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Activación por Lípidos de Proteínas Quinasas C

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A mis padres y hermana

A José Manuel

AGRADECIMIENTOS

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PRÓLOGO

Resumen en castellano.

I. INTRODUCCIÓN.

I.1. Proteínas quinasas C: Estructura y clasificación.

Las Proteínas Quinasas C (PKC) constituyen un grupo de enzimas con actividad fosfotransferasa que fosforilan específicamente residuos de Ser y Thr de sus proteínas sustrato. Están involucradas en diversas vías de transducción de señales en las células, participando en multitud de funciones fisiológicas, tales como la mitogenesis y proliferación celular, la regulación del metabolismo, la apoptosis, la activación de plaquetas, el reordenamiento del citoesqueleto de actina, la modulación de canales iónicos, la secreción y la diferenciación de las células nerviosas, así como en diferentes procesos patológicos como el cáncer, enfermedades de pulmón y determinadas cardiopatías *(Coleman and Wooten, 1994; Dekker and Parker, 1997; Dempsey et al., 2000; Griner and Kazanietz, 2007; Mangoura et al., 1995; Mellor and Parker, 1998; Nishizuka, 1986; Nishizuka, 1992)*. Su ubicuidad y diversidad funcional, las hacen una familia de proteínas muy atractiva de estudiar por la gran relevancia que esto representa.

Esta familia de enzimas se compone de 10 isoenzimas, que están codificadas por 9 genes en mamíferos. Cada isoenzima se expresa en una amplia variedad de células y además cada célula expresa un gran número de estas proteínas, siendo así una familia muy ubicua, además del gran número de funciones celulares que desempeña.

Las isoenzimas de la PKC se han clasificado en tres subfamilias en base a su estructura primaria y dependencia de distintos cofactores para obtener un incremento de su actividad enzimática (Figura I.1):

a) PKC clásicas o convencionales: incluye las isoenzimas PKCα, βI, βII y γ *(Coussens et al., 1986; Inoue et al., 1977)*. Presentan un dominio pseudosubstrato, el dominio C1 capaz de interaccionar con DAG y esteres de forbol, y un dominio C2 capaz de unir Ca2+ y fosfolípidos aniónicos.

b) PKC nuevas: en esta subfamilia se agrupan las isoenzimas ε, δ *(Ono et al., 1987; Osada et al., 1990; Osada et al., 1992)*. Este grupo posee los mismos dominios reguladores que las PKC clásicas, pero el dominio C2 es insensible a

Ca2+, por eso este grupo de enzimas solo necesita de fosfolipidos anionicos y DAG o esteres de forbol para activarse totalmente.

c) PKC atípicas: es una subfamilia compuesta por dos miembros, la PKCζ y la PKC ι/λ [PKCι (isoforma en humanos) *(Selbie et al., 1993)*/ PKCλ (isoforma en ratón) *(Akimoto et al., 1994)*]. Este grupo solo posee el dominio pseudosubstrato, un dominio C1 incapaz de interaccionar con DAG o esteres de forbol y otro llamado PB1 encargado de interaccionar con otras proteínas.

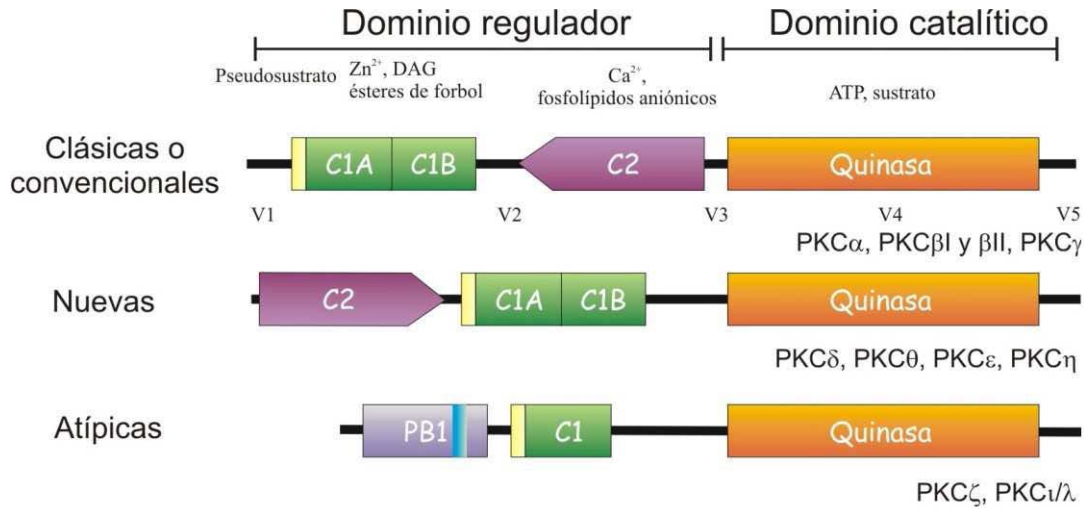

Figura I.1. Representación esquemática de la estructura primaria de los isoenzimas de la PKC. Se muestran los distintos dominios que lo forman, así como los requerimientos de cofactores para su regulación. En el extremo amino terminal se encuentra la región reguladora, con el dominio pseudosustrato, y los dominios C1 y C2, que unen diferentes cofactores según el tipo de isoenzima. La PKC atípicas poseen un dominio PB1. El extremo carboxilo terminal, contiene la región catalítica del enzima con el dominio quinasa de unión al ATP y al sustrato. Las regiones variables se muestran nombradas como V1, V2, V3, V4 y V5. (Tomado de *Corbalán García y Gómez Fernández, 2006*).

El dominio C1 suele presentarse en *tándem* formando 2 subdominios, cada uno de los que posee una secuencia consenso del tipo **H**X12**C**X2**C**X13/14**C**X2**C**X4**H**X2**C**X2**C**X7**C** siendo imprescindible para la coordinación de dos iones de zinc.

Presentan una estructura globular, donde los aminoácidos se agrupan en una distribución polarizada; en la parte superior, por donde se unen al DAG o esteres de forbol, se encuentran residuos aromáticos, mientras que en la parte media se encuentran aminoácidos catiónicos *(Bittova et al., 2001; Sanchez-Bautista et al., 2009)*.

El otro dominio de la región reguladora es el dominio C2, cuya estructura es un β-sándwich compuesto por ocho cadenas β dispuestas de forma antiparalela y que están conectadas por medio de lazos de estructura flexible tanto en la parte alta como baja del dominio *(Cho and Stahelin, 2006)*. En las PKCs clásicas el principal papel de este dominio es actuar como motivo de anclaje a la membrana activado por Ca^{2+} *(Corbalan-Garcia and Gomez-Fernandez, 2006)*.

El dominio presenta dos regiones funcionales importantes; la primera es la *región de unión a calcio* localizada en la parte superior y encargada de unir dos o tres iones Ca^{2+} dependiendo de la isoenzima, además de PtdSer *(Conesa-Zamora et al., 2000; Corbalan-Garcia and Gomez-Fernandez, 2010; Ochoa et al., 2002)*. La otra región importante es la *región polibásica* o *rica en lisinas*, que une específicamente PtdIns(4,5)P_2 *(Marin-Vicente et al., 2008b; Sanchez-Bautista et al., 2006a)*.

Para que ocurra la activación de la PKC, antes debe localizarse en membranas biológicas donde se encuentren sus cofactores. En el caso de las PKCs clásicas, esta localización esta mediada por los dominios C1 y C2 siendo primeramente la interacción del dominio C2 con la PtdSer y el PtdIns(4,5)P_2 de la membrana a través de Ca^{2+}, lo que favorece que el dominio C1 interaccione con DAG, permitiendo un mejor anclaje de la PKC en dicha membrana *(Corbalan-Garcia and Gomez-Fernandez, 2006)*.

I.2. Objetivos.

Los objetivos concretos de esta tesis son:

- Determinación de la función del dominio regulador C2 de la PKCα en la activación provocada por Ca^{2+}, PtdSer y PIP_2.
- Caracterizar la interacción entre los dominios C1B de las PKC nuevas y fosfolípidos negativos, su especificidad y su dependencia de DOG y DOcPA.
- Caracterizar los residuos aminoácidos involucrados en la interacción entre el dominio C1Bε y fosfolípidos negativos (POPS y POPA).
- Estudio lipidómico por espectrometría de masas en células RBL-2H3 de la variación en la composición lipídica de la bicapa después de la estimulación antígeno-anticuerpo, y previo al uso de diferentes inhibidores de fosfolipasas.

II. MATERIALES Y MÉTODOS.

II.1. Construcciones de ADN plasmídico.

Los ADN correspondientes a diferentes isoenzimas de la PKC fueron cedidos por los Drs. Nishizuka y Ono. Estos fueron amplificados mediante PCR y expresados en *Escherichia coli* siguiendo el protocolo propuesto por *(Cohen et al., 1973)*, con el fin de producir grandes cantidades de plásmidos. Las construcciones que se generaron fueron las siguientes:

CONSTRUCTION USED	OLIGONUCLEOTIDES USED	RESTRICTION ENZYMES
PKCα-WT	5'-ATTCTCGAGCTATGGCTGACGT 3'-CCGCCTACCTACGCACTTTGCAAGAT	XbaI/XpnI
PKCε-WT-EGFP (PKCε-EGFP)	5'- GCCAGATCTATGGTAGTGTTCAATG 3'- GAAAAGTCTACGTGATCATCGATC	BglII/BamHI
C1Bε-WT-ECFP (C1Bε-ECFP)	5'-CGGAATTCTAAACATGCCCCACAAG 3'-CCCAAGCTTTCAGTCCACCCCACAATTG	BglII/BamHI
ECFP-C1BεK251A	5'-AAGTTCGGGATCCACAACTACGCGGTCCCCACGTTCTGTGAC 3'-GTCACAGAACGTGGGGACCGCGTAGTTGTGGATCCGAACTT	HindIII/XmaI
ECFP-C1BεK251M	5'-TTCGGGATCCACAACTACATGGTCCCCACGTTCTGTGAC 3'- GTCACAGAACGTGGGGACCATGTAGTTGTGGATCCCGAA	HindIII/XmaI
ECFP-C1BεR268A	5'-TCCCTGCTCTGGGGCCTCTTGGCGCAGGGCCTGCAGTGTAA 3'-TTTACACTGCAGGCCCTGCGCCAAGAGGCCCCAGAGCAGGG	HindIII/XmaI
ECFP-C1BεR282H	5'-TGCAAAATGAATGTTCACCATCGATGCGAGACCAACGTG 3'-CACGTTGGTCTCGCATCGATGGTGAACATTCATTTTGCA	HindIII/XmaI
ECFP-C1BεR282A/R283A	5'- GTCTGCAAAATGAATGTTCACGCGGCGTGCGAGACCAACGT 3'-AGCCACGTTGGTCTCGCACGCCGCGTGAACATTCATTTTGCA	HindIII/XmaI
ECFP-C1Bθ	5'-CGGAATTCATCGCTTTAAAGTGTAT 3'-CCCAAGCTTTCAGCACAGGTTCGCCAC	EcoRI/ BamHI
ECFP-C1Bδ	5'- TATAAGCTTGACATGCCTCACCGA 3'- GACACACCATAGTTGACTCCTAGGAA	HindIII/BamHI
ECFP-C1Bγ	5'-GGATCCCGGGTCAGTCCACGCCGC 3'-CCGAAGCTTCGCAACAAGCACAAG	HindIII/XmaI

II.1. Cultivos celulares y transfecciones.

Para esta Tesis Doctoral se usaron tres líneas celulares:

- Las *células RBL-2H3* son una línea celular de *leucemia* basofílica de rata Wistar aislada y clonada en 1978.
- La línea celular HEK293, que son células epiteliales de riñón humano.

- Las *SH-SY5Y* son una sub-*línea c*lonada 3 veces (SK-N-SH -> SH-SY -> SH-SY5 -> *SH-SY5Y*) de una biopsia de médula ósea humana derivada de la *línea*.

Las células RBL-2H3 se crecieron en medio de cultivo DMEM 4,5 g/l glucosa suplementado con 15% de suero bovino fetal, antibiótico (penicilina 50 unidades/ml y estreptomicina 50 µg/ml) y 4mM glutamina, mantenidas a 37 ºC en calor húmedo con 7.5% CO_2 . Las células HEK293 se incubaron a 37 ºC en calor húmedo con 7.5% CO_2 en medio de cultivo DMEM 4,5 g/l glucosa suplementado con 10% de suero bovino fetal, antibiótico (penicilina 50 unidades/ml y estreptomicina 50 µg/ml) y 2 mM glutamina. Las células SH-SY5Y crecieron en medio de cultivo DMEM 4,5 g/l glucosa suplementado con 15% de suero bovino fetal, antibiótico (penicilina 50 unidades/ml y estreptomicina 50 µg/ml), 2 mM glutamina y 1 mM piruvato sódico, manteniéndose a 37 ºC en una estufa de calor húmedo con5% CO_2.

Para la transfección de células RBL-2H3 se realizó la electroporación utilizando los siguientes parámetros: Protocolo Onda Cuadrada: 1 pulso de 200 V de 10 ms. Las células HEK293 fueron transfectadas por el método de precipitación con fosfato cálcico *(Wigler et al., 1977)*. Para la transfección de células SH-SY5Y también se utilizó la electroporación en modo de decaimiento exponencial usando como parámetros: 250 V y 950 µF.

II.3. Microscopía confocal.

Se uso el microscopio confocal Leica TCS SP2 AOBS (Leica, Heidelberg, Germany) con los objetivos Nikon PLAN APO-CS 63x 1.4 W (agua) para visualizar las células con proteínas marcadas con construcciones fluorescentes.

Las células transfectadas con construcciones de EGFP, fueron excitadas con el laser Ar/ArKr a 488 nm y la emisión se recogió entre 500-520 nm. En el caso de construcciones ECFP se utilizo el laser diodo azul a 405 nm para la excitación y 470-490 nm para la detección.

Se recogieron series de imágenes respecto al tiempo y los datos de localización se analizaron con el programa Imagen NIH (http://rsb.info.nih.gov/ij/ 1997-2011).

II.4. Transferencia de energía por resonancia (FRET).

Para llevar a cabo los experimentos de FRET en esta memoria de tesis doctoral, se expresaron las diferentes construcciones C1B-ECFP en células HEK293 y los lisados celulares fueron añadidos a la cubeta de fluorescencia, alcanzando la misma intensidad de fluorescencia inicial en todos los casos. En los experimentos de enlace se utilizaron vesículas unilamelares pequeñas, que contenían las concentraciones deseadas de lípidos de estudio y la sonda Oregon Green 488® unida a fosfatidiletanolamina. La transferencia de energía entre el fluoróforo donador (CFP) y el aceptor (Oregon Green 488®) fue medida utilizando una longitud de onda de excitación de 433 nm y 473 nm de emisión.

II.5. Purificación y medidas de actividad quinasa.

Para la obtención de proteínas parcialmente purificadas (concretamente la PKCε-EGFP y el mutante PKCεK251A/R268A/R282A/R283A-EGFP) se transfectaron células HEK-293 mediante la técnica de fosfato de calcio. En el caso de la PKCα se infectaron con baculovirus recombinante células de insecto Sf9. Después de expresar la proteína de interés, se lisaron las células y se introducía el sobrenadante en la columna . Para la elución de la proteína de interés se utilizó la columna His-Gravi TrapTM® (Amersham Biosciences, Uppsala, Suecia) por medio de un gradiente salino.

Con estas enzimas parcialmente purificadas se llevaron a cabo experimentos de actividad enzimática utilizando el isótopo radiactivo ^{32}P. El proceso consiste en simular las condiciones óptimas para la activación de la enzima, es decir, presencia de vesículas lipídicas, Ca^{2+} (en el caso de la PKCα), ATP (una parte marcado con radiactividad) y como sustrato se utilizó la histona III-S. Tras 20 minutos de actuación de la PKC, se para la reacción con BSA y tricloroacético para finalmente medir, mediante un contador de centelleo, el grado de marcaje de la histona con el isótopo radiactivo.

II.6. Espectrometría de masas.

Estos estudios se realizaron en el laboratorio del Profesor Bernd Helms, en el Departamento de Bioquímica y Biología Celular de la Facultad de Veterinaria en la Universidad de Utrecht, (Países Bajos).

Los experimentos de lipidómica se realizaron mediante LC-MS/MS en un espectrómetro de masas 4000 Q TRAP (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands), un híbrido QqLIT con una fuente de ionización APCI (AB Sciex Instruments, Toronto, ON, Canadá).

II.7. Análisis estadístico.

Los análisis estadísticos se realizaron por medio de los test de la chi-cuadrado, Krustal-Wallis, Man-Whitney y de la varianza según cada caso. Se utilizó el paquete estadístico SPSS y se consideró una diferencia significativa cuando $p<0.05$.

III. EL FOSFATIDILINOSITOL 4,5-BIFOSFATO DISMINUYE LA CONCENTRACIÓN DE CA^{2+}, FOSFATIDILSERINA Y DIACILGLICEROL REQUERIDAS PARA QUE LA PROTEÍNA QUINASA C α ALCANCE SU MÁXIMA ACTIVIDAD.

La isoenzima PKCα se encuentra dentro de la subfamilia de PKCs clásicas. Su activación viene determinada por segundos mensajeros como el incremento de la concentración de Ca^{2+} citoplasmático en la célula, o la presencia de diacilglicerol en la membrana, donde tienen lugar interacciones específicas con fosfatidilserina y PIP_2 *(Corbalan-Garcia and Gomez-Fernandez, 2006)*. Esta translocación a la membrana está mediada principalmente en las PKC clásicas por las interacciones dominio C2-membrana, seguidas de las interacciones del dominio C1-diacilglicerol.

El dominio C2 de la PKCα posee dos sitios de unión diferentes, uno para Ca^{2+} y fosfatidilserina y un segundo lugar para la unión a PIP_2 con una alta afinidad.

La actividad enzimática de la PKCα fue estudiada con ensayos de actividad quinasa utilizando vesículas unilamelares grandes y variando la concentración de Ca^{2+} y del contenido de diacilglicerol (DOG), fosfatidilinositol 4,5-bifosfato (PIP_2) y fosfatidilserina (POPS) en estas membranas modelo.

Los resultados mostraron que el PIP_2 aumentaba la V_{max} de PKCα y, cuando la concentración de PIP_2 fue de 5 mol% del total de lípidos de la membrana, la adición de 2 mol% de DOG no incrementaba la actividad. La adición de PIP_2 disminuía la $K_{0.5}$ de Ca^{2+} más de tres veces, la del DOG más de 5 y la del POPS la mitad.

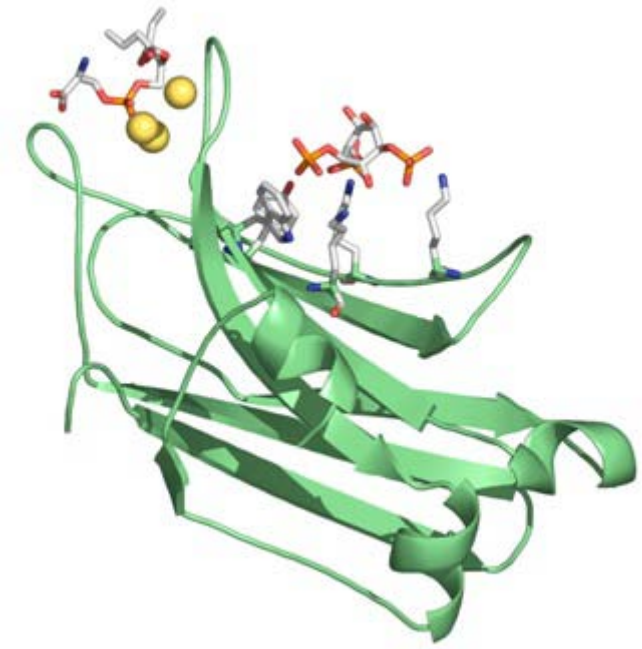

Figure III.1. Representación del dominio C2 de la PKCα unido a Ca^{2+}-POPS-PIP_2. Se observa la unión a la PtdSer por la región de unión de Ca2+ en la parte superior del esquema, y a una molécula de PtdIns(4,5)P2 por la región polibásica en el centro del esquema, unido en las cadenas β3-β4 *(Guerrero-Valero et al., 2009a).*

El valor de la $K_{0.5}$ para el PIP_2 es de 0.11 µM en presencia de DOG y de 0.39 µM en ausencia, encontrándose dentro del rango fisiológico de la monocapa interna de la membrana plasmática en mamíferos, y como consecuencia, la PKCα podría actuar cercana a su máxima actividad incluso en ausencia de diacilglicerol. Los resultados obtenidos en este trabajo son compatibles con el mecanismo secuencial previamente propuesto *(Corbalan-Garcia et al., 2003a)* y posteriormente confirmado in vivo *(Marin-Vicente et al., 2008b)* (Fig. III.1), en el que elevaciones de Ca^{2+} intracitosólico desencadenan la translocación de la PKCα a la membrana, continuando con dos posibles situaciones. La primera es que en la membrana se encuentren microdominios enriquecidos únicamente con fosfatidilserina, y que el docking del dominio C2 no sea suficiente para liberar del dominio catalítico el sitio de unión al substrato, como ha sido visto recientemente en la estructura 3D resuelta *(Leonard et al.,*

2011), en el que el dominio C1B se encuentra bloqueando el dominio catalítico, y sería necesaria la presencia de DAG para que la enzima tuviera su máxima activación *(Ananthanarayanan et al., 2003b)*. La segunda situación es que los microdominios estén enriquecidos con PIP_2 y fosfatidilserina. En este caso, el dominio C2 se uniría a la membrana con una diferente orientación al anclarse en dos diferentes puntos (Ca^{2+}/PS en el sitio de unión a calcio, y a PIP_2 en la región rica de lisinas), y como consecuencia, induciría un cambio conformacional que liberaría al dominio C1 del centro catalítico de la enzima, consiguiéndose la activación completa de la enzima.

IV. LOS DOMINIOS C1B DE LAS PKC NUEVAS ε Y η TIENEN UNA MAYOR AFINIDAD DE UNION A MEMBRANAS QUE LAS PKC NUEVAS θ Y δ.

Tanto las PKC clásicas como las nuevas poseen dos dominios C1 diferentes, que forman un tándem en el dominio regulador, C1A y C1B, aunque la función exacta de cada uno de ellos no está del todo claro. Un gran número de estudios sugieren que ambos dominios son funcionalmente distintos, como es evidente si nos fijamos en su desigual afinidad por diacilgliceroles o ésteres de forbol *(Corbalan-Garcia and Gomez-Fernandez, 2006; Irie et al., 2002a; Irie et al., 1997; Shindo et al., 2001)*.

Debe también tenerse en consideración que ambos dominios C1 muestran afinidad por lípidos negativos, y la imposibilidad de medir la actividad de la enzima completa para detectar cual es el dominio en el que se presenta la afinidad observada, hace que tome importancia el estudio de estos dominios de forma independiente.

Los cuatro dominios C1B de las PKC nuevas (δ, θ, η y ε) fueron estudiados con diferentes mezclas lipídicas que contenían los fosfolípidos negativos de estudio, así como diacilglicerol o ésteres de forbol. Los resultados mostraron que en presencia de 5 mol% de diacilglicerol, C1Bε-ECFP y C1Bη-ECFP y exhibían una substancial mayor propensión en la unión de vesículas que contenían fosfolípidos con carga negativa en comparación de los dominios C1Bθ-ECFP y C1Bδ-ECFP.

Estas diferencias observadas entre los dominios C1B de las PKCs nuevas, y que pueden ser agrupadas a su vez en dos grupos de dos, fueron también evidentes en células RBL-2H3 utilizadas como membranas modelo, en las que los dominios C1Bε-ECFP y C1Bη-ECFP se translocaban a la membrana con la adición de ácido fosfatídico soluble sin diacilglicerol o ésteres de forbol. En cambio, C1Bθ-ECFP y C1Bδ-ECFP requería de la presencia de diacilglicerol para la unión a membranas cuando se añadía ácido fosfatídico soluble fuera detectable.

Con todo ello podemos concluir diciendo que los dominios C1B de las PKC nuevas tienen diferentes afinidades de unión por membranas modelo, dependiendo de que haya presencia o no de diacilglicerol, como también fue observado en células RBL-2H3, y en base a estas diferencias podemos establecer dos grupos de dominios C1B dentro de las PKCnuevas: un primer grupo formado por PKCε y PKCη, con una alta afinidad de unión a membranas; y un segundo grupo compuesto por PKCθ y PKCδ, que presentan una baja afinidad, que vendría a confirmar la homología de secuencias existente entre los dominios ε y η de un 84%, mientras que solo exhiben un 62% de homología con δ y θ, cuando la homología de estos dos últimos es de un 80%.

