ratón knockout de LPA2 han demostrado que este receptor tiene efectos antiapoptóticos y de protección contra radiación de rayos x, o daño intestinal producido por drogas antitumorales. Estos ratones también presentan menor tumorigénesis colorectal. (Deng W et al 2007) (Lin S et al 2009). Se ha observado también que la estimulación prolongada con LPA promueve la degradación del receptor LPA_2.

El LPA_2 se ha asociado a la agresividad de tumores debido a que se ha encontrado sobreexpresado en tejidos cancerosos de estómago, de ovario, de tiroides y de pecho (Shida D et al. 2014). Esta sobreexpresión se asocia con un aumento de la migración en dichas células.

En cuanto a la regulación particular del LPA_2 se sabe muy poco. Un trabajo previo del laboratorio describe sitios blanco putativos para diversas cinasas tanto en sus asas intracelulares como en su región carboxilo terminal (figura 5) (Hernández-Méndez A. et al 2014). Sin embargo hasta este trabajo no se había comprobado la fosforilación de este receptor (LPA_2), qué cinasas mediaban dichas fosforilaciones ni los efectos que esto pudiera tener en las respuestas mediadas por este receptor.

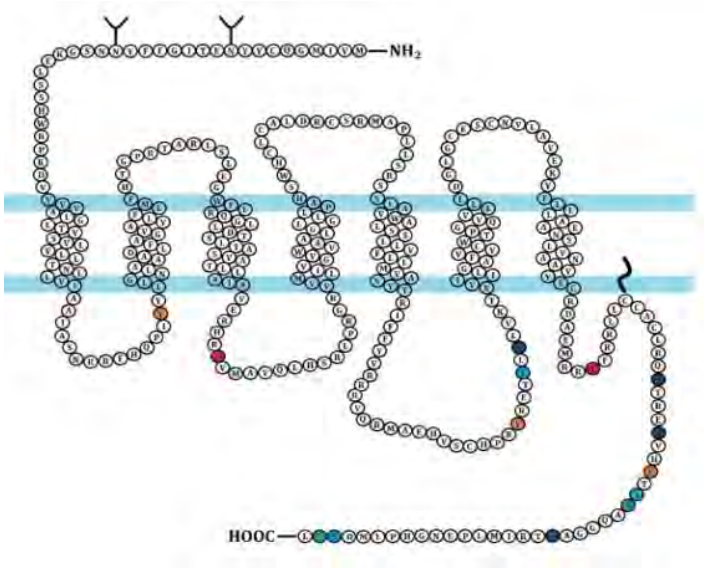


Figura 5. Representación esquemática del receptor LPA_2, los puntos con color son blancos putativos de diversas cinasas. (Tomada de Hernández-Méndez et al 2014).

Objetivos.

Objetivo general:

Estudiar las respuestas inmediatas del receptor dos para ácido lisofosfatídico (LPA_2) y como éstas se afectan durante la desensibilización del receptor.

Objetivos particulares:

- Cuantificar el cambio de la concentración intracelular de calcio en respuesta a distintas concentraciones de LPA.
- Evaluar la desensibilización homóloga y heteróloga del receptor LPA2 analizando al menos dos respuestas fisiológicas; el incremento en la concentración de calcio intracelular y la fosforilación de ERK.
- Evaluar la fosforilación de otras proteínas río abajo (cinasas Akt y p38) del receptor a diversos tiempos y compararla con las cinéticas de internalización y fosforilación del LPA_2.
- Determinar la interacción del LPA_2 con proteínas cinasas C (PKC) y su relación con su desensibilización.

Materiales y métodos

Materiales

El L-α-LPA (oleoil-sn-glicerol-3-fosfato) y el PMA (forbol miristato acetato) fueron obtenidos de Sigma-Aldrich. Los kits de purificación de DNA, el medio F12K de Ham (modificado de Kaighn, F12K), tripsina, antibióticos, y otros agentes usados para el cultivo celular fueron comprados a LifeTechnologies (Gibco BRL) Thermo Fisher Scientific. El suero de bovino fetal fue obtenido de Multicell (Gibco). Fura2AM (fura2 acetoximetil ester) fue obtenido de Invitrogene. Plásmidos para la expresión de los receptores de LPA, anticuerpos contra p38, pp38, ERK1/2, pERK1/2, PKCα, PKCβ_1, GFP, Akt/PKB, pAkt/pPKB, membranas de polifluoruro de vinilideno (PVDF) y nitrocelulosa fueron obtenidas de BioRadyel. El kit de quimioluminiscencia fue comprado a Pierce. La línea celular de epitelio de hígado de rata, C9, fue comprada a la American Type Culture Collection. El suero anti-GFP fue generado en el laboratorio. La protenia A agarosa fue obtenida de Millipore.

Líneas celulares y transfección.

La línea celular, derivada de la línea C9, que sobreexpresa de manera estable el receptor para LPA1 fusionado con eGFP (enhancer green fluorescent protein) fue generada previamente en el laboratorio. (Avendaño-Vázquezetal.,2005;Colín-Santanaetal.,2011; González-Arenas etal.,2008).

Para generar las líneas que sobreexpresaran de manera estable los receptores LPA2 o LPA3 se transfectaron células C9 silvestres con los plásmidos independientes pEGFPN1 que contienen la secuencia que codifica para LPAR2 o LPAR3 fusionados a la proteína verde fluorescente (GFP) en el carboxilo de cada receptor, utilizando lipofectamina 2000.

Cultivo celular

Las células C9 se cultivaron en cajas de Petri con medio F12K suplementado con 10% de suero fetal de bovino, 100µg/ml de estreptomicina, 100 unidades/ml de penicilina, 0.25 µg/ml anfotericina B, a 37ºC en una atmósfera con 95% de aire y 5% de CO_2. Al medio de las células transfectadas se le agregan 300 µg/mL de G418.

Medición de calcio intracelular

Las células se preincubaron con 2.5 µM del indicador fluorecente de Ca^{2+}: Fura-2/AM, en Krebs-Ringer-HEPES con 0.05% de albumina sérica, a un pH de 7.4 por 1hora a 37ºC. Después de eso es retirado el fura extracelular con lavados del mismo medio. La medición de la fluorescencia es con 340 a 380nm de onda de excitación y a 510nm de emisión. Con mediciones cada 0.5 segundos utilizando un espectrómetro de luminiscencia Aminco-Bowman Series 2 (Rochester, NY,USA). La concentración de calcio intracelular ($[Ca2^{+}]i$) se midió de acuerdo con el método de Grynkiewicz et al.(1985).

Transformación bacteriana

Brevemente, para la transformación ocupamos 20 µL de *E coli* competentes y 2 µL de plásmido, esta mezcla se incubó en hielo durante 20 minutos, posteriormente se realizó un choque térmico a 42°C durante 30 segundos. Se colocaron en el hielo y se les agregó 150 ml de medio LB. Esta preparación se incubó a 37°C durante una hora y transcurrido ese tiempo las bacterias se sembraron Agar-LB con 100 µg/ml de Ampicilina como agente de selección.

Cultivo de bacterias

Las cajas antes mencionadas se dejaron en incubación a 37°C durante toda la noche. Por la mañana se inoculaban las clonas observadas en 3ml de medio LB-antibiótico (en tubos de ensayo) y se dejaron crecer durante 8 horas.

Aislamiento y purificación de ADN plasmídico “Miniprep”

Se realizo con un kit de columnas de invitrogen y para realizarlo de siguió el protocolo de la misma compañía.

Para comprobar que el DNA plasmídico se obtuvo de manera correcta se realizaron electroforesis de todas las muestras (Figura 1A). Este DNA se utilizó para realizar las transfecciones en células C9.

Western Blot

Posterior al estímulo indicado las células son lavadas con buffer salino de fosfatos, lisadas con 0.3 ml de Laemmli a 4ºC por 1 hora. Los lisados son hervidos a 90ºC y centrifugados a 12,700g por 5 minutos. 20 µL de esta proteína se corre en una electroforesis en un gel de bisacrilamida al 10% La proteína pasa por una electro transferencia a una membrana de PVDF, se bloquea con TBS-tween con 5% de leche por una hora, y el inmunoblot se realiza con su respectivo anticuerpo primario toda la noche a 4ºC y secundario por 1hr a temperatura ambiente. La señal se detecta por kits de quimioluminiscencia y se cuantifica por densitometría con el programa Image J.

Inmunoprecipitación

Para la inmunopecipitación se utilizó RIPA (con inhibidores de proteasas) como buffer de lisis. Posterior a una centrifugación (12,000 rpm a 4ºC 15 minutos) el sobrenadante se recupera en tubos de 1.5 mL que contienen 30 µL de perlas de proteína A-agarosa y 10 µL de suero anti-GFP. Ésta mezcla se deja en agitación toda la noche. Al día siguiente las perlas se lavan con RIPA y se les agrega SDS urea y Laemmli para proseguir con el Western Blot.

Microscopía Confocal

Las células fueron sembradas en cajas de petri con fondo de cristal y las imágenes fueron obtenidas con el microscopio fv1000 olympus de la unidad de microscopía del Instituto de Fisiología Celular y procesadas con el programa ImageJ.

Análisis estadístico

El análisis estadístico se realizó con el programa GraphPad Prism 5. Dependiendo del experimento se utilizó una prueba de T de Student ó ANOVA y la prueba post-hoc de Bonferroni.

Resultados y discusión

Para poder estudiar las funciones de los receptores de LPA decidimos utilizar una proteína de fusión del receptor dos para ácido lisofosfatídico (LPA_2) fusionado a eGFP (del inglés *Enhanced Green Fluorescense Protein*) ya que esto nos permite observar la localización subcelular del receptor y nos da un antígeno que nos permite inmunoprecipitar el receptor con anticuerpos comerciales. Al transfectar el plásmido que contenía dicho gen en células C9 se generó una línea celular que expresa de manera estable el LPA_2 fusionado a la eGFP, en lo posterior me referiré a ésta línea como C9-LPA_2. Elegimos esta línea celular de epitelio hepático de rata (C9) debido a que el objetivo del trabajo es entender la regulación de este receptor en células de mamífero y se sabe que estas células expresan de forma endógena este receptor, por lo que poseen un sistema de señalización en el que participa este receptor. Verificamos la expresión adecuada del receptor por medio de Western Blot y microscopía confocal observamos la presencia de GFP principalmente en membrana (figura 6 y 16).

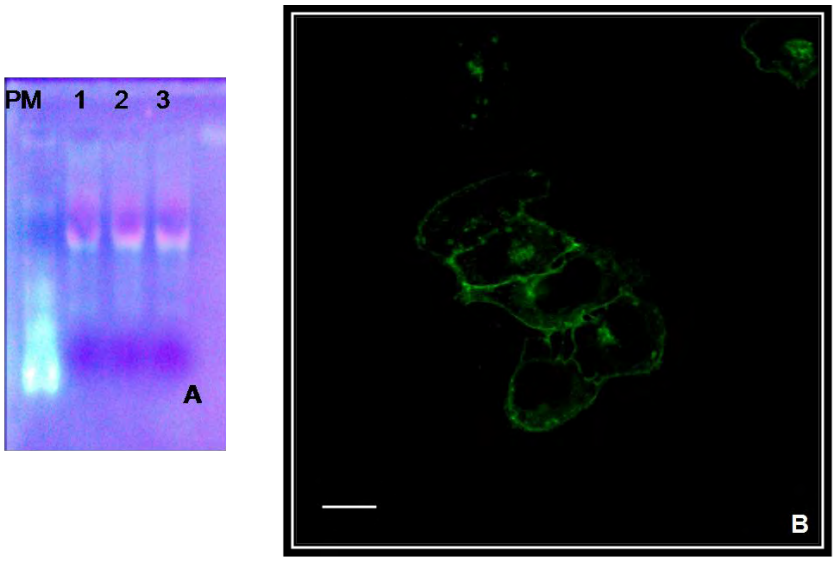


Figura 6. Expresión estable del LPA2-eGFP en las células C9. A) Plásmido LPA2-eGFP purificado. PM (Marcador de peso molecular), 1-3 purificaciones de tres cultivos distintos. B) Células C9 transfectadas con el plásmido LPA2-eGFP observadas con microscopía confocal. La línea representa 15 µm

En el laboratorio previamente se había evaluado previamente la presencia de receptores endógenos de la familia EDG en células C9. Se encontró que las células C9 expresan LPA_1 y LPA_2 (Avedaño-Vázques E. 2005) y que son capaces de liberar calcio de las pozas de calcio al citoplasma cuando son estimuladas con LPA. En este trabajo evaluamos éste proceso tanto en células silvestres (C9-Wt) como en las células C9-LPA_2 y comparamos las respuestas. Atribuimos que las diferencias entre ambas líneas celulares son causadas por la presencia del receptor que nosotros introdujimos al sistema. Observamos una diferencia importante en la magnitud de la inducción de la liberación de calcio. Mientras que las células silvestres aumentan su concentración de calcio intracelular en aproximadamente 200 nM las células C9-LPA2 presentaron un cambio de 600 nM (figura 7).

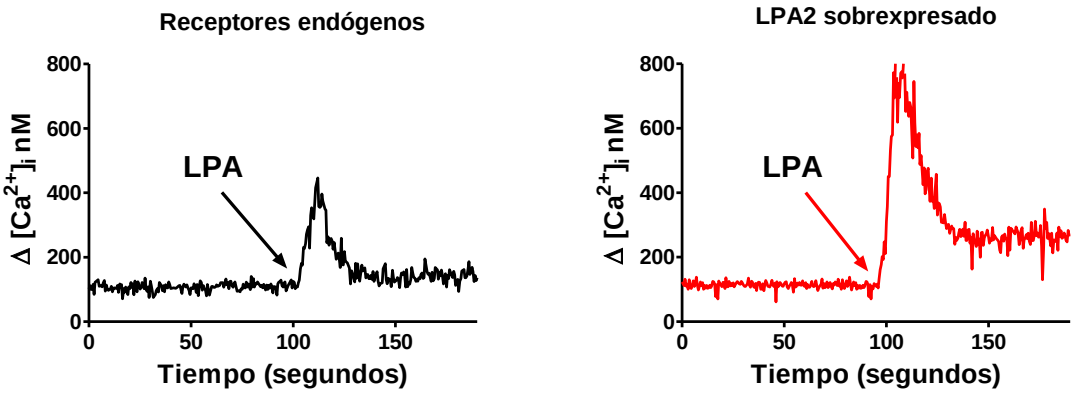


Figura 7. Aumento en la liberación de calcio mediado por LPA2. Se muestran los trazos representativos de la liberación de calcio intracelular al estimular con LPA a los 100 segundos en células sin transfectar (izquierda) y transfectadas con el receptor LPA2 (derecha).

Continuando con la caracterización de la respuesta de los LPA_2 se llevó a cabo un experimento utilizando distintas concentraciones de LPA para evaluar la sensibilidad a LPA midiendo la liberación de calcio en nuestro modelo. En la figura 8 se presentan en forma gráfica los datos obtenidos; únicamente se muestran los errores en el caso del LPA_2 porque es el principal objeto de estudio de éste trabajo. La curva muestra que el LPA_2 no llega a saturación, aún a elevadas concentraciones del ligando, por lo que no pudimos determinar la dosis efectiva 50 (EC50), a pesar de ello es evidente que el LPA_2 posee mayor eficacia pero menor sensibilidad al LPA

con respecto a la respuesta que se observa a las mismas dosis en los receptores LPA1 y LPA3 incluso a concentraciones tan altas como 100 µM.

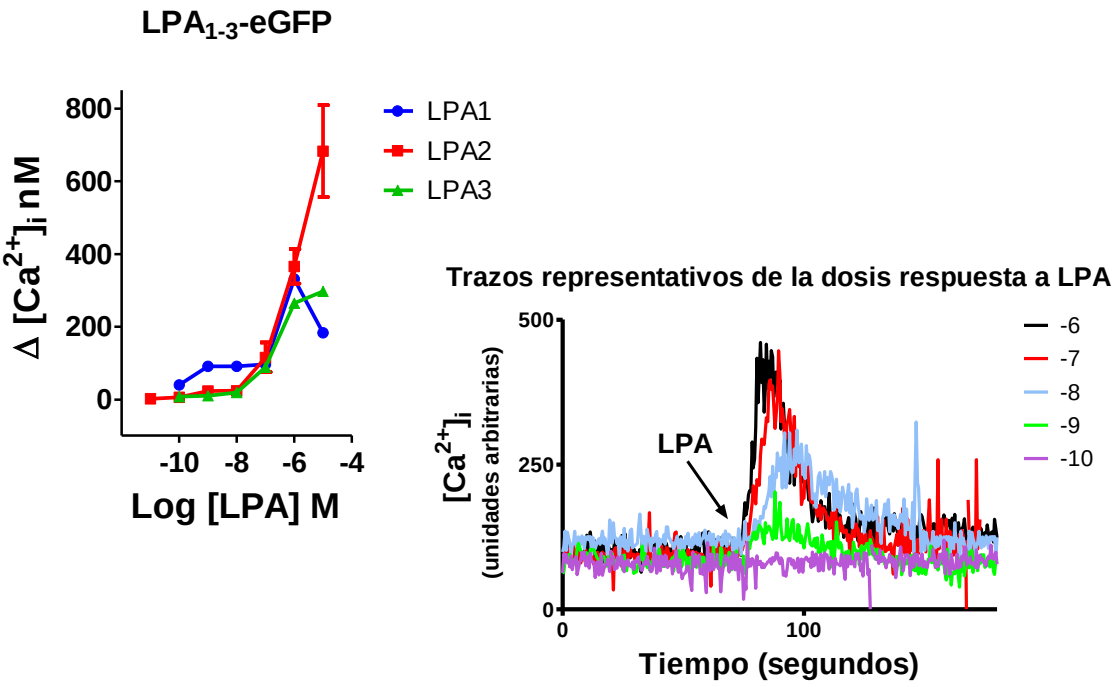


Figura 8. Dosis respuesta a LPA A) Cinética de la respuesta en las líneas celulares que expresan cada uno de los receptores de LPA de la familia edg. Los puntos representan el promedio y las barras representan el error estándar (SEM) B) Empalme de los trazos representativos de un solo experimento con las células que sobreexpresan el receptor para LPA2.

Posteriormente estudiamos la inhibición de estos receptores utilizando un antagonista específico de LPA_1 y LPA_3, el Ki16425, el cual también logra inhibir al LPA_2 pero de manera menos efectiva (Hideo et al. 2002). Efectivamente la respuesta en calcio mediada por LPA_2 fue inhibida por el Ki16425 en una menor proporción al compararlo con LPA_1. Sin embargo inhibió al receptor en una mayor proporción a lo esperado (figura 9). Es posible que este resultado se deba a la diferencia en el contexto celular; las células en las que se probó previamente el efecto del Ki16425 son en su mayoría células derivadas de diversos tipos de canceres o células transformadas por virus, mientras que nuestro modelo es una línea celular derivada de tejido sano.