V. FUNCIÓN DE RESIDUOS CON CARGA POSITIVA EN EL DOMINIO C1B DE LA PKCε EN LA TRANSLOCACIÓN A LA MEMBRANA Y ACTIVIDAD ENZIMÁTICA.

No está totalmente claro por qué las PKC clásicas y nuevas contienen dos dominios C1 con una alta homología en sus secuencias, y cuál es el rol específico que tiene cada uno de ellos. Lo que sí es conocido es la diferencia de afinidades por diacilglicerol, esteres de forbol o fosfolípidos con carga negativa.
Como vimos en el capítulo anterior, C1Bη y C1Bε, pero mucho mas esta última, mostraron una mayor afinidad por lípidos negativos, y en especial por ácido fosfátidico, que los otros dos dominios C1B de la PKCδ and PKCθ.
A la luz de estos resultados, se realizó un estudio de la estructura del dominio C1Bε para averiguar qué aminoácidos serían los responsables de su alta afinidad por fosfolípidos ácidos. De entre todos ellos, los residuos con carga positiva

K251, R268, R282 y R283 podrían interaccionar con la membrana debido a que quedarían expuestos sobre la superficie del dominio.
Mediante mutagénesis dirigida, estos residuos fueron sustituidos por alaninas, y el efecto de mutaciones simples, dobles, triples y cuádruple sería caracterizado mediante diferentes técnicas.

Los diferentes constructos fueron expresados en células HEK293 y los lisados se utilizaron para los ensayos de FRET usando membranas modelo que contenían una concentración de 20 mol% de POPS o POPA en presencia o ausencia de 5 mol % de DOG. Los mutantes simples mostraron una mayor inhibición a membranas en ausencia de DOG, pero serían los dobles, y en mucha mayor medida los triples, los que inactivarían casi por completo la afinidad del dominio por membranas, incluso en presencia de DOG.

Además, células RBL-2H3 fueron transfectadas con los diferentes mutantes de dominios C1Bε aislados y de la proteína completa corroborando los resultados obtenidos en los ensayos de FRET. Los mutantes simples en los dominios aislados C1Bε disminuían en gran medida la translocación de los dominios a membranas, en cambio, en la proteína completa sería necesaria la presencia de 3 mutaciones para ver descensos de afinidad comparables a los del dominio aislado. Este hecho sería debido a que en la proteína completa el dominio C2 también podría favorecer el anclaje de la enzima a la membrana.

Para comprobar si esas mutaciones podrían producir la inhibición de la actividad enzimática de la PKCε, se estudió la capacidad de la PKCε para fosforilar un substrato *in*-vivo bajo condiciones POPA-dependientes. La actividad quinasa fue también medida en el mutante PKCεK251AR/268A/R282A/R283A-EGFP en las mismas condiciones que el tipo silvestre. Este mutante mostró una actividad catalítica residual con una inhibición del 80% de la actividad a altas concentraciones de POPA.

Por último, se realizó un estudio lipidómico de la variación en la composición de ácido fosfatídico y diacilgliceroles en células RBL-2H3 estimuladas tras la unión antígeno-receptor, utilizando la técnica de espectrometría de masas en el Departamento de Bioquímica y Biología Celular, en la Facultad de Veterinaria, Universidad de Utrecht.

Todos estos resultados confirman nuestra hipótesis de que el dominio C1B de la PKCε es necesario para la translocación de la proteína a membranas y para la activación de la enzima.

VI. EL DOMINIO C1B DE LA PKCε TAMBIÉN INTERACCIONA CON CARDIOLIPINA, UN LÍPIDO DE LA MEMBRANA MITOCONDRIAL INTERNA.

La cardiolipina es un fosfolípido aniónico localizado de manera casi exclusiva en la membrana mitocondrial interna. Tiene importancia a nivel estructural y también ha sido descrita su asociación con transportadores y grandes complejos proteicos de la membrana mitocondrial interna, donde parece esencial para su correcto funcionamiento (*Claypool et al., 2008; Hoffmann et al., 1994; Pfeiffer et al., 2003; Schlattner et al., 2009*).

La cardiolipina podría también actuar como anclaje de la PKC en la membrana mitocondrial debido a la afinidad por lípidos negativos que está presente en esta familia de proteínas, y así explicar por qué en episodios de isquemia y reperfusión la PKCε y PKCδ se transloca a la mitocondria en cardiomiocitos *(Duquesnes et al., 2011)* o las isoenzimas ε y γ en isquemia cerebral *(Bright and Mochly-Rosen, 2005a)*.

En este trabajo hemos fijado nuestra atención en la isoenzima PKCε porque como ya comentamos en capítulos previos, la mayor afinidad a membranas era la establecida entre el dominio C1Bε y fosfolípidos cargados negativamente. Aunque el mecanismo que permite la translocación mitocondrial de la PKCε no es conocido todavía, la hipótesis de trabajo en este trabajo era que la translocación y anclaje de la PKCε a la mitocondria podría ser llevada a cabo no solo por las interacciones entre el dominio C2 y proteínas mitocondriales descritas por *Budas et al., 2010*, sino también por las interacciones proteína-cardiolipina a través del dominio C1B.

Se llevaron a cabo ensayos de FRET transfectando células HEK293 con los diferentes dominios C1B de las PKC nuevas y el dominio C1B de la PKCγ utilizando membranas modelo que contenían concentraciones crecientes de cardiolipina en presencia de 5 mol% de DOG. C1Bε-ECFP fue el dominio que mayor afinidad mostró en el rango fisiológico en el que se encuentra la

cardiolipina (~15%) *(van Meer et al., 2008)*. Para conocer si los residuos con carga positiva mutados en el Capítulo V también intervenían en la unión del dominio a cardiolipina, ensayos de FRET con mutantes simples, dobles y triples del dominio C1Bε fueron llevados a cabo utilizando membranas con 20 mol% de cardiolipina en ausencia o presencia de 5 mol% de DOG, y tal y como ocurría en el Capítulo V, la afinidad por membranas disminuía cuando el número de residuos en el dominio aumentaba, llegando a inactivarse incluso cuando las vesículas contenían 5 mol% de DOG.

Los ensayos de actividad quinasa con el mutante PKCεK251A/R268A/R282A/R283A-EGFP mostraron que la actividad catalítica de la enzima era del 20% de la registrada por el tipo de silvestre.

Por último, para comprobar si la cardiolipina inducía la localización de la PKCε a la membrana mitocondrial interna bajo condiciones de isquemia, se transfectaron células SH-SY5Y con la PKCε silvestre y con el mutante cuádruple, confirmando que cuando se sometían las células a la privación de oxígeno y de glucosa, la PKCε mutante permanecía en su mayoría en el citosol, mientras que la tipo silvestre se repartía en partes iguales entre la fracción de membranas y la fracción mitocondrial.

Estos resultados coinciden con los obtenidos con los ensayos de actividad quinasa y FRET, sugiriendo que los residuos mutados en el dominio C1B, son esenciales para la translocación y activación de la PKCε en membranas que contengan cardiolipina.

VII. CONCLUSIONES

Las conclusiones más importantes de esta memoria de tesis doctoral son:

1. La actividad enzimática de la PKCα puede operar cercana a su máxima capacidad dentro del rango fisiológico de concentraciones de PIP_2 sin necesidad de que ninguna otra señalización celular produzca diacilglicerol.

2. Los dominios C1B de las PKC nuevas presentan diferentes afinidades de unión a membranas dependiendo de la presencia o no de diacilglicerol y de fosfolípidos negativos.
3. Dos grupos de dominios C1B de PKC nuevas pueden establecerse en función de su afinidad de unión a membranas, estos serían PKCε y PKCη con una alta afinidad, y PKCδ y PKCθ con una baja afinidad.
4. El dominio C1B de la PKCε es necesario para la translocación de la proteína a la membrana y de la activación de la enzima.
5. El dominio C1B de la PKCε interacciona con una gran afinidad con cardiolipina, que es el lípido negativo mayoritario presente en la membrana mitocondrial interna.
6. La actividad enzimática de la PKCε obtenida en membranas que simulan la concentración de cardiolipina en la membrana mitocondrial interna es dos veces mayor que en membranas que contienen fosfatidilserina en el rango fisiológico presente en la membrana plasmática.
7. La unión del antígeno con el receptor de alta afinidad IgE (FcεRI) en células RBL-2H3 estimula varias rutas de señalización de lípidos con la generación de diferentes especies de diacilglicerol y ácido fosfatídico vía PI-PLCγ y PLD respectivamente.

ABBREVIATIONS

COMMON ABBREVIATIONS USED

AA	Arachidonic acid
ADN	Ácido desoxirribonucleico
APCI	Atmospheric pressure chemical ionization
BSA	Bovine serum albumin
CBR	Calcium Binding Region
CCP	Ceramide cholinephosphotransferase
CMV	Cytomegalovirus promoter
cpm	Counts per minute
CT	Carboxy-terminal tails
DAG	Diacylglycerol
DAPS	1,2-diacetyl-*sn*-phosphatidyl-L-serine
DGK	Diacylglycerol kinase
DHA	Docosahexaenoic acid
DHPE	Dihexadecanoyl-*sn*-glycero-3-phospho-ethanolamine
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	Dimethyl sulfoxide
DNP-HSA	Dinitrophenyl-human serum albumin
DPG	1,2-*sn*-Dipalmitilglicerol
DOcG	Dioctanoylglycerol
DOcPA	Phosphatidic acid
DTT	Dithiothreitol
ECFP	Enhanced cyan fluorescent protein
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FBS	Fetal bovine serum
FcεRI	High affinity IgE receptor
FPLC	Fast protein liquid chromatography
FRET	Fluorescence resonance energy transfer
HBS	Hepes Buffer Saline
HEK293	Human Embryonic Kidney 293 cell line
HEPES	N-(2-Hidroxietil)piperacina-N'-(2-ácido etanosulfónico)
HSP90	Heat shock protein-90
$Ins(1,4,5)P_3$	Inositol-1,4,5-trisphosphate
K_D	Apparent equilibrium dissociation constant
kDa	Kilodalton

LB	Lysogeny broth
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LUV	Large unilamellar vesicles.
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site
ML	Membrane localization
MRM	Multiple-reaction monitoring
MS	Mass spectrometry
OG-dPE	Oregon Green® 488-dansil fosfatidiletanolamina
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
PBS	Phosphate buffer sline
PC-PLC	Phosphatidylcholine-dependent phospholipase C
PC-PLD	Phosphatidylcholine-dependent phospholipase D
PCR	Reacción en cadena de la polimerasa
PKD	Protein kinase D
PDK-1	Phosphinositide-dependent protein kinase-1
PI-PLC	Phosphatidylinositol-dependent phospholipase C
PH domain	Pleckstrin homology domain
PLA	Phospholipase A2
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulfonyl fluoride
PNS	Postnuclear supernatant fraction
POPA	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)
POPS	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine
PSA	Persulfato amónico
PtdCho	Phosphatidylcholine
PtdIns(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
RACK	Receptor for activated C-kinase
rasGRP	Ras guanyl-releasing protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revoluciones por minuto
RT	Room temperature
RTK	Receptor tyrosine kinase
SAG	1,2-*sn*-esterail-araquidonilglicerol
SDS-PAGE	Sodium dodecil sulphate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TRIS	Tris(hidroximetil)aminometano
RasGRPs	Guanyl nucleotide-releasing proteins

RBL-2H3	Rat Basophilic Leukemia 2H3 cell line
TRPC	Transient Receptor Potencial Canonical
(p/v)	Relación peso/volumen
(v/v)	Relación volumen/volumen

CHAPTER 1

Introduction and Objectives.

In cells, signal transmission between the plasma membrane and nucleus is mediated by different enzymatic pathways, and protein kinase C (PKC) is an essential component in many of them.

PKC is a large family of phospholipid-dependent serine/threonine kinases, which is activated by many extracellular signals and have a crucial role in signaling transmission, being involved in an enormous variety of physiological functions, including mitogenesis and cell proliferation, metabolism regulation, apoptosis, platelet activation, reorganization of actin cytoeskeleton, ion channel modulation, secretion and neural differentiation, and also in many illness like cancer, lung and heart diseases *(Bell, 1986; Corbalan-Garcia and Gomez-Fernandez, 2006; Dekker and Parker, 1997; Dempsey et al., 2000; Inagaki et al., 2006; Mellor and Parker, 1998; Newton, 2001; Nishizuka, 1986; Nishizuka and Nakamura, 1995).*

This family contains 10 members encoded by 9 genes in mammals. Each PKC isoenzyme is expressed in a large variety of tissues, and multiple PKC isoenzyme are expressed in a single cell, making it a ubiquitous family of enzymes and to possess wide functional diversity, which underlines the enormous importance of their study (Bell and Burns, 1991; Corbalan-Garcia and Gomez-Fernandez, 2006; Newton, 1995a; Nishizuka, 1992).

1. STRUCTURE.

The primary structure of mammals PKCs consists of an amino-terminal regulatory domain that maintains the enzyme in an inactive conformation and a highly conserved carboxy-terminal catalytic domain (kinase domain) *(Coussens et al., 1986)*, tethered together by a hinge region.

The differences in the regulatory domain and their second messenger requirements are used to subdivide them into three subfamilies: conventional (or classical), novel, and atypical.

1.1. The regulatory domain.

This domain is located in the amino terminal region containing four different conserved domains depending on the isoenzyme:

1.1.1. Pseudosubstrate domain.

Every PKC isoform has an auto-inhibitory pseudosubstrate domain, which is a short sequence at the N-terminus of PKC and has a high affinity for the active site of the kinase, able to maintain PKC in an inactive state by sterically blocking the active site of the kinase and preventing substrate interaction *(House and Kemp, 1987; Makowske and Rosen, 1989; Orr et al., 1992).*

The sequence of this domain is highly conserved in all isoforms (Fig. I.1.) which is also very similar to target sequences in proteins that are susceptible to phosphorylation by PKC. The difference is the substitution of Ser/Thr residue in the target protein by an Ala *(House and Kemp, 1987; Nishikawa et al., 1997).*

PKC isozymes	Position												
	−7	−6	−5	−4	−3	−2	−1	0	1	2	3	4	5
PKCα													
optimal.	R	R	R	R	R	K	G	**S**	F	R	R	K	A
pseudo.	N	R	F	A	R	K	G	**A**	L	R	Q	K	N
PKCβI	F	K	L	K	R	K	G	**S**	F	K	K	F	A
	V	R	F	A	R	K	G	**A**	L	R	Q	K	N
PKCβII	Y	K	L	K	R	K	G	**S**	F	K	K	K	A
	V	R	F	A	R	K	G	**A**	L	R	Q	K	N
PKCγ	R	R	R	R	R	K	G	**S**	F	K	R	K	A
	P	L	F	C	R	K	G	**A**	L	R	Q	K	V
PKCδ	A	R	R	K	R	K	G	**S**	F	F	Y	G	G
	P	T	M	N	R	R	G	**A**	I	K	Q	A	K
PKCε	Y	Y	X	K	R	K	M	**S**	F	F	E	F	F
	R	P	R	K	R	Q	G	**A**	V	R	R	R	V
PKCη	A	R	R	R	R	R	R	**S**	F	R	R	X	R
	F	T	R	K	R	Q	R	**A**	M	R	R	R	V
PKCζ	R	R	F	K	R	Q	G	**S**	F	F	Y	F	F
	K	S	I	Y	R	R	G	**A**	R	R	W	R	K

Fig. I.1. Comparison of the optimal sequence of each PKC isoenzyme determined by the peptide library with the pseudosubstrate region of each isoenzyme. The optimal sequence of each PKC isoenzyme determined by the peptide library (optimal) and the pseudosubstrate region of each isoenzyme (pseudo) are presented by the one-letter codes, respectively. Bold letters indicate the phosphorylated Ser in the optimal sequence and the corresponding Ala in the pseudosubstrate region. Boxed amino acids emphasize positions that are similar between optimal sequence and pseudosubstrate region. (Taken from *Nishikawa et al., 1997)*

Some studies suggest that the coordinate C1/C2 domain engagement with membranes leads to high-affinity membrane binding of PKCs, promoting a conformation change that release the pseudosubstrate domain from the

substrate binding site, facilitating the PKC activation *(Johnson et al., 2000).* In that moment, this domain could interact with anionic lipids, being bond to the plasma membrane while the enzyme is in its active conformation *(McLaughlin et al., 2002; Mosior and McLaughlin, 1991).* (Fig. I.2.)

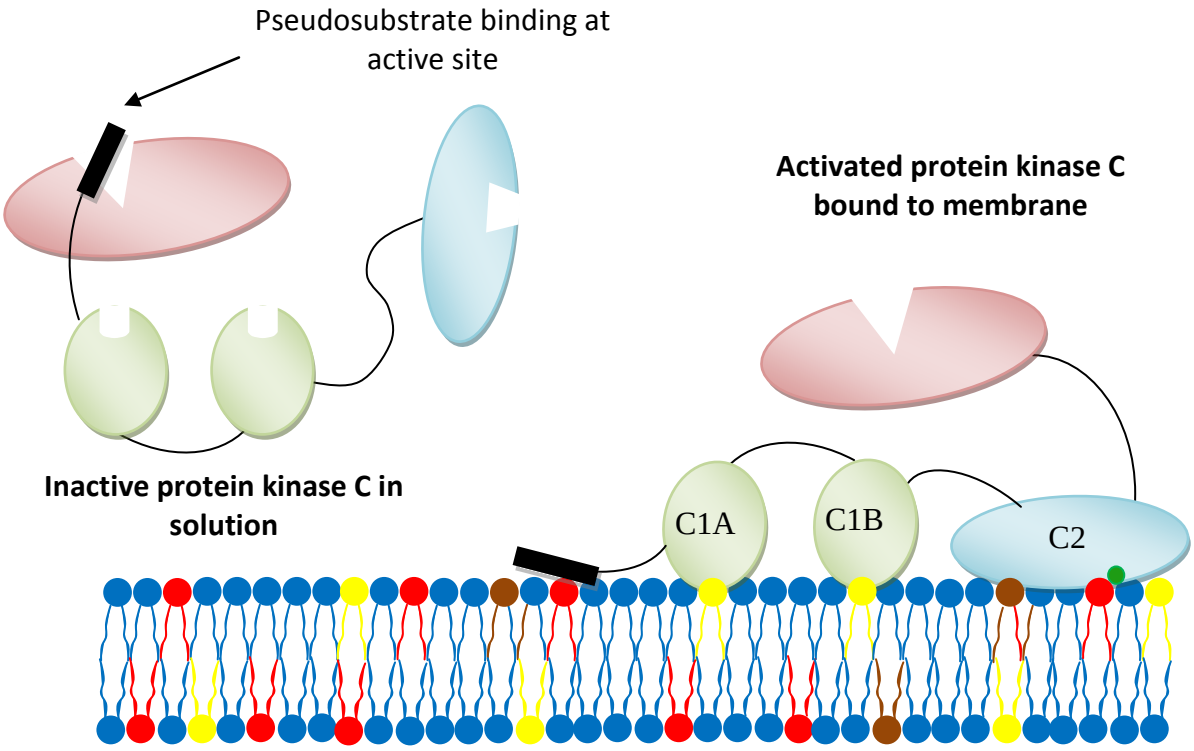

Fig. I.2. Pseudosubstrate domain during the Protein kinase C activation. The inactive protein kinase C is soluble in the cytoplasm, where the pseudosusbtrate domain is blocking the kinase site. When an increased of calcium is produced in the cell (green), the C2 domain binds to the anionic phospholipids phosphatidylinositol (4,5)-bisphosphate (brown) and phosphatidylserine (red), the C1 domains bind to diacylglycerol (yellow) in the membrane, and the pseudosubstrate domain is pulled from the active site, permitting catalysis.

1.1.2. C1 Domain.

The C1 domain is in the regulatory region of each PKC isoform and is a member of a superfamily of Cys-rich domains that it is composed by approximately 50 amino acids that were originally discovered as lipid-binding modules in PKCs *(Colon-Gonzalez and Kazanietz, 2006).*

In general, these domains are classified in two groups, depending on their capacity to bind DAG or phorbol esters.

▪ Typical C1 domains can bind effectors and are present in cPKC and nPKC *(Bell and Burns, 1991; Bittova et al., 2001; Dekker and Parker, 1994; Kazanietz et al., 1994; Ono et al., 1989; Quest and Bell, 1994b; Wender et al., 1995).*

Along with the PKC family, there are six additional families of proteins that contain a DAG-responsive C1 domain: the chimaerins, the guanyl nucleotide-releasing proteins RasGRPs, the protein kinase D (PKD), myotonic dystrophy kinase-related Cdc42-binding kinases (MRCK), the Unc-13 scaffolding proteins and the DAG kinases β and γ *(Colon-Gonzalez and Kazanietz, 2006; Yang and Kazanietz, 2003)* (Fig. I.3.)

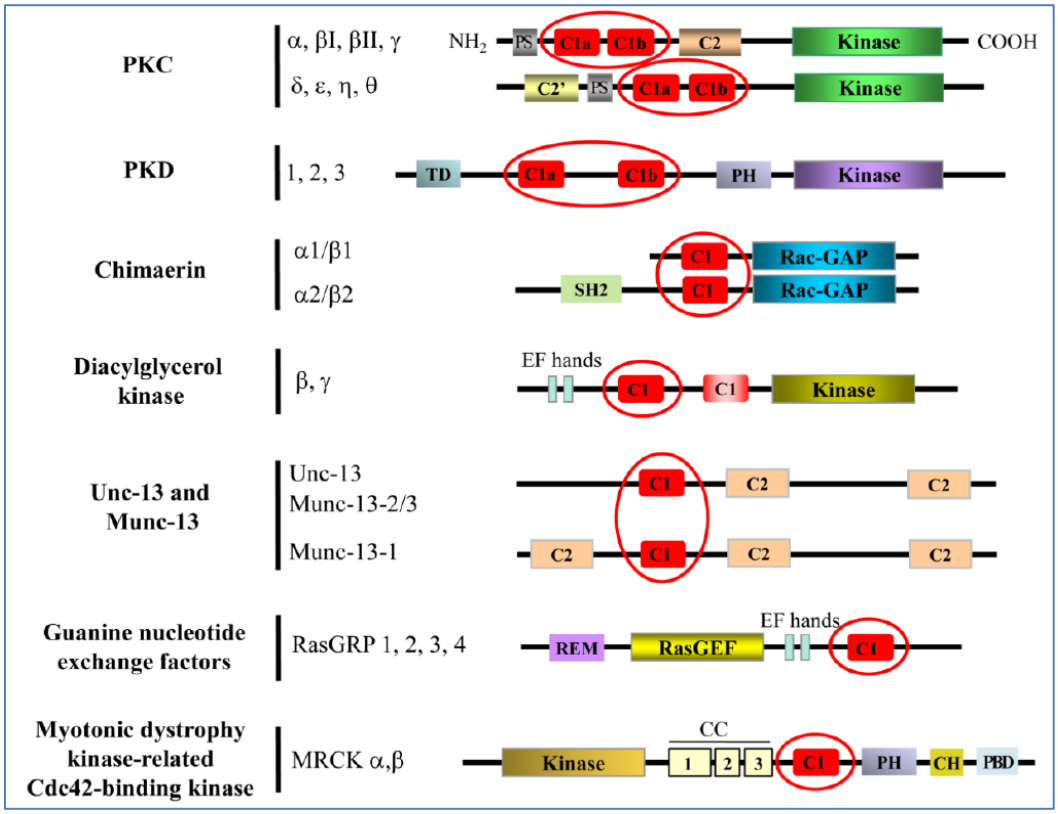

Fig. I.3. Structure of the C1 domain containing proteins. PS, pseudosubstrate domain; TD, transmembrane domain; PH, plekstrin homology domain; SH2, Src homology 2 domain; Rac-GAP, Rac GTPase-activating protein domain; REM, Ras exchange motif; GEF, guanine nucleotide exchange factor; CC, coiled –coil domain; CH, citron homology domain; PBD, p21 GTPase binding domain. Taken from *Blumberg et al., 2008*).

▪ Atypical C1 domains with only one cysteine-rich membrane targeting structure that binds PIP3 or ceramide (not DAG or phorbol esters). They are present in aPKC *(Mellor and Parker, 1998; Newton and Johnson, 1998).*

The structural features that distinguish DAG-/PMA-sensitive C1 domains in cPKCs and nPKCs from PMA-insensitive aPKC C1 domains have recently been identified. The NH2-terminal half of aPKC C1 domains contain a cluster of basic residues (HLFQAKRFNRRAYCGQCSERI) that are not found in PMA-sensitive C1 domains *(Pu et al., 2006)*.

In cPKC and nPKC, the C1 domain is composed of a tandem of two subdomains, called C1A and C1B according to their position in amino-terminal end *(Hurley et al., 1997)*. Each of these subdomains shows a conserved and characteristic motif **H**X_{12}**C**X_2**C**X_n**C**X_2**C**X_4**H** X_2**C**X_2**C**X_7**C**, where "H" is His, "X" is any amino acid, "C" is Cys and "n" is 13 or 14, which is essential to coordinate two Zn^{2+} *ions (Ahmed et al., 1991; Bell and Burns, 1991; Quest et al., 1992)*. Both of these subdomains can bind DAG or phorbol esters, although some studies have demonstrated that each subdomain does so with a different affinity *(Ananthanarayanan et al., 2003b)*, while the presence of only one is sufficient to ensure the smooth running of this part of the protein *(Bell and Burns, 1991; Bittova et al., 2001; Kazanietz et al., 1994; Quest et al., 1994; Wender et al., 1995)*.

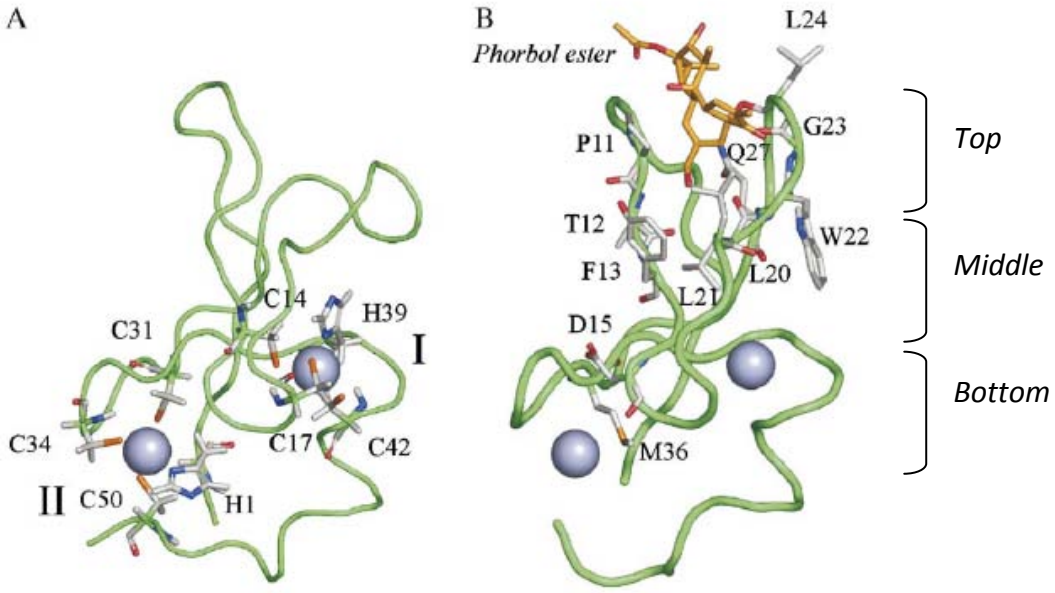

Fig. I.4. Overall structure of the C1B domains of PKCγ and PKCδ. (A) Overall structure of the C1B domain of PKCγ. The zinc atoms are represented by big purple balls and the residues involved in coordinating these two zinc ions are represented by stick models with carbon in grey, nitrogen in blue and sulphur in yellow. (B) Overall structure of the C1B domain of PKCδ in complex with phorbol 13-acetate. The phorbol ester is represented as a stick model with carbon in yellow and oxygen in red (Taken from *Corbalán-García and Gómez-Fernández, 2006*).

High-resolution crystal structure of PKCδ *(Zhang et al., 1995b)* and NMR structures of PKCα C1B *(Hommel et al., 1994)*, PKCγC1B *(Xu et al., 1997)* domains have been published and it could see that these C1 domains adopt similar tertiary structures suggesting that typical C1 domains present a polarized distribution of hydrophobic and ionic amino acids (Fig. I.4.).

The upper third of the C1 domain contains aromatic and aliphatic residues forming a hydrophilic binding cleft surrounded by hydrophobic surface where ligands like DAG/phorbol esters are bound. This hydrophilic cleft is capped by the binding of DAG/phorbol esters to the C1 domain, which forms a continuous hydrophobic surface and lets the penetration of the C1 into the membrane *(Zhang et al., 1995b)*. Cationic residues are exposed on the middle third, leading the nonspecific electrostatic interactions of the domain with anionic lipids. This initial interaction positions the C1 domain to penetrate the membrane bilayer and bind DAG, which is located more deeply within the membrane structure *(Steinberg, 2008)*. The two Zn^{2+}-coordinating sites that are required for proper C1 domain folding are located in the bottom third *(Hurley and Meyer, 2001; Pak et al., 2001)*.

Several studies have demonstrated that typical C1 domains inside the same isoenzyme, are not equivalent and have different affinities for DAG and phorbol esters. Likewise, different isoenzymes show different responses; therefore the results for one isoenzyme will not necessarily be the same for another even if they are from the same family *(Corbalan-Garcia and Gomez-Fernandez, 2006)*. Biologically, these differences reflect the huge variety of situations in which different PKC isoenzymes may participate *(Steinberg, 2008)*.

Besides DAG and phorbol esters, C1 domains can bind other lipids, such as phospholipids, ceramides, fatty acids and other tumor promoters like teleocidins and ingenols *(Kedei et al., 2004; Kozikowski et al., 1997)*. Each C1 domain shows different affinities for these compounds, and with the selective interactions between them, PKC is anchored to different subcellular compartments with the subsequent consequences of these interactions *(Becker and Hannun, 2003; Kashiwagi et al., 2002; Sanchez-Bautista et al., 2009)*.

Moreover, a potential of C1 domains as drug targets has emerged during the last years. The high degree of sequence homology and structural similarity of their catalytic region among PKC isozymes and the similarity of this region in

other protein kinases suggests that generating isozyme-selective inhibitors for the ATP-binding site is extremely challenging. On the other hand, the number of C1 domain containing proteins is considerably smaller than the number of kinases, thus the C1 domain provides an attractive target for designing selective PKC. While the structure of the binding cleft in the C1 domain is highly conserved, selectivity can be achieved by various strategies *(af Gennas et al., 2009; Blumberg et al., 2008)*.

1.1.3. C2 Domain.

The whole structure of several C2 domains from several proteins, including synaptotagmins and PKC, has been elucidated. In PKCs, the C2 domains were initially discovered as a Ca^{2+} binding site of cPKC *(Coussens et al., 1986; Kashiwagi et al., 2002; Knopf et al., 1986; Ono et al., 1986)*.

C2 domain is present in numerous proteins involved in cellular signalling, for example phospholipase C (PLC) *(Rebecchi and Pentyala, 2000)* small GTPase regulation or vessel transport *(Nalefski and Falke, 1996; Rizo and Sudhof, 1998)*, or phosphoinositide 3-kinases (PI3Ks) *(Walker et al., 1999)*. They contain approximately 130 amino acids and together with C1 domain, they anchor the enzyme to the membrane in a correct position. Besides, this domain has other functions, including protein-protein interactions, mainly through the *lysine-rich cluster (Conesa-Zamora et al., 2000; Corbalan-Garcia et al., 1999; Marin-Vicente et al., 2008b; Medkova and Cho, 1998; Mochlyrosen et al., 1992; Ochoa et al., 2001; Ron et al., 1995; Sanchez-Bautista et al., 2006a; Verdaguer et al., 1999)*.

The 3D structure of the PKCα *(Verdaguer et al., 1999)*, βII *(Sutton and Sprang, 1998)*, ε *(Ochoa et al., 2001)*, δ *(Pappa et al., 1998)* and η *(Littler et al., 2006)* C2-domains have been published and these C2 domain has a common overall fold: a single compact Greek-key motif organized as an eight-stranded anti-parallel β-sandwich consisting of a pair of four-stranded β-sheets with flexible loops on top and at the bottom *(Corbalan-Garcia and Gomez-Fernandez, 2006; Rizo and Sudhof, 1998; Shao et al., 1996; Sutton and Sprang, 1998; Verdaguer et al., 1999)*.

Unlike C1 domain, C2 domains have a high degree of variability among themselves *(Nalefski and Falke, 1996; Rizo and Sudhof, 1998)*. The sequence of

amino acids is much conserved in β-strands and in some residues that have an essential role in this domain. However, there is great variability in the binding regions between β-strands, where residues like Pro and His are very common in these zones, which confer flexibility to them. As a result, a structural role is attributed to β-strands, while the binding regions reflect the specific function of the domain *(Nalefski and Falke, 1996; Rizo and Sudhof, 1998)*.

C2 domains can be classified in two groups according to their ability to bind Ca^{2+} ions and its topology *(Ochoa et al., 2001; Pappa et al., 1998; Sutton and Sprang, 1998; Verdaguer et al., 1999)*.

- The synaptotagmin-like variants, also referred to as the S-family or type I topology, which include the C2 domains of conventional PKCs.

- The PLC-like variants, also known as the P-family or type II topology, which include the C2 domains of novel PKCs.

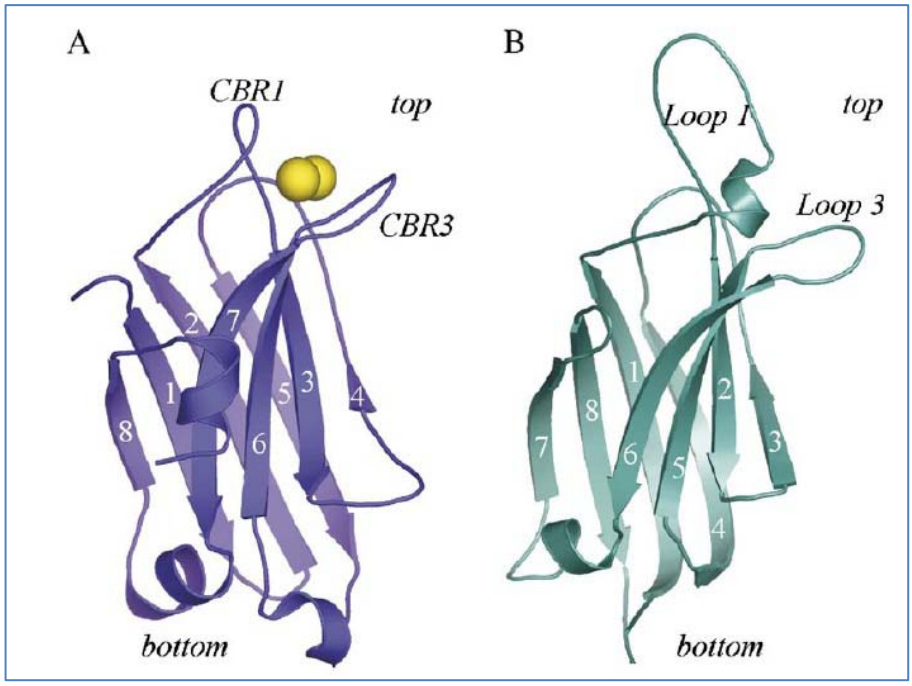

Figure I.5. Ribbon diagrams of the C2 domain structures of PKCα (A) and PKCε (B) as representative members of topology I and II, respectively. The two different topologies result from a circular permutation of the β-strands that leaves the N- and C-termini either at the top or at the bottom of the β-sandwich, respectively. It is important to note that the membrane interaction area (CBR1 to 3 in topology I, and loops 1 to 3 in topology II) is located at the top of each domain independent of the topology exhibited (Taken from *Corbalán García and Gómez Fernández*, 2006).

The main difference between both topologies is that the first strand in the C2 domain with topology I occupies the structural position of the eighth β-strand in the C2-domain with topology II (Fig. I.5.). As a result, domains with topology II represent a circular permutation of those with topology I, and both are inter-convertible, since topology I becomes II when its amino- and carboxyl-terminal are linked and new termini are generated, cutting the connection between β1 and β2-strands *(Nalefski and Falke, 1996; Rizo and Sudhof, 1998)*.

The classical ligands to C2 domains are Ca^{2+} and anionic phospholipids like PtdSer *(Newton and Johnson, 1998; Verdaguer et al., 1999)*. Besides PtdSer, other anionic phospholipids can activate PKC in the presence of Ca^{2+}, such as phosphatidylglycerol, phosphatidic acid and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) *(Lee and Bell, 1991; Marin-Vicente et al., 2008b; Newton, 1993a)*. Others lipids also intervene in the regulation of PKC: for instance, ceramides *(Kashiwagi et al., 2002; Yakushiji et al., 2003)*, unsaturated fatty acids like arachidonic acid *(Lopez-Nicolas et al., 2006; O'Flaherty et al., 2001)* and retinoid compounds *(Boskovic et al., 2002; Lopez-Andreo et al., 2005; Radominska-Pandya et al., 2000)*.

The main role of C2 domain in cPKCs is to act as the Ca^{2+}-activated membrane-targeting motif *(Cho and Stahelin, 2006; Corbalan-Garcia and Gomez-Fernandez, 2006)*. This C2 domain displays two functional regions. The first is the *Ca^{2+}-binding region*, located in the flexible top loops, that binds two or three Ca^{2+} ions, depending on the isoenzyme and also interacts with anionic phospholipids, but with a certain specificity for PtdSer (Fig. I.6.A). *(Conesa-Zamora et al., 2000; Corbalan-Garcia and Gomez-Fernandez, 2010; Kohout et al., 2002; Ochoa et al., 2002; Sutton and Sprang, 1998; Torrecillas et al., 2004b; Verdaguer et al., 1999)*.

The second functional region of the C2 domain of PKCα is the *polybasic cluster* or *lysine-rich cluster (Ochoa et al., 2002)*, located in a groove of this domain formed by strands β3 and β4 (Fig. I.6.B) *(Ochoa et al., 2002)*.

The C2 domain is responsible for binding Ca^{2+}-dependent activation of cPKC, and three different binding sites (Ca1, Ca2 and Ca3) have been characterized at the top of the β-sandwich *(Ca^{2+}-binding region)* where Ca^{2+} ions coordination occurs, specifically in the connection loops between β-strands *(Ochoa et al., 2001; Verdaguer et al., 1999).* For this reason, the loops located in

this part of the C2 domain are called CBR1 (Calcium binding region 1), CBR2 and CBR3, in order of the amino-terminal of the protein (Fig. I.5.B).

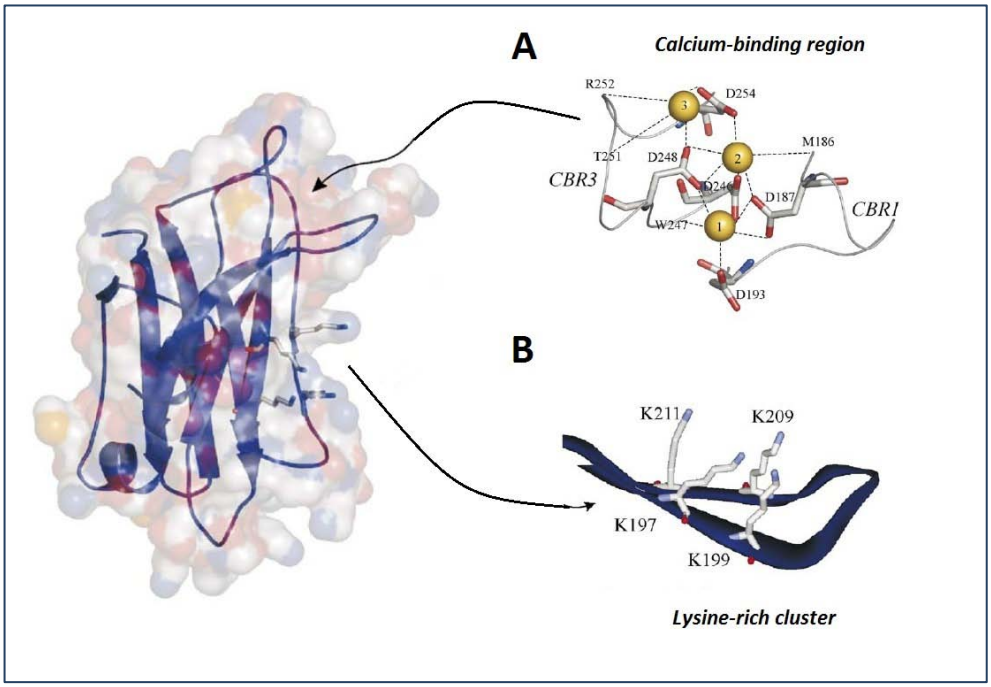

Figure I.6. Overall structure of the C2 domain of PKCα as representative member of topology I and localization of the Ca^{2+} binding region and the Lysine-rich cluster in the domain. Overall structure of the C2 domain of PKCα represented as a cartoon scheme over its molecular surface where acidic surfaces are represented in red and basic in blue. Note how the Ca^{2+}-binding region is an area rich in negatively charged amino acids, while the lysine-rich cluster forms a basic surface. The lysine residues involved in the cluster, which are located in β3 and β4 strands, are represented as stick models with carbon in gray and nitrogenin blue. **(A) Coordination scheme of the calcium ions in the structure determined for PKCα in complex with Ca^{2+} and DCPS.** Dotted lines represent the coordination established between the different carboxylate and oxygen groups, and Ca1, Ca2 and Ca3. (**B**) **Amplification of β3 and β4 strands of PKCα with the Lys residues forming the polybasic cluster represented by stick models**. The lysine residues are represented as stick models with carbon in grey and nitrogen in blue. (Taken from *Corbalán García and Gómez Fernández, 2006*).

CBRs provide all the necessary residues involved in Ca^{2+} coordination: five aspartates (Asp187, 193, 246, 248 and 254), which are highly conserved among all classical PKCs (Fig. I.5). Asp187 and Asp193 are located in the CBR1 (loop between β2- and β3-strands), where one of the calcium ions (Ca1) is lodged, whereas CBR3 (loop between β6- and β7-strands) includes Asp246, Asp248 and Asp254, and it is the site where Ca2 is lodged *(Sutton and Sprang, 1998; Verdaguer et al., 1999)*. (Fig. I.6).

Further mutagenesis studies at the *Ca^{2+}-binding site* have demonstrated that individual Ca^{2+} ions and their ligands play different roles in membrane

binding and PKCα activation. The model suggested that Ca1 is involved in initial membrane anchoring, whereas Ca2 and Ca3 are involved in conformational changes *(Bolsover et al., 2003; Conesa-Zamora et al., 2000; Corbalan-Garcia et al., 1999; Edwards and Newton, 1997; Garcia-Garcia et al., 1999; Medkova and Cho, 1998)*.

Crystallization of the C2 domain of PKCα in complex with Ca^{2+} and 1,2-diacetyl-*sn*-phosphatidyl-L-serine (DAPS) demonstrated the presence of an additional binding site for anionic phospholipids in the vicinity of the conserved *lysine-rich cluster* in strands β3 and β4 in cPKCs *(Ochoa et al., 2002)*.

More specifically, it was found that Lys197 and Lys199, located in β3 strand, and Lys209 and Lys211, in β4 strand, establish a series of electrostatic interactions with a second DAPS molecule, suggesting that this site participates in the interaction of the C2 domain with the membrane, and forming the conserved *lysine-rich* cluster. Other studies demonstrated that this area could bind other negatively charged molecules such as phosphate, phosphatidic acid, all-*trans*-retinoic acid or phosphoinositides like PtdIns(4,5)P_2. *(Lopez-Andreo et al., 2005; Marin-Vicente et al., 2008b; Ochoa et al., 2002; Ochoa et al., 2003; Verdaguer et al., 1999)*, suggesting that this site also participates in the interaction of the C2 domain with the membrane.

Recent studies have demonstrated that the C2 domains of three classical PKCs exhibit different affinity for the PtdIns(4,5)P_2, which is bound through the *lysine-rich cluster (Guerrero-Valero et al., 2007b; Sanchez-Bautista et al., 2006a)*. PKCγ-C2 domain shows the lowest affinity for this lipid, while PKCα-C2 domain needs a lower amount of this lipid to localize in membranes *(Guerrero-Valero et al., 2007b)*.

It can be concluded that both PtdSer and PtdIns(4,5)P_2 are very important for regulating the localization and activation of classical PKCs through C2 domain interaction, specifically through the *Ca^{2+}-binding site* and the *lysine-rich cluster (Bolsover et al., 2003; Landgraf et al., 2008; Marin-Vicente et al., 2008b)*.

In novel PKCs, the C2 domain shows some differences from classical isoforms; for example, it is the first conserved domain of amino-terminal; it presents a type II topology and it anchors to membranes in a Ca^{2+}-independent manner (Corbalan-Garcia et al., 2003c; Ochoa et al., 2001).

This subfamily also contains an important region at the top of β-sandwich, although now, the connection chains between β-strands are called *loops* and no *Calcium Binding Regions* since these isoforms do not bind Ca^{2+} ions. *Loop 1* and *loop* 3 are located in this region and are formed by β-1 and β-2, and β-5 and β-6 connections, respectively. Unlike the *Calcium Binding* Region (in cPKC), this region exhibits significant differences among novel isoforms (Fig. I.5). The binding mechanisms of nPKCs differ from those of cPKCs, not only due to the absence of appropriate residues to form the *Ca^{2+} Binding Region*, but also due to the structure of the lipid docking motif formed by *loops 1 and 3 (Corbalan-Garcia et al., 2003c)* (Fig. I.7)

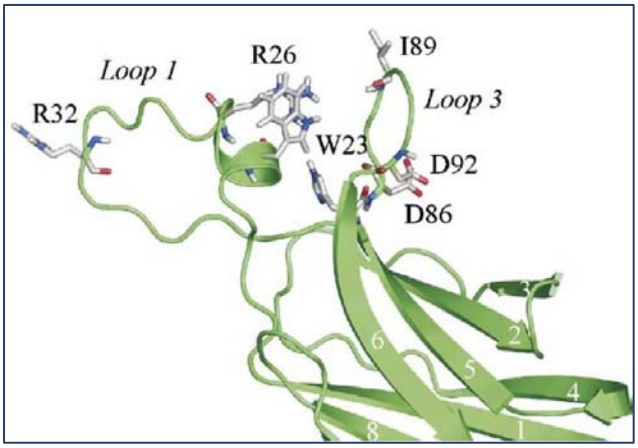

Figure 1.7. Residues involved in lipid binding of PKCε–C2 domain. The cartoon shows the 3D structure of PKCε. The critical residues involved in phospholipid binding have been represented as stick models with carbon in grey, nitrogen in blue and oxygen in red (Trp23, Arg26 and Arg32 in loop1 and Ile89 in loop 3). Additional residues not involved in lipid binding are also shown: side chains of Asp86 and Asp92 are represented as stick models; observe how these chains point to the surface of the molecule, in the opposite direction to the side chains of the residues involved in phospholipid binding (Taken from *Corbalán García and Gómez Fernández, 2006*).

The data structure of the C2 domain of PKCε further shows that this domain maintains only two of the five Asp that coordinate calcium ions in the C2 domain of PKCα**.** Specifically, the conserved Asp residues in the PKCε, corresponding to positions 39 and 93, which are homologous to those occupied by the Asp193 and 254 in the PKCα**.** However, site-directed mutagenesis studies and lipid binding assays have shown that in PKCε, the role of these residues (Asp39 and 93) is merely structural *(Corbalan-Garcia et al., 2003c; Ochoa et al., 2001)***.**

In the light of the results, a model has been proposed for the interaction of the C2 domain of nPKC with membranes, where the interaction of the C2

domain of PKCε with the membrane is carried out through a combination of electrostatic and hydrophobic forces, integrated in a two-step mechanism**.** Firstly the loop 1, establish electrostatic interaction with the anionic membrane phospholipids, and then produce a second hydrophobic interaction mediated type loop 3, and which would C2 domain anchoring to the membrane *(Ochoa et al., 2001*).