Por otra parte estudiamos la desensibilización heteróloga del LPA_2 mediada por PMA (forbol 12-miristato 13-acetato) (Figura 9). El PMA, también conocido como TPA (Tetradecanoil forbol acetato), es un éster de forbol que activa a la Proteína Cinasa C (PKC) debido a su parecido con el 1,2-diacilglicerol (DAG). Observamos que los tres tipos de receptores para LPA de la familia EDG fueron sensibles a la acción del PMA resultando en la desensibilización de éstos. En el caso del LPA_2 la inhibición de la liberación de calcio fue casi total después de estar en contacto con PMA durante 5 minutos.

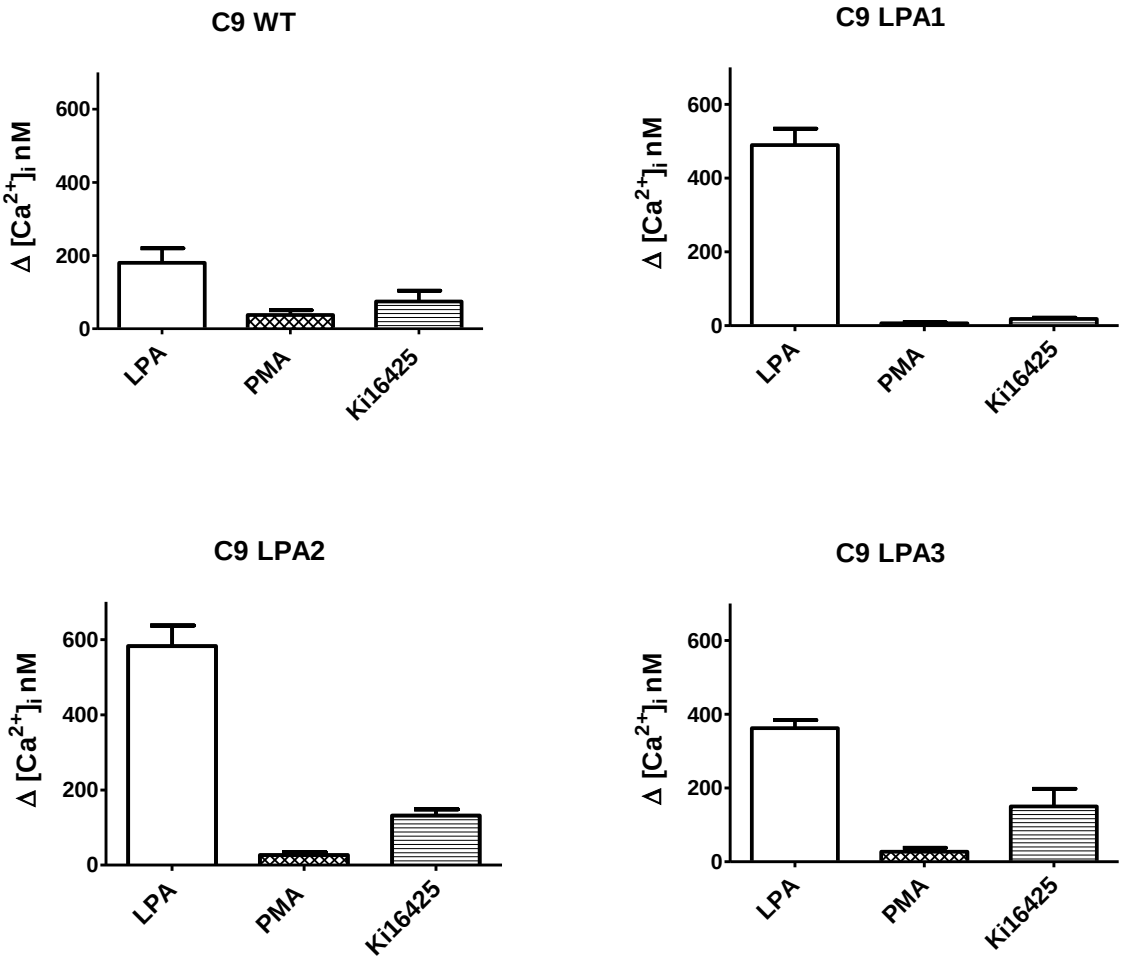


Figura 9. Desensibilización de los receptores para LPA mediada por PMA o por Ki16425. Las diferentes líneas celulares fueron estimuladas con LPA [1 µM] para medir la diferencia de la concentración de calcio intracelular. Sin pretratamiento o pretratados 5 minutos con 1µM de PMA o 10µM de Ki16425.

En estudios ulteriores nos enfocamos en las respuestas mediadas únicamente por el LPA2.

Primero estudiamos la acción del PMA sobre la desensibilización del LPA_2 evaluando la diferencia en la concentración de calcio al estimular células C9-LPA_2 con una misma concentración de LPA, después de un pretratamiento de cinco minutos de dosis logarítmicamente crecientes de PMA (figura 10).

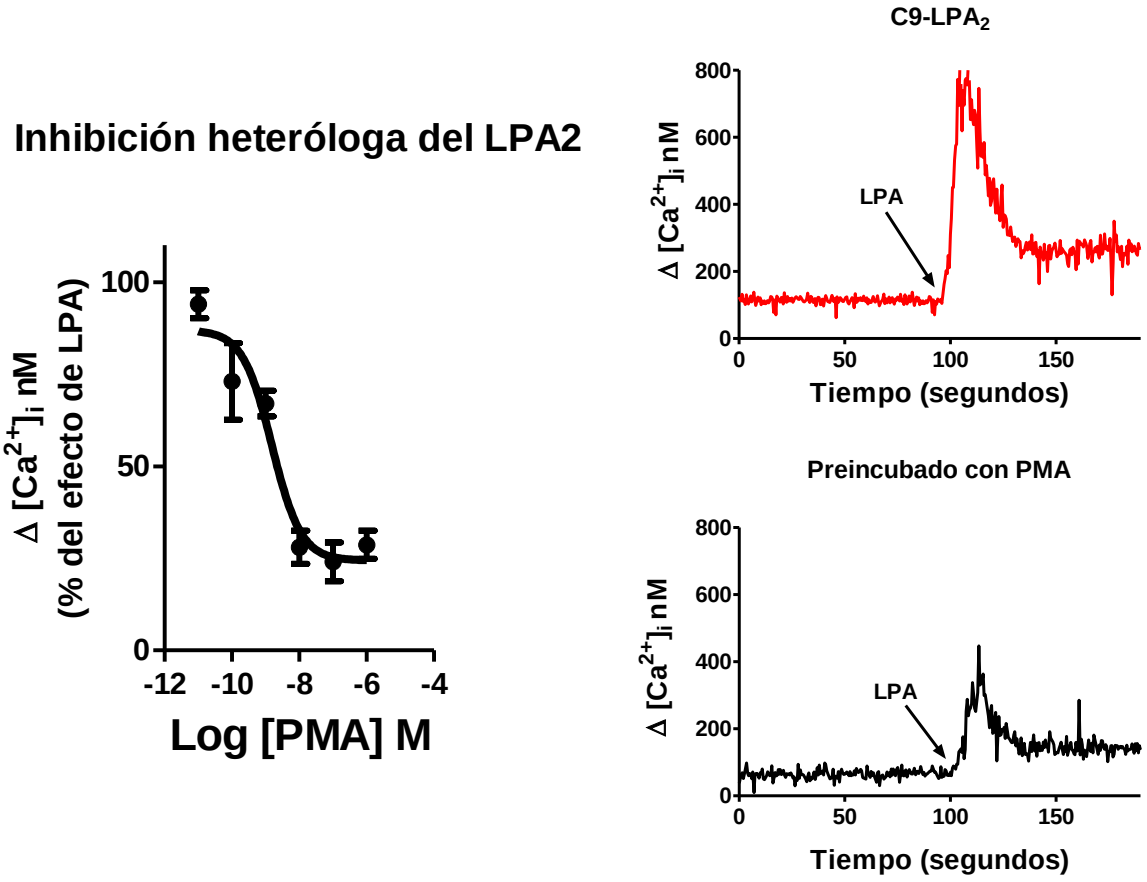


Figura 10. Inhibición heteróloga del LPA2. Dosis respuesta a PMA del lado izquierdo. Los trazos representativos que ilustran la inhibición a 1µM de PMA. Los puntos representan el promedio y las barras el error estándar (SEM).

Para entender cómo otras vías (ERK, p38 y Akt/PKB) son afectadas por la activación de este receptor evaluamos por medio de la técnica de Western blot la

fosforilación de estas proteínas como parámetro de su activación. Los resultados fueron los siguientes:

A diferencia de lo que pasa en la concentración de calcio la fosforilación de ERK en las células silvestres no cambia con respecto al basal al estimular las células con LPA ni con PMA. El antagonista Ki16425 tampoco tiene efecto en ninguna condición. En contraste la fosforilación de ERK que se observa en las células C9-LPA_2 aumenta con respecto al basal al estimular las células con LPA, esta respuesta no es reducida por el antagonista Ki16425, esto mismo se observa en la estimulación por PMA. El ki16425 por sí solo no tiene efecto (figura 11). De lo anterior podemos concluir que la activación del LPA_2 en induce la fosforilación de ERK. El curso temporal sugiere que el LPA_2 induce la activación de ERK1/2 de forma rápida y transitoria, ya que su pico máximo de fosforilación se da a los cinco minutos y este empieza a decaer inmediatamente, incluso llega a niveles por debajo del basal (figura 12).

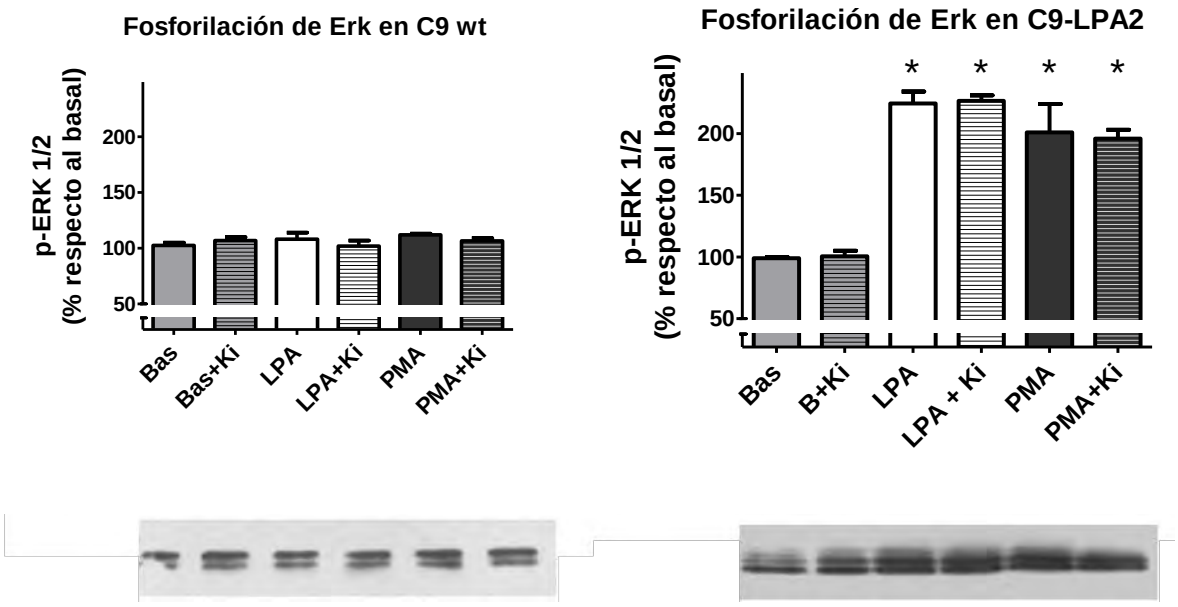


Figura 11. Fosforilación de ERK por diferentes estímulos. Fosforilación de ERK mediada por LPA. A) En células sin transfectar. B) En células transfectadas con LPA2-eGFP. Sin estimular (bas), al incubar con el inhibidor (+ki), al estimular con LPA después de preincubar con ki16425, con PMA o ambos 5 minutos. En la parte inferior de cada gráfica se muestran los blots representativos.

Una cinasa cuya fosforilación se asocia comúnmente a la activación de ERK es la proteína p38, otra MAPK. En este caso la cinética de fosforilación es totalmente

distinta a la de ERK, en nuestro sistema celular. La activación de p38 fue máxima a los dos minutos, a diferencia de ERK cuyo pico de fosforilación es a los cinco minutos. Sumado a esto, la fosforilación de p38 se mantiene aproximadamente al mismo nivel hasta los 60 minutos posteriores al estímulo (figura 13). Otro trabajo ha demostrado igualmente que la activación de LPA_2 induce la fosforilación de p38 (Huang L. S. et al 2013).

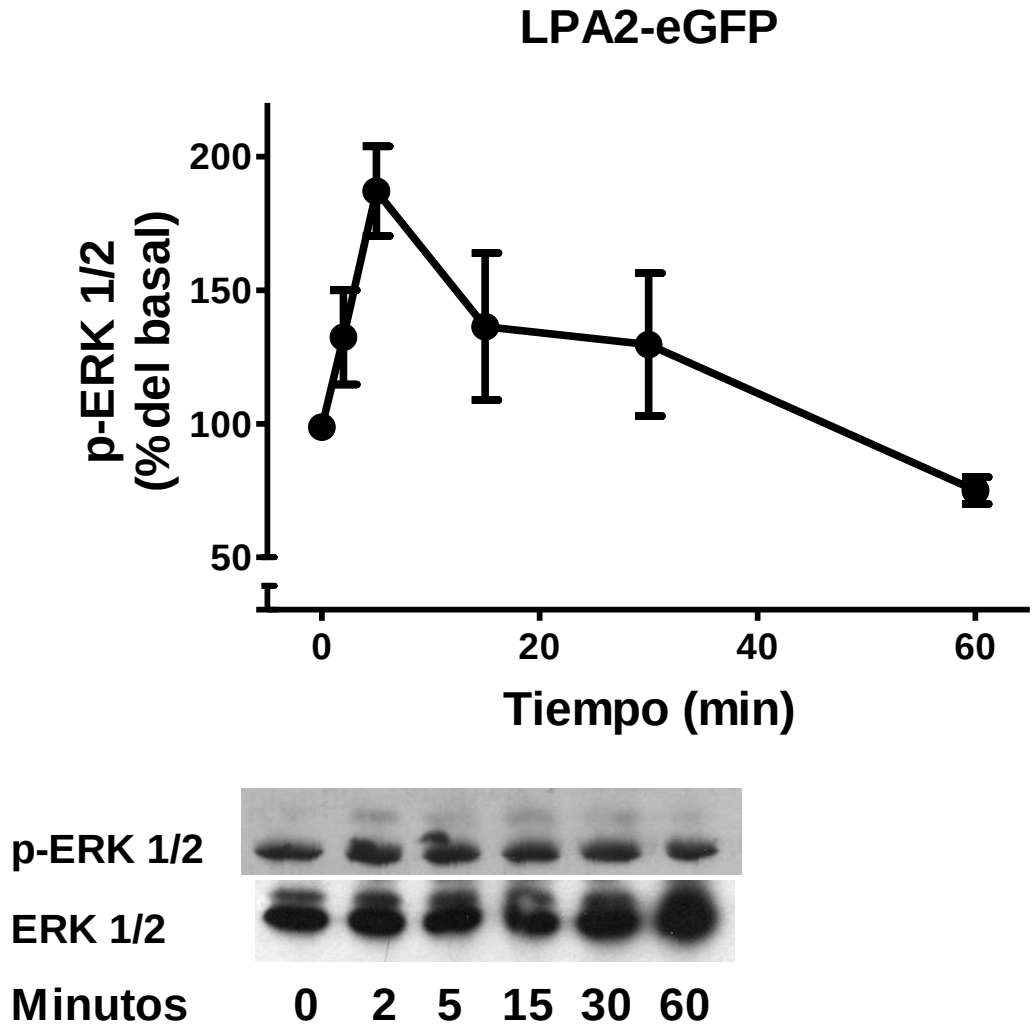


Figura 12. Curso temporal de la fosforilación de ERK. En la parte superior se gráfica el cambio en la fosforilación de al menos 3 experimentos. Los puntos representan el promedio y las barras

representan el error estándar (SEM). En la parte inferior se muestran los blots representativos de p-ERK y ERK total como control de carga.

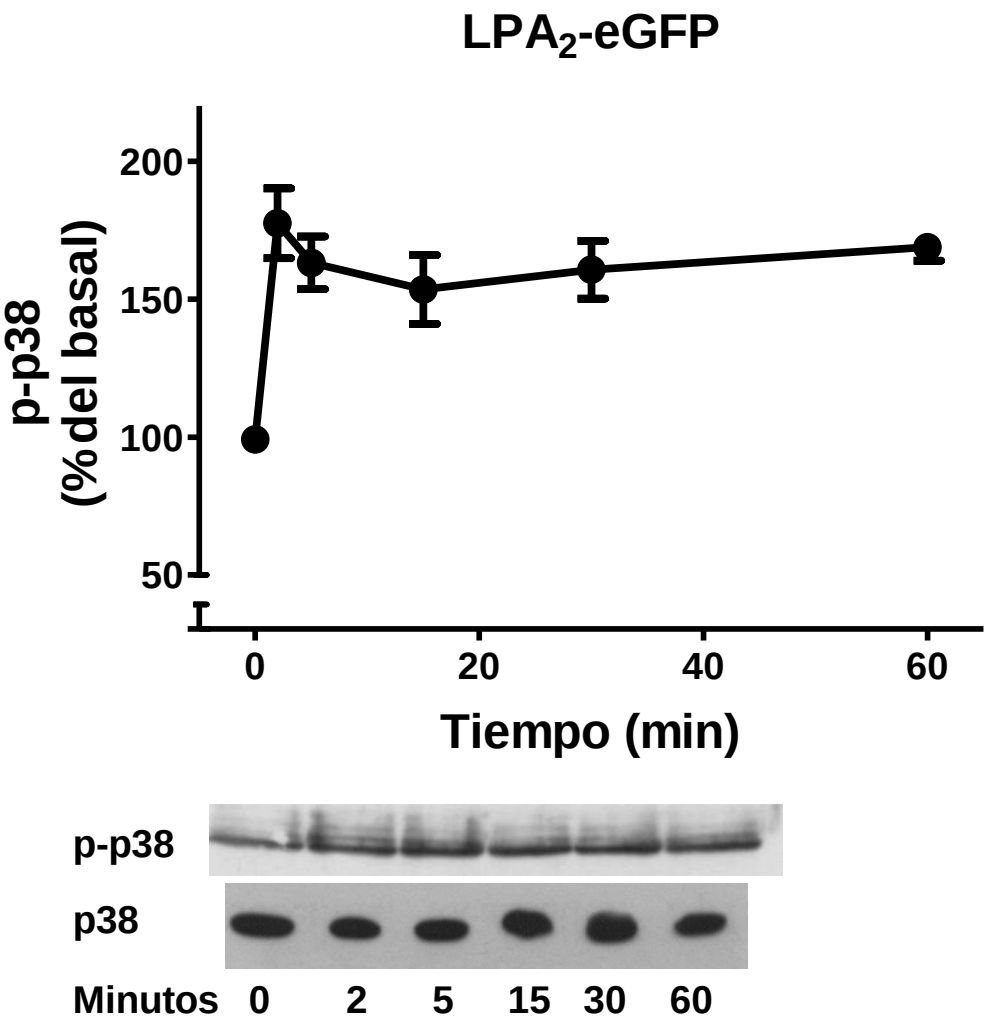


Figura 13. Curso temporal de la fosforilación de p38. En la parte superior se gráfica el cambio en la fosforilación de p38 de al menos 3 experimentos. Los puntos representan el promedio y las barras representan el error estándar (SEM). En la parte inferior se muestran los blots representativos de p-p38 y p38 total como control de carga.