Recent studies in cells RBL-2H3 transfected with the C2 domain of PKCε and after stimulation with IgE, have shown that this domain plays an essential role in anchoring the protein to the membrane *(Lopez-Andreo et al., 2003)*. The C2 domain residues involved in this interaction were located in loops 1 and 3 of the domain. The combination of different family specific inhibitors of Phospholipase C and Phospholipase D has shown that both the phosphatidic acid as the diacylglycerol are necessary for membrane localization and in the activation of the enzyme, showing that the domains C1 and C2 play cooperative roles in the function of the enzyme, being required for PKCε binding to the membrane *(Lopez-Andreo et al., 2003)*.

1.1.4. PB1 Domain.

These domains appear just in a few PKC isoenzymes (not in classical PKCs) and are involved in Protein: Protein interactions. These PB1 (Phox and Bem 1) domains mediate interactions with PB1-containing scaffolding proteins, through the formation of PB1 domain heterodimers *(Moscat et al., 2006a; Moscat et al., 2006b)*.

1.2. The Catalytic Domain.

This domain is highly conserved among the different isoforms (show 60% sequence homology) (Fig. I.8). It contains the ATP binding site and the place where target proteins are susceptible to be phosphorylated by PKC. It interacts with substrates and is responsible for phosphotransferase activity *(Corbalan-Garcia and Gomez-Fernandez, 2006; Hanks et al., 1988; Kemp and Pearson, 1990; Newton, 2003)*.

To date, the 3D structures of the catalytic domain of some PKC have been solved: classical PKCα (*Wagner et al., 2009*) and PKCβII *(Grodsky et al., 2006)*, novel PKCθ *(Xu et al., 2004)* and atypical PKCι *(Messerschmidt et al., 2005; Takimura et al., 2010)*. All of them show these PKC catalytic domains.
Protein kinase fold is separated into two subdomains or lobes. Between them, there is a cleft where the ATP- and substrate-binding sites are located (Fig. I.9).

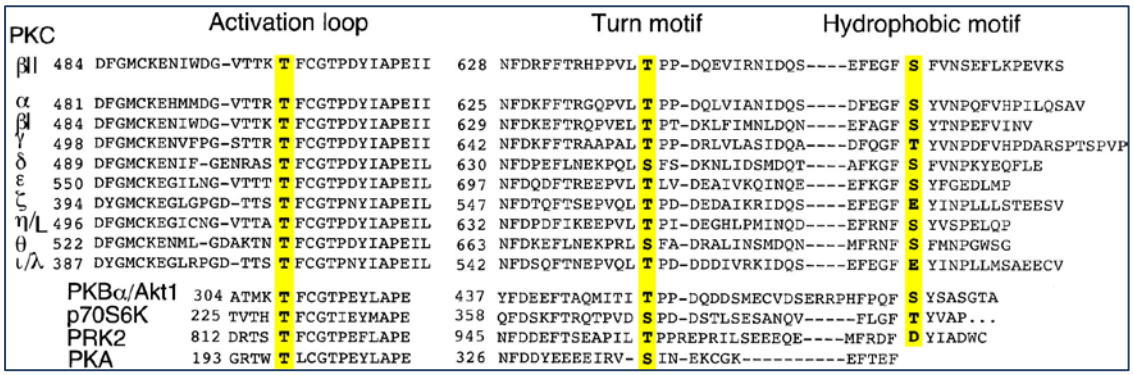

Figure I.8. Alignment of the activation segment, turn motif and hydrophobic motif phosphorylation sequences for the PKC isoenzymes, PKBα/Akt1, p70S6 kinase, PRK2 and PKA. Sequences shown are for human PKC isoenzymes α, ε, ζ, η/L, θ and ι/λ and rat PKC γ and δ, rat PKC βI and βII, murine PKB a/Akt1, rat p70S6 kinase and murine PKA. Amino acid residue numbers are indicated to the left of the sequences. (Taken from *Newton, 2003*).

The smaller N-terminal lobe, or N lobe, is composed of a five-stranded β sheet and one prominent α helix. It contains a preserved region in all kinases called the glycine-rich loop, whose the consensus sequence is $XGXGX_2GX_{16}KX$, where "X" is any amino acid and "G" is glycine. This Region is located between β1 and β2 strands and it is included in the ATP binding site. Its function consists of orienting the ATPγ-phosphate correctly for transferring to target protein.

The larger lobe is called the C lobe and is predominantly helical *(Johnson and Lewis, 2001; Taylor and Radzioandzelm, 1994)* and includes *Activation loop, Turn motif* and *Hydrophobic motif*. It is responsible for target peptide union and the beginning of phosphate transfer.

The *Activation loop* is a variably structured region located near the active site entrance and includes the region where the target proteins are phosphorylated. This zone, together with the C1 domain, the ATP binding region and the variable region 5 (V5), is involved in the specific recognition of substrates *(Pears et al., 1991)*.

The *Turn motif* possesses a Pro rich sequence which includes the phosphorylable residue (Thr for cPKCs and nPKCs; Ser for aPKCs). Structural models suggest that this sequence corresponds to a turn in the molecule, which is why it is called the *turn motif (Cenni et al., 2002)*.

The *Hydrophobic motif* is so-named because the Ser/Thr phosphorylable residues are surrounded by hydrophobic amino acids *(Cenni et al., 2002)*. This region is less preserved than the other two. Its consensus sequence is FXXFS/T/E, where "X" is any amino acid, and the end represents, depending on the isoenzyme considered, a residue of Ser, Thr or Glu. Classical and novel PKCs possess a preserved residue of Ser or Thr which is phosphorylable, whereas atypical isoforms possess a Glu that mimics, although not perfectly, the phosphorylation site *(Messerschmidt et al., 2005)* (Fig. I.9).

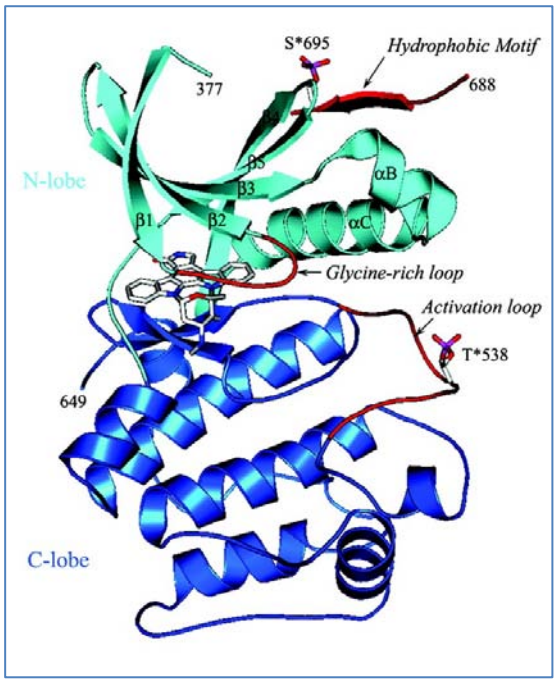

Figure I.9. Overall structure of the PKCθ-staurosporine complex and comparison with the structure of the PKA-staurosporine-PKI complex. *A*, ribbon representation of the PKCθ kinase domain structure. The N-lobe is *cyan* and the C-lobe is *blue*. The glycine-rich loop, activation loop, and HM segment are highlighted in *red*. Staurosporine and phosphorylated residues are shown in *stick* representation. (Taken from *Xu et al., 2004*).

1.3. Variable regions.

In PKC isoforms several variable regions exist along the amino acids chain; a particular isoenzyme may contain up to five of such regions. They are called V1, V2, V3, V4 and V5 from amino to carboxyl-terminal and the function of which is very important for regulating PKC functions and interactions with other proteins.

V1 is located at the N-terminal end and is present in all PKC isoforms, although it differs slightly in each one, which has been related to substrate specificity of the isoenzymes *(Pears et al., 1991; Schaap et al., 1990; Zidovetzki and Lester, 1992)*.

V2 is only present in classical and novel PKCs, where connects C1 and C2 domains, while V3, also called a hinge region, is very flexible *(Flint et al., 1990)* and appears in every PKC, connecting the regulatory and catalytic regions. This motif is essential for proper protein functioning, since it possesses a sequence sensitive to some proteases like trypsin and calpain, which may release the regulatory from the catalytic domain, generating a constitutive active enzyme *(Newton, 2001)*.

The V4 region is located at the centre of the catalytic domain, while at the C-terminal we find the V5 region, which consists of a 50 amino acid sequence. V5 has a regulatory function due to its proximity to the catalytic *centre (Bornancin and Parker, 1997; Edwards and Newton, 1997)* and its inclusion of two phosphorylation sites involved in PKC phosphoregulation *(Parekh et al., 2000)*. The importance of this V5 region is reflected in βI- and βIIPKC, members of the classical family of PKCs, are differentially spliced products of the same gene and therefore differ only in their C-terminal variable domain, the V5 domain, and this difference varying the localization in the active or inactive state *(Keranen and Newton, 1997)*.

2. CLASSIFICATION.

Classification of the ten PKC isoenzymes that exist is based on their primary structure of the regulatory region and their respective cofactor

requirements (Fig. I.10.) for the regulation of their enzymatic activity *(Corbalan-Garcia and Gomez-Fernandez, 2006; Leonard et al., 2011; Mellor and Parker, 1998; Newton, 2001; Nishizuka, 1995; Ohno and Nishizuka, 2002)*. These ten isoenzymes can be distributed in three groups:

- Classical or conventional PKCs (cPKC): these include PKCα, βI, βII and γ. Two isoforms of PKCβ result from differential RNA processing. Isoenzymes included in this family are regulated by DAG or phorbol ester, anionic phospholipid and Ca^{2+} *(Newton and Johnson, 1998)*.

Three domains can be distinguished in the regulatory region of cPKCs: a pseudosubstrate sequence which is able to block the catalytic centre and so inhibit enzymatic activity *(House and Kemp, 1987)*; the C1 domain appears in tandem (C1A and C1B), has a cysteine-rich region and it is responsible for binding diacylglycerol (DAG) and phorbol esters *(Bell and Burns, 1991)*; and a C2 domain, which is of type I and has binding sites for acidic phospholipids, Ca^{2+} and also for PtdIns(4,5)P_2 *(Conesa-Zamora et al., 2000; Corbalan-Garcia et al., 1999; Medkova and Cho, 1998; Sanchez-Bautista et al., 2006a; Verdaguer et al., 1999)*.

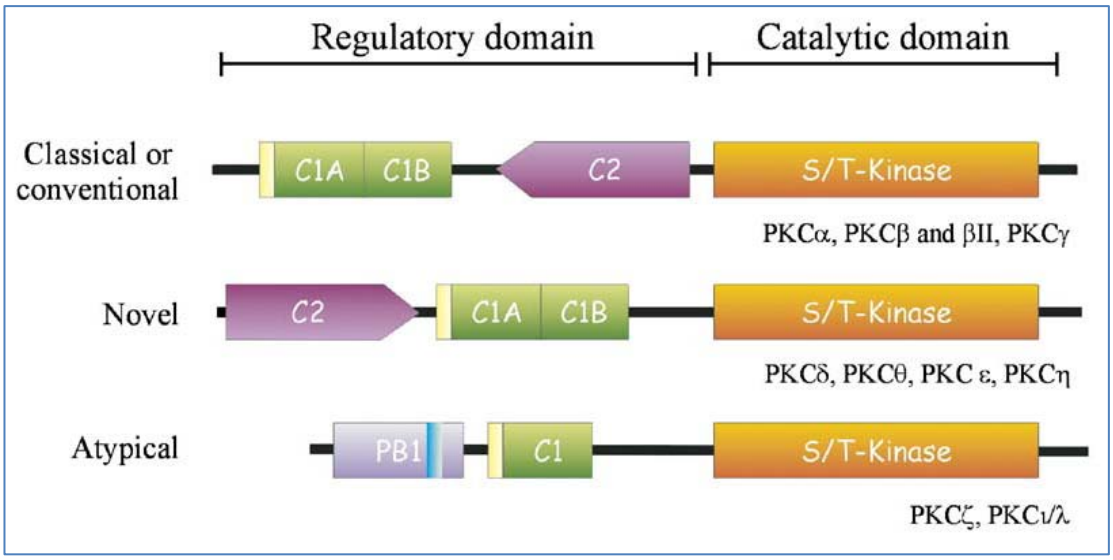

Figure I.10. Scheme of primary structures of members of Protein Kinase C family, showing domain composition. The regulatory domain is located in the amino terminal region containing different domains, depending on the isoenzyme. Pseudosubstrate (yellow); the C1 domain (green); the C2 domain (purple). The PB1 domain (violet), present in atypical PKC, is represented by a blue box [OPCA (Octicosapeptide repeat domain) is the motif where aPKC interact]. All kinases have a conserved kinase core (orange) that conains the ATP binding, the substrate binding and the phosphotransfer sites. (Taken from *Corbalán García and Gómez Fernández, 2006*).

- Novel PKCs (nPKC): this sub-family brings together isoenzymes ε, δ *(Ono et al., 1989)*, η *(Osada et al., 1990)* and θ *(Osada et al., 1992)*. These isoforms require anionic phospholipids and DAG or phorbol esters and they are Ca^{2+}-independent.

Structurally, nPKCs have two C1 domains in tandem, but the C2 domain is type II and is nearer the amino-terminal region than the C1 domain (Nalefski and Falke, 1996); it interacts with anionic phospholipids and does not present a Ca^{2+} *binding-site (Garcia-Garcia et al., 2001; Ochoa et al., 2001)*. Because these isozymes are regulated by only one membrane-targeting module, their affinity for diacylglycerol is two orders of magnitude higher than that for the cPKCs *(Giorgione et al., 2006)*.

- Atypical PKCs (aPKC): this sub-family is composed by only two members, PKCζ and PKC ι/λ [PKCι for human *(Selbie et al., 1993)* / PKC λ for mouse *(Akimoto et al., 1994)*. Enzymes in this group only require acidic phospholipids to be activated, although they can be regulated by ceramides and protein-protein interactions. They do not have a C2 domain and only contain one C1 domain called PB1 which is located in amino-terminal region.

3. CLASSICAL PKC ACTIVATION MECHANISM.

Newly-synthesized PKC is inactive in the cytoplasm next to membranes, where it suffers three phosphorylations *(Newton, 2003; Parker and Parkinson, 2001; Rodriguez-Alfaro et al., 2004; Tsutakawa et al., 1995)* (Fig. I.11.)

The maturation process of conventional and novel PKCs begins with the binding of the chaperone HSP90 and the co-chaperone Cdc37 to a molecular clamp in the kinase domain formed by a conserved PXXP motif. The integrity of this clamp is essential to allow the phosphorylation to occur: either inhibition of HSP90 or mutations of residues that maintain the clamp result in PKC that cannot be phophorylated and is degraded *(Gould et al., 2009)*.
The enzyme is processed by a series of ordered phosphorylation events during its maturation *(Tsutakawa et al., 1995)*.

Firstly, the *activation loop* Ser/Thr is phosphorylated by PDK1 *(Chou et al., 1998; Le Good et al., 1998)*. Phosphorylation by PDK-1 is likely the first phosphorylation event in the processing of PKC by phosphorylation. As mentioned above, replacement of the Thr at the activation loop of cPKCs isoenzymes with neutral, nonphosphorylable residues (Ala or Val) prevents the subsequent phophorylations on the carboxy-terminal tails (CT) sites, indicating that phosphorylation of the activation loop is necessary to allow CT phosphorylation *(Bornancin and Parker, 1996; Orr and Newton, 1994)*.

After *activation loop* phosphorylation, two more phosphorylations take place, specifically in the *turn motif (Hauge et al., 2007)* and in the *hydrophobic motif (Behn-Krappa and Newton, 1999)*, the structure of mature PKC stabilizing.

It has recently been shown that phosphorylation of the *turn motif* depends on the mTORC2 complex *(Facchinetti et al., 2008; Ikenoue et al., 2008)*. However, an auto-phosphorylation through an intramolecular reaction at the *hydrophobic motif* occurs *(Behn-Krappa and Newton, 1999)*, which is also controlled by the interaction of HSP90 with the PXXP clamp described above. This phosphorylation step produces a catalytically competent, thermo-stable and phosphatase-resistant enzyme *(Bornancin and Parker, 1996; Edwards et al., 1999)*. In that moment the pseudosubstrate domain occupies the active centre of the catalytic domain, PKC remaining catalytically competent but inactive.

A variety of stimuli may reach the plasma membrane, where they activate PKC along several pathways. Phospholipase C (PLC) is involved in the main pathway for classical and novel PKCs. This enzyme hydrolyzes PtdIns(4,5)P_2 to generate DAG and Ins(1,4,5)P_3, *(Parker, 1999; Swannie and Kaye, 2002)* increasing the level of cytoplasmic [Ca^{2+}]. Ca^{2+} ions allow the binding between C2 domain and PtdSer through the *calcium binding region,* in cPKCs, acting Ca^{2+} ions acting like a bridge.

Furthermore, C2 domain interacts with PtdIns(4,5)P_2 through its *lysine-rich cluster* (Fig. I.11.). DAG binding to the C1 domain confers a high-affinity interaction between PKC and the membrane, leading to a massive conformational change that releases the pseudosubstrate domain from the substrate binding site, allowing for substrate binding, phosphorylation and the activation of downstream signalling effectors *(Griner and Kazanietz, 2007)*.

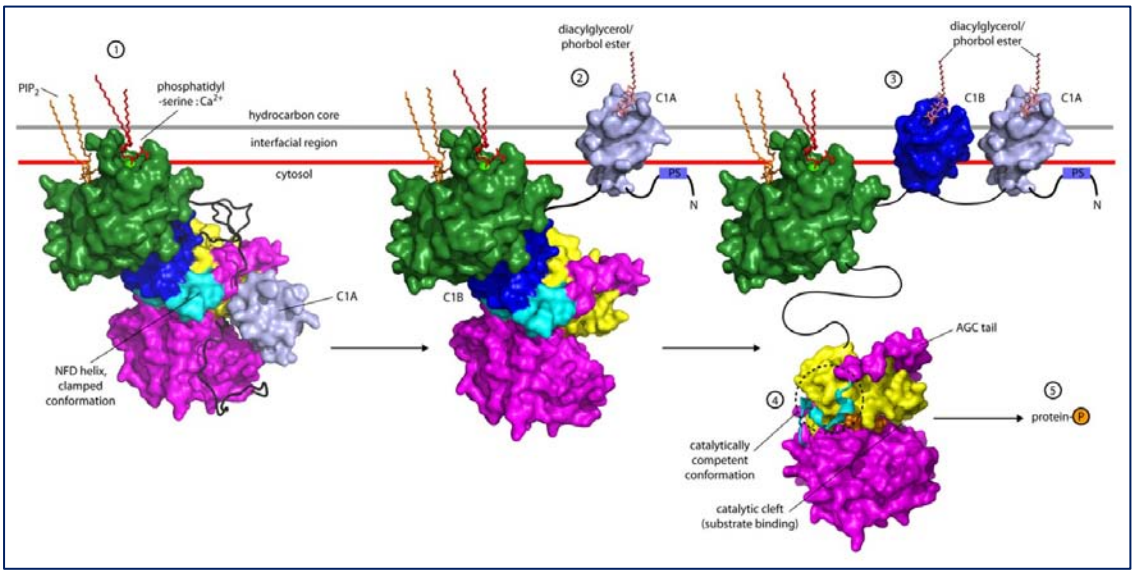

Figure I.11. **Model for Multistep Activation of PKCβII.** PKCβII translocates to the membrane upon Ca^{2+} release in the cell, where the calcium-binding regions of its C2 domain mediate bridging to the anionic phopholipid phosphatidylserine, with an adjoining site on the C2 domain-binding phophatidylinositol (4,5)-bisphosphate (1). Subsequent binding of DAG to the C1A domain results in disengagement of the C1A domain (2), wich in turn forces the removal of the pseudosubstrate (PS) from the catalytic cleft. Binding of a second molecule of DAG by the C1B domain results in unclamping of the kinase: C1B assembly (3) and rearrangement of the NFD helix into the catalytically competent state (4), triggering the phophorylation of cellular targets (5) and subsequent downstream signaling. (Taken from *Leonard et al., 2011).*

Novel PKCs might translocate to membranes slower than classical PKCs because they are not pre-targeted by calcium, but this is compensated for by an increased affinity for DAG *(Ananthanarayanan et al., 2003b)*.

If any of these requirements is not fulfilled, the cell signalling pathways through this enzyme will be interrupted *(Newton, 2001)*, presenting diseases, like cancer. Now, PKC obtains an active conformation ready to phosphorylate its substrates, triggering downstream signaling *(Leonard et al., 2011; Newton, 2010; Rodriguez-Alfaro et al., 2004)*.

After this interaction, pseudosubstrate domain is released from active centre, favoring interaction of C1 domain with DAG generated in plasma membrane. After the peseudosubstrate released from the substrate-binding site, allowing for the enzyme must be in the correct intracellular compartment in order to carry on their catalytic role.

4. LIPID ACTIVATION OF PROTEIN KINASE C.

PKC isoenzymes are subject to precise structural and spatial regulation: their phosphorylation state, conformation and subcellular localization must be defined in a very precise way to preserve their correct physiological function. These kinases must be phosphorylated, the pseudosubstrate released from the active site and the enzyme must be in the correct intracellular compartment in order to carry on their catalytic role. If any of these requirements is not fulfilled, the cell signalling pathways through this enzyme will be interrupted *(Newton, 2001)*, giving place to pathologies, like cancer.

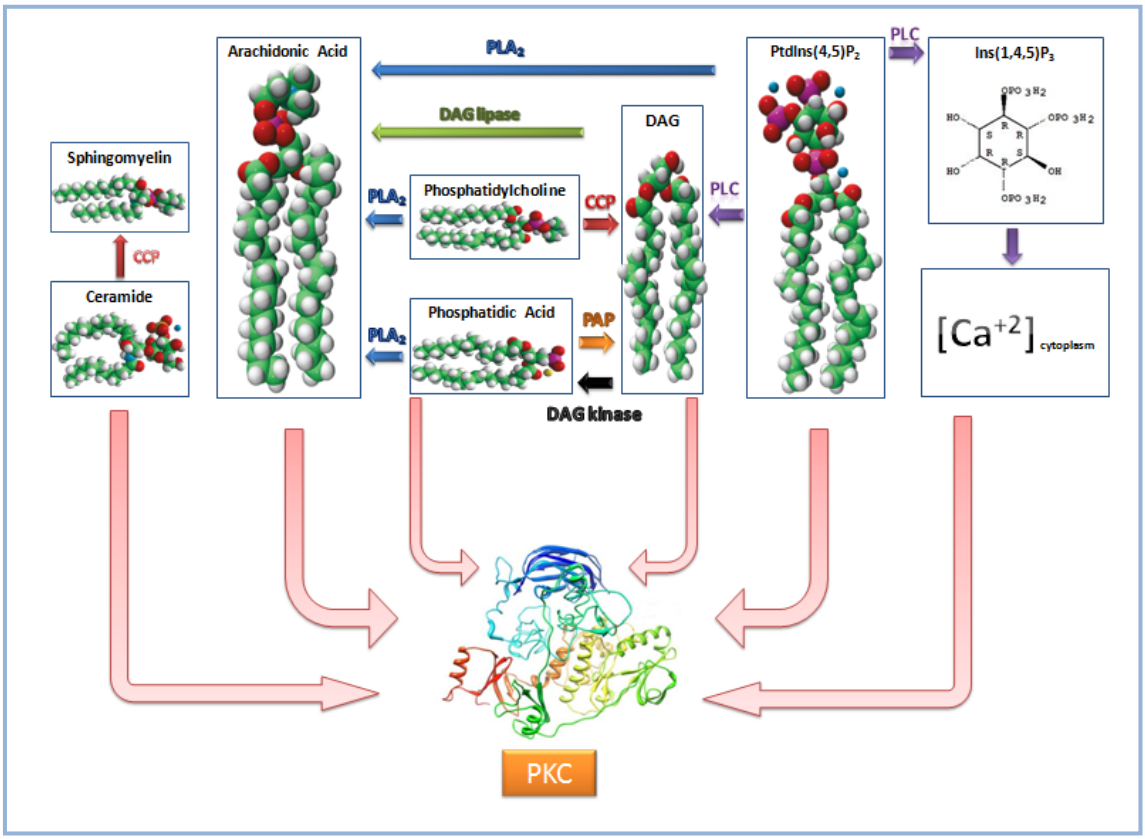

Figure I.12. Schematic draw of PKC activation pathways. Main pathway of PKC activation is mediated by PLC, marked with purple arrows. In the diagram is represented other enzymatic reactions mediated by Phospholipase A2 (PLA2), Ceramide cholinephosphotransferase (CCP), DAG kinase, Phosphatidylcholine-dependent phospholipase D (PC-PLD), Phosphatidic acid phosphatase (PAP) and DAG lipase, which also synthesizes PKC activators. All PKC activators represented in this diagram are joined with indicative box through red arrows.

4.1. Main phospholipases involved in PKC activation.

A variety of stimuli may reach the plasma membrane, where they activate PKC along several pathways.

The main pathway for classical and novel PKCs involves Phospholipase C (PLC). This enzyme hydrolyzes PtdIns(4,5)P_2 to generate DAG and Ins(1,4,5)P_3, *(Parker, 1999; Swannie and Kaye, 2002)* which activate PKC binding through the C1 domain and increase the level of cytoplasmic [Ca^{2+}], respectively.

There are other secondary activation pathways in which DAG is produced constantly and where phosphatidylcholine (PtdCho) plays an important role, since it may act as a precursor of DAG in three different pathways (Figure I.12): the first following phospholipase C (PLC) activation, the second one due to ceramide cholinephosphotransferase (CCP) activation, and the third one catalyzed by PtdChodependent phospholipase D (PC-PLD), although this pathway is unusual in mammals *(Wakelam, 1998)*.

Besides classical cofactors like DAG and PtdCho, other compounds generated by these enzymatic activities may activate PKC, for example arachidonic acid, ceramides, phosphatidic acid and PtdIns(4,5)P_2 *(Kashiwagi et al., 2002; Lopez-Andreo et al., 2003; Lopez-Nicolas et al., 2006; Marin-Vicente et al., 2008b; Wakelam, 1998)* among others (Figure I.12).

Below, we will summarize briefly the characteristics and mechanism of activation of each phospholipase which take part in the lipid-signaling events as result of the antigen stimulation in RBL-2H3, and whose understanding will be necessary for the Chapter VII.

4.1.1. Characteristics and mechanism implicated in phosphatidylinositol-specific phospholipase C activity.

It is known that the metabolism of phophoinositides plays an important role in the control of cell growth, differentiation, and survival, as well as cell architecture *(Noh et al., 1995)*. The various isoforms of phospholipase C (PLC) catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers, Ins(1,4,5)P_3 and diacylglycerol (DAG) (Figure I.12). It has been shown that Ins(1,4,5)P_3 mediates the release of Ca^{2+} from

intracellular stores, while DAG functions as a intracellular ligand of protein kinase C (PKC) *(Schlessinger, 1997)*.

All PLC enzymes identified up to now are single polypeptides that can be divided into three types (β, γ, and δ) exemplified by the 150 kDa PLC-β, the 145 kDa PLC-γ , and the 85 kDa PLC-δ, on the basis of their size and amino acid sequence *(Rhee et al., 1989)*. Ten mammalian enzymes have been identified, including four β, two γ, and four δ members. Only two regions of high homology are shared by the three types: designated X and Y, they are ~60% and ~40% identical, respectively, among the isoenzymes *(Lee and Rhee, 1995)*. It is generally accepted that there are at least two distinct mechanisms to link receptor occupancy to the activation of PLC isozymes: tyrosine phosphorylation for PLC-γ isoenzymes and the action of heterotrimeric G proteins for PLC-β *(Murthy et al., 2004; Rhee et al., 1989)*. Although four distinct PLC-δ isoforms are known, the mechanism by which these isozymes are coupled to membrane receptors remains unclear *(Murthy et al., 2004; Rhee and Bae, 1997)*. The β-isoenzymes are recognized to mediate the regulation of PI breakdown by G-protein linked receptors that are activated by certain hormones, neurotransmitters and related agonists. In contrast, the PLCγ1 and PLCγ2 isoforms mediate PIP_2 hydrolysis induced by the tyrosine kinase activity intrinsic to the receptors for many growth factors or by soluble tyrosine kinases activated by certain receptors *(Exton, 1994; Falasca et al., 1998)*. There are evidences that PIP_2 is a poor substrate for all types of PLC isozymes, but that tyrosine phosphorylation of PLC-γ overcomes that fact *(Goldschmidtclermont et al., 1991)*.

The specific inhibitor of PLC, U73122 can be used to block this enzyme (Figure I.12). U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) has been extensively used as a pharmacological inhibitor of PLC to elucidate the importance of this enzyme family in signal transduction pathways. U73122 has an electrophilic maleimide group, which readily reacts with nucleophiles such as thiols and amines and is firmly established as the archetypal inhibitor of PLC *(Wilsher et al., 2007)*.

4.1.2. Characteristics and mechanism implicated in phospholipase D activity.

Another enzyme that is also activated by the cross-linking of the high-affinity IgE receptor (FcεRI) with antigen in RBL-2H3 cells, is the phospholipase D (PLD). PLD is a phosphodiesterase that cleaves the distal phosphodiester bond of phospholipids, phosphatidylcholine in our case, to produce a water-soluble product (choline, in this case) and a simple phospholipid called phosphatidic acid (DOcPA) (Figure I.12). This activity has been proposed to play a role in mediating a wide range of cellular processes, including hormone action, membrane trafficking, cell proliferation, cytoskeletal organization, defense responses, differentiation, and reproduction *(Cockcroft, 1997; Colley et al., 1997; Exton, 1997; Rose et al., 1995)*.

The reaction catalyzed by PLD is a phosphatidyl transfer reaction in which the phospholipid substrate acts as a phosphatidyl group donor. Normally, water acts as the phosphatidyl acceptor resulting in a simple hydrolytic reaction. However, a unique and very useful property of PLD is its ability to utilize also primary short-chain alcohols as phosphatidyl group acceptors. The resultant lipid product is, in this case, the corresponding phosphatidic acid alkyl ester or phosphatidyl-alcohol *(Heller, 1978; Liscovitch, 1996)*.

Biochemical evidences confirm that there are multiple PLD isoenzymes in mammalian cells: A membrane-bound PLD activity, first identified in rat brain *(Kanfer, 1980*). This enzyme seems to be an integral membrane protein that is highly specific for phosphatidylcholine as substrate. A cytosolic PLD was characterized *(Balsinde et al., 1989)* which is less strict in its substrate specificity, because it can hydrolyze phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol in that order of preference *(Wang et al., 1991)*. In addition, a membrane-bound PLD that behaves as a peripheral membrane protein was described in HL-60 cell membranes *(Brown et al., 1993; Liscovitch, 1996)*.

PLD is rapidly and dramatically activated in response to a wide variety of stimuli in many different cell types *(Billah, 1993; Liscovitch et al., 1994)*. The nature of the activating signals is diverse. Stimuli that activate PLD include hormones, growth factors, neurotransmitters, cytokines, extracellular matrix constituents, antigens, and even physical stimuli such as radiation and mechanical stretch *(Liscovitch, 1996)*. In virtually all cell types studied, PLD

activity can be stimulated by phorbol esters. These agents are known to stimulate protein kinase C (PKC) isoenzymes, implicating the activation of PKC as an upstream event in PLD activation. However, there are a PKC-independent pathway of PLD activation in some cell types, and the co-existence of PKC-dependent and PKC-independent activation of PLD by different agonists in the same cell type activate the PLD enzyme by a PKC-independent mechanism such as a G protein that act on PLD directly *(Cockcroft, 1992)* or an elevated cytosolic Ca^{2+} concentration *(delPeso et al., 1997; Huang et al., 1991)*. A PKC-independent pathway of PLD activation would be present in RBL-2H3 cells upon the activation with the antigen.

PLD activity can be manipulated by applying the specific inhibitor 1-butanol *(Munnik et al., 1995)*. Generally, PLD-catalysed hydrolysis removes the head group of structural phospholipids, forming a short-lived phosphatidyl-PLD intermediate *(Munnik et al., 1998)*. Under normal conditions the PLD then transfers the phosphatidyl moiety to H_2O, forming DOcPA. In the presence of 1-butanol, however, the alcohol is used as an alternative trans-phosphatidylation substrate, resulting in the formation of phosphatidylbutanol *(Munnik et al., 1995)*. Because phosphatidylbutanol has no known biological activity, 1-butanol is a useful inhibitor of formation of DOcPA by PLD *(Gardiner et al., 2003)*.

4.1.3. Characteristics and mechanism implicated in diacylglycerol kinase activity.

The mammalian diacylglycerol kinases (DGK) are a group of enzymes, which have important roles in regulating many biological processes. Both the product and the substrate of these enzymes, diacylglycerol (DAG) and phosphatidic acid (DOcPA) (Figure I.12), are important lipid signalling molecules. Each DGK isoform appears to have a distinct biological function as a consequence of its location in the cell and/or the proteins with which it associates *(Topham and Epand, 2009)*. Thus, the intracellular DAG levels are tightly regulated. Under most circumstances, conversion of DAG to DOcPA by the DGKs is the major route to terminate DAG signalling. DOcPA, itself, has a set of signalling properties that are distinct from DAG *(Merida et al., 2008)*, indicating that DGKs might modulate lipid signalling either by terminating DAG or by producing DOcPA *(Topham and Epand, 2009)*.

Ten mammalian DGKs have been identified and all of them have two common structural elements: a catalytic domain and at least two C1 domains. All DGKs, except for DGKθ which has three, have two cysteine-rich regions (C1a and C1b domain) in the regulatory domain at N-terminus. Although DGK's C1 domains are homologous to PKC, only those of DGKβ and γ bind to DAG *(Shindo et al., 2003)*. All DGKs have catalytic domain at C-terminus, but catalytic domains of Type II DGKs (δ, η and κ) are separated. In addition to these domains, they have different structures depending on their groups. Type I DGKs, DGKα, β and γ, have EF-hand motif and recoverin homology (RVH) domain. Type II DGKs, DGKδ, η and κ have pleckstrin homology (PH) domain instead of these two domains at N-terminus. DGKδ and η have sterile α motif (SAM) domain at C-terminus. Type III DGK, DGKε, has only C1 domain. Type IV DGKs, DGKζ and ι, have myristoylated alanine rich protein kinase C substrate phosphorylation site like region (MARCKS homology domain) at N-terminus and four ankyrin repeats and PDZ binding domain at C-terminus. Finally, Type V DGK, DGKθ has proline and glycine rich domain and PH domain with overlapping Ras associating domain *(Shirai et al., 2012)*.

Based on their structural diversity, it is quite possible that each DGK will regulate a distinct set of DAG signalling events, a concept that is supported by mouse knockout studies showing that mice with targeted deletion of individual DGK isoforms have distinct phenotypes *(Regier et al., 2005; Zhong et al., 2003)*. As an example of complexity in the regulation of the activation of DGKs isoenzymes, we will make a brief description of DGKα regulation, one of the best characterized mammalian isoenzymes. DKGα can be activated by Ca^{2+} *(Sakane et al., 1991)* and binding to membranes that contains phosphatidylserine *(Abe et al., 2003)* or in a calcium-independent manner through the lipid products of phosphoinositide 3-kinase, such as phosphatidylinositol(3,4)bisphosphate and phosphatidylinositol(3,4,5) trisphosphate *(Cipres et al., 2003)* or is able to bind to membranes that contain lipids with poorly hydrated head groups such as phosphatidylethanolamine or cholesterol in the absence of Ca^{2+} *(Fanani et al., 2004)*. Another way of activation of DGKs is by phosphorylation, and in particular, the mechanism to activate DGKα is by the phosphorylation of Tyr^{335}, and is mediated by Src tyrosine kinases. This phosphorylation promotes membrane localization of the enzyme and increases its activity *(Chianale et al., 2007; Merino et al., 2008)*.

It is not known what isoenzymes of DGK are present in RBL-2H3 cells or involved in the lipid signalling pathways upon activation of antigen. For that reason, to block the DGK is used the inhibitor R59022 type II, a more potent analog of diacylglycerol kinase inhibitor type I (6-[2-[4-[(4-fluorophenyl)phenylmethylene)-l-piperidinyl]ethyl]-7-methyl-5H-thiazolo[3,2-alpyrimidin-5-one) which was found to inhibit diacylglycerol kinase in human red blood cell membranes *(Decourcelles et al., 1985)*. Recent studies have shown that R59022 strongly inhibited DGKs type I (DGKα, β and γ) and moderately attenuated type III (DGKε) and type V (DGKθ) *(Sato et al., 2013)*.

4.1.4. Characteristics and mechanism implicated in phosphatidate phosphohydrolase.

Phosphatidate phosphohydrolase (PAP) is an important enzyme in signal transduction. It catalyzes the conversion of phosphatidic acid (DOcPA) into diacylglycerol (DAG) which is an activator of protein kinase C (Figure I.12). The PAP reaction constitutes part of the phospholipase D pathway where DOcPA is generated by the hydrolysis of phosphatidylcholine. *(Brindley and Waggoner, 1996)*.

Initially the importance of this pathway was thought to be the generation of a second phase of diacylglycerol from phosphatidylcholine in order to sustain the activation of protein kinase C. The first phase of diacylglycerol is produced from phosphoinositides. Phosphoinositide-specific PLCγ degrade the relatively small amount of phosphatidylinositol 4,5-bisphosphate in the plasma membrane and thus the amount of diacylglycerol that can be produced to maintain this signal is relatively limited. The activation of PLD produces diacylglycerol indirectly from the more abundant phosphatidylcholine. Subsequent works have demonstrated that phosphatidic acid itself is a potent second messenger, which has added a further dimension to the PLD pathway. Consequently, the regulation of PAP activity could determine the balance of the signal that the cell receives in terms of DOcPA and DAG *(Brindley and Waggoner, 1996; Day and Yeaman, 1992)*.

For the inhibition of this enzyme is used propranolol *(Koul and Hauser, 1987)*. Besides being used clinically as a β-adrenergic receptor antagonist, has been found to inhibit the conversion of DOcPA to DAG by PAP. Thus, inhibition of a cell function by propranolol has been interpreted to indicate that DAG

formed from DOcPA is an important second messenger for that cell function. In contrast, enhancement of a cell function by propranolol has been used to support a direct second messenger role for DOcPA *(Sozzani et al., 1992)*.

4.2. Lipid regulators of PKC activity.

4.2.1. DAG.

Diacylglycerol is an important lipid second messenger in metazoan cellular signaling *(Carmelo Gomez-Fernandez and Corbalan-Garcia, 2010; Dekker and Parker, 1994; Kajikawa et al., 1989)* and is generated in the cell following receptor-dependent activation of phospholipase C and D isoenzymes *(Dekker and Parker, 1994; Hodgkin et al., 1998; Wakelam, 1998)*.

It has been described that the diacylglycerol generated in response to cell stimulation comes from several sources including PtdIns(4,5)P_2 hydrolyzed by phospholipase C isoenzymes, phosphatidylcholine hydrolyzed by phospholipase D isoenzymes and sphingomyelin synthases; and phosphatidic acid hydrolyzed by phosphatidic acid phosphohydrolase *(Corbalan-Garcia and Gomez-Fernandez, 2006; Hodgkin et al., 1998; Huitema et al., 2004; Wakelam, 1998)*.

Upon its synthesis, DAG remains within the plasma membrane, due to its hydrophobic properties, where it is a physiological activator of conventional and novel PKC isoenzymes (this event is assisted by Ca^{2+} for the conventional PKC isozymes).

Diacylglycerols are recognized by these PKCs through the C1 domain and established models propose that they will stabilize the translocation of the protein to the membrane *(Battaini, 2007; Burns and Bell, 1991b; Gallegos and Newton, 2008; Newton, 2009; Quest et al., 1994; Steinberg, 2008)*. This is done by modulating the physical properties of the membrane through alteration of the membrane surface curvature, dehydration of the surface and the separation of phospholipid surface groups *(Gomez-Fernandez et al., 2004)*.

Diacylglycerol levels at the Golgi are significantly elevated compared with the plasma membrane under basal conditions, and, in addition, agonist-evoked

increases of this lipid second messenger are much more sustained at the Golgi compared with the plasma membrane. The unique profile of diacylglycerol at Golgi produces, in turn, a PKC signature unique to Golgi; not only is there preferential recruitment of novel PKCs, which have an intrinsically higher affinity for diacylglycerol because of a C1 domain tuned for tighter binding to diacylglycerol *(Dries et al., 2007; Giorgione et al., 2006)*, but the agonist-evoked activity at Golgi is much more prolonged than at the plasma membrane *(Gallegos et al., 2006; Newton, 2009)*.

In addition to activating PKC, diacylglycerol has a number of other functions in the cell, as a source for prostaglandins, precursor of the endocannabinoid 2-arachidonoylglycerol *(Ueda et al., 2010)*, or activator of a subfamily of TRPC (Transient Receptor Potential Canonical) cation channels, TRPC3/6/7 *(Soboloff et al., 2007)*, among others.

DAGs mediates a wide array of crucial physiological and pathophysiological events, including cell proliferation, differentiation, cell cycle progression and malignant transformation by activating PKC family of enzymes and other family of proteins including Ras guanyl nucleotide-releasing protein (RasGRPs), chimerins, protein kinase D (PKD), Unc-13 scaffolding proteins, myotonic dystrophy kinase-related Cdc42-binding kinases (MRCK), and the DAG kinases *β* and *γ (Gomez-Fernandez and Corbalan-Garcia, 2007)*.

Another product of PtdIns(4,5)P_2 hydrolysis, inositol-1,4,5-triphosphate ((1,4,5)IP_3) releases Ca^{2+} from endoplasmic reticulum (ER) which also activate various proteins, including Ca^{2+}-dependent protein kinase C (PKC) isoforms *(Griner and Kazanietz, 2007; Nishizuka, 1992; Rhee, 2001)*.

4.2.2. PtdIns(4,5)P_2.

Phosphatidyl-Inositol-4,5-Bisphosphate (PtdIns(4,5)P_2) is the most abundant phosphoinositide in the plasma membrane of a typical mammalian cell (it represents 1% of the total lipid) which regulates many important cellular processes (Figure I.13) *(Stephens et al., 1993)*.

Several mechanisms have been described for the interaction of PtdIns(4,5)P_2 with its protein partners, where it exhibits different degrees of specificity and affinity. In some cases, these proteins interact with the

phosphoinositide through an electrostatic mechanism involving membrane bound clusters of basic residues that laterally sequester the multivalent (z= -4) acidic lipid PtdIns(4,5)P_2, however, these proteins do not share a common structural fold *(McLaughlin and Murray, 2005; McLaughlin et al., 2002)*. Examples of such proteins are those of the WASP family, actin-binding proteins, GAP-43, CAP23 and MARCKS *(Downes et al., 2005; Lemmon, 2003; Toker, 1998)*. In other cases, these proteins have been shown to bind to this lipid with varying degrees of specificity. These include proteins containing C2, ENTH, ANTH and PH domains *(Cho and Stahelin, 2006; Lemmon, 2003; Li and Gobe, 2006; Liu et al., 2006; Parker, 2004; Sanchez-Bautista et al., 2006a)* among which, the PH domain of PLCδ-1 is one of the most widely used as a probe to study PtdIns(4,5)P_2 dynamics in cells *(Nahorski et al., 2003; Stauffer et al., 1998)*.

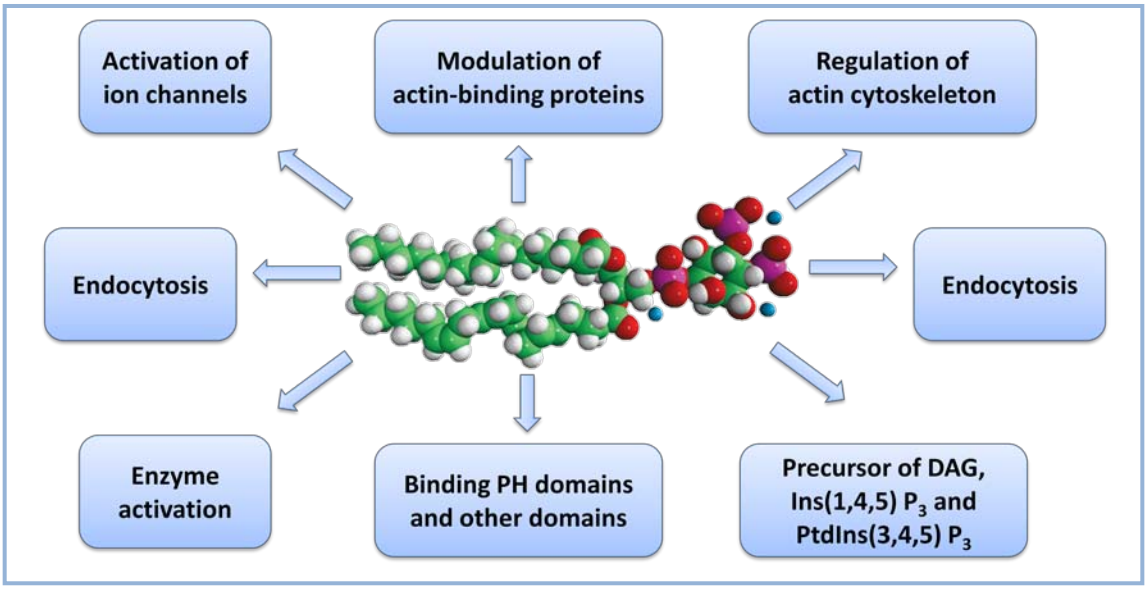

Figure I.13. Overall structure of the PtdIns(4,5)P2 molecule, consisting of a glycerol backbone, with two fatty acid and three phosphate groups bonded. Carbon atoms are shown as green, hydrogen atoms as white, oxygen atoms as red and phosphorus atoms as purple balls **and representation of the different roles PtdIns(4,5)P_2.** This phosphoinositide possesses a large number of cellular functions at the level of the membrane such as activation of ion channels, endocytosis, exocytosis, binding proteins that regulate the actin cytoskeleton and activation of enzymes, among others. These functions are developed by itself, but its metabolism also generates DAG, Ins(1,4,5)P_3 and PtdIns(3,4,5)P_3, which carry out other actions as second messengers.

For a long time, it was supposed that the only role of PtdIns(4,5)P_2 was to act as source of three second messengers, Ins(1,4,5)P_3, DAG and PtdIns(3,4,5)P_3, leading to the activation of a wide range of membrane-related events *(Czech, 2000; Lemmon, 2003; McLaughlin and Murray, 2005)*.

However, a myriad of recent works has revealed additional signaling roles not only for PtdIns(4,5)P_2 itself but also for the whole family of phosphoinositides, which function as site-specific signals on membranes that recruit and/or activate proteins for the assembly of localized functional complexes *(Di Paolo and De Camilli, 2006; Downes et al., 2005; Lemmon, 2003; Martin, 1998; McLaughlin and Murray, 2005; Toker, 1998; Wenk and De Camilli, 2004)*. Therefore, PtdIns(4,5)P_2 appears to be a crucial regulator of exocytosis, endocytosis, focal adhesion complexes, actin polymerization and ion channels in the plasma membrane, among others *(Downes et al., 2005; McLaughlin and Murray, 2005; Wenk and De Camilli, 2004)*.

4.2.3. Phosphatidic acid.

Phosphatidic acid (DOcPA) is the simplest diacyl-glycerophospholipid, and the only one with a phosphomonoester as the head group. The molecule is acidic and carries two negative charges (it is an anionic lipid), being important both as an intermediate in the biosynthesis of triacylglycerols and phospholipids, as a signalling molecule.

DOcPA has been implicated in cell proliferation, differentiation, transformation, tumor progression and survival signalling. It appears to regulate some membrane trafficking events (vesicle trafficking, secretion and endocytosis), and it is involved in activation of the enzyme NADPH oxidase, which operates as part of the defense mechanism against infection and tissue damage during inflammation. It may have a role in promoting phospholipase A2 activity, and it appears to function in vesicle formation and transport within the cell. By binding to targeted proteins, including protein kinases, protein phosphatases and G-proteins, it may increase or inhibit their activities *(English, 1996; Wang et al., 2006)*.

One of the main ways in which DOcPA is generated in the cell is by the activation of PLD, which hydrolyzes PtdCho to produce DOcPA and choline. The DOcPA itself is an activator of PKC that also becomes DAG by action of the phosphatidic acid phosphatase (PPA). This reaction can be reversed through the action of DGK, synthesizing DOcPA from DAG *(Alcon et al., 2002); Makhlouf and Murthy, 1997; Murthy and Makhlouf, 1995)*.

Several studies with PKCα *(Ochoa et al., 2002)* have shown that DOcPA can participate in the membrane anchorage of the enzyme through the lysine-rich cluster of its C2 domain.