En cuanto a la activación de la vía Akt, la gráfica muestra que la cinasa tiene un máximo de activación a los cinco minutos y, al igual que p38, es una fosforilación

sostenida que se mantiene hasta al menos los primeros 60 minutos posteriores al estímulo (figura 14). No se probaron tiempos más largos.

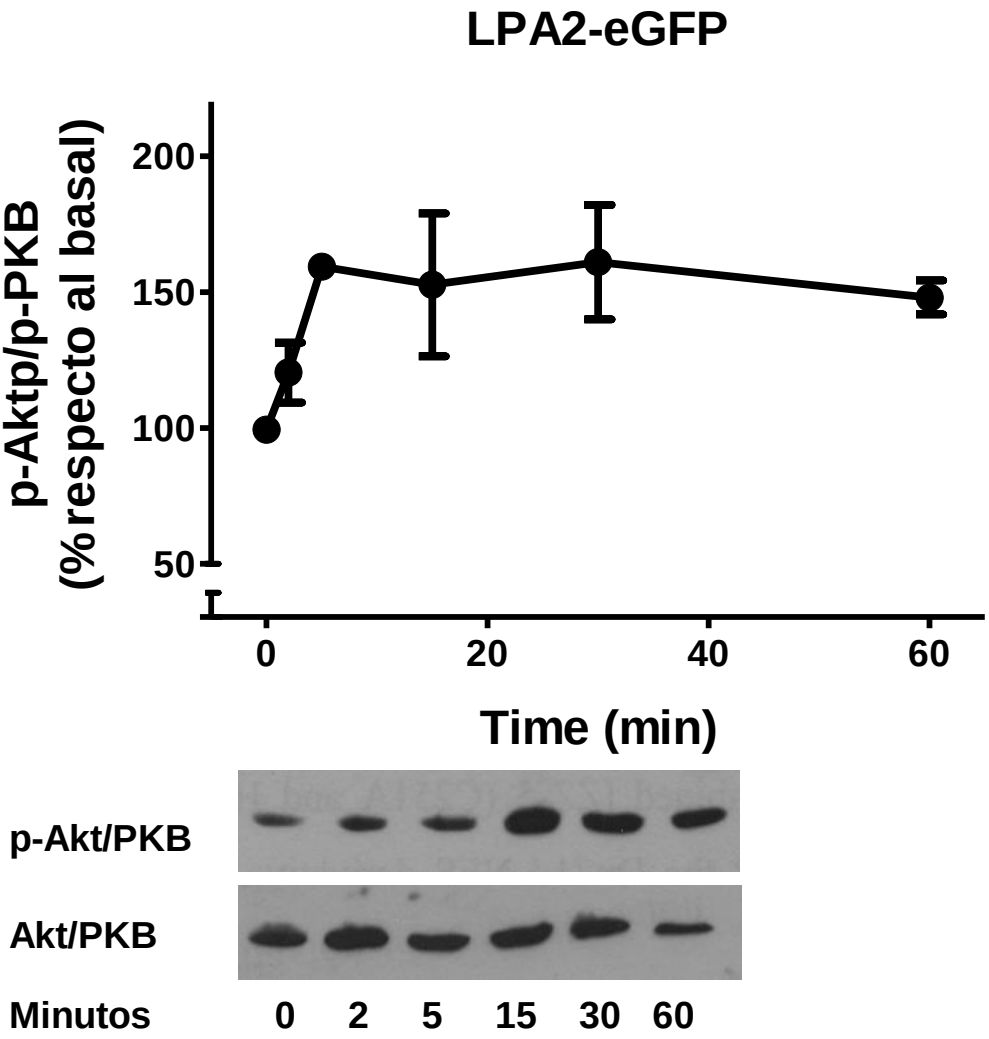


Figura 14. Curso temporal de la fosforilación de Akt/PKB. En la parte superior se grafica el cambio en la fosforilación de Akt/PKB de al menos 3 experimentos. Los puntos representan el promedio y las barras representan el error estándar (SEM), donde no hay barras de error están dentro del símbolo. En la parte inferior se muestran los blots representativos de p-Akt/p-PKB y Akt/PKB total como control de carga.

Los resultados anteriores muestran que el receptor LPA_2 fue desensibilizado por la acción de PMA ya que disminuyó el cambio en la concentración de calcio intracelular producido en respuesta al LPA. Sin embargo, también observamos que

el PMA, sin la activación del LPA_2, induce la fosforilación de ERK. Nos preguntamos si estos efectos son mediados por PKC. Para responder esta pregunta pretratamos por 15 minutos a las células C9-LPA2 con un inhibidor general de PKC, el Bim I (Bisindolilmaleimida I). Posteriormente agregamos PMA durante 2 minutos y finalmente medimos la liberación de calcio intracelular estimulando a las células con LPA 1µM. La figura 15 muestra que la liberación de calcio del RE se realizó de forma normal a pesar del pretratamiento con PMA, lo que sugiere que la inhibición de PKC permite que el LPA_2, en respuesta a LPA, induzca la liberación de calcio, mostrando que PKC participa en su desensibilización. Para comprobar esto realizamos un ensayo de *down-regulation* de la PKC, en el que se pretratan las células con PMA durante toda la noche (TN), lo cual se sabe promueve la degradación de la PKC. Los resultados muestran que un segundo pretratamiento con PMA ya no tiene efecto sobre la desensibilización del receptor, observándose incluso que la respuesta del receptor fue exacerbada al compararlo incluso con células estimuladas sin ningún pretratamiento. Lo que confirma la participación de PKC en la desensibilización del LPA_2.

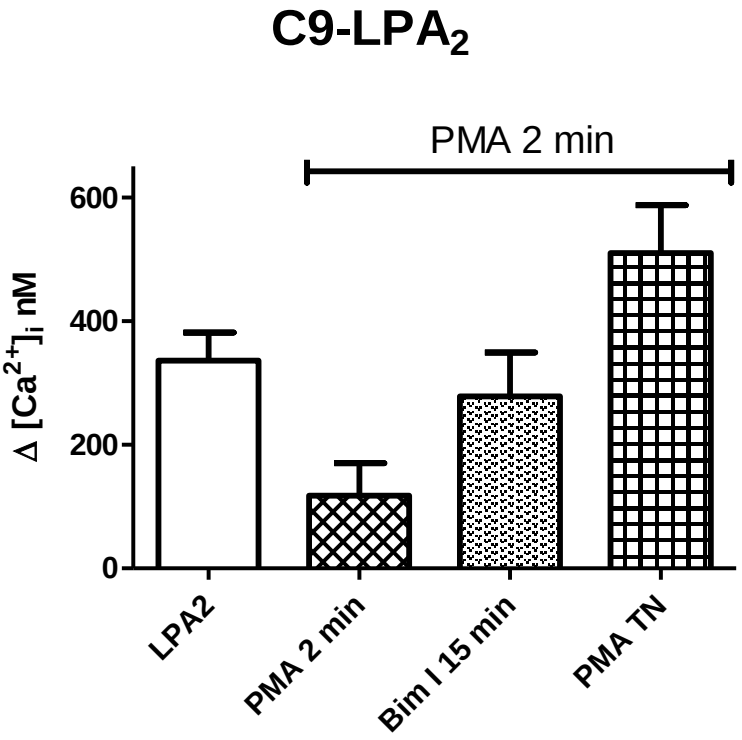


Figura 15. Participación de las PKCs en la respuesta a LPA. Las células C9-LPA2 fueron estimuladas con 1µM de LPA y se midió la diferencia en la concentración de calcio intracelular para cuantificar la respuesta en células sin pretratamiento (LPA2) o pretratadas con: 1 µM de PMA 2 minutos (PMA), Bim I 15 minutos o 1µM de PMA toda la noche (PMA TN) + PMA 2 minutos.

Por último nos preguntamos cuál podría ser la isoforma de PKC implicada en la desensibilización del LPA_2 mediada por PMA (Fig. 4 y 5). Para responder esta pregunta realizamos ensayos de co-inmunoprecipitación en los cuales se inmunoprecipita al LPA_2, con ayuda de un suero anti-GFP producido en el laboratorio, y se detecta por medio de western blot la cantidad relativa de proteínas asociadas, en este caso isoformas de PKC, asociada al receptor LPA_2. Utilizamos anticuerpos específicos que reconocen las isoformas convencionales de PKC (PKCα, PKCβI, PKCβII, PKCγ) las cuales son dependientes de calcio. Nuestros resultados muestran que aún probando tres distintas concentraciones de anticuerpos no detectamos la presencia de las isoformas PKCβII ni PKCγ, únicamente fuimos capaces de detectar la asociación de PKCα y PKCβI en todos los estímulos probados. La asociación del LPA_2 con estas isoformas está presente incluso antes del estimulo y sólo detectamos un aumento significativo en el caso de la asociación LPA_2-PKCβI a los 30 minutos después de ser estimulado con LPA. Por lo que podemos sugerir que PKCβI juega al menos un papel en la regulación tardía del receptor LPA_2. Sin embargo, dada la variabilidad de los datos obtenidos de este experimento sería conveniente realizar más repeticiones del mismo para llegar a una conclusión más certera. Por otro lado, los efectos observados durante el desarrollo de este trabajo ocurrían en tiempos más cortos (dos a cinco minutos) y no observamos cambios evidentes a los 30 minutos, por lo que es poco probable pensar que esta asociación tenga algún efecto sobre las respuestas estudiadas. Ya que se observó que la PKC participa en este proceso es posible que sean otras isoformas las que participen en la regulación temprana del receptor.

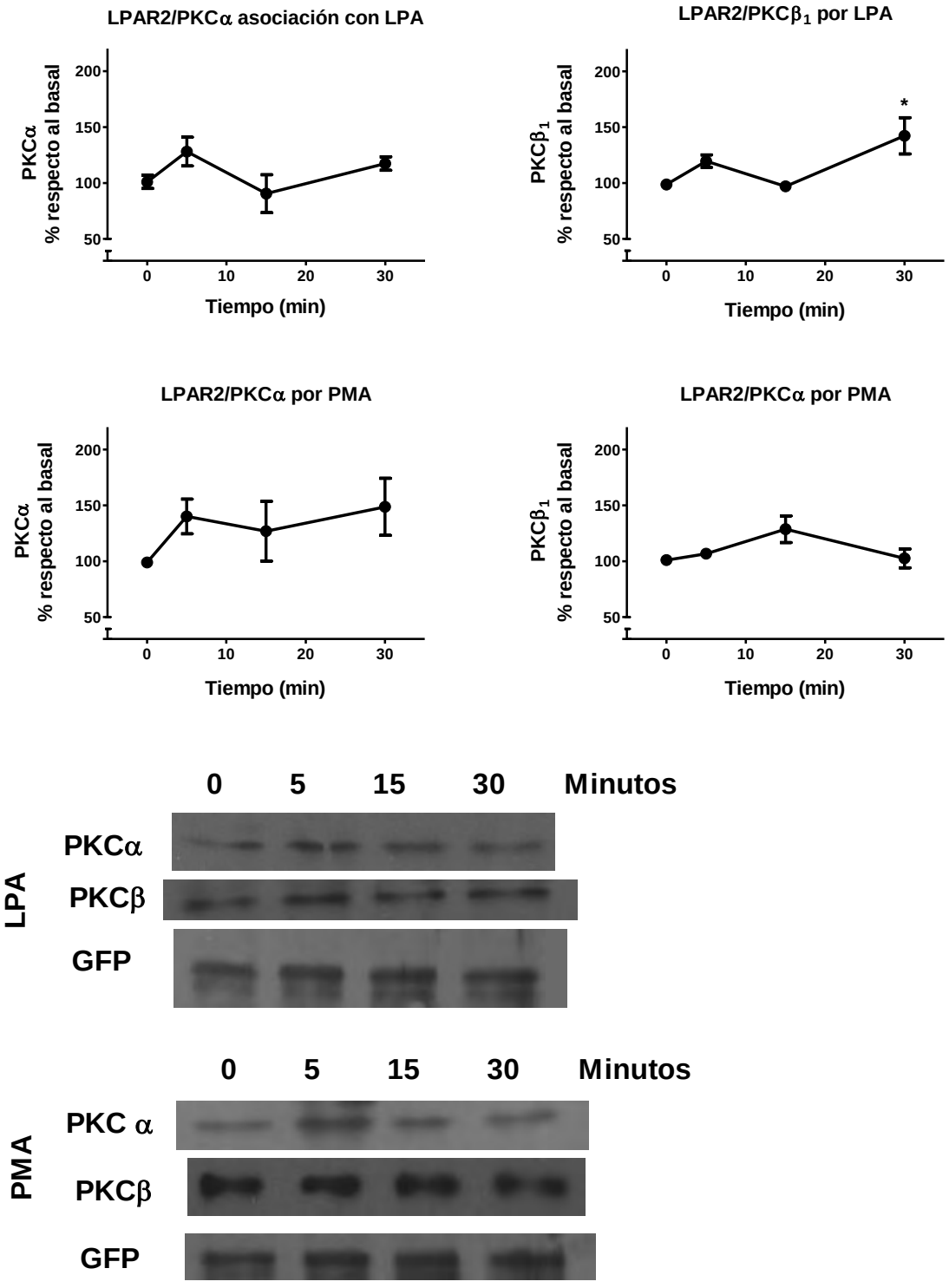


Figura 16. Coinmunoprecipitación de LPA2 con PKCα o PKCβ$_1$. Las gráficas representan de al menos 3 experimentos independientes. Los puntos representan el promedio y las barras representan el error estándar (SEM). En la parte inferior se muestran los blots representativos de cada gráfica y GFP como control de carga.

Conclusiones

Al término de este trabajo podemos concluir que el LPA_2 es sensible a la desensibilización mediada por PMA y al antagonista Ki16452. Éste receptor también es capaz de activar la vía de ERK de manera rápida y transitoria, activa las vías de p38 y Akt/PKB de manera sostenida. La desensibilización mediada por PMA es bloqueada por Bim I por lo que sabemos que la PKC está participando en este proceso.

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PLOS ONE

RESEARCH ARTICLE

Phosphorylation and Internalization of Lysophosphatidic Acid Receptors LPA_1, LPA_2, and LPA_3

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Abstract

Results

The lysophosphatidic acid receptors LPA_1, LPA_2, and LPA_3 were individually expressed in C9 cells and their signaling and regulation were studied. Agonist-activation increases intracellular calcium concentration in a concentration-dependent fashion. Phorbol myristate acetate markedly inhibited LPA_1- and LPA_3-mediated effect, whereas that mediated by LPA_2 was only partially diminished; the actions of the phorbol ester were inhibited by bisindolylmaleimide I and by overnight incubation with the protein kinase C activator, which leads to down regulation of this protein kinase. Homologous desensitization was also observed for the three LPA receptors studied, with that of LPA_2 receptors being consistently of lesser magnitude; neither inhibition nor down-regulation of protein kinase C exerted any effect on homologous desensitization. Activation of $LPA_{1–3}$ receptors induced ERK 1/2 phosphorylation; this effect was markedly attenuated by inhibition of epidermal growth factor receptor tyrosine kinase activity, suggesting growth factor receptor transactivation in this effect. Lysophosphatidic acid and phorbol myristate acetate were able to induce $LPA_{1–3}$ phosphorylation, in time- and concentration-dependent fashions. It was also clearly observed that agonists and protein kinase C activation induced internalization of these receptors. Phosphorylation of the LPA_2 subtype required larger concentrations of these agents and its internalization was less intense than that of the other subtypes.

Conclusion

Our data show that these three LPA receptors are phosphoproteins whose phosphorylation state is modulated by agonist-stimulation and protein kinase C-activation and that differences in regulation and cellular localization exist, among the subtypes.

Introduction

Lysophosphatidic acid (LPA) is one of the so-called, "bioactive lipids", that participates not only in cell metabolism but also as an autacoid or local hormone, communicating cells. LPA is involved in a very large number of physiological processes, modulating the function of many organs and systems (gastrointestinal apparatus, nervous, immune, and urogenital systems, and others); this lipid takes part in embryonic development and also has a "dark side" being involved in the pathogenesis of diseases (fibrosis, inflammation, and cancer, among many others); at the cellular level, it modulates migration, chemotaxis, proliferation, survival, and other processes (see [1–5] and references therein). LPA actions are mainly exerted through a family of G protein-coupled receptors (GPCRs), that is, the LPA receptors, comprising six members that are currently designated LPA_{1-6}; the possibility that GPR87 could be also a member of this family has been suggested, i. e., such as LPA_7 [1–6]. Of these receptors, LPA_1, LPA_2 and LPA_3 are phylogenetically related among themselves and also with those of other bioactive lipids (the endothelial differentiation gene ["edg"] family); the remaining LPA receptors are distant phylogenetically from these and are more closely related with the purinergic receptor family [1–7]. Evolutionary aspects of these receptors, among vertebrates, have been recently reported [7]. It is also known that LPA can modulate transcription through nuclear receptors, such as the peroxisome proliferator-activated receptor γ [8]. LPA also activates the TRPV1 ion channel involved in the control of body temperature and nociception [9].

Our present work deals exclusively with the LPA_{1-3} receptors. The actions of these receptors have been studied using many different natural (i. e. endogenously expressed) and transfected cellular and systemic models. However, few studies have analyzed LPA_{1-3} desensitization and internalization employing the same cellular model. In particular, the phosphorylation of these receptors has been scarcely studied. To the best of our knowledge, solely LPA_1 receptor phosphorylation has been reported and only by our own group [4, 10–14]. The present work was designed to fulfill this gap in knowledge.

Desensitization, defined as a stage of reduced sensitivity to a particular stimulus, can involve a large number of processes with different time scales. It is generally accepted that GPCR sensitivity (desensitization/ resensitization) involves phosphorylation/ dephosphorylation cycles controlled by particular protein kinases and phosphatases [15–20]; although there is evidence also for phosphorylation-independent desensitization [21]. The majority of current data indicate that agonist-induced receptor desensitization (homologous desensitization) involves receptor phosphorylation by G protein-coupled receptor kinases (GRKs) whereas desensitization of unoccupied receptors, i. e. agonist-independent (heterologous desensitization) mainly involves signaling activated kinases such as the second messenger-activated kinases, protein kinase A and protein kinase C (PKC), among others [15–20]. Receptor internalization appears to be related with receptor phosphorylation. Current ideas indicate that phosphorylated receptors interact with β-arrestins and act as molecular bridges with clathrin, clustering receptors that internalize in coated vesicles; such internalization can lead receptors to plasma membrane recycling, trafficking to other compartments or to degradation. Variation in the phosphorylation pattern of a given receptor has been observed and it has been suggested that such phosphorylation "bar code" might determine receptor's destination and function [19, 22, 23]. Recently, we reported differential association of α_{1B}-adrenergic receptors to Rab proteins during internalizations induced by agonists (homologous) or unrelated (heterologous) stimuli [24].

In silico analysis showed that these three receptors, i. e., LPA_1, LPA_2, and LPA_3, possess putative phosphorylation sites for a variety of protein kinases, particularly GRKs and PKC isoforms, with marked differences among them [4]. These receptors fused to the enhanced green

fluorescent protein (eGFP) were expressed in C9 cells and their signaling, sensitivity to PKC activation, phosphorylation, and internalization were studied comparatively. Our results clearly indicate that LPA_1, LPA_2, and LPA_3 receptors are phosphorylated and internalize in response to LPA and PKC activation. Differences in agonist sensitivity and degree of internalization/ desensitization were also observed.

Materials and Methods

1. Materials

LPA (oleyl-sn-glycerol 3-phosphate), phorbol myristate acetate (PMA), G418, Ham's F12 Kaighn's modification medium, protease inhibitors and DNA purification kits were purchased from Sigma Chemical Co. Phosphate-free Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin, antibiotics, and other reagents used for cell culture were from Life Technologies. Fura-2 AM was obtained from Invitrogen and agarose-coupled protein A, from Upstate Biotechnology. Bisindolylmaleimide I and AG1478 were purchased from Calbiochem whereas EGF was obtained from Preprotech. [^{32}P]Pi (8,500–9,120 Ci/mmol) was obtained from Perkin Elmer Life Sciences. The plasmid construction for the expression of mouse LPA_1 receptor fused to the eGFP was previously described [11] and those used for the expression of human LPA_2 and LPA_3, also fused to the eGFP at the carboxyl termini, were obtained from GeneCopoeia and OriGene, respectively. Rabbit polyclonal antibodies against ERK 1/2 and phospho-ERK 1/2 were from Cell Signaling Technology. Secondary antibodies were obtained from Zymed. For Western blotting an anti-GFP monoclonal antibody from Clontech was employed. Rabbit antisera against eGFP were generated at our laboratory using standard procedures [25] by immunizing New Zealand rabbits with *E. coli*-overespressed GST-fused eGFP (1 mg/ kg, each 2 weeks for at least 6 times) and have been previously characterized and compared with commercial antibodies [11, 12, 14]. Animals were handled and maintained in individual cages, with free access to water and rabbit chow, in rooms with controlled air temperature and lighting (12 h/12 h); handling and bleeding (marginal ear vein) were performed under the direct supervision of one of the Veterinary Doctors in change of the animal facility.

2. Cell culture and transfection

C9 cells (Clone 9, rat hepatic epithelial cells, CRL-1439™), obtained directly from American Type Culture Collection, were cultured in Ham's F12 Kaighn's modification medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 units/ml penicillin and 0.25 μg/ml amphotericin B at 37°C under a 95% air and 5% CO_2 atmosphere, as described previously [11]. The medium was replaced with one containing 1% fetal bovine serum, 12–16 h before the experiment. C9 cells stably expressing the mouse LPA_1 receptor fused at the carboxyl termini with eGFP were those previously described [11]. Stable expression of the human LPA_2 and LPA_3 receptors fused to eGFP was obtained by transfecting wild type C9 cells with the plasmid constructs described above using lipofectamine 2000, following the manufacturer's instructions; cells were transfected three times to increase the gene transfer efficiency [26]. Clones expressing these constructs were selected by resistance to incubation with G418 (600 ng/ml) and by their robustness of response to LPA (increase in intracellular calcium concentration). After this selection (3 cell passages) the cells were cultured in media containing 300 ng/ml of G418. As depicted in"Fig A in S1 File", expression of LPA_1 and LPA_2 receptors was similar (~80%) whereas that of LPA_3 was consistently lower (~60%), as evidenced by fluorescence imaging and flow cytometry. Flow cytometry was performed using an Attune NxT Acoustic Focusing Cytometer, employing excitation of 488 nm; data were analyzed using the Attune cytometric software included with the equipment.

3. Intracellular calcium concentration ($[Ca^{2+}]_i$)

This procedure has been described previously [11]. Briefly, cells were loaded with 2.5 μM Fura-2 AM in Krebs–Ringer–Hepes containing 0.05% bovine serum albumin (pH 7.4) for 1 h at 37°C and then washed to eliminate unincorporated indicator. Determinations were carried in an AMINCO-Bowman Series 2 luminescence spectrometer, employing 340 and 380 nm excitation wavelengths and an emission wavelength of 510 nm; chopper interval was 0.5 sec. The intracellular calcium concentration was calculated as described by Grynkiewicz et al. [27]. In the experiments where prolonged incubation was required (resensitization studies), cells were stimulated, washed, and the incubation was continued in the presence of Fura-2 AM, to avoid dye depletion. Cells were washed again to remove extracellular fluorescent dye, and determinations made as indicated above; this procedure did not alter the magnitude of the control calcium responses.

4. ERK 1/2 phosphorylation

Cells were cultured to near confluence in 6 well plates and stimulated with the agents tested for the times indicated. Total cellular extracts were obtained by lysing the cells in Laemmli's sample buffer containing 5% β-mercaptoethanol. The cell extracts were subject to 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes from samples obtained in parallel were incubated overnight at 4°C, with anti-pERK 1/2 (1:2000) or anti ERK 1/2 (1:2000). The membranes were washed and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (1:10,000) for enhanced chemiluminescence detection.

5. Phosphorylation of LPA_{1-3} receptors

The procedure was very similar to that previously described to study LPA_1 receptor phosphorylation [11, 12, 14]. In brief, cells were incubated in phosphate-free Dulbecco's modified Eagle's medium containing 100 μCi/ml [^{32}P]Pi for 3 h at 37°C. LPA_1- and LPA_2-expressing cells were cultured in 6 well plates, whereas those expressing LPA_3 receptors were cultured in 10 cm dishes. Labeled cells were stimulated with the agents tested and then washed twice with ice-cold phosphate buffered saline and solubilized in buffer containing 10 mM Tris, 150 mM NaCl, 1% sodium cholate, 1% Nonident P40, protease and phosphatase inhibitors, pH 7.4 [11, 12, 14]. The lysates were incubated overnight with protein A-agarose and anti-GFP serum with constant agitation at 4°C. Samples were washed and the pellets containing the immunocomplexes were solubilized in Laemmli's sample buffer containing 5% β-mercaptoethanol. Proteins were separated using 10% SDS-PAGE and electrotransferred onto polyvinyliden fluoride membranes. Receptor phosphorylation was analyzed with a Molecular Dynamics Typhoon PhosphorImager and the ImageJ software (http://rsb.info.nih.gov/ij/).

6. Receptor internalization- Imaging

Cells were seeded at approximately 30% confluence onto glass-bottomed Petri dishes and cultured for 3 h at 37°C in media containing 1% serum. After treatment, cells were washed three times with phosphate buffered saline and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min at room temperature; samples were then washed three additional times with phosphate buffered saline. The fluorescent images were acquired with an Olympus Fluoview FV10 confocal microscope with a water-immersion objective (60X). To determine receptor internalization, the plasma membrane was delineated utilizing the differential interference contrast imaging, and fluorescence in this region was quantified employing the ImageJ software. At least 5 or 6 images of different cultures were taken for each condition. Data were normalized

as follows: for each experiment, fluorescence (arbitrary units) at the plasma membrane of baseline samples were pooled and the average was considered as 100%.

7. Statistical Analysis

EC_{50} and IC_{50} values were calculated from the individual concentration-response curves employing the software included in the GraphPad Prism 6 program and reported as the rounded range of values observed. Similarly, analysis of variance with the Bonferroni's post-test was performed using the statistical software included in the Prism 6 program. A p value < 0.05 was considered statistically significant.

8. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Mexican Laws and Regulations on this matter. Protocol AGS29-14 was approved to our work by the Institutional Committee for the use of laboratory animals, Instituto de Fisiología Celular.

Results

In agreement with previous results [10, 11] wild-type C9 cells increase intracellular calcium concentration in response to LPA; this is likely due to the activation of LPA_1 and LPA_2 that are expressed in these cells [11]. The effect was concentration-dependent with a maximal calcium concentration increase of ~150–200 nM and EC_{50} values in the range of ~ 200–400 nM (Fig 1, panel A). Expression of LPA_{1-3} receptors markedly augmented this calcium response to increases the cation's concentration to ~ 300 nM (in cells over-expressing LPA_3 receptors) and to 450–600 nM (cells over-expressing LPA_1 or LPA_2 receptors); concentration-response curves yielded EC_{50} values in the range of 200–400 nM in LPA_1- or LPA_3- overexpressing cells. In cells overexpressing LPA_2, the LPA concentration response curve was slightly, but consistently, shifted to the right and no clear saturation was achieved at the concentrations tested (Fig 1, panels B-D; representative images in "Fig B in S1 File").

In order to evaluate heterologous desensitization (PMA-induced) cells were incubated with different concentrations of PMA for 2 min and then challenged with 1 μM LPA. As illustrated in Fig 2 (panel A), cells overexpressing LPA_1 receptors were very sensitive to PMA (IC_{50} 1–3 nM) whereas those overexpressing LPA_2 and LPA_3 receptors were slightly less sensitive (IC_{50} values in the range of 3–10 nM). Interestingly, in LPA_2-overexpressing cells PMA was unable to completely blunt the calcium response to the bioactive lipid, i. e., a remaining ~ 30–40% response was consistently observed, even at the highest PMA concentration tested (Fig 2, panel A; representative tracings are presented in "Fig B in S1 File"). In the case of LPA-induced (homologous) desensitization, cells expressing the different LPA receptors were incubated for 10 min in the presence of different LPA concentrations; after this, the cells were washed twice to remove LPA and were then challenged with 1 μM LPA. The results showed that cells expressing any of the three receptors were affected by the preincubation, even at very low concentrations of the agonist (Fig 2, panel B); the preincubation and washing procedures were not responsible for this as evidenced by the control responses (baseline, vehicle during the preincubation). Agonists-mediated decreases occurred in a concentration-dependent fashion, with a maximum at the concentration of ~ 100 nM. Interestingly, the magnitude of this desensitization process was $LPA_1 \geq LPA_3 > LPA_2$ (Fig 2, panel B). When cells were incubated for 10 min with 1 μM LPA, washed as indicated above and then challenged with 1–100 μM LPA the calcium response increased. This indicated that rather than decreasing the maximal response, homologous desensitization reduced the cell's sensitivity to LPA (Fig 3, left panels). This was

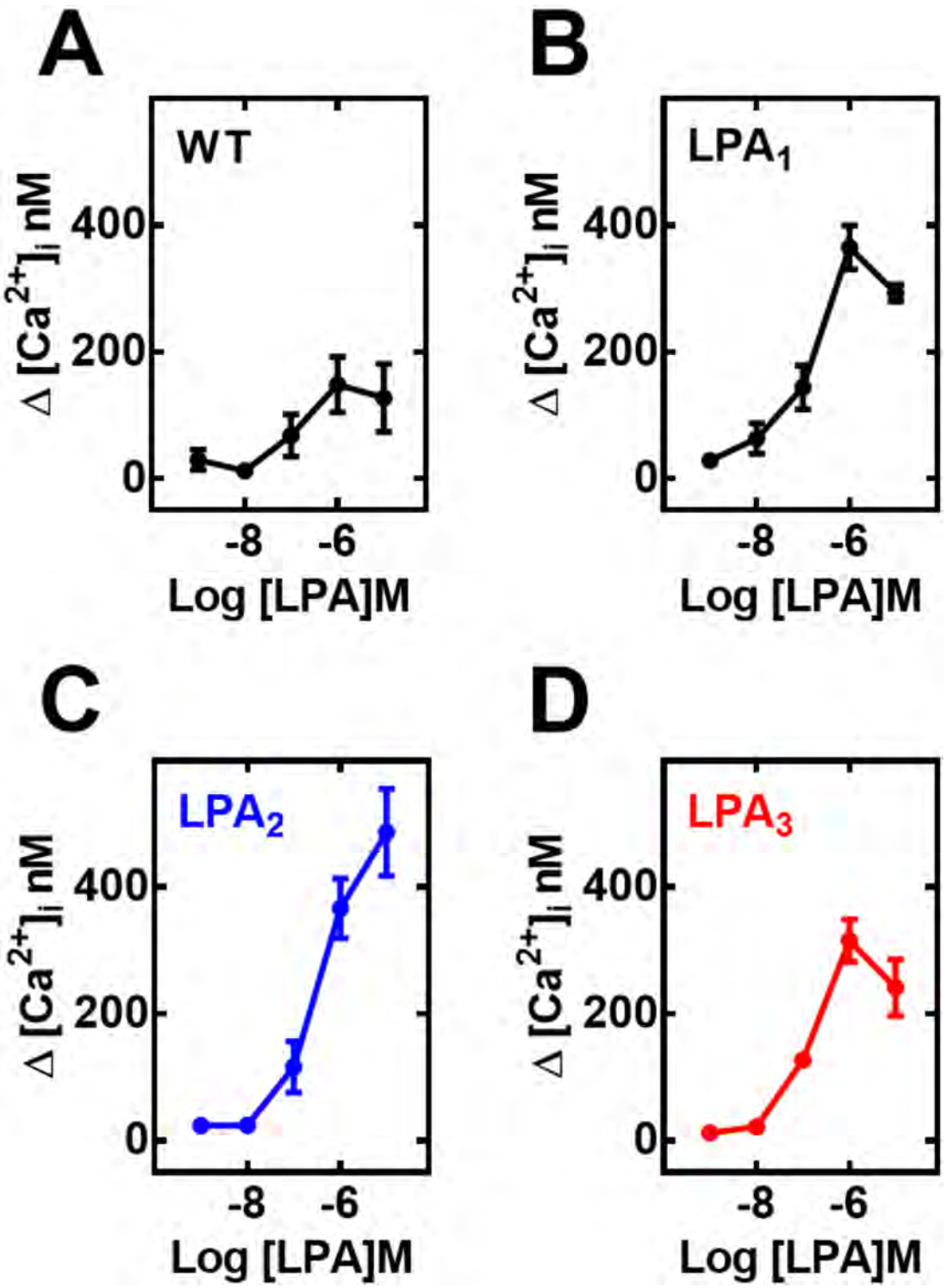

Fig 1. Effect of LPA on intracellular calcium concentration ($[Ca^{2+}]_i$). Wild type C9 cells (panel A) or overexpressing LPA_1 (panel B), LPA_2 (panel C) or LPA_3 (panel D) were stimulated by different concentrations of LPA. Plotted are the increases in intracellular calcium as mean ± S. E. M. of 4–5 experiments using different cell preparations.

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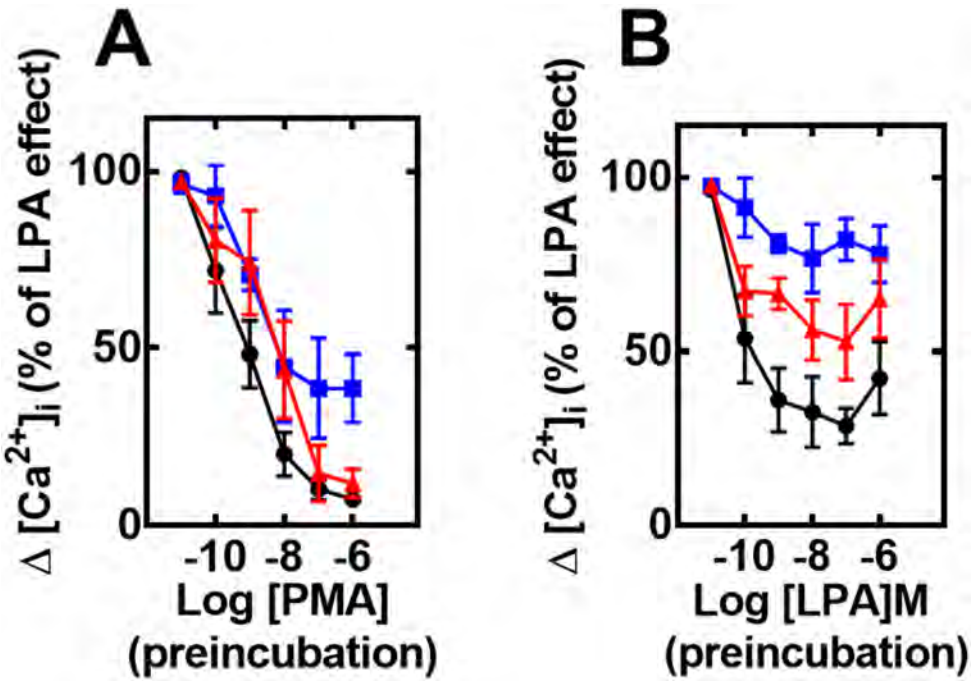

Fig 2. Effect of preincubation with PMA (heterologous desensitization) or LPA (homologous desensitization) on LPA-induced intracellular calcium concentration ([Ca^{2+}]$_i$). Cells overexpressing LPA$_1$ (black, circles), LPA$_2$ (blue, squares) or LPA$_3$ (red, triangles) receptors were preincubated in the absence or presence of different PMA concentrations for 2 min and then challenged with 1 μM LPA (panel A) or with different concentrations of LPA for 10 minutes, washed 3 times and then challenged with 1 μM LPA (panel B) and the increase in intracellular free calcium concentration was determined. Plotted are the increases in calcium as the percentage of that obtained in cells preincubated without any agent as mean ± S. E. M. of 6 experiments using different cell preparations.

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more clearly shown when the concentration-response curves to LPA in control and agonist-pretreated cells were normalized and plotted (Fig 3, right panels). It is worth noticing that the shift in the curves was more pronounced in LPA$_1$- or LPA$_3$-expressing cells, than in those that expressed the LPA$_2$ subtype. The response to 100 μM LPA of PMA-treated cells was very small (Fig 3, right panels).