The C2 domain in PKCε also showed interacting with the DOcPA in the plasma membrane, being essential for anchoring the enzyme in this cellular compartment *(Lopez-Andreo et al., 2003)*. However, it would be the C1B domain in PKCε which would play a more important role in the anchoring of the protein to the plasma membrane (Chapter V) .

4.2.4. Arachidonic acid.

Arachidonic acid (AA) and its precursor, linolenic acid, are common dietary ω-6cis-polyunsaturated fatty acids that are sometimes found in the extracellular microenvironment *(Larsson et al., 2004)*. Arachidonic acid is one of the unsaturated fatty acids released in the plasma membrane when cPLA2 is activated *(Luo et al., 2003)* and by the action of diacylglycerol lipase from diacylglycerol *(Chakraborti et al., 2004)*.

AA is generated in a wide variety of cell types and participates, either directly or indirectly, in many cellular processes, including the inflammatory response, the contraction of cardiac myocytes, the regulation of ionic channels, cytokine synthesis, cell growth, eicosanoid metabolism, apoptosis, secretion and the regulation of gene expression *(Bordin et al., 2003; Liu et al., 2001; Patino et al., 2003; Priante et al., 2002)*.

This lipid has been demonstrated to be a direct activator of PKCα *(Anfuso et al., 2007; Lopez-Nicolas et al., 2006)* and needs the Ca^{2+} cooperation to exert its function through the Ca^{2+}-binding region in the C2 domain . It was also demonstrated that the C1 domain cooperates in the arachidonic acid-dependent localization and activation of PKCα, and however, the β-groove played no relevant role in either the membrane localization or activation of the enzyme, suggesting a very precise mechanism for PKCα activation by arachidonic acid, involving a sequential model of activation in which an increase in intracytosolic Ca^{2+} leads to the interaction of arachidonic acid with the Ca^{2+}-binding region; only after this step, does the C1A subdomain interact with

arachidonic acid, leading to full activation of the enzyme *(Lopez-Nicolas et al., 2006)*.

PKC regulates the AA synthesis and this fatty acid only affects some PKC isoforms depending on cell type *(Bordin et al., 2003; Mackay and Mochly-Rosen, 2001a)*. Thus, it has been reported that PKC participates, either directly or through the phosphorylation of MAP Kinases, in the activation of phospholipase A2, which is one of the pathways leading to the production of arachidonic acid (Akiba et al., 2002; Xu et al., 2002).

4.2.5. Retinoic acid.

Another molecule that binds to the C2 domain of PKCα is retinoic acid. High resolution structural studies have shown that this molecule binds to the lateral site localized on the β-groove and also to the Ca^{2+}-binding region *(Ochoa et al., 2003)*. Since retinoic acid binds to the same sites than phospholipids it might be thought that some competition may take place. It was observed, in this way, that whereas retinoic acid may activate PKCα through the Ca^{2+}/phosphatidylserine-binding site of the C2 domain, it may also inhibit the activity of this enzyme when displacing the phospholipid from the β-groove *(Lopez-Andreo et al., 2005)*, underlining again the importance of this site and demonstrating that a single domain can function in different ways depending on the target lipid available at the membrane *(Corbalan-Garcia and Gomez-Fernandez, 2010)*.

4.2.6. Ceramide.

Ceramides are derivatives of sphingomyelin and are generated either by sphingomyelinase, via de novo synthesis using palmitate as a precursor, or by the salvage pathway that uses sphingosine through reacylation to produce ceramides *(Summers, 2010)*. Ceramides are relatively minor components of cell membranes but are known to be mediators of very important cellular processes such as apoptosis, proliferation and differentiation, cell senescence, cell migration and adhesion *(Hannun and Obeid, 2008; Holland and Summers, 2008)*. Roles for ceramide and its downstream metabolites have also been suggested in a number of pathological states including cancer, neurodegeneration, diabetes,

microbial pathogenesis, obesity, and inflammation *(Wu et al., 2007; Zeidan and Hannun, 2007)*.

Several studies have demostrated the activation of PKCα translocation , by interacting of ceramide with the C2 domain of PKCα *(Aschrafi et al., 2003; Huwiler et al., 2003)*, and the inhibition of PKCβII juxtanuclear translocation *(Becker et al., 2005)*. On the other hand, it has seen that a derivative of ceramide, ceramide-1-phosphate, stimulates translocation of PKCα from the cytosol to the cell membranes *(Gangoiti et al., 2010)*. So, ceramide is involved in the regulation of cPKC activity, but the detailed mechanism remains unclear.

5. OBJECTIVES.

The specific objectives established at the beginning of this work were:

- Determination of the importance of PIP_2 in the activation of PKCα through its C2 domain in presence of different lipids and concentrations of calcium ion.

- Characterization of the different binding affinities of C1B domains of novel PKCs, their specificity and dependence by DOG and negatively charged phospholipids.

- Identification of the amino acidic residues involved in the interaction of C1Bε domain with negatively charged phospholipids.

- Determination of the role of the C1B domain of PKCε localization in neuroblastoma cells after being subjected to oxygen glucose deprivation.

- The lipid signaling events that take place in RBL-2H3 will be studied using a mass spectrometry technique. Basically, lipid composition of stimulated cells with antigen-receptor binding, subjected or not with different phospholipases, will be analyzed in a LC/MS/MS system.

CHAPTER II

Materials and Methods.

1. CONSTRUCTION OF EXPRESSION PLASMIDS.

The DNAs of PKC isoforms were amplified by PCR using specific primers. the cDNA obtained was digested with same restriction enzymes than the vector in order to ligate them, using the T4 ligase, obtaining a recombinant vector. Later DH10B *Escherichia coli* strain was transformed with the ligation vector by heat shock *(Cohen et al., 1973)* . Then it was plated on LB plates containing the appropriate antibiotic to select the antibiotic-resistant clones.
Finally, these clones were amplified and purified to confirm the positive clones by sequencing them.

1.1. Plasmid constructions.

PKCε and PKCα cDNA was a gift from Drs. Nishizuka and Y. Ono (Kobe University, Kobe, Japan).

1.1.1. Constructions in plasmid pEGFP-N3.

The N-terminal fusions of rat PKCε and mutant to EGFP were generated by inserting cDNAs into the multiple cloning site of the pEGFP-N3 (BD Biosciences Clontech, Palo Alto, CA) mammalian expression vector which contains, besides EGFP sequence, two replications origins (f1 and SV40), a cytomegalovirus transcription promoter (PCMV), a kanamycin resistant gene and a multiple cloning site (MCS) (Figure II.1).

Full-length PKCε mutant were generated using the Quikchange XL site-directed mutagenesis Kit (Stratagene, La Jolla, CA, USA). The mutant performed was the follow: PKCεK251A/R268A/R282A/R283A.

The cDNAs were amplified by PCR using as template the wild-type cDNA and the corresponding mutants with the following primers:

PKCε: 5.' GCCAGATCTATGGTAGTGTTCAATG
PKCε: 3.' GAAAAGTCTACGTGATCATCGATC

BglII/BamHI-digested PKCε and mutated fragments were ligated with the BglII/BamHI digested vector generating the different fusion constructs.

All constructs were confirmed by DNA sequencing, which was carried out in the Research and Development Support Center (CAID), University of Murcia (Spain).

Previous studies have shown that a C-terminal GFP tag does not affect the localization, catalytic activity or the cofactor dependence of PKCε *(Shirai et al., 1998)*. The stability and viability of the mutated proteins have been already studied by using specific activity measurements *(Lopez-Andreo et al., 2003)*.

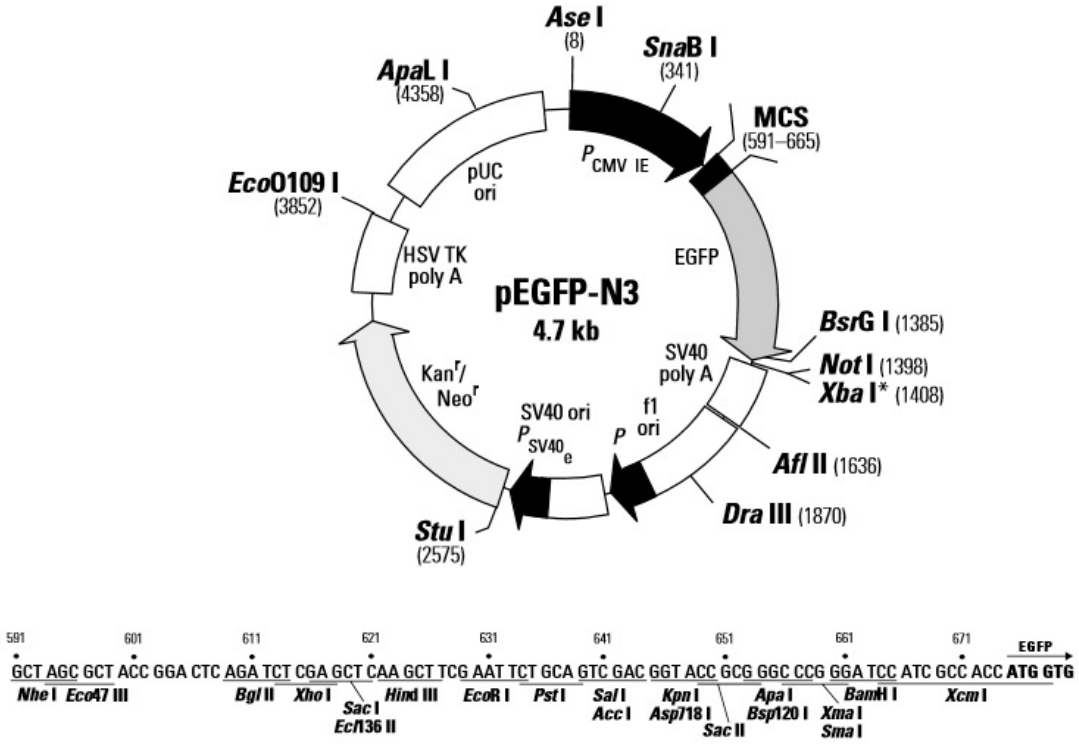

Figure II.1. pEGFP-N3 mammalian expression vector and its multiple cloning site. It is shown restriction sites along plasmid sequence and the multiple cloning site. This plasmid also possesses a cytomegalovirus promoter (PCMV), two replication origins (f1 and SV40) and two genes of antibiotic resistance, kanamycin (Kanr)and neomycin (Neor).

1.1.2. Constructions in plasmid pECFP-N3.

This vector is similar to pEGFP, the difference is that pECFP possesses a gene which codifies for Enhanced Cyan Fluorescent Protein (ECFP); instead of Enhanced Green Fluorescent Protein (EGFP).

This plasmid was used for cloning the isolated C1B domains of novel isoenzymes, the mutants of the PKCε-C1B domain, the isolated C1A domain of PKCε and the C1AB domain of PKCε. Steps were similar to pEGFP, but template and primers were different.

C1B-PKCε mutants were generated using the Quikchange XL site-directed mutagenesis Kit (Stratagene, La Jolla, CA, USA). The mutants performed were as follow: C1B-PKCεK251A, C1B-PKCεR268A, C1B-PKCεR282A, C1B-PKCεR282H, C1B-PKCεR283A, C1B-PKCεK251A/R268A, C1B-PKCεR282A/R283A, C1B-PKCεR268A/R283A, C1B-PKCεK251M/R282H, C1B-PKCεK251A/R268A/R282A, C1B-PKCεK251A/R282A/R283A and C1B-PKCεR268A/R282A/R283A.

Briefly, cDNAs encoding C1B domain of PKCθ, δ, ε and C1Bε-PKCε mutants were amplified by PCR using the following primers:

C1Bθ: 5´ CGGAATTCATCGCTTTAAAGTGTAT
C1Bθ: 3´ CCCAAGCTTTCAGCACAGGTTCGCCAC

C1Bδ: 5' TATAAGCTTGACATGCCTCACCGA
C1Bδ: 3' GACACACCATAGTTGACTCCTAGGAA

C1Bε: 5' CCGAAGCTTAACATGCCCCACAAG
C1Bε: 3' GTTAACACCCCACCTGACTGGGCCCTAAA

C1Bε and mutated fragments were digested with HindIII/XmaI, C1Bδ was digested with HindIII/BamHI and C1Bθ was digested with EcoRI/ BamHI. C1Bη was synthesized and cloned into EcoRI and BamHI sites of the plasmid vector pUC57 by GenScript Corporation (Piscataway, New Jersey, USA). The resulting fragments were ligated with their corresponding digested pECFP-N3 vectors to generate the different fusion constructs.

All constructs were confirmed by DNA sequencing in the Research and Development Support Center (CAID), University of Murcia (Spain).

1.1.3. Constructions in plasmid pFASTBAC HTb.

PKCα was subcloned into the expression vector pFASTBAC HTb (Invitrogen, Life Technologies, CA, US) as was described by A. Torrecillas, Ph.D. Thesis, University of Murcia, 2003.

The cDNAs were amplified by PCR using the following primers:

PKCα: 5'ATTCTCGAGCTATGGCTGACGTT
PKCα: 3'CCGGGTACCTACTGCACTTTGCAAGAT

XbaI/XpnI-digested PKCα fragment was ligated with the XbaI/XpnI digested vector generating the fusion construct.

1.1.4. Site-directed Mutagenesis.

Site-directed mutagenesis was performed by using the Quikchange XL site-directed mutagenesis Kit (Stratagene, La Jolla, CA, USA).

2. CELLS CULTURE.

2.1. RBL-2H3 cells.

Rat Basophilic Leukemia 2H3 (RBL-2H3) is a rat cell line derived from a basophilic leukemia. One important feature of RBL-2H3 cells is the high expression of the receptor FcERI (Fc of IgE), making them a model for studies of different aspects of secretion in cells including the role of changes in intracellular calcium, the activation of phospholipases, protein kinases and small G proteins. Besides, this cell line is a suitable transfection host.

2.1.1. RBL-2H3 culture conditions.

RBL-2H3 cells were thawed in a 37ºC water-bath from one vial kept in liquid nitrogen. One vial, approximately 3 millions of cells, was grown in a 75 cm^2 ventilated flask with 10 ml of the appropriate growth medium.

The growth medium consisted of DMEM (Dulbecco's modified Eagle's medium) with 4500 mg/L glucose, supplemented with 15% (v/v) fetal bovine serum (FBS), antibiotic (penicillin 50 units/ml and streptomycin 50 µg/ml), and 4 mM glutamine. This culture was kept at 37ºC humidity heater with 7.5% CO_2.

When cells reached 80-85% confluency, approximately in 2 days, the culture was diluted in ratio 1:4 (3 millions/ml). For subculturing, cells were detached from the culture flask using a cell scraper and centrifuged at 200 g for 5 minutes. After the centrifugation, the supernatant was removed and the cells were resuspended by gentle pipetting in fresh growth medium in order to spread the dislodged cells in new ventilated flasks.

2.1.2. RBL-2H3 transfection.

Firstly, cells were harvested, and once counted in Neubauer chamber, an aliquot of 12 millions cells was taken and centrifuged separately. The pellet was resuspended in 400 µl of electroporation buffer (120 mM NaCl, 25 mM glucose, 5.5 mM KCl, 2.8 mM $MgCl_2$, 20 mM Hepes pH 7.2) and, together 15-30 µg DNA, were put into an electroporation cuvette (0.4 cm) to carried out an electro-shock using a Gene Pulser Xcell (BioRad, Hercules, CA), in square wave mode, 200 V, 1 pulse of 10 ms. Immediately, every 3 million of transfected cells were plated on a 6 cm plate.

2.1.3. RBL-2H3 sample preparation for *in vitro* stimulation experiments.

To perform these experiments, $3x10^6$ RBL-2H3 transfected cells were plated on 24x40 mm culture coverslips over a 6 cm plate, and incubated at 37°C for 4–6 h, after which the growth medium was renewed. RBL-2H3 cells were used 16 h later. The coverslips were washed with 3 ml of extracellular buffer HBS (120 mM NaCl, 25 mM glucose, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES pH 7.2). All added substances were dissolved or diluted in HBS. 1,2-dioctanoylglycerol (DOcG) and phosphatidic acid (DOcPA) were dissolved in dimethyl sulfoxide and diluted to the final concentration with extracellular buffer shortly before the experiment. During the experiment, the cells were not exposed to dimethyl sulfoxide concentrations >1%.

In case of the activation of the IgE receptor experiments, cells were used 16-24 h later after priming overnight with 500 ng/ml IgE-anti-dinitrophenyl (mouse monoclonal; Sigma-Aldrich Quimica, S.A., Madrid, Spain), being stimulated with 2 µg/ml with the antigen dinitrophenyl-human serum albumin (DNP-HSA).

All the experiments were carried out at room temperature and, unless otherwise stated, on at least four different occasions. In each experiment, recordings were obtained from two to six cells.

2.1.4. RBL-2H3 lysis and isolation of plasma membrane.

The plasma membrane fraction was prepared from 9 cm plate of confluent culture cells (approximately $9x10^6$ cells per plate). Cells were washed twice with 5 ml of ice-cold extracellular buffer HBS (120 mM NaCl, 25 mM glucose, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES pH 7.2) and harvested by scraping in 3 ml of HBS. The cells were centrifuged at 1000 x g 5 min at 4ºC, the supernatant was discarded and the pellet was resuspended in 200 µl of Buffer B (0.25 mM sucrose, 1 mM EDTA, 20 mM Tris-HCl, pH 7.8 and mini protease EDTA free cocktail inhibitors (Roche Diagnostic, GmbH, Germany) and phosphatase inhibitor cocktail (Roche Diagnostic, GmbH, Germany). They were sonicated during 2 s for 5 times and transferred the suspension to a centrifuge tube and centrifuged at 1000 x g for 10 min. The postnuclear supernatant fraction (PNS) was transferred to another centrifuge tube, and layered on the top of 30% Percoll® (Sigma-Aldrich Chemistry, S.A., Madrid, Spain) in buffer A (0.25 mM sucrose, 1 mM EDTA, 20 mM Tris-HCl, pH 7.8) and centrifuged at 84.000 x g for 30 min at 4ºC.
The plasma membrane was collected with a Pasteur pipette.

2.2. HEK293 cells.

Human Embryonic Kidney 293 (HEK293 cells) is a specific cell line originally derived from human embryonic kidney cells grown in tissue culture (Figure II.2). HEK293 cells are very easy to grow and transfect very readily and have been widely used in cell biology research for many years. They are also used by the biotechnology industry to produce therapeutic proteins and viruses for gene therapy.

2.2.1. HEK293 culture conditions.

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/L glucose supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotic (penicillin 50 units/ml and streptomycin 50 µg/ml). This culture was kept at 37ºC humidity heater with 7.5% CO_2.

Cells were seeded in 75 cm^2 flasks with 10 ml of growth medium. When cells reached 80-85% confluence, approximately in 4 days, a dilution was performed. To harvest HEK293 cells a 0.25% trypsin-0.25% EDTA solution was

used during 3 minutes at 37ºC. After that, trypsin was neutralized with growth medium and centrifuged at 200 g for 5 minutes. Finally, the supernatant was removed and the cells were resuspended in fresh growth medium in order to spread 1.5 millions cells in a new ventilated flask.

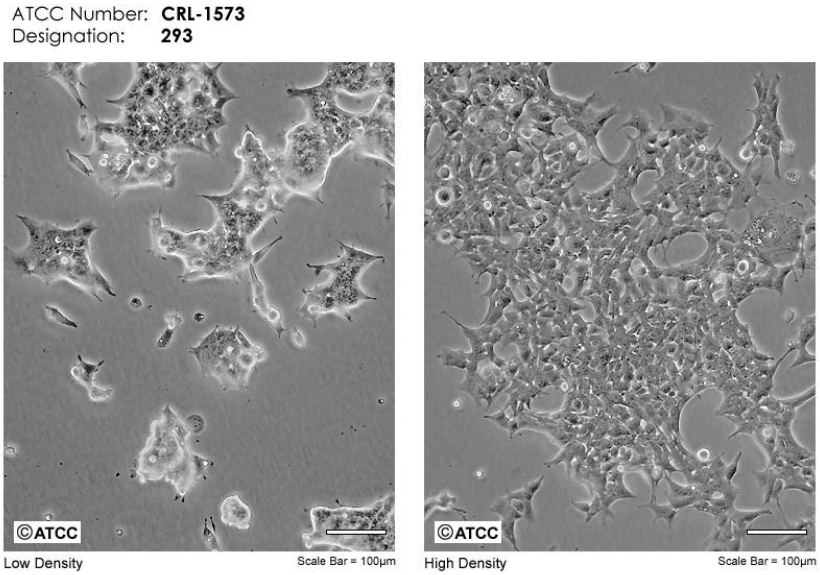

Figure II.2. HEK293 cells. Left picture shows cells after a subculture (low density), whereas in the right one we can observe cells at high density.

2.2.2. HEK293 transfection.

To perform HEK293 transfection, 2 million cells were sowed on a 9 cm plate. At 24 h after seeding, the growth medium was removed and the transfection initiated using the Ca^{2+} phosphate method *(Wigler et al., 1977)*.

Transfection of each plate required the preparation of two different reagents (A and B).
Reagent A included 0.54 ml of Hepes 50 mM pH 7.05 and 11 µl of $Na_2HPO_4 \cdot 2H_2O$ 1.5 mM. On the other hand, reagent B was performed adding 10 µg of the corresponding plasmid DNA to 0.48 ml of TE buffer (Tris-HCl, 1 mM pH 8.0, EDTA 0.1 mM), and once both reagents were mixed, we added 66 µl of $CaCl_2$ 2 M.

After 5 minutes of the reagents preparation, both reagents were mixed forming a precipitate corresponding to calcium phosphate. The resulting mixture was incubated at room temperature for 20 minutes before being added

to the cell plate. Once the transfection mixture had been added to the cell plate, this plate was incubated at 37ºC humidity heater with 7.5% CO_2. After 14 hours, the HEK293 cells were washed with PBS (phosphate buffer saline) and incubated in DMEM culture medium supplemented as described previously.

2.2.3. HEK293 lysis and subcellular fractionation.

The cells were lysed 24 hours after transfection in ice-cold hypotonic buffer (10 mM Tris-HCl pH 7.4, 10 mM NaF, 10 mM NaCl, 5 mM Na_3VO_4, 1 mM PMSF (Sigma-Aldrich Chemistry, S.A., Madrid, Spain), 10 µg/ml leupeptin (Sigma-Aldrich Chemistry, S.A., Madrid, Spain) and 10 µg/ml aprotinin (Sigma-Aldrich Chemistry, S.A., Madrid, Spain) and incubated on ice 20 min. Cells were lysed by 15 passages through a 30-gauge needle. The resultant lysate was centrifuged at 15000 x g for 15 min at 4ºC to remove nuclei and cell debris. Supernatants were collected and used in the fluorescence experiments.

2.3. SH-SY5Y cells.

SH-SY5Y is a thrice-cloned sub-line of bone marrow biopsy-derived line SK-N-SH, which it was subcloned and isolated from a bone marrow biopsy from a human with neuroblastoma. SH-SY-5Y has dopamine-β-hydroxylase activity and can convert glutamate to the neurotransmitter GABA. They will form tumours in nude mice in approximately 3-4 weeks. (Figure II.3)

2.3.1. SH-SY5Y culture conditions.

SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/L glucose supplemented with 15% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and antibiotic (penicillin 50 units/ml and streptomycin 50 µg/ml). This culture was kept at 37ºC humidity heater with 5% CO_2.

These cells grow as a mixture of floating and adherent cells, thus the growth medium with the floating cells was removed and cells were recovered by centrifugation. The adherent cells were rinsed with fresh 0.25% trypsin, 0.53 mM EDTA solution at 37ºC until the cells were detached. Fresh medium was added and combined with the floating cells recovered previously. Then, the cells were

centrifuged at 200 x g during 5 minutes, and the pellet was resuspended with new growth medium and dispensed into 75 cm^2 flasks.