The possibility that the cell responsiveness to LPA could resensitize was considered. To study this, cells were incubated with 1 μM PMA for 2 min or with 1 μM LPA for 10 min and then extensively washed. After this procedure, the incubation continued for the times indicated (up to 2 h) and then cells were challenged with 1 μM LPA. As shown in Fig 4 the cell responsiveness to LPA recover after washing, during the subsequent incubation but not when the active phorbol ester, PMA, was employed. These resensitization patterns were similar for the three LPA receptor subtypes studied (Fig 4). The resenstization time-courses after homologous desensitization were inversely correlated with the desensitization magnitudes, i. e., cells expressing LPA$_2$ receptors resensitize clearly faster that those expressing the LPA$_1$ or LPA$_3$ subtypes (Fig 4). We were unable to detect changes in receptor density (degradation) in response to LPA or PMA during these incubations times, as evidenced by anti-eGFP Western blotting of extracts from cells incubated in the presence cycloheximide (50 μM) to prevent new protein synthesis; cycloheximide was added 30 min before addition of LPA or PMA and was present during the whole incubation period ("Fig C in S1 File").

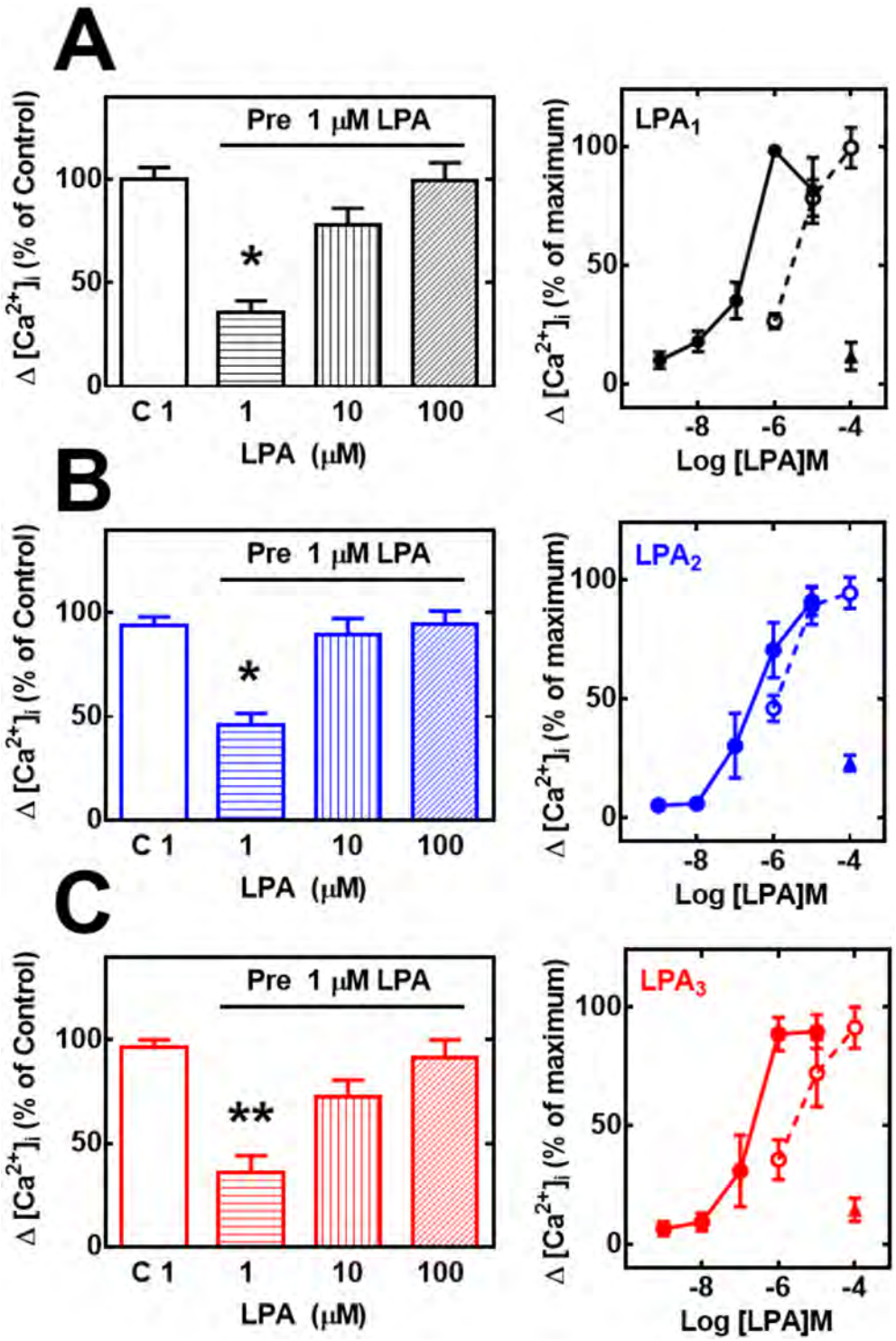

Fig 3. Effect of preincubation with PMA (heterologous desensitization) or LPA (homologous desensitization) on LPA-induced intracellular calcium concentration ([Ca^{2+}]$_i$) using high agonist concentrations. Cells overexpressing LPA$_1$ (panel A, black), LPA$_2$ (panel B, blue) or LPA$_3$ (panel C, red) receptors were preincubated in the absence or presence of 1 μM LPA for 10 min, extensively washed, then challenged with the indicated concentrations of LPA, and the increase in intracellular free calcium concentration was determined. Plotted are the increases in calcium as the percentage of that obtained in cells preincubated without any agent and challenged with 1 μM LPA (C 1 in the abscisa) (% of control) as

mean ± S. E. M. of 6 experiments using different cell preparations. In the right panels concentration response curves are plotted. Data from Fig 1 (without pre-stimulation) were normalized and re-plotted as percentage of the maximal response (solid symbols and continuous connecting lines) together with those in the left panels of this figure, normalized in the same way (open symbols, dotted connected lines). The response of cells preincubated with 1 μM PMA for 2 min, washed, and then challenged with to 100 μM LPA is also presented (solid triangles).

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The PKC inhibitor, bisindolylmaleimide I, markedly diminished PMA-induced desensitization in cells expressing any of the LPA receptor subtypes (Fig 5, panel A) but was unable to alter LPA-induced desensitization (Fig 5, panel B).

It is well-known that over-night treatment with 1 μM PMA markedly down-regulates conventional and novel PKC isoforms [14, 28]. Consistent with the previous data, this treatment markedly reduced or abolished PMA-induced desensitization (Fig 6, panel A) but was completely unable to alter agonist-induced desensitization (Fig 6, panel B).

We next examined another further downstream functional response: ERK phosphorylation. Phosphorylation of this key enzyme has been observed in response to LPA_{1-3} receptor activation [29–32]. As shown in Fig 7, activation of any of the three receptors studied was able to activate ERK as reflected by its phosphorylation state. The magnitude of the response was somewhat different, with that induced by LPA_1 receptors greater and with a more prolonged duration than that induced by LPA_3 receptor activation and that due to LPA_2 activation was clearly smaller and lesser in duration (Fig 7). Elegant work by Ullrich and coworkers has shown that many GPCRs, including LPA receptors (subtype(s) not defined) can transactivate EGF receptors, through sequential metalloproteinase activation and HB-EGF shedding and that joint signaling through GPCRs and the EGF tyrosine kinase activity participates in some of the actions ([33] reviewed in [34–37]). Previously, we showed that activation LPA_1 receptors induce Akt/PKB phosphorylation through the previously-mentioned EGF receptor transactivation process [12]. In the present experiments, the possible role of EGF receptor transactivation was evaluated, by inhibiting the EGF receptor kinase with the selective tyrphostin, AG1478 [38]. As presented in Fig 6, both LPA (1 μM) and EGF (100 ng/ml) increase ERK phosphorylation (~ 2- and ~ 4-fold, respectively). AG1478 clearly diminished the effect of both growth factors and, in some cases, to below the baseline signal (Fig 8). The baseline phospho-ERK signal was very low and AG1478 either did not alter it (cells expressing LPA_1 receptors) or decrease it (cells overexpressing LPA_2 (statistically insignificant) and LPA_3 receptors (statistically significant) ("Fig D in S1 File").

Agonist- and PMA-induced receptor phosphorylation was examined next. Data showed that the three LPA receptors studied are phosphoproteins whose phosphorylation states are increased by LPA and the active phorbol ester, PMA (Figs 9 and 10). The time-course of the effect of 1 μM LPA (Fig 9, panel A) showed that in cells expressing any of the receptors studied, the agonist increased receptor phosphorylation, and this reached its maximum during the first 15 min and remained at the same level for up to 60 min. The effect of 1 μM PMA (Fig 9, panel B) took place faster than that of the agonist, reaching its maximum during the first 5 min and remaining at a plateau during the time studied (60 min). Interestingly, the relative magnitudes (percentage of baseline labeling) and temporal patterns were similar for all three receptor subtypes, although in some experiments LPA_2 receptor phosphorylation was slightly delayed. The concentration-response curves to LPA and PMA are presented in Fig 10 (panels A and B, respectively). It can be observed that the curves for LPA and PMA were very similar for LPA_1- and LPA_3-overexpressing cells; saturation was obtained at ~ 1 μM LPA (EC_{50} 10–30 nM) and 100 nM PMA (EC_{50} ~ 3–10 nM) (Fig 10). In the studies utilizing cells that overexpress LPA_2 receptors, no clear saturation was obtained for either LPA or PMA (Fig 10; panels A and B, respectively); under these conditions EC_{50} values could not be estimated, but the

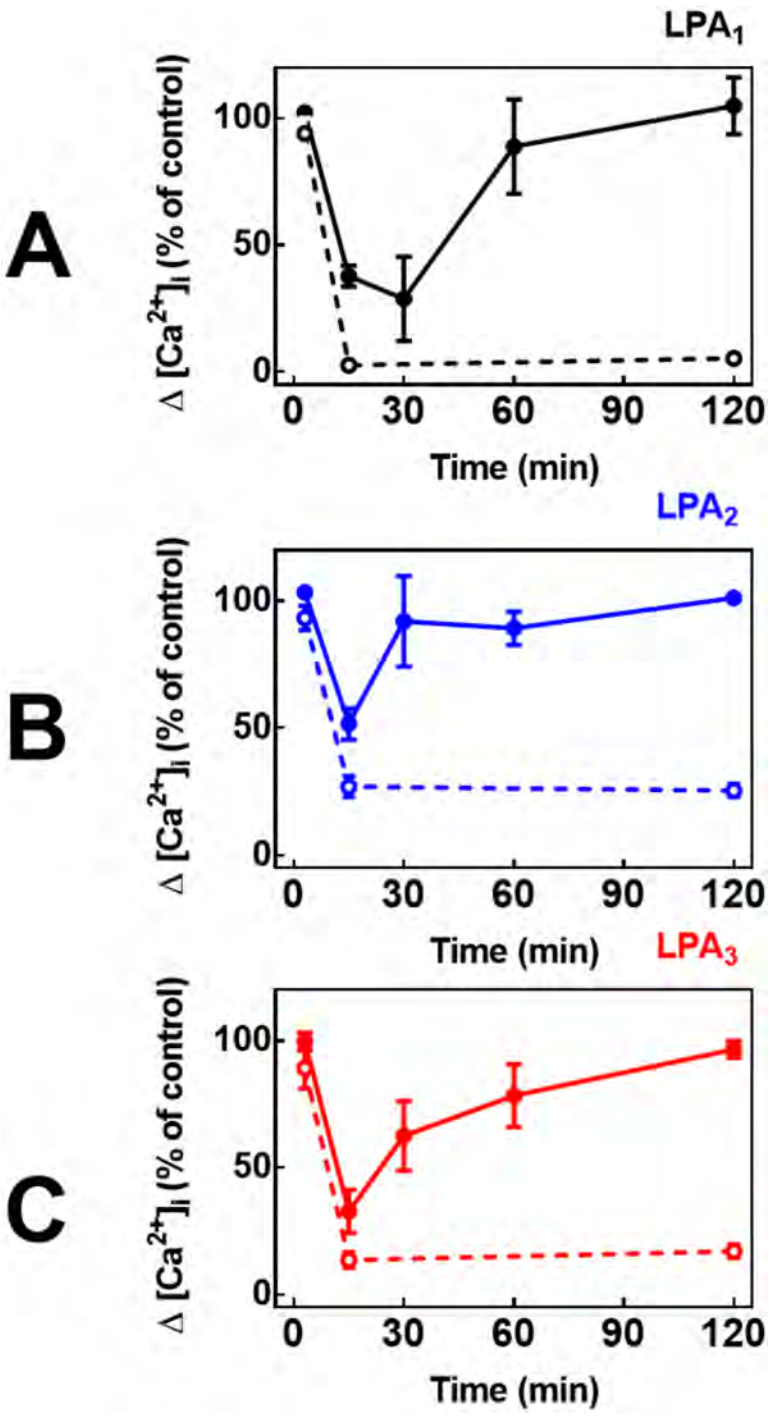

Fig 4. Reversibility (homologous) and persistency (heterologous) of the desensitizations of the intracellular calcium response to LPA. Cells overexpressing LPA$_1$ (panel A, black symbols and lines), LPA$_2$ (panel B, blue symbols and lines) or LPA$_3$ (panel C, red symbols and lines) receptors were preincubated in the presence of 1 μM PMA for 2 min (open symbols, dotted lines) or with 1 μM LPA for 10 minutes and then extensively washed. Incubation was continued for the times indicated and cells were challenged with 1 μM LPA and the increase in intracellular free calcium concentration was determined. Plotted are the increases in calcium as the percentage of that obtained in cells preincubated without any agent (control, time 0) as mean ± S. E. M. of 5–6 experiments using different cell preparations.

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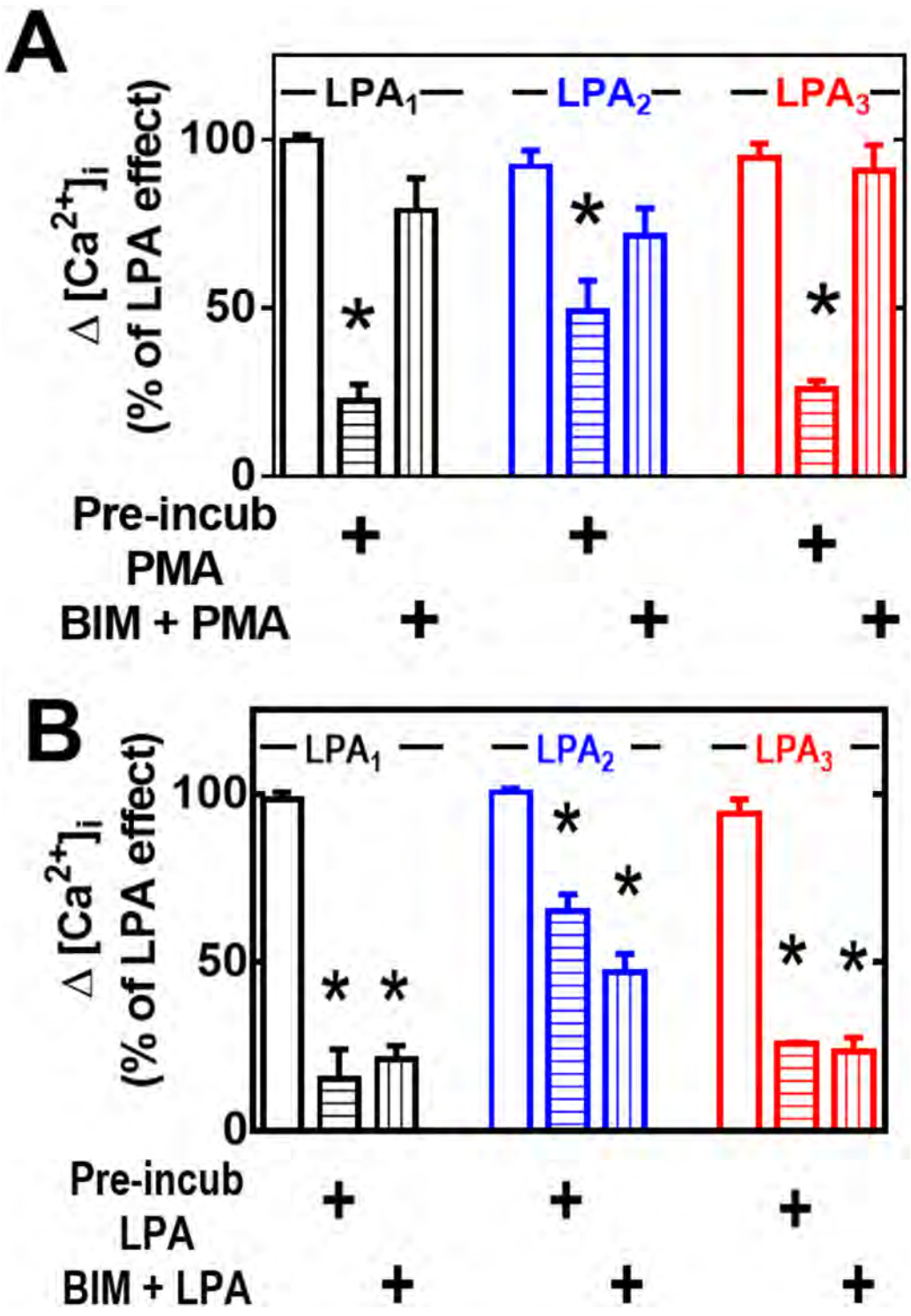

Fig 5. Effect of PKC inhibition on heterologous (PMA-induced) or homologous (LPA-induced) desensitization. Cells overexpressing LPA_{1-3} receptors were preincubated for 15 min in the presence of the PKC inhibitor, bisindolylmaleimide I (BIM), and then subjected to the desensitization protocols (indicated under the Experimental section and in Fig 2), using 1 μM PMA or 1 μM LPA. Cells were challenged with 1 μM LPA and the increase in intracellular free calcium concentration was determined. Plotted are the increases in calcium as the percentage of that obtained in cells preincubated without any agent as mean ± S. E. M. of 6–8 experiments using different cell preparations. *$p < 0.001$ vs. baseline.

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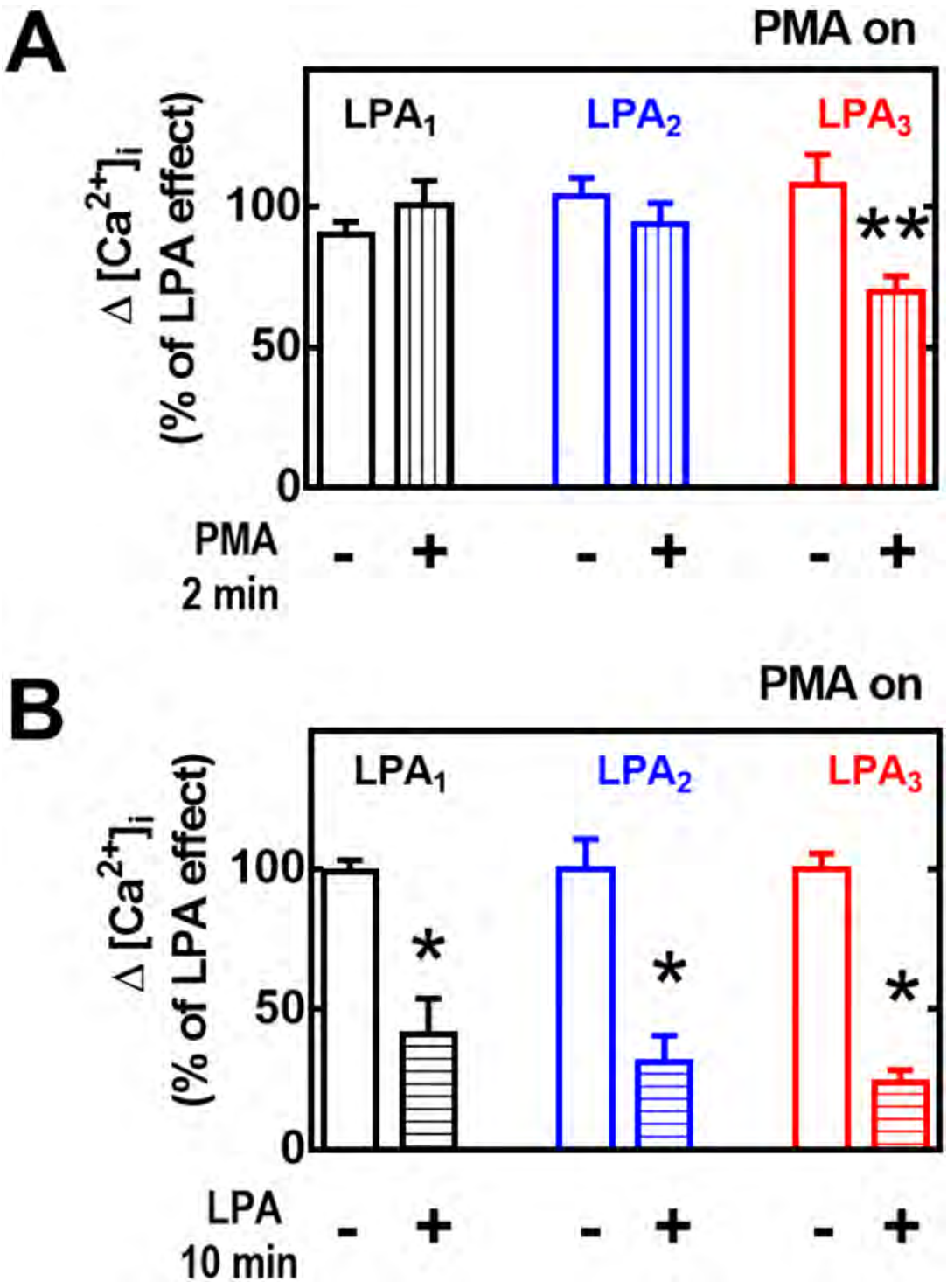

Fig 6. Effect of PKC down regulation on heterologous (PMA-induced) or homologous (LPA-induced) desensitization. Cells overexpressing LPA$_{1-3}$ receptors were preincubated overnight with 1 μM PMA, and then subjected to the desensitization protocols (indicated under the Experimental section and in Fig 2) using 1 μM PMA (2 min) or 1 μM LPA (10 min). Cells were challenged with 1 μM LPA, and the increase in intracellular free calcium concentration was determined. Plotted are the increases in calcium as the percentage of that obtained in cells preincubated without any agent as mean ± S. E. M. of 5–7 experiments using different cell preparations. *p < 0.001 vs baseline; **p <0.05 vs baseline.

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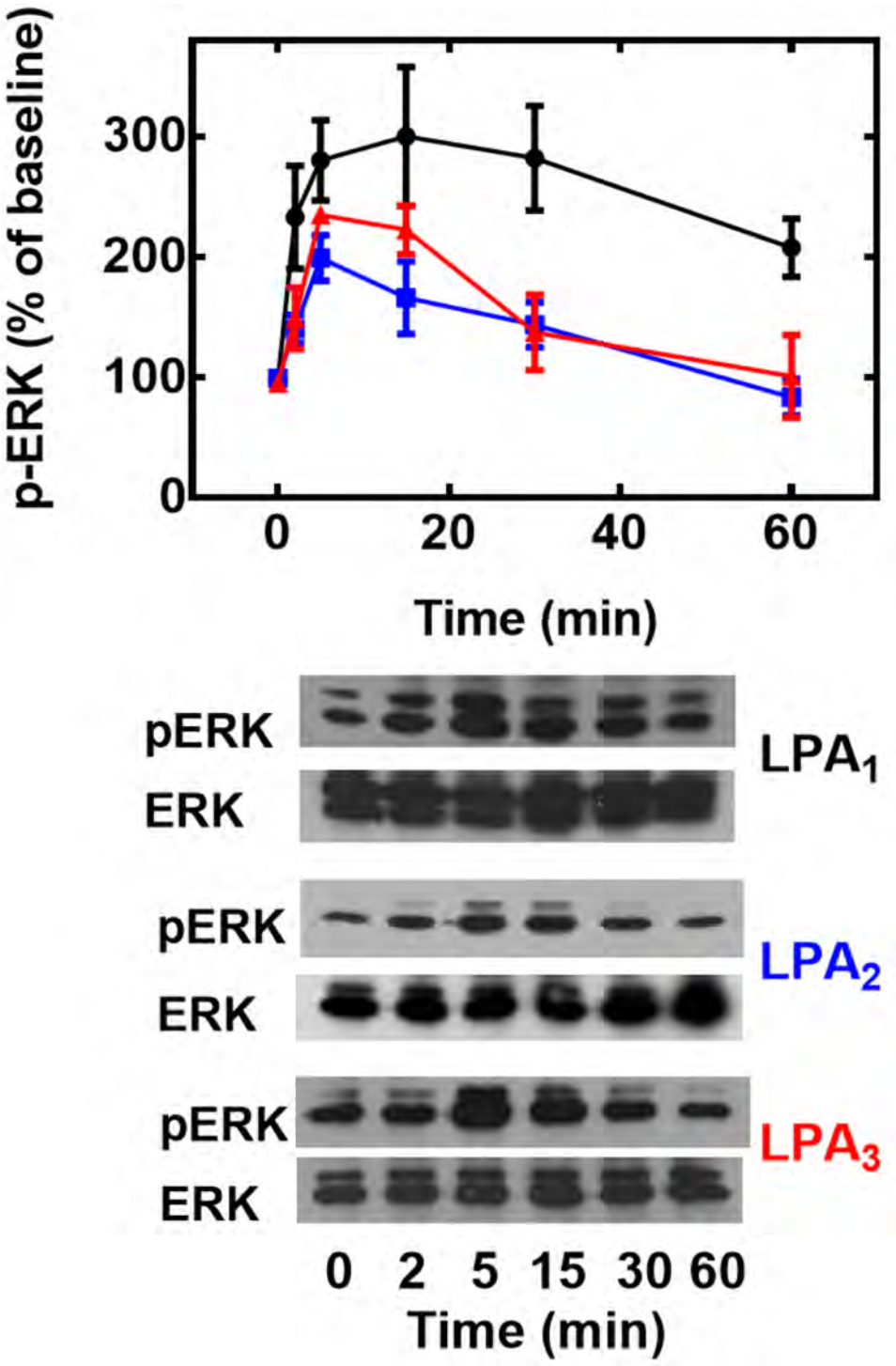

Fig 7. Effect of LPA on ERK 1/2 phosphorylation. Cells overexpressing LPA$_1$ (black, circles), LPA$_2$ (blue, squares) or LPA$_3$ (red, triangles) receptors were incubated for the times indicated in the presence of 1 μM LPA, incubation was terminated and phospho-ERK 1/2 (pERK) and total ERK 1/2 (ERK) were assayed by Western blotting. Plotted are the increases in phospho-ERK 1/2 as mean ± S. E. M. of 4–5 experiments using different cell preparations. Representative Western blots are presented for the different receptor subtypes.

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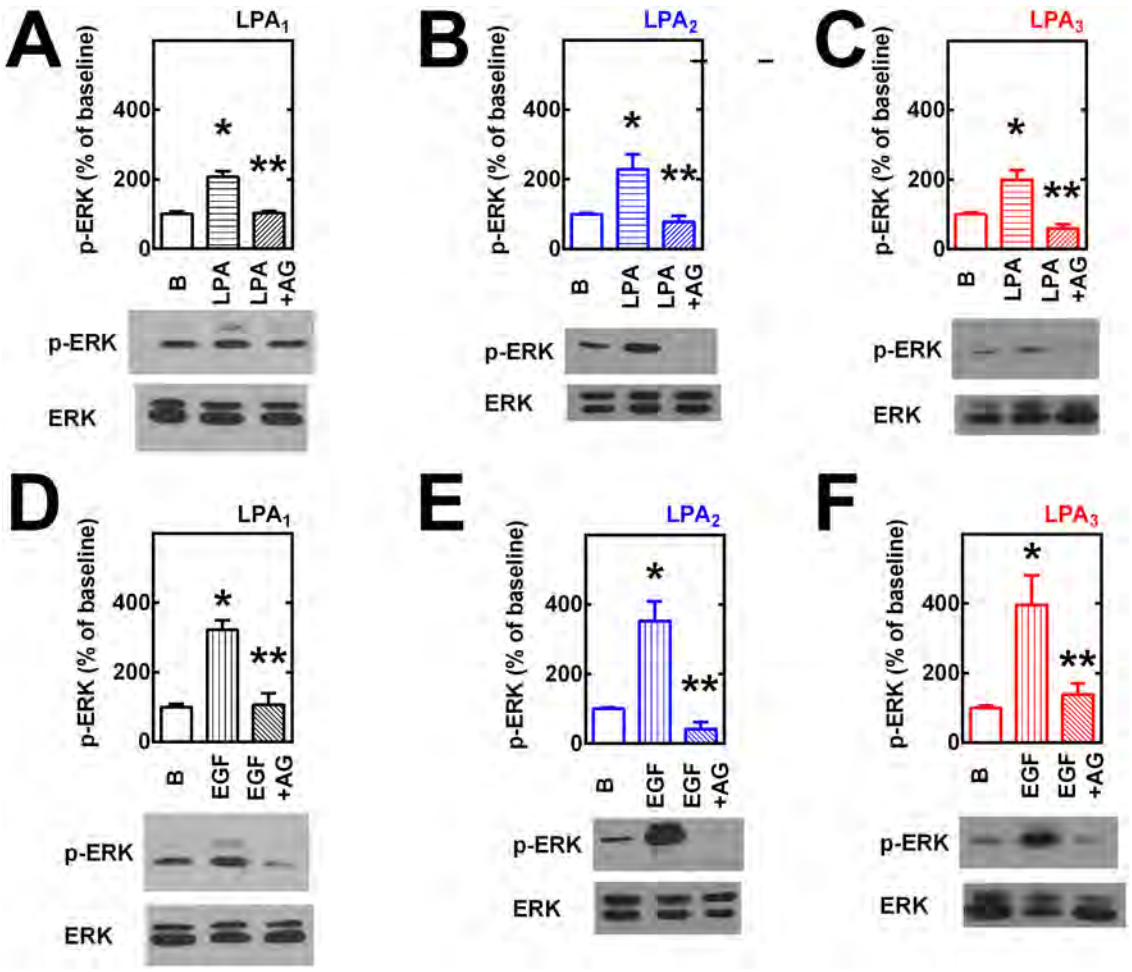

Fig 8. Transactivation of EGF receptors in LPA-induced ERK 1/2 phosphorylation. Cells overexpressing LPA$_1$ (panels A and D), LPA$_2$ (panels B and E) or LPA$_3$ (panels C and F) receptors were incubated in the absence or presence of 10 μM AG1478 (AG) for 30 min and then challenged with 1 μM LPA (panels A-C) or 100 ng/ml EGF (panels D-F) for 5 min; incubation was terminated and phospho-ERK 1/2 (pERK) and total ERK 1/2 (ERK) were assayed by Western blotting. Plotted are the increases in phospho-ERK 1/2 as mean ± S. E. M. of 4–5 experiments using different cell preparations. Representative Western blots are presented for the different receptor subtypes. *p < 0.001 vs. baseline (B); ** p <0.001 vs. LPA or EGF alone.

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concentration-response curves exhibited a clear shift to higher concentrations as compared with those studying the remaining receptor subtypes.

It has been observed that EGF receptor transactivation plays a role in the phosphorylation of some GPCRs, such as the α_{1B}-adrenergic receptor [39–42]. As shown in Fig 11, this was also the case in agonist-induced LPA$_{1-3}$ receptor phosphorylation, i. e., the EGF receptor tyrosine kinase inhibitor, AG1478, markedly reduced (but did not abolish) LPA-induced phosphorylation of the three receptor subtypes studied. The inhibitor by itself did not alter basal receptor phosphorylation (data not shown). As already mentioned, prolonged treatment (over-night) with 1 μM PMA markedly down-regulates conventional and novel PKC isoforms [14, 28]. Consistent with previous data on LPA$_1$ receptors [14], this treatment markedly reduced or abolished PMA-induced LPA$_{1-3}$ phosphorylation but did not alter LPA-induced receptor phosphorylation (Fig 12). GPCR phosphorylation appears to be associated with receptor internalization. Current ideas suggest that GPCR phosphorylation increases the receptor's affinity for β-arrestins, which associates with clathrin favoring the formation of receptor-enriched

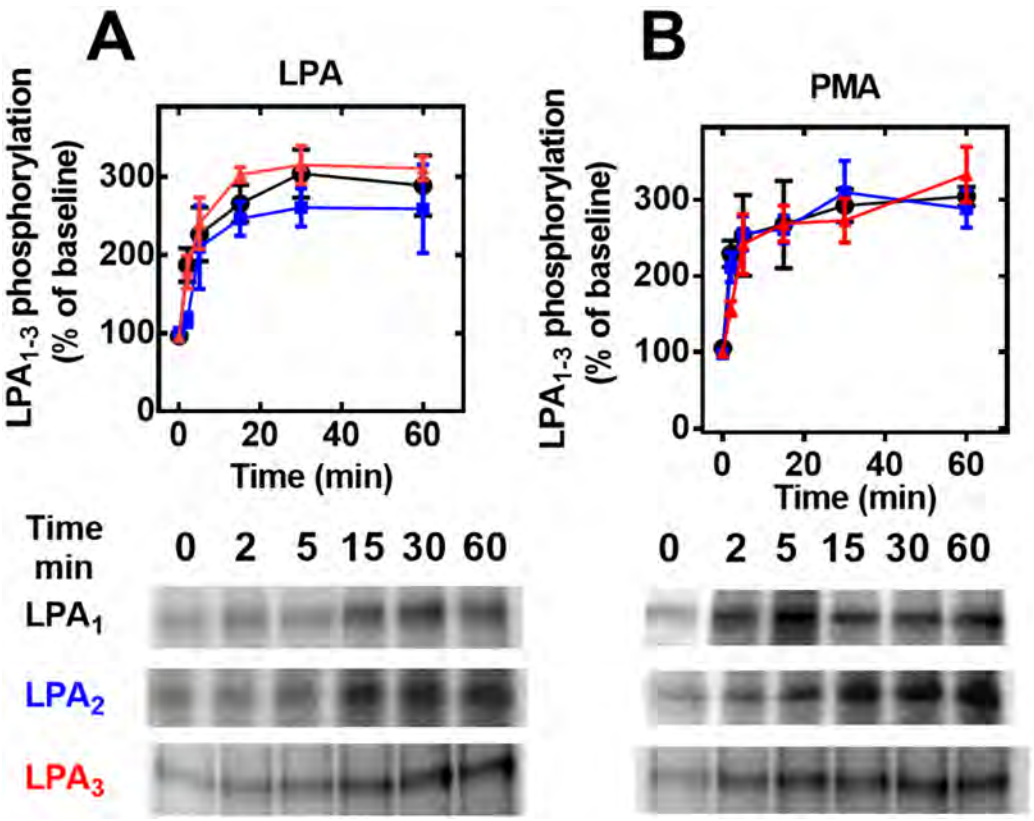

Fig 9. Time-courses of the effects of LPA and PMA on LPA$_{1-3}$ receptor phosphorylation. Cells overexpressing LPA$_1$ (black, circles), LPA$_2$ (blue, squares) or LPA$_3$ (red, triangles) receptors were incubated for the times indicated in the presence of 1 μM LPA (Panel A) or 1 μM PMA (Panel B). Plotted are the percentage of baseline phosphorylations as mean ± S. E. M. of 4–5 experiments using different cell preparations. Representative autoradiographs are presented for the different receptor subtypes.