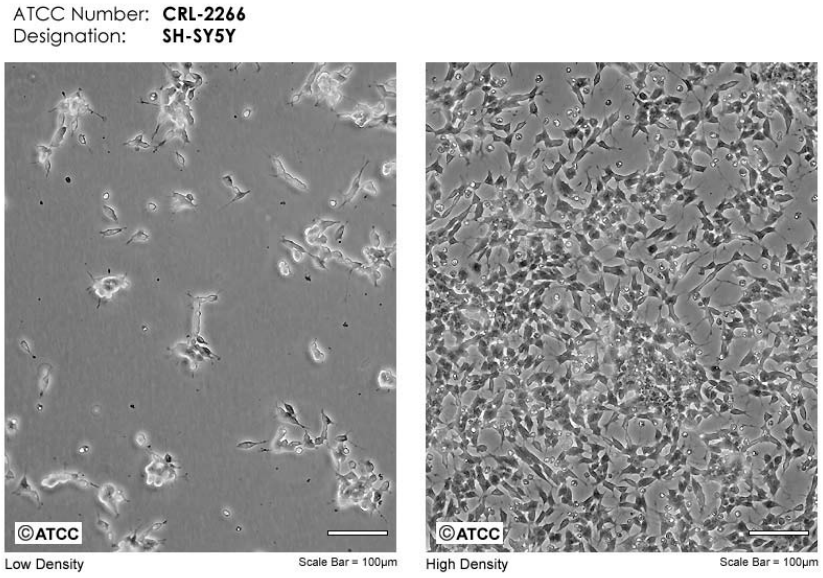

Figure II.3. SH-SY5Y cells. Left picture shows cells after a subculture (low density), whereas in the right one we can observe cells at high density.

2.3.2. SH-SY5Y transfection.

Once the cells had been harvested and counted, an aliquot with $8x10^6$ cells was taken and centrifuged separately. The pellet was resuspended in 400 µl Ingenio® electroporation buffer (Mirus Bio LLC, Madison, WI, USA) and, together 30 µg DNA, were put into an electroporation cuvette (0.4 cm) to carried out an electro-shock using a Gene Pulser Xcell (BioRad, Hercules, CA, USA), in exponential decay mode, 250 V and 950 µF. Immediately, every 8 million of transfected cells were plated on a 9 cm plate.

2.3.3. SH-SY5Y sample preparation for *in vitro* model of hypoxic-ischemia.

For the experiments we used an in vitro model of ischemia as described previously *(Agudo-Lopez et al., 2011)*. Briefly, the in vitro model of hipoxic-ischemia was achieved maintaining the cells under oxygen–glucose deprivation for 2 h without recovery. The standard culture medium was replaced with a glucose-free buffer (154 mM NaCl, 5.6 mM KCl, 5.0 mM Hepes, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$; pH 7.4), and bubbled with 95% N_2 and 5% CO_2 for at least 2 h before use. The culture plates were then placed into an hypoxia

humidified incubator Thermo Electron Corporation (Model 3141), under atmosphere of 1% O_2, 5% CO_2 at 37 °C for 2 h. After the appropriate time, the culture plates were removed from the anaerobic chamber and cells were collected using cold phosphate buffer saline (PBS), and stored at -80ºC.

2.3.4. SH-SY5Y lysis and subcellular fractionation.

$16x10^6$ cells were resuspended in 300 µl ice-cold lysis buffer (20 mM Hepes pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM Na-EDTA, 250 mM sucrose, 1 mM EGTA, 1 mM DTT, 10 mM NaF, 10 mM NaCl, 5 mM Na_3VO_4, 1 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin) and incubated on ice during 5 minutes. Cells were lysed by 15 passages through a 25-gauge needle. The resultant lysate was centrifuged at 1000 x g for 10 min at 4ºC to remove nuclei and cell debris. Supernatant were collected and centrifuged 10.000 x g 20 min at 4ºC. The pellet corresponded with the *mitochondrial fraction* and the supernatant was centrifuged again at 100.000 x g for 1 h at 4ºC. The supernatant and pellet were used as *cytosolic fraction* and *membrane fraction* respectively. Either the mitochondrial fraction like the membrane fraction pellets were resuspended in 300 µl lysis buffer to maintain the same volume in all the fractions.

3. CONFOCAL MICROSCOPY

Transfected cells with fluorescent constructs were examined under a confocal microscope type Leica TCS SP2 AOBS (Leica, Heidelberg, Germany) (Figure II.4), which has several lasers: ArKr (emits at 488 and 568 nm), GrHe (excites at 543 nm), HeNe (excites at 633 nm) and blue diode, which excites at 405 nm. The objective used were Nikon PLAN APO-CS 63X 1.2 NA (water) for *in vivo* stimulation experiments or 1.4 NA (immersion oil) when we used fixed samples.

The LEICA TCS SP 2 system makes it possible to image a single, in-focus plane (horizontal or vertical) as well as a series of planes. A single vertical section, or xz-scan, allows you to see your sample as though from the side.

Besides, this confocal model was designed as an integrated system. Optical and mechanical elements work seamlessly with computer hardware and software. The integrated Leica Confocal Software package supports the

complete imaging process, from optical sectioning, through image processing and analysis (which is the main application), to hard copy output.

For the capture of images from EGFP constructs, the sample was excited with Ar/Arkr laser at 488 nm, while emission waves were captured to 500-525 nm.

Cells transfected with ECFP constructs were obtained by excitation with the diode blue laser at 405 nm, and emission wavelengths at 475-490 nm.

Series of 200-300 confocal images were recorded for each experiment at time intervals of 3 seconds. The time series were subsequently analyzed using ImageJ-NIH software (http://rsb.info.nih.gov/ij/, 1997-2011).

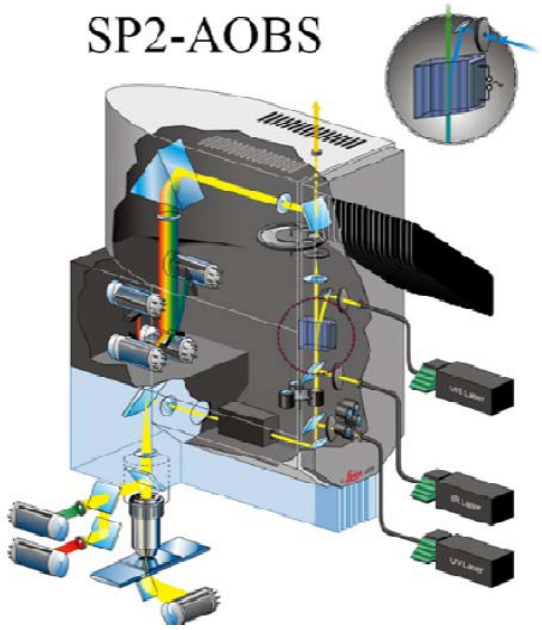

Figure II.4. SP2 AOBS Leica Confocal Microscope. Diagram of the optical circuit of confocal microscope used along this work. Note that it is represented a vertical microscope, while an inverted one has been used in this work, although light circuit is the same in both cases: the light from the excitant laser is polarized after passing through mirrors and later, when it go through a pinhole, is unified to excite the specimen. The emitted light go to detector and only the desired wavelength, selected by AOBS system, reach it. In the detail picture is shown the AOBS system (Acousto Optical Beam Splitter). In this case, wavelength of excited or emitted light from specimen is controlled by this system independently of the fluorophore used (Taken from www.leicamicrosystems.com).

3.1. Fluorophores used.

For subcellular localization of PKCε isoform and C1B domains of different PKC isoforms (wild type and mutants in both cases), some GFP variants were used, like EGFP and ECFP (Fig II.5 and Table II.1).

Fluorescent tag	λ excitation max. (nm)	λ emission max. (nm)	Emission range (nm)
EGFP	488	520	500 - 540
ECFP	405	475	475 - 550

Table II.1. Fluorescent substances used.

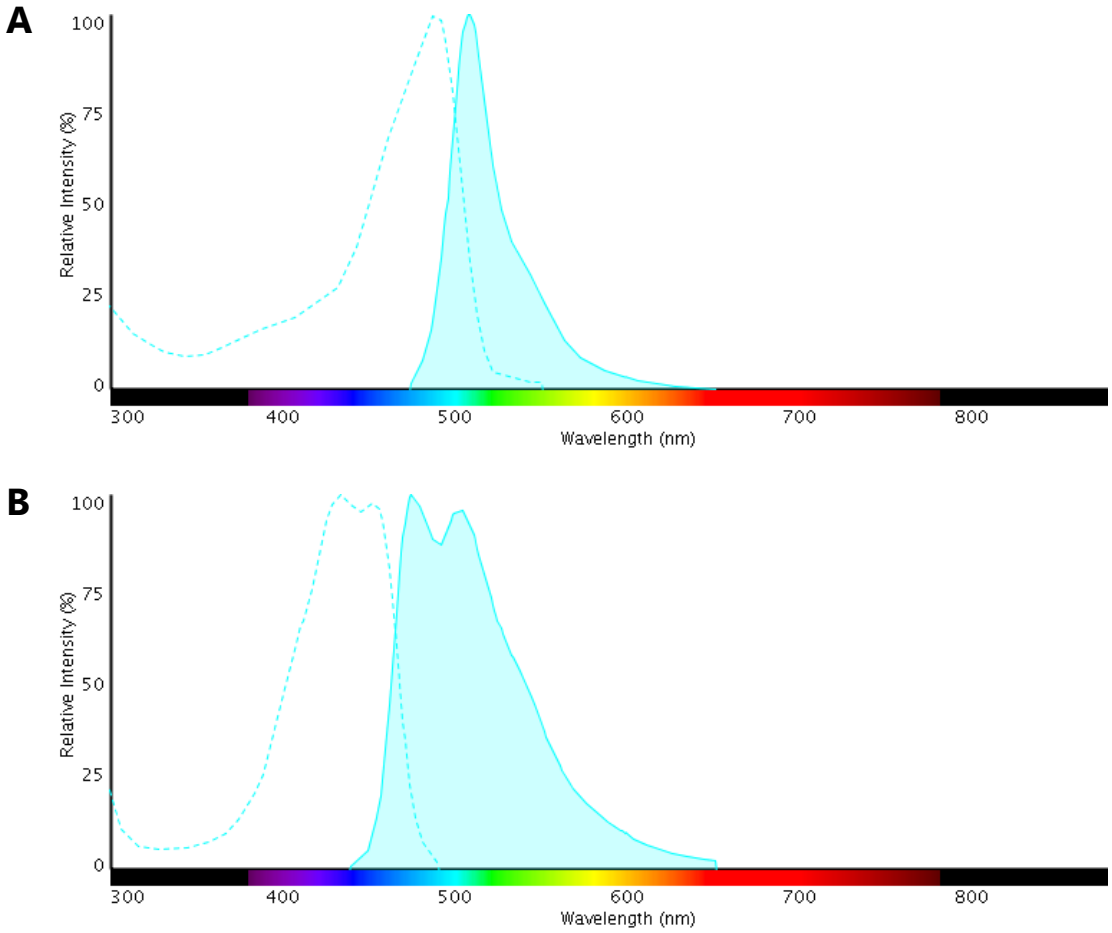

Figure II.5. Fluorophores spectra. Absorption (doted lines) and fluorescence emission (continue lines) spectra of the single fluorescent substances, employed along this Doctoral Thesis. **(A) EGFP and (B) ECFP fluorescent proteins.**

GFP, originally obtained from jellyfish called *Aequorea Victoria*, is known like Green Fluorescent Protein whose chromophore, is formed by the cyclation of 3 aminoacids (Ser-Tyr-Gly) located between the residues 65-67 and it is able to absorb blue light and emit green light (Figure II.6). GFP is incorporated in different plasmid in order to create proteins fused to it.

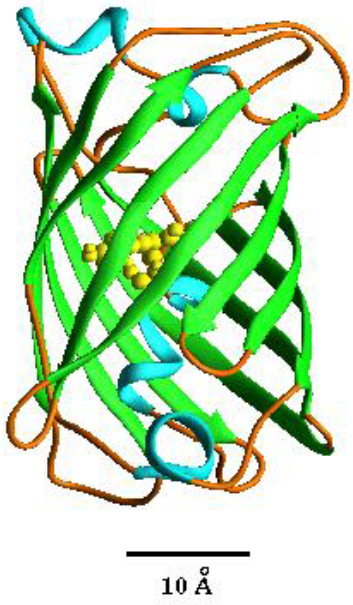

Figure II.6. GFP terciary structure. Eleven strands of β-sheet (green) form the walls of a cylinder. Short segments of α-helices (blue) cap the top and bottom of the "can" and also provide a scaffold for the fluorophore which is near the geometric center of the can. The sequence of three aminoacids (Ser-Tyr-Gly) is essential to produce fluorescence and it has been changed by means of site directed mutagenesis in order to enhance and obtain more excitation and emission ranges, providing different applications in microscopy. *(Taken from Fan Yang et al, 1996).*

3.2. Media used in stimulation of living cells in concocal microscope.

To observe cells in confocal microscope, the coverslips were mounted in a special holder and washed with 3 ml Hepes Buffer Saline (HBS) consisting of 120 mM NaCl, 25 mM glucose, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM Hepes pH 7.2.

Although all added substances were dissolved or diluted in 2 ml of HBS, they were resuspended in different media; while dinitrophenylhuman serum albumin (HSA-DNP) was resuspended in HBS, 1,2-dioctanoyl-sn-glycerol (DiC8) and phosphatidic acid (DOcPA) were resuspended in DMSO. Anyway, all of them were diluted to the final concentration with HBS extracellular buffer, shortly before the experiment. During the experiment, the cells were not exposed to DMSO concentrations higher than 1%.

All the experiments were carried out at room temperature and, in every assay, image series of 200-300 images were recorded in intervals of 3 seconds, unless otherwise stated.

3.3. Image processing and analysis.

First confocal microscopes consisted of a detector connected to an oscilloscope with long-persistence phosphor, which would display an image as it was being scanned.

Nowadays, the signal is digitized and recorded in a computer, what makes the image enhancement possible in many aspects such as improved contrast thresholds, gamma correction (curvature of the image intensity value versus source intensity graph) or noise suppression, among others.

Along this Doctoral Thesis, images were analyzed with two different softwares: ImageJ-NIH (http://rsb.info.nih.gov/ij/, 1997-2011, *Rasband, 1997*) and Leica software (Leica Confocal Software Lite). Both programs allowed us to select areas of interest of every individual cell and determine the average pixel intensity in each area and every time point analyzed.

3.3.1. Analysis of C1B domains of novel PKCs and full lenght PKCε localization in the plasma membrane.

The time-series were analyzed using Leica software (Leica Confocal Software Lite). An individual analysis of protein translocation for each cell was performed by tracing a line intensity profile across the cell *(Meyer and Oancea, 2000)*. The relative increase in plasma membrane localization of the enzyme for each time-point was calculated by using the ratio:

(1) $$R = \frac{I_{mp} - I_{cit}}{I_{mp}}$$

where R is the percentage of protein in plasma membrane, I_{mb} is the fluorescence intensity at the plasma membrane and I_{cyt} is the average cytosolic fluorescence intensity (Figure II.7).

After analyzing every frame of time-series cell by cell, R values and standard deviations were represented versus time showing the localization profiles of different isoenzymes and mutants. Directly from the chart we can calculate the localization halftime (t½), which means the necessary time to get 50% of maximum localization (R_{max}).

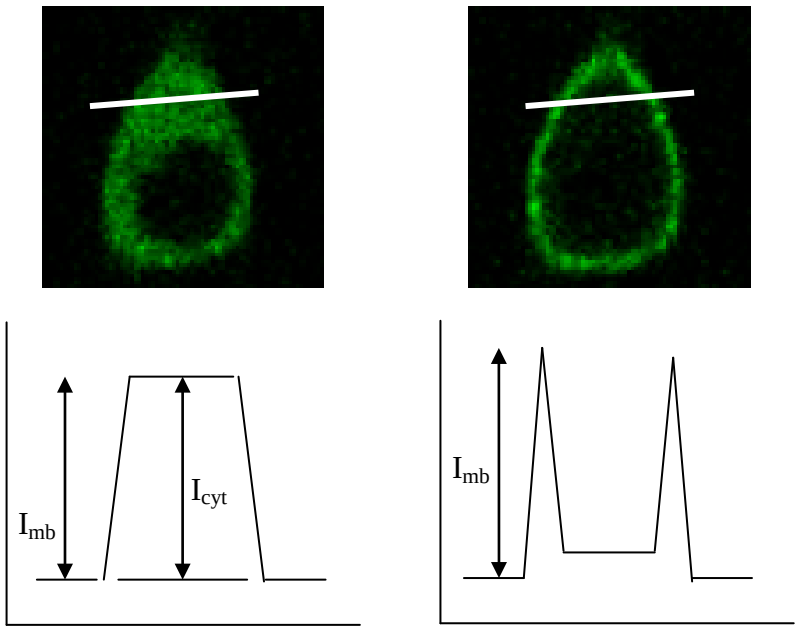

Figure II.7. Representation of protein translocation analysis in cells recorded in the confocal microscope. At the top of the picture cells transfected with PKCε-EGFP are shown, at the beginning and final of a localization series. A line was traced, using software, across the cell and we obtained the fluorescence intensity of every pixel along the line (it is shown at the bottom). After applying the formula R= (Imb - Icyt)/ Imb, we obtain R=0 (on the left) and R=1 (on the right).

4. FLUORESCENCE RESONANCE OF ENERGY TRANSFER (FRET).

FRET is a very useful technique to detect interactions between bio-molecules. Basically it consists of fluorescence energy transfer from a donor to an acceptor, so we excite a fluorescence donor and detect the emission fluorescence from the acceptor molecules whenever donor and acceptor are closer than 100 Å *(Teale, 1984)*.

The efficiency of energy transfer depends on spectra overlap of fluorescence emission donor and excitation acceptor, relative orientation and distance between both molecules (0-100 A). Specifically in this work, FRET was measured between ECFP donors in each C1B domain and phospholipid vesicles containing acceptor phosphatidylethanolamine labeled with DHPE (Figure II.8).

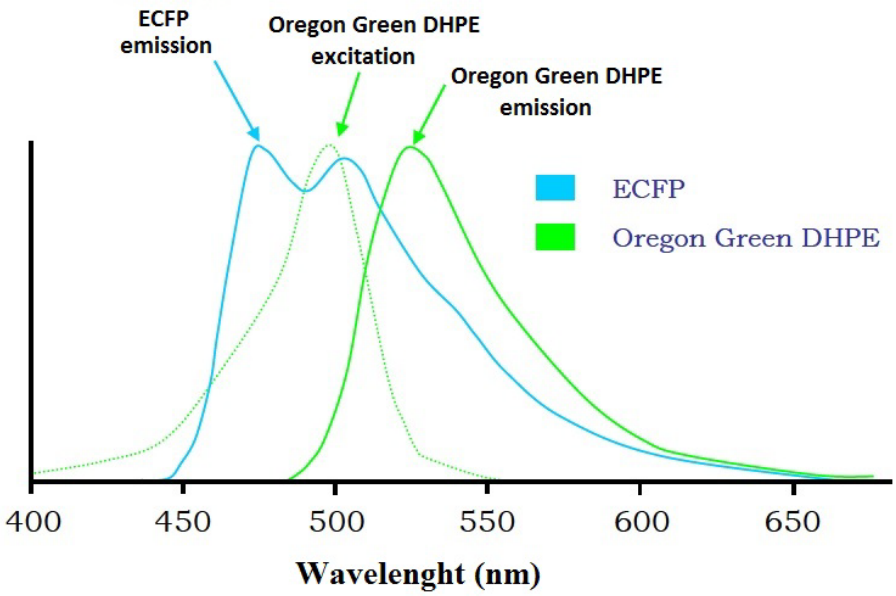

Figure II.8. Overlap of emission and excitation spectra. Emission spectra of the donor, ECFP in this case, (blue line) and excitation and emission spectra of the acceptor, dansyl- derivative compound, (green line) are shown.

This efficiency of fluorescence energy transfer (*E*) can be measured with next equation:

(1) $$E = \frac{\tau_D - \tau}{\tau_D} \approx \frac{F_D - F}{F_D}$$

where F and F_D are the donor fluorescence emission in the presence and in the absence of acceptor.

The efficiency energy transfer allows us to calculate the distance between donor and acceptor (τ) as follows *(Förster, 1996):*

(2) $$r = R_0 \left(\frac{1-E}{E}\right)^{1/6}$$

being R_0 a known parameter like *critical distance of Förster*, where the energy transfer is 50%.

Knowing the dependence of the distance between donor and acceptor to transfer energy, this technique has been widely used to measure lenghts as well as to detect interactions between molecules *(Corbalangarcia et al., 1994; Hovius et al., 2000; Singleton and Xiao, 2001)*. In this way, we can measure the union between a specific protein and model membranes in a huge variety of

conditions, for instance the presence of different ligands in several concentrations. In this case, the carboxy termini addition of ECFP is used like donor and Oregon Green® 488 DHPE (Invitrogen Co., CA, USA), which is a fluorescent compound in membranes, like an acceptor (Figure II.9).

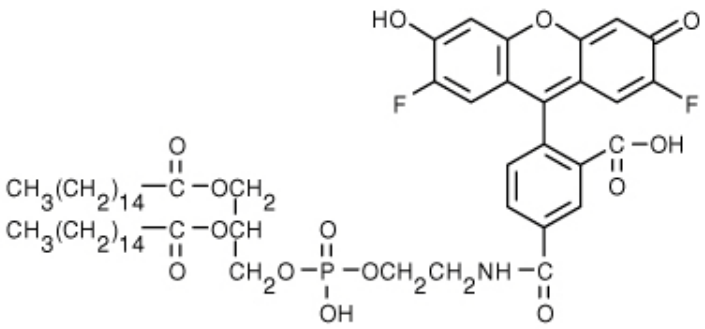

Figure II.9. Molecular structure of Oregon Green® 488 DHPE. It is labeled on the head group with the green-fluorescent Oregon Green® fluorophore with excitation/emission maxima ~501/526 nm.

To carry out FRET experiments in this work, C1B-CFP constructs were used to measure the interaction of C1B domains from novel PKCs to membrane by FRET.

The lipids used for the reaction were dried under a stream of N_2 and the last traces of organic solvent were removed by keeping the samples under vacuum for 1 h. Lipids were suspended in 20 mM Hepes (pH 7.4) and vortex mixed vigorously to form multilamellar vesicles. They were sonicated for 10 s for ten times to form small unilamellar vesicles.

Equilibrium fluorescence experiments were carried out on a Fluoromax-3 fluorescence spectrometer at 25ºC in a standard assay buffer composed of 20 mM Hepes (pH 7.4) and 100 mM NaCl. The excitation and emission slits widths were both 2 nm for all equilibrium fluorescence experiments.

Quantitation of the negative lipid dependent increase in protein-to-membrane FRET for the C1B domains binding to membranes was performed according to the method described below. Basically, for the normalization of the amount of protein between different experiments, cell lysates were added to the fluorescence cuvette to reach the same starting ECFP fluorescence intensity.
FRET was measured as the fluorescence variation of ECFP as a function of lipid concentration. The intensity of ECFP fluorescence was measured using an

excitation wavelenght of 433 nm and collected at 473 nm. To correct for the fluorescence attenuation produced by the lipidic vesicles, a control was measured in which vesicles containing only POPC and OG-PE were added to a cuvette with C1B-ECFP, whereas the values obtained were subtracted from these of the above experiments. Additionally, the dilution effect produced by the addition of vesicles was corrected by subtracting the values obtained after adding the same volumes of buffer without lipid vesicles to a cuvette containing the C1B domain.

Equilibrium binding data were best-fitted using the Hill equation (3):

(3) $$\Delta F = \Delta F_{max} \left(X^H / K_D^H + X^H\right)$$

whereby ΔF_{max} represents the calculated maximal fluorescence change (normalized to unity to simplify graphical representation), H is the Hill coefficient, X represents the free diacylglycerol concentration corrected for the leaflet effect (for phospholipid titrations), and K_D represents the apparent equilibrium dissociation constant for lipid binding and corresponds to the inverse of the affinity constant. Note that the amount of protein added is low because it comes from the cell lysates of C1B-overexpressing HEK293 cells, and from the first lipid additions the [protein]<<[lipid].

All total lipid concentrations were divided by 2 to take into account the inaccessibility of one leaflet of the lipid vesicles to protein, due to the sealed nature of the bilamellar sonicated vesicles.

5. PKCα PURIFICATION.

A 0.5 L scale culture of Sf9 insect cells at 2.1×10^6 cells/ml was infected with the recombinant baculovirus. Cells were harvested at 60 h postinfection (cell viability 80%), pelleted at 4500 rpm for 20 min, and resuspended in buffer containing 25 mM Tris-HCl pH 7.5, 100 µM EGTA, 50 mM NaF, 100 µM $NaVO_3$, 1% Triton X-100, 10 % glycerol, 150 mM NaCl, 1 mM PMSF, 10 µg/ml leupeptin y 10 mM benzamidine. The pellet was disrupted by sonication (6×10 s) and the resulting lysate was centrifuged at 15000 rpm for 20 min. The supernatant was applied to a 1 ml His-Gravi TrapTM® column (Amersham Biosciences, Uppsala, Sweden) and equilibrated with buffer 25 mM Tris-HCl pH 7.5, 150 mM NaCl and

20 mM imidazole. The bound proteins were eluted by the application of an imidazole gradient (20-500 mM).