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coated pits, triggering internalization [43–46]. As depicted in the confocal images presented as in Fig 13, fluorescence (i. e. the eGFP-tagged receptors) was present in both, the plasma membrane and intracellular vesicles. Treatment with LPA or PMA clearly altered receptor distribution, markedly decreasing fluorescence at the plasma membrane level and increasing that in intracellular vesicles. Such changes were clearly observed only after 20–30 min indicating that desensitization precedes internalization. Differences were observed among the LPA receptor subtypes after continuous exposure to these agents for 30 or 60 min. The decrease in fluorescence at the plasma membrane was much less intense in LPA$_2$-overexpressing cells as compared with that in those overexpressing the other subtypes (Fig 13) (see also images overlapping fluorescence and differential interference contrast in "Fig E in S1 File"); quantitative analysis also clearly evidenced this (Fig 14). Differences were also observed in the morphology (small punctuated or large vesicles) and localization (concentrated in perinuclear region or distributed in the whole cell) of the internalized fluorescence both among the distinct receptors studied and also depending on the stimulus. Analysis of such differences will require a systematic work with different approaches. In an effort to get further insight into the receptor traffic dynamics, images were obtained in cells treated with 1 μM LPA (10 min), exhaustively washed, and further incubated to complete 60 or 120 min of incubation (Fig 15). It was observed that under these conditions fluorescence recovered rapidly and completely at the membrane level in cells expressing LPA$_1$ receptors; slowly in cells expressing LPA$_2$ receptors, and only partially and also slowly in cells expressing LPA$_3$ receptors (Fig 15, see also "Fig F in S1 File");

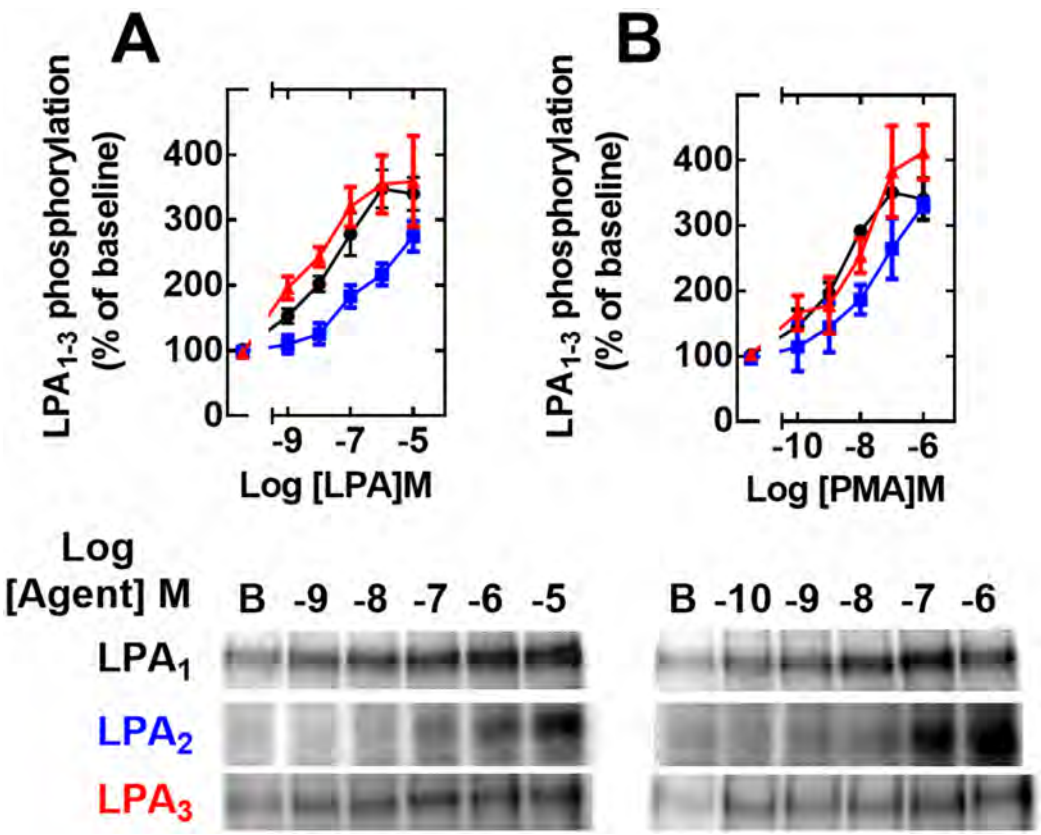

Fig 10. Concentration-response curves to LPA and PMA on LPA$_{1-3}$ receptor phosphorylation. Cells overexpressing LPA$_1$ (black, circles), LPA$_2$ (blue, squares) or LPA$_3$ (red, triangles) receptors were incubated for 15 min in the presence of different concentrations of LPA (Panel A) or PMA (Panel B). Plotted are the percentage of baseline phosphorylations as mean ± S. E. M. of 4–5 experiments using different cell preparations. Representative autoradiographs are presented for the different receptor subtypes.

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quantitative analysis is presented in Fig 16. Fluorescence recovery at the plasma membrane could represent, receptor recycling, incorporation of receptors previously present in vesicles and newly synthesized ones, and, more likely a mixture of all these. Distinction among such processes will also require a systematic work with different approaches.

Discussion

In the present work, we comparatively analyzed LPA $_{1-3}$ receptor signaling and desensitization in the same cellular model, C9 cells. The actions and mechanisms of action of these receptors have been extensively studied but data on their phosphorylation are scarce. LPA-, PMA- and angiotensin II-induced LPA$_1$ receptor phosphorylation has been observed [4, 10–14] and in an elegant work, Kuriyama et al. reported that LPA signaling is required, during *Xenopus* development, for neural crest migration and that such action involves LPA$_2$ receptor phosphorylation at serine 324 [47]. We are not aware of any publication describing LPA$_3$ phosphorylation. In the present work eGFP tagged-LPA receptors were employed because it allowed us to use fluorescence confocal microscopy to follow the receptors' locations within the cells and to immunoprecipitate the receptors, using anti-eGFP antisera. This is a common strategy and the eGFP itself does not appear to be a phosphorylation substrate [11, 48, 49]. Our data clearly showed that the three receptors studied, i. e., LPA$_{1-3}$, are phosphoproteins whose phosphorylation state is modulated by the natural agonist, LPA, and by pharmacological activation of PKC by PMA. These

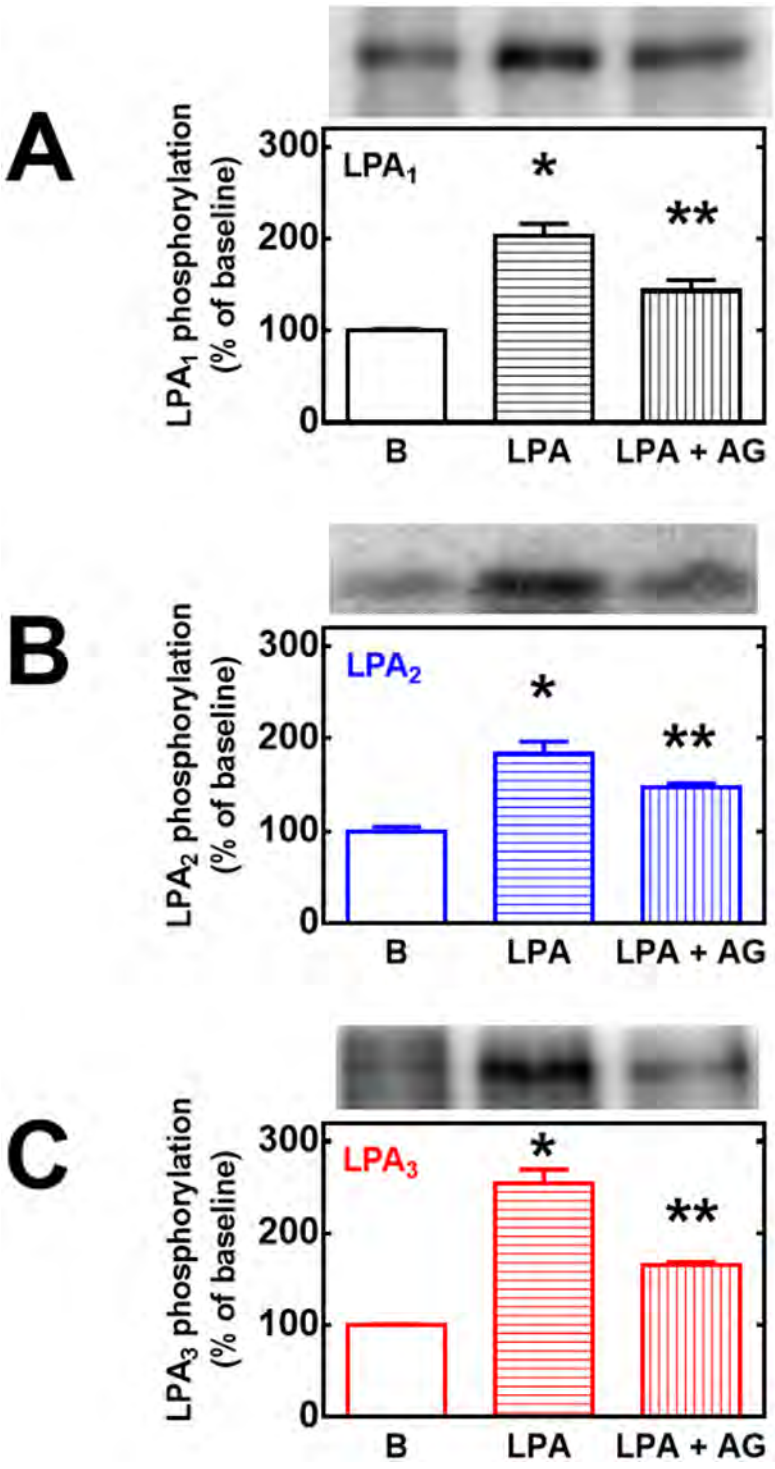

Fig 11. Role of EGF receptor transactivation on LPA-induced LPA$_{1–3}$ receptor phosphorylation. Cells overexpressing LPA$_1$ (panel A, black bars), LPA$_2$ (panel B, blue bars) or LPA$_3$ (panel C, red bars) receptors were preincubated for 30 min in the absence or presence of 10 μM AG1478 (+AG) and then incubated for 15 min in the absence or presence of 1 μM LPA. Plotted are the percentage of baseline (B) phosphorylations as mean ± S. E. M. of 4–5 experiments using different cell preparations. Representative autoradiographs are presented on the top of the figures for the different receptor subtypes. $p < 0.001$ vs. baseline; $p < 0.05$ vs. baseline and vs. LPA alone.

doi:10.1371/journal.pone.0140583.g011

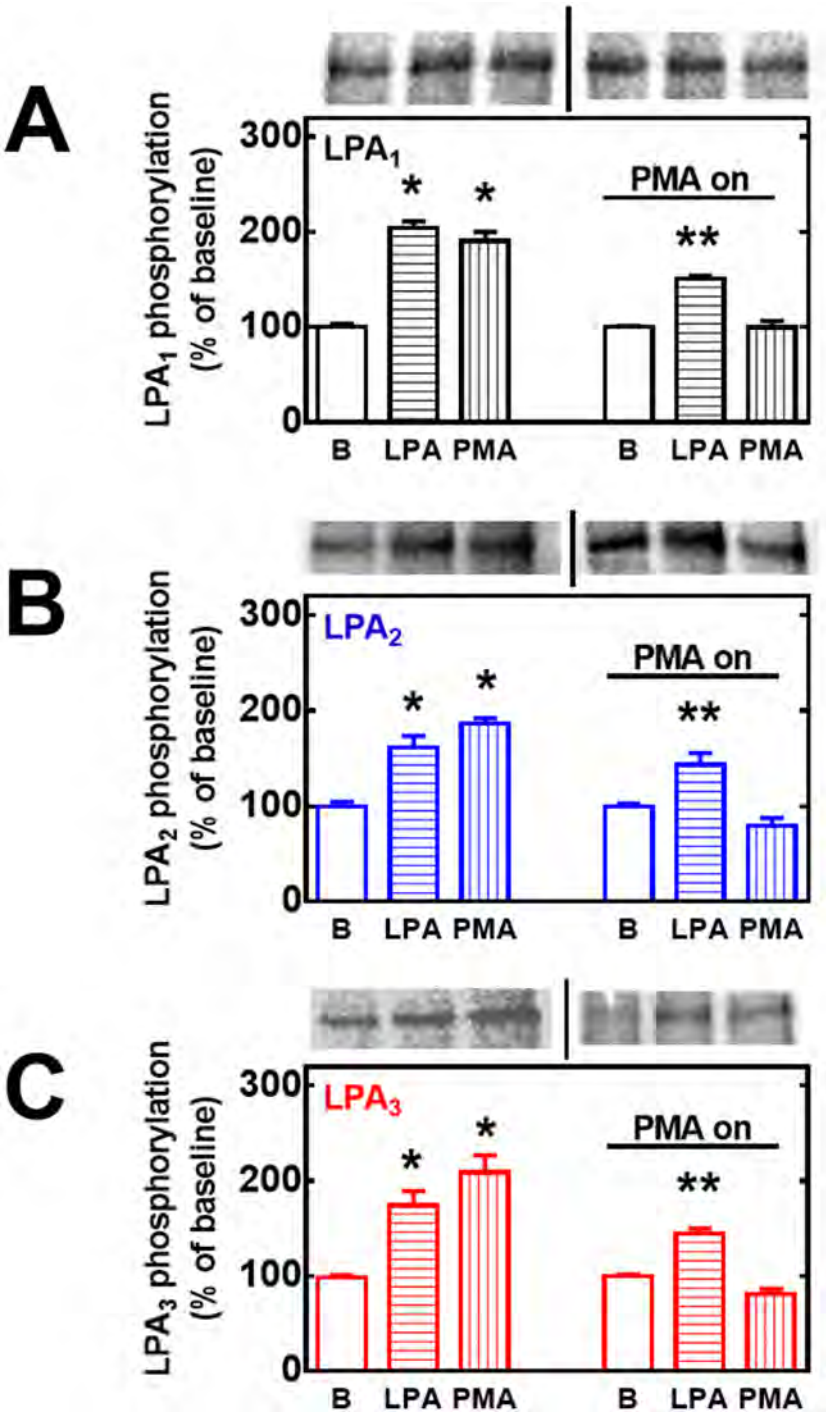

Fig 12. Role of PKC down-regulation on LPA- and PMA-induced LPA_{1-3} receptor phosphorylation. Cells overexpressing LPA_1 (panel A, black bars), LPA_2 (panel B, blue bars) or LPA_3 (panel C, red bars) receptors were incubated in the absence or presence of 1 μM PMA overnight, washed and subjected to the receptor phosphorylation protocol. Cells were incubated for 15 min in the absence or presence of 1 μM LPA or 1 μM PMA. Plotted are the percentage of baseline (B) phosphorylations as mean ± S. E. M. of 3–4 experiments using different cell preparations. Representative autoradiographs separated by vertical lines are presented on the top of the figures for the different receptor subtypes. $p < 0.001$ vs. baseline; ** $p < 0.05$ vs. baseline.

doi:10.1371/journal.pone.0140583.g012

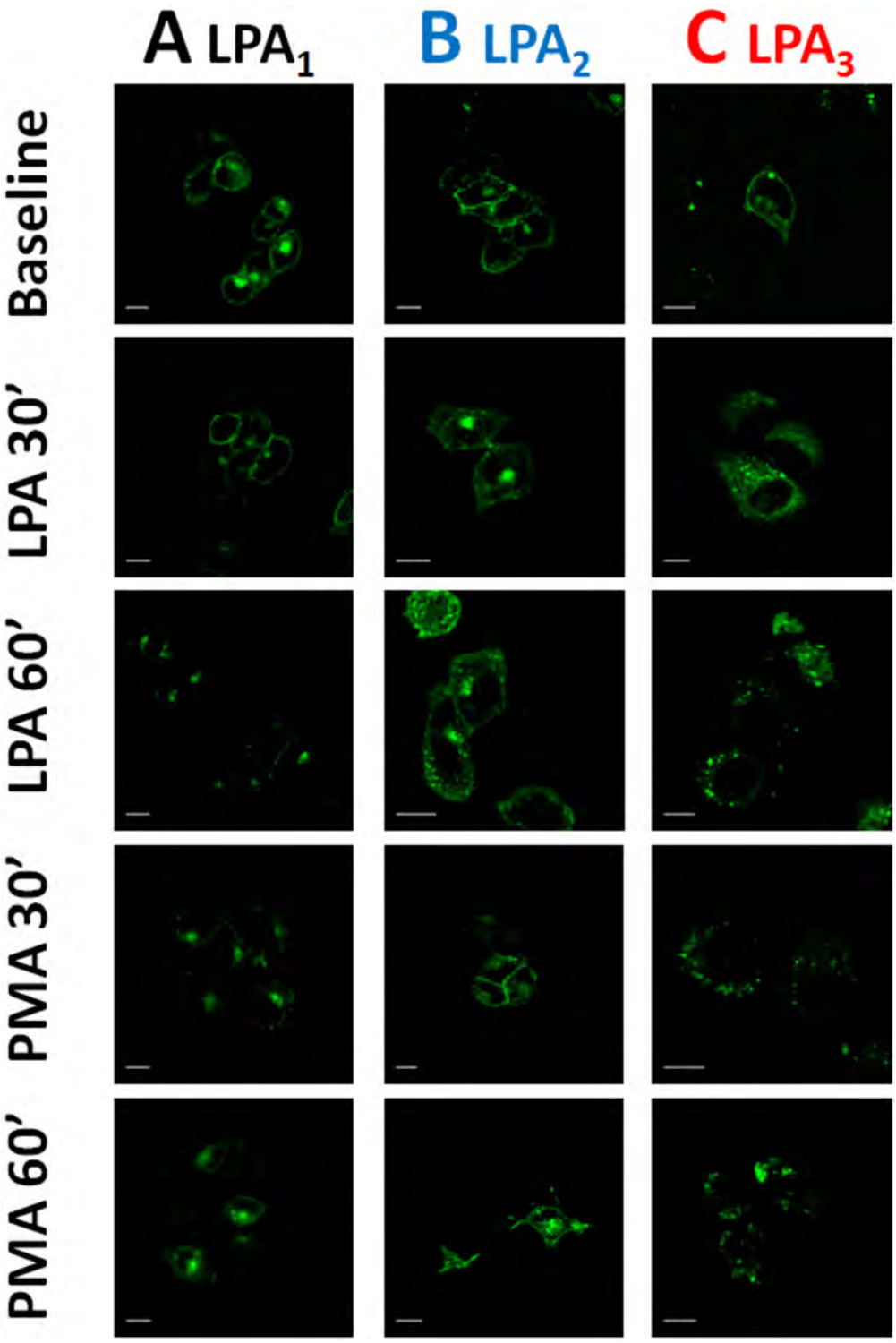

Fig 13. Images of the effects of LPA and PMA on LPA$_{1-3}$ receptor internalization. Fluorescent confocal images of cells overexpressing LPA$_1$ (column A), LPA$_2$ (column B) or LPA$_3$ (column C) receptors were incubated in the absence of any agent (Baseline) or for 30 or 60 min in the presence of 1 μM LPA or 1 μM PMA. Images are representative of data of 3–4 experiments using different cell preparations. Bars 15 μm.

doi:10.1371/journal.pone.0140583.g013

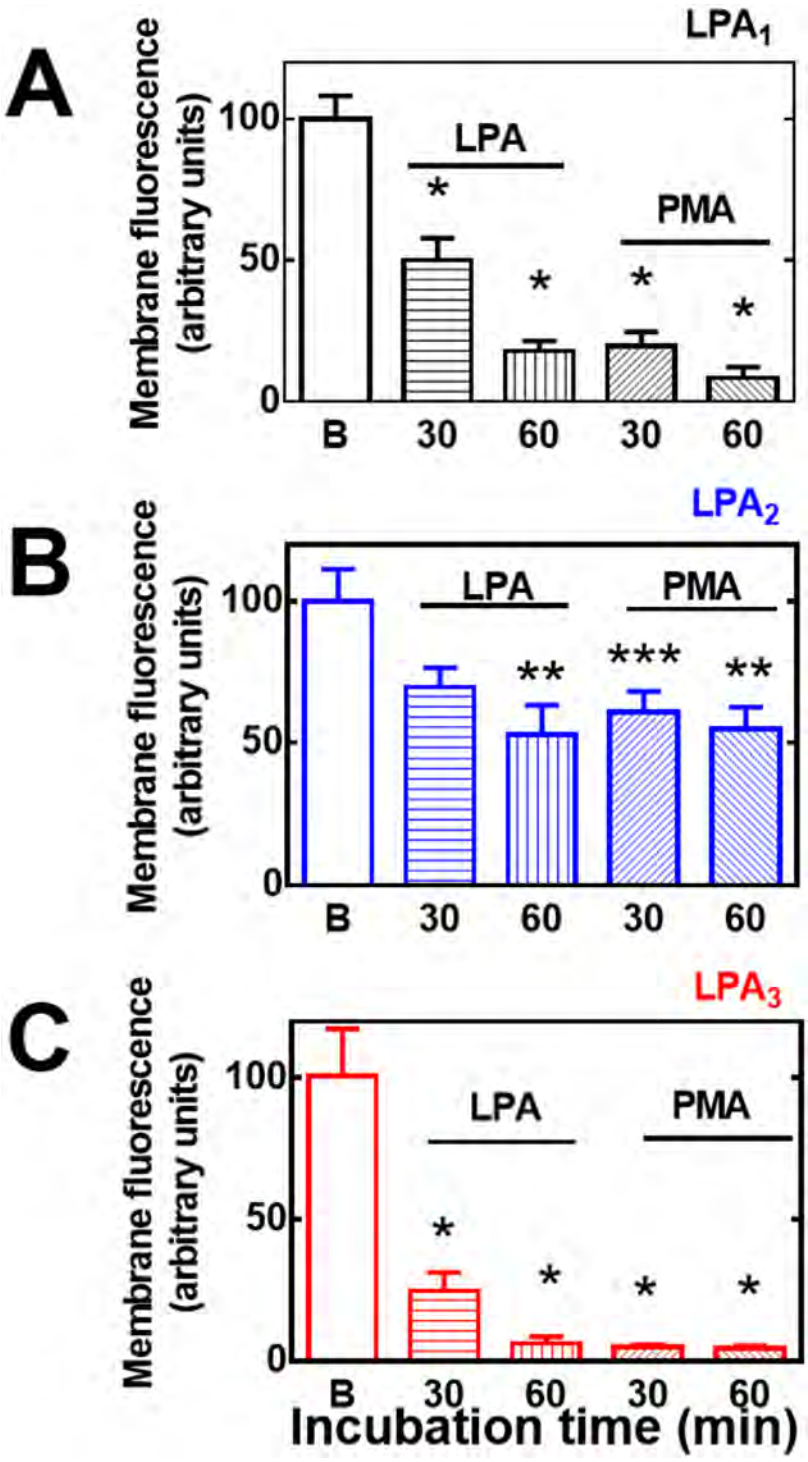

Fig 14. Effects of LPA and PMA on LPA$_{1-3}$ receptor internalization. Cells overexpressing LPA$_1$ (panel A), LPA$_2$ (panel B) or LPA$_3$ (Panel C) receptors were incubated for 30 or 60 min in the presence of 1 μM LPA or 1 μM PMA. Plotted is membrane-associated fluorescence (arbitrary units) as the mean ± S. E. M. of 5 different fields of 3–4 experiments using different cell preparations. * $p < 0.001$ vs. baseline (B), ** $p < 0.01$ vs. baseline (B), *** $p < 0.05$ vs. baseline (B).

doi:10.1371/journal.pone.0140583.g014

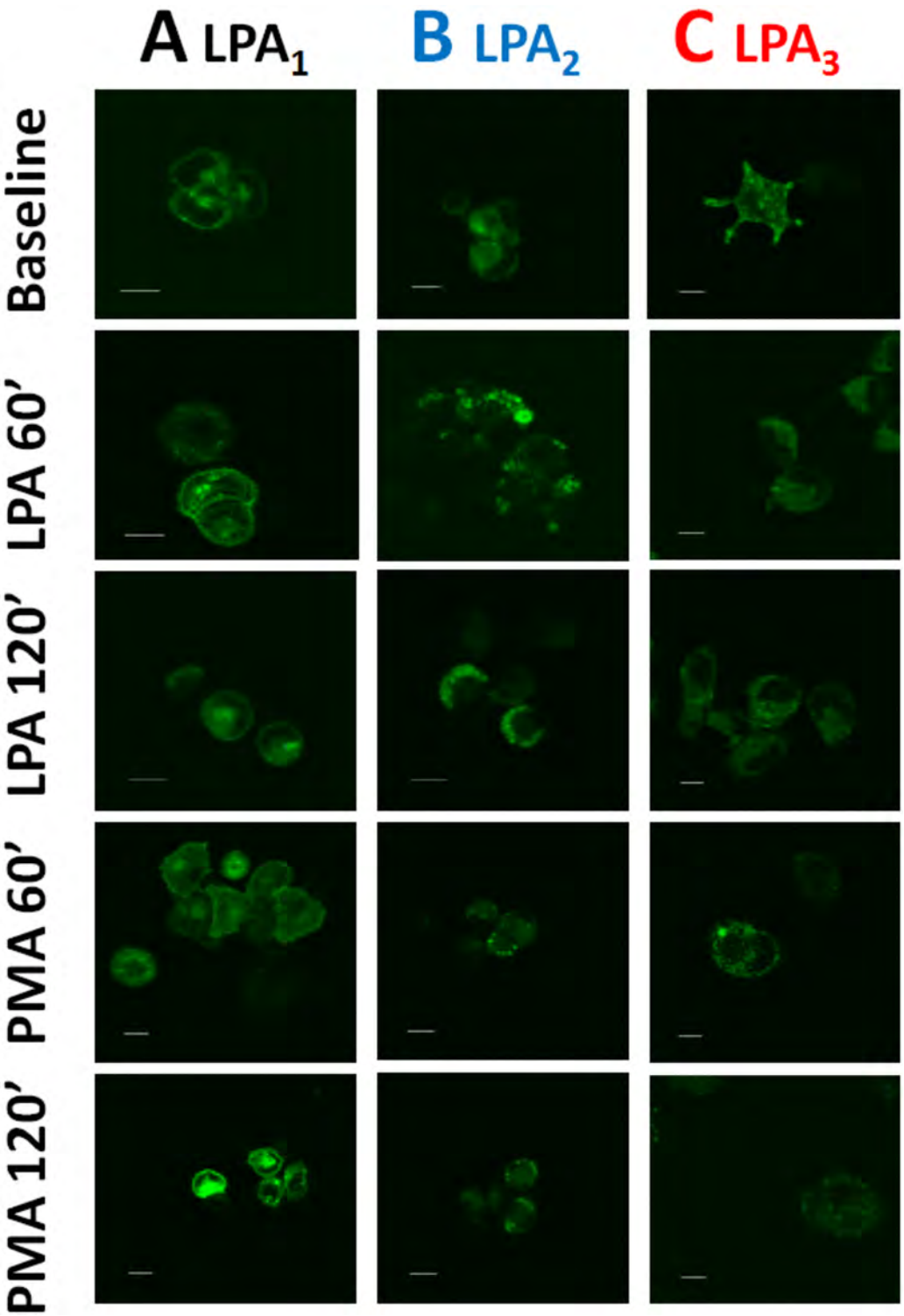

Fig 15. Images of the effects of LPA and PMA on LPA$_{1-3}$ receptor internalization (60 and 120 min). Fluorescent confocal images of cells overexpressing LPA$_1$ (column A), LPA$_2$ (column B) or LPA$_3$ (column C) receptors. Cells were incubated in the absence of any agent (Baseline), for 10 min in the presence of 1 μM LPA, or for 2 min in the presence of 1 μM PMA. After this incubation cells were extensively washed and further incubated for the times indicated. Images are representative of data of 3 experiments using different cell preparations. Bars 10 μm.

doi:10.1371/journal.pone.0140583.g015

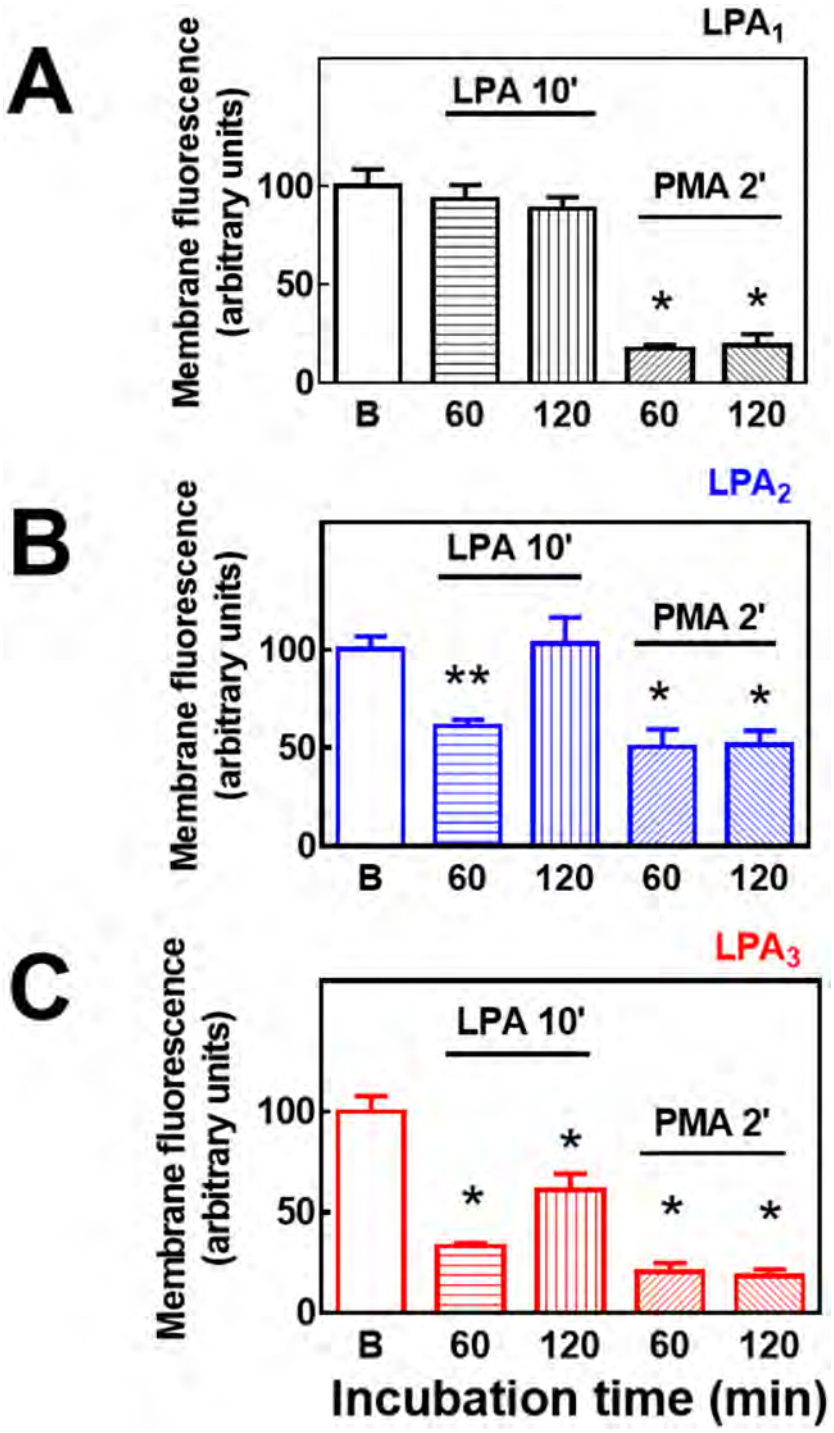

Fig 16. Effects of LPA and PMA on LPA$_{1-3}$ receptor internalization (60 and 120 min). Cells overexpressing LPA$_1$ (panel A), LPA$_2$ (panel B) or LPA$_3$ (Panel C) receptors were incubated in the absence of any agent (Baseline), for 10 min in the presence of 1 μM LPA, or for 2 min in the presence of 1 μM PMA. After this incubation cells were extensively washed and further incubated for the times indicated (60 or 120 min) Plotted is membrane-associated fluorescence (arbitrary units) as the mean ± S. E. M. of 5 different fields of 3 experiments using different cell preparations. * $p < 0.001$ vs. baseline (B), ** $p < 0.01$ vs. baseline (B), *** $p < 0.05$ vs. baseline (B).

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data are consistent with *in silico* analysis, which revealed potential phosphorylation sites in the structure of these three receptors [4]. Alignment of these receptors' intracellular loops 2 and 3 and of the carboxyl termini showed that some of these putative phosphorylation sites are conserved but many of them are not [4]. Interestingly, many of the putative sites for GRKs were located in the carboxyl termini, whereas those for PKC were mainly in the third intracellular loop; however, sites for these protein kinase families were located both in intracellular loops and in the carboxyl tail [4]. Putative phosphorylation sites for other protein kinases were also present in these receptors; these include sites for protein kinase A, Akt/PKB, calcium/calmodulin protein kinase, AMP-dependent protein kinase, and receptor and non-receptor tyrosine kinases [4]. Future structural work will be required to determine the site(s) that are real target(s) of theses protein kinases *in cellulo* and the functional repercussion of such covalent modifications. The importance of studying receptor phosphorylation sites is multiple. There is evidence that GPCR phosphorylation is important in favoring their interaction with β-arrestins, and that such interaction participates in both receptor internalization and signaling ([50–52] and references therein, see also [53, 54]). Evidence indicating that internalized GPCRs continue signaling in endosomes is accumulating [55–58] and this appears to vary among different receptor types [58]. Additionally, receptors are phosphorylated in different residues depending on the stimulus (i. e., the protein kinases involved, such as GRKs, second messenger-activated kinases and others) and the cell context (likely reflecting the repertoire of protein kinases and other interacting proteins expressed). This has been denominated the "phosphorylation bar code" and has been suggested to define the interaction of receptors with other proteins and hence the internalization processes involved, the receptors' fate (recycling/ degradation) and their endosomal signaling [19, 23, 59]. Our group has recently provided evidence that a GPCR, the α_{1B}-adrenergic receptor, interacts with different proteins and internalizes into distinct endosomal compartments during homologous and heterologous desensitizations [24]. At this point, little is known on these aspects for LPA receptors and for many other GPCRs.

It is clear from our data that the phosphorylation of the LPA receptors studied and its functional repercussion differ among subtypes and the triggering process, i. e. agonist-stimulated vs. PMA action. PKC appears to be involved in PMA action, as evidenced by the use of the inhibitor, bisindolylmaleimide I, and PKC down-regulation (overnight incubation with the active phorbol ester). In contrast, PKC does not appear to play a significant role in agonist-induced desensitization. Work by Iacovelli and coworkers [45] have shown that in FRTL-5 cells, which endogenously express LPA_{1-3} receptors, LPA markedly inhibits forskolin-stimulated cyclic AMP accumulation and increases ERK 1/2 phosphorylation; these effects were attenuated by overexpression of GRK2 or β-arrestin 1[45]. Similarly, it has been observed that GRK2 is required for agonist-induced desensitization of LPA_1 and LPA_2 receptors transfected into HEK293 cells [60]. With these data and our present findings, it appears probable that major roles might be played by GRKs (likely GRK2) and β-arrestin in homologous desensitization/ phosphorylation.

LPA-induced desensitization of the three studied receptors was characterized by a decreased sensitivity to the agonist, as evidenced by a shift to the right of the concentration-response curves; this was less pronounced in cells expressing the LPA_2 subtype. In contrast, PKC activation with PMA resulted in a much diminished response even to high LPA concentrations. In addition, the effect of LPA was reversible, whereas that of PMA was not, during the times explored. I should be considered that LPA interacts with GPCRs at the external surface of the plasma membrane, is rapidly metabolized and can be removed by extensive washing whereas PMA acts intracellularly (mainly on PKC), is very lipophilic and its complete removal by washing is rather unlikely. This might explain some of the differences observed. However, angiotensin II is also able to induce LPA_1 phosphorylation and desensitization, with the involvement of PKC [11, 12]. Work in progress in our laboratory indicates that angiotensin II-induced LPA_1

desensitization is not readily reversible after extensive washing. These data suggest that the differences among the mechanisms involved in these desensitization processes likely play also a role. Agonist-induced LPA_1 internalization has been reported by several groups [10, 11, 60–64] and appears to involve membrane cholesterol, β-arrestin, dynamin, and Rab 5 [62–64]. A cluster of serine residues in the receptor's carboxyl terminus seems to be required for β-arrestin translocation to the plasma membrane [64]. Interestingly, it has been observed that β-arrestin is not required for PMA-induced LPA_1 receptor internalization [64], which further emphasizes the differences between LPA-mediated processes and those induced by pharmacological activation of PKC. Agonist-triggered internalization of LPA_2 receptors has also been studied [60, 65]. In one of these works clear agonist-induced receptor internalization was observed [60] whereas in the other, internalization was slow and very limited in magnitude [65]; marked differences in the experimental conditions (i. e., cell types, conditions for LPA exposure and in the detection of membrane receptors) might explain the disparate results. Interestingly, LPA_2 receptors are key elements in the formation of the macromolecular complexes that mediate LPA gradient sensing in fibroblasts [65]. In our work, we consistently observed that LPA- and PMA-induced LPA_2 receptor internalizations were of lesser magnitude than those observed with the remaining subtypes studied. Similarly, we observed that phosphorylation of the LPA_2 receptor subtype required higher concentrations of agonist or PMA. A link between receptor phosphorylation and internalization might exist, but no causal relationship can be defined at this point and, as mentioned, different approaches will be required. To the best of our knowledge internalization of LPA_3 receptors has not been previously reported.

As already mentioned, many GPCRs, including LPA receptors can transactivate EGF receptors, an effect important for many of the actions of this lysophospholipid [34–37]. Numerous studies have highlighted this action for LPA_1 receptors (see for example [12, 31, 66]) and there is evidence that LPA_2 [67–69] and LPA_3 [30, 32] receptors also employ in their signaling this transactivation process. EGF receptors transactivation is a complex process that can or cannot involve, changes in intracellular calcium, metalloproteinase activation, shedding of membrane-bound EGF activators (TGF-α, HB-EGF, amphiregulin, betacellulin, and epiregulin, among others) non-receptor tyrosine kinases (such as Src and Pyk), second messenger-activated kinases (such as PKC o PI3K) and other molecular elements. Two major processes have been defined. One of them involves the release of membrane-bound EGF ligands and autocrine/paracrine cell communication, whereas the other takes place intracellularly without the release of a messenger. These processes are not mutually exclusive and can coexists in the same cell (see [34–37] and references therein).

Our data also showed that the ability to transactivate EGF receptors is shared by the three LPA receptors studied, and that such action is required for some actions (such as ERK phosphorylation and agonist-induced receptor phosphorylation). In a previous work, we observed that EGF induces LPA1 desensitization, consistent with LPA1-EGFR functional crosstalk [12]. Recent work has shown that antidepressants and LPA induce tyrosine phosphorylation of insulin-like growth factor receptors and insulin receptor substrate-1, involving LPA_1 receptors and Src activation [66]. It is clear that further work is necessary to fully understand the regulation of LPA_{1-3} receptors; this has medical and biological importance, considering the many functions in which these lysophosphospholipid-activated receptors are involved and their roles in the pathogenesis of morbid entities.

Supporting Information

S1 File. Supplementary Figs A-F are included in this file.
(PDF)

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Author Contributions

Conceived and designed the experiments: JAG-S RA-H AH-M. Performed the experiments: RA-H AH-M GAC-M AM-H. Analyzed the data: RA-H AH-M GAC-M AM-H JAG-S. Contributed reagents/materials/analysis tools: JAG-S. Wrote the paper: JAG-S. Reviewed and corrected the manuscript: RA-H AH-M GAC-M AM-H.

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