Fractions containing protein kinase Cα from His-Gravi TrapTM® column were pooled, concentrated by ultrafiltration to a 2 ml volume and adjusted by the addition of 5 M NaCl to give a NaCl concentration of 1 M.

This fraction was directly applied to a SOURCE 15PHE 4.6/100 PE ® column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equilibrated with 25 mM Tris-HCl pH 7.5, 1 mM DTT and 10 % glycerol. After unbound proteins had passed through the column, PKC was eluted with a gradient of 1-0 M NaCl. High pure PKCα was obtained, as determined by SDS-PAGE (12.5 %). Protein was aliquoted and stored at -80 °C in the presence of 10% (w/v) glycerol and 0.05% (v/v) Triton X-100.

6. PKCε PURIFICATION.

PKCε wild type and the mutant (K251A/R268A/R282A/R283A) were partially purified from HEK-293 cells in order to run the kinase activity experiments.

HEK-293 cells were grown following ATCC recommendation and when cells reached 80% confluency, they were transfected with DNA constructs in pEGFP plasmid following the Ca^{2+} phosphate method *(Wigler et al., 1977)* in 9 cm ø plates.

Plates were grown during 14 hours incubated with the calcium phosphate precipitate in a 5% CO_2 atmosphere, and afterwards the medium was refreshed and cells were grown for another 48 hours in a 7.5% CO_2 atmosphere in order for them to express the maximum amount of protein as possible.

After this time, cells were washed with PBS and collected to start the purification protocol described by *(Conesa-Zamora et al., 2001)*. Cell pellets were resuspended in lysis buffer (composed by 20 mM Tris HCL pH 8, 10 mM EGTA, 2 mM EDTA, 0.25 mM sucrose, 1 mM PMSF, 10 μg/ml leupeptin, 100 μM Na_3VO_4 and 50 mM NaF) with a ratio: 5 ml lysis buffer / g of cells. They were sonicated for 6 seconds 16 times controlling that pH keeps constant at 7.5.

The resultant lysate was centrifuged to 13000 rpm for 30 minutes and 4ºC. The supernatant was collected and the pellet was resuspended again in the same volume of lysis buffer to sonicate and centrifuged it in the same way.

Both supernatant were mixed and it was ultra-centrifuged to 35.000 x g during 30 minutes and 4ºC. The sediment was discarded and the supernatant was added to a SOURCE 15PHE 4.6/100 PE ® column (GE Healthcare Bio-Sciences AB, Uppsala, Suecia) previously equilibrated with Buffer E (20mM Tris HCl pH 8, 0.5 mM EGTA, 0.5 mM EDTA and 10 μM β-mercaptoethanol).

To elute proteins from the column, an FPLC (Äkta, Amersham Pharmacia Biotech AB, Uppsala, Sweden) was used. Particularly, proteins were eluted from the column using a saline gradient from 0 to 1 M of KCl dissolved in buffer E; and they were collected in the fraction collector with a flux of 0.5 ml per minute.

Whole elution process was monitored with ultraviolet detector and the fluorescence of each fraction was measured on a Fluoromax-3 fluorimeter (Jobin Yvon, Edison, NJ) with excitation and emission wavelenghts of 488 nm and 530 nm, respectively, in order to determine the fractions which contain the constructions with the pEGFP plasmid. Later every fraction with a high value of fluorescence was checked using Western Blot. The fractions, which contained higher amount of proteins, were put together and were concentrated in a concentrator filter 30K (Ultrafree Millipore) and finally, they were aliquoted and frozen to -80ºC.

7. KINASE ACTIVITY ASSAYS.

These assays were carried out using a partially purified PKCα wild type, PKCε wild type and the mutant PKCεK251A/R268A/R282A/R283A.

It was used a technique described previously *(Lopez-Nicolas et al., 2006*) in which it was measured the incorporation of radioactive phosphate [γ^{-32}P] to kinase substrate (histone III-S) in order to detect enzymatic activity.

The lipids used for the reaction were dried under stream of N_2 and the last traces of organic solvent were removed by keeping the sample under

vacuum for several hours. Lipids were suspended in 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.05 mM EGTA and vortex vigorously to form multilamellar vesicles.

Large unilamellar vesicles (LUVs) were prepared using the extrusion technique, where MLVs suspensions were extruded 10 times through two stacked 100-nm polycarbonate filters (Millipore Inc., Bedford, USA) and then added to the reaction to give a final total lipid concentration of 625 μM. The reaction mixture contained 20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.650 mM LUVs with different concentrations of lipid, 0.4 mg/mL histone III-S, 40 μM [γ^{-32}P]ATP (300000 cpm/nmol), 0.4 μg of protein and 200 μM $CaCl_2$.

The reaction was started by addition of 4 μl of the purifed protein (0.004 μg/ml). After 20 min at 25 ºC, the reaction was stopped with 1 ml of ice-cold 25% trichloroacetic acid (TCA) and 1 ml of ice-cold 0.05% bovine serum albumin (BSA).

After precipitation on ice for 30 min, protein was collected on a 2.5 cm glass fiber filter (Sartorius AG, Goettingen, Germany) and washed three times with 6 mL of ice-cold 10% TCA. The level of incorporation of ^{32}P into histone was measured by liquid scintillation counting. For the PKCα, basal kinase activity was measured in the presence of 5 mM EGTA instead of lipids and $CaCl_2$ and subtracted from the sample results. The linearity of the assay was confirmed by a 30 min time-course of histone phosphorylation.

Additional control experiments were performed with mock cell lysates to estimate the endogenous PKCε and non specific activities, which represented less than 1% of the total enzyme activation measured.

Kinase activity assays were repeated at least for three times.

8. ELECTROPHORESIS TECHNIQUES.

Two electrophoretic methods have been used in this work; one for the separating fragments of nucleic acids (DNA) and another for proteins. In both cases the separation depends on the size of macromolecules.

8.1. Agarose gel preparation.

The suitable amount of agarose is dissolved in TAE buffer, which contains 24.2% trizma base, 5.7% glacial acetic acid (v/v) and 50 mM EDTA pH 8.0.

Before agarose polymerizes, an intercalating agent (etidium bromide) was added (2 µg/ml) in order to visualize DNA fragments in the later steps. DNA is loaded in the gel with the help of loading buffer (0.25% brome-phenol blue (2/v), 30% glycerol (v/v) and Tris 0.5 mM pH 8.0).

After a suitable running time, the gel was exposed to ultraviolet (UV) light to visualize the DNA fragments.

8.2. Acrylamide gel preparation.

Polyacrylamide gels were elaborated under denaturalization conditions with SDS (SDS-PAGE), following *(Laemmli, 1970)* method. Mini-PROTEAN Tetra Cell (BioRad Laboratories, CA, USA) was used to run this kind of gels.

The running gel was composed by 10% acrylamide-bisacrylamide (2/v) dissolved in 0.375 M Tris-HCL buffer pH 8.8 and 0.1% SDS (w/v). It was added 0.064% ammonic persulfate (PSA) (w/v) and 0.64% TEMED (w/v) to the mix in order to polymerize it.

Stacking gel was made above running gel. In this case, the mix contains 5% acrylamide-bisacrylamide (2/v) dissolved in 0.13M Tris-HCl pH 6.8 and 0.1% SDS (w/v). As before, PSA and TEMED were added to polymerize de mix, although in this occasion 0.1% (w/v). The function of this gel is to concentrate proteins in order for then to penetrate at the same time in the running gel.

Samples were dissolved in Sample buffer (40 mM Tris-HCL pH 6.8, 50 mM DTT (w/v), 1% SDS (w/v), 7,5% glycerol (w/v) and 0.003% bromophenol blue (w/v)) before loading them in stacking gel.

The running buffer used contained 25 mM Tris-HCL pH 8.3, 192 mM glycine and 0.1% SDS (w/v).

The gel was stained with Coomassie Blue (0.1% Coomassie blue R-250 (w/v) dissolved in 40% methanol (v/v) and 10% acetic acid (v/v)) for 20 minutes and follow on destaining step with 40% methanol (v/v) and 10% acetic acid (v/v).

8.3. Western blot.

After running a polyacrylamide gel with appropriate samples, proteins are transferred to 0.2 µm pore nitrocellulose membrane. To do that, a semidry system from BioRad (BioRad Laboratories, CA, USA) was used with a continue potential of 20 V during 45 minutes, and cold transfer buffer composed by 1x of NuPAGE commercial transfer buffer (v/v) and 10% methanol (v/v).

Non-specific interaction sites of the antibody were blocked with a solution of 2% BSA (w/v) in TBT (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20 (v/v)) during one hour with gently agitation at room temperature.

After that, membranes were incubated overnight with primary antibody in the appropriate dilution in blocking buffer (2% BSA in TBST) at 4ºC. PKCε was detected by using a polyclonal mouse antibody (dilution 1:2000) (BD Biosciences Clontech, Palo Alto, CA, USA), while to detect GAPDH (dilution 1:1000) a monoclonal mouse antibody was used (Abcam, Cambridge Science Park).

Next morning, membrane was rinsed for three times with TBST in agitation before a secondary blocking incubation (2% BSA in TBST) for 10 minutes three times.

Afterwards, the secondary antibody, which is marked with Alexa Fluor 488, (goat anti-mouse) (Invitrogen, Oregon, USA) was applied to membrane in a dilution of 1:10000 (in blocking buffer). Next, the membrane was again washed for three times.

Finally, immunoblotting was detected with a fluorescence scanner (Thyphoon, Amersham Bioscience). To reveal the membrane, it was excited at 488 nm and detected at 520 nm. Captured images were analyzed in several ways thanks to software incorporated by Amersham.

9. MASS SPECTROMETRY OF LIPIDS.

Mass spectrometry (MS) has increasingly become an analytical tool of choice in lipidomics studies owing to its speed, wide dynamic signal range, quantitative capability and the facility to interface with chromatographic separation methods. Due to its specificity and complexity, lipidomics research is quite challenging. As opposed to genomics and proteomics, there is no available information that can predict the number of individual lipid molecules present in an organism. Current technologies are therefore still unable to map lipidomes.

9.1. General structure of Mass Spectrometer.

Generally, a typical Mass Spectrometer consists of three parts: an ion source (for producing gaseous ions from the substance being studied), a mass analyzer (for resolving the ions into their characteristics mass components according to their mass-to-charge ratio) and a detector system (for detecting the ions and recording the relative abundance of each of the resolved ionic species).

Besides this, previous steps related with the sample preparation and the separation by chromatographic techniques of the sample to study may increase the success of the analysis.

9.1.1. Chromatographic device.

Liquid chromatography (LC) coupled to tandem mass spectrometry, called LC-MS/MS (sometimes abbreviated simply as LC-MS), is a powerful technique for the analysis of lipids. This methodology combines efficient separations of biological materials and sensitive identification of the individual components by mass spectrometry. Complicated mixtures containing hundreds of different species of lipids can be analyzed directly even when concentration levels of different lipids vary by orders of magnitude. Including liquid chromatography enables analysis of the phospholipidome at a level of detail that is not achievable by mass spectrometry alone. Furthermore, the use of a chromatographic separation of phospholipids has benefits when quantitative analysis is required such as the reduction of ion suppression *(Brouwers, 2011)*.

9.1.2. Ion source.

Mass spectrometry relies on the ability to manipulate ions in the gaseous state on the basis of mass to charge ratios. This implies that a crucial step in mass spectrometry is to get analytes both ionized and in the gas phase. This dual process in MS is called ionization and is performed by an ion source.

The two primary methods for ionization of whole lipids are electrospray ionization (ESI) and atmospheric pressure chemical ionisation (APCI).

The development of electrospray ionization (ESI) has revolutionized the analysis of mass spectrometric analysis of biomolecules, including lipids. ESI starts by a spray of charged lipid droplets from an emitter and subsequent evaporation of the solvent from these droplets. This then leads to an increase of charge at the droplets surface, and when the surface tension of the droplet no longer suffices to overcome the repulsive forces of surface ions, droplets break up in smaller droplets, thus increasing that available surface area.

With APCI source, ionization takes place in the gaseous phase, in contrast to ESI where ionization occurs in the fluid phase. To achieve vaporization and desolvation of the neutral analyte, the effluent from an HPLC column is nebulized from a nozzle with assistance of a nebulizing gas. Droplets that are thus formed are quickly evaporated in a heating chamber (Figure II.10).

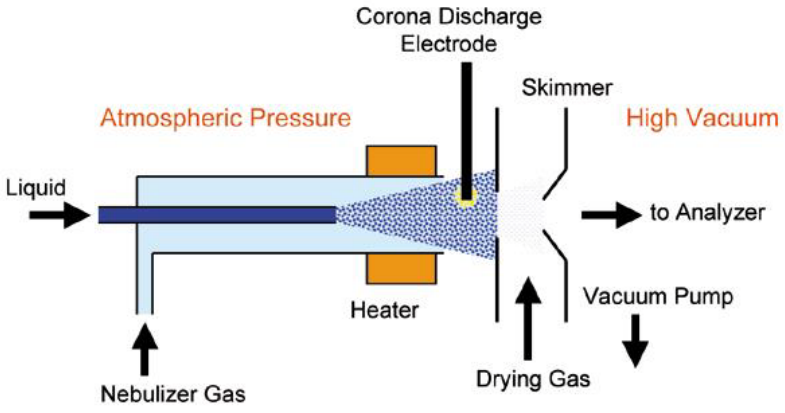

Figure II.10. Atmospheric pressure chemical ionization schematic.

Modern, optimized heating chambers are operated at temperatures well above the Leidenfrost temperature. At these temperatures, droplets that are directed at the heating chamber's wall will not make contact because of the

formation of a thin layer of vapor between droplet and wall, comparable to a few drops of water that fall onto a hot plate. This will let the droplet 'bounce' off, losing a significant part of the solvent by evaporation and leading to droplet disintegration. Repetitive contacts with the wall, induced by the turbulent gas flow in the chamber lead to vaporized analyte molecules *(Brouwers, 2011)*.

9.1.2. Mass analyzer.

The mass analyzer or analyzers sort and separate the ions according to their mass-to-charge ratio (m/z).

There are two broad categories of mass analyzers: the scanning and ion-beam mass spectrometers, such as time-of-flight (TOF) and quadrupoles (Q); and the trapping mass spectrometers, such as ion trap (IT), Fourier transform ion cyclotron resonance (FTICR), and Orbitrap. IT, Orbitrap, and FT-ICR mass analyzers separate ions based on their m/z resonance frequency, quadrupoles (Q) use m/z stability, and time-of-flight (TOF) analyzers use the flight time of the ions. Each mass analyzer has unique properties, such as mass range, analysis speed, resolution, sensitivity, ion transmission, and dynamic range.

In this work, the lipidomics analysis was carried out using The 4000 Q TRAP, a hybrid triple quadrupole in tandem with the linear ion trap (LIT).

The hybrid quadrupole linear ion trap (QqLIT) is based on an API 4000 triple quadrupole platform commercially named Q TRAP (AB/MDS Sciex).

A triple quadrupole mass spectrometer is a tandem mass spectrometer consisting of two quadrupole mass spectrometers in series, with a (non mass-resolving) radio frequency (RF)-only quadrupole between them to act as a cell for collision-induced dissociation (Figure II.11).

The first (Q_1) and third (Q_3) quadrupoles serve as mass filters. Precursor ions selected in Q1 are dissociated in the collision cell in the presence of an inert gas such as Ar, He, or N_2 collision-induced dissociation. Resulting fragments are passed through to Q_3 where they may be filtered or scanned. This configuration is often abbreviated QqQ.

The linear ion trap is an extremely fast but low resolution and low mass accuracy mass analyzer (+/- 0.5 m/z typical accuracy). This type of mass spectrometer confines and manipulates ions through the use of quadrupolar radio-frequency (RF) fields (as quadropole system). Linear ion traps are extremely flexible and are used for collisional dissociation, ion-ion interactions, and ion storage prior to higher resolution mass analysis. Series of linear ion traps can even be strung together to create more flexible mass spectrometers, that are coupled to higher resolution mass spectrometers in "hybrid" instruments.

The combination of triple quadrupole MS with LIT technology in the form of an instrument of configuration QqLIT, using axial ejection, is particularly interesting, because this instrument retains the classical triple quadrupole scan functions such as selected reaction monitoring (SRM), product ion (PI), neutral loss (NL) and precursor ion (PC) while also providing access to sensitive ion trap experiments.

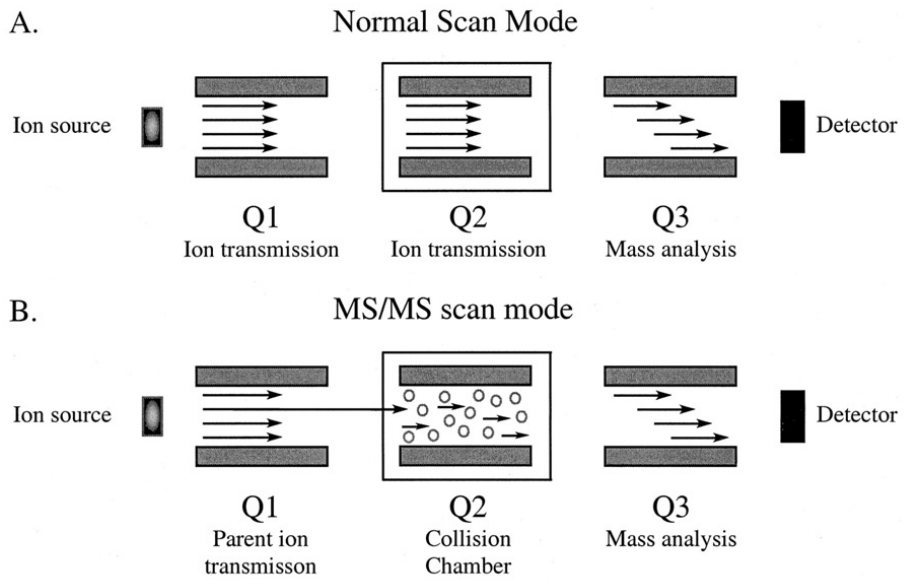

Figure II.11. Conventional and MS/MS modes of analysis in a triple-quadrupole mass spectrometer. (A) In the normal scanning mode, all ions of a certain m/z range are transmitted through the first two quadrupoles for mass analysis in the third quadrupole. From this MS spectrum, a parent ion is selected for fragmentation in the collision cell. (B) In MS/MS mode, the parent ion is selectively transmitted into the collision chamber and fragmented, and the resulting daughter ions are resolved in the third quadrupole. *(Taken from Graves P R et al 2002).*

Also, the QqLIT allows two particular modes to perform MS/MS experiment in a time-delayed fashion (TDF) and the selection of multiply charged ions in the trap mode (EMC). The particularity of the system is that

basically they are no new scan functions, but scan combinations of triple quadrupole mode and trap mode can be performed in the same LC/MS run, which is unique and offers new possibilities for quantitative and qualitative analysis *(Hopfgartner et al., 2004)*.

9.1.3. Computer.

The computer registers the data rendered by the detector and converts them either into values of masses and peak intensities or into total ionic current, temperatures, acceleration potential values, etc. The computer can control the mass spectrometer by a feedback circuit.

9.1.4. Detector.

The final element of the mass spectrometer is the detector. The detector records either the charge induced or the current produced when an ion passes by or hits a surface. In a scanning instrument, the signal produced in the detector during the course of the scan versus where the instrument is in the scan (at what *m/Q*) will produce a mass spectrum, a record of ions as a function of *m/Q*.

9.2. Experiments to determine the lipidomics changes in RBL-2H3 cells

9.2.1. Sample preparation.

Rat basophilic leukemia (RBL-2H3) cells were cultured under the culture conditions described previously. Cells were counted and aliquots of $9x10^6$ cells (for the isolation of plasma membrane) or $3x10^6$ cells (for complete pellets) were taken apart and centrifuged, resuspended and plated on 9 or 6 cm^2 dishes respectively.

The different conditions of study were the followed:
A. *Control cells*: Cells were growing during three days before being harvested.
B. *Control - Antibody cells:* After being one day plated, cells were primed overnight with 500 ng/ml IgE-anti-dinitrophenyl (mouse monoclonal; Sigma-Aldrich Quimica, S.A., Madrid, Spain) growing for another 24 hours.

C. *Stimulated cells:* Once the cells were primed overnight with Ig-E-anti-dinitrophenyl, they were stimulated with the antigen (DNP-HSA) during different times of exposure (1 and 5 min) and at different concentrations of DNP-HSA (2 µg/ml and 100 ng/ml).
D. *Use of inhibitors:* The various phospholipase inhibitors were present 10 min before stimulation at the following concentrations: 30 µM U73122, 50 mM 1-butanol, 100 µM propranolol, and 20 µM DGK inhibitor II (R59022). In all cases, we used a concentration of antigen of 2 µg/ml during 1 or 5 min.

9.2.2. Extraction and separation of lipids.

For the extraction of lipids from RBL-2H3 plasma membrane isolations or total lipids from RBL-2H3 pellets, we used the method described previously by *(Bligh and Dyer, 1959)*. The phospholipids were separated from neutral lipids (cholesterol, diacylglycerols, ceramides and cholesterol esters) by fractionation on a 1 ml silica column prepared from 10 mg of 0.063–0.200mm silica 60 (Merck, Darmstadt, Germany) according to the method of *(Rouser et al., 1976)*. Lipid standards were obtained from Avanti Polar Lipids (Allabaster, AL, USA). All solvents were supplied by Biosolve (Valkenswaard, The Netherlands).

9.2.3. HPLC Mass Spectrometry Analysis of Neutral Lipids.

For the analysis of neutral lipids we used the method described previously by *(Aardema et al., 2013)*. Briefly, isolated neutral lipids were dissolved in 1000 µl of methanol/chloroform (1:1, v/v) and 20 µl was injected on a Halo C8 (150 × 3.0 mm, particle size of 2.7 µm) HPLC column (Advanced Material Technology, Inc., Wilmington, DE) maintained at 40°C. Lipids were eluted by a linear gradient from methanol/water (50:50, v/v) to methanol/2-propanol (80:20, v/v) in 5 min, followed by isocratic elution with the latter solvent for 20 min and regeneration of the column for 5 min, all at a flow rate of 0.3 ml/min. The column effluent was introduced by an atmospheric pressure chemical ionization (APCI) interface (AB Sciex Instruments, Toronto, ON, Canada) into a 4000 QTRAP mass spectrometer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Source temperature was set to 450°C, and nitrogen was used as curtain gas. The declustering potential was set to 100 V and the needle current to 3 µA. For maximal sensitivity and for linearity of the response, the mass spectrometer was operated in multiple-reaction monitoring (MRM) mode

at unit mass resolution and selected ion transitions of the most representative neutral lipid species were monitored. Peaks were identified by comparison of retention time and mass spectrum with authentic standards of the diacylglycerol described (1,3-di-(9E-Octadecenoyl)-glycerol) and 3-Ketocholesterol (Merck). Cholesterol levels were determined in APCI multiple reaction monitoring mode and 3-Ketocholesterol was used as an internal standard using the transitions 369.3 → 161.1 and 385.3 → 109.1 at a collision energy of 32 V for cholesterol and internal standard, respectively.

Ion transitions monitored for ceramides were the followed:

Ceramide SPECIES	[M+H]+ Q1	[M+H]+ Q3
d18:1/16:0	520.5	264.25
d18:1/18:1	546.5	264.25
d18:1/18:0	548.5	264.25
d18:1/20:4	568.5	264.25
d18:1/20:5	576.5	264.25
d18:1/22:6	592.5	264.25

Ceramide SPECIES	[M+H]+ Q1	[M+H]+ Q3
d18:1/22:5	594.5	264.25
d18:1/24:0	632.7	264.25
d18:1/24:1	630.7	264.25
d18:1/26:1	656.6	264.3
d18:1/26:0	658.6	264.3

The transitions for the diacylglycerols (DAG) were the followed:

DAGs SPECIES	[M+H]+ Q1	[M+H]+ Q3
15:0/15:0	523.5	225.2
16:0/16:1	549.5	237.2
14:0/18:1	549.5	265.2
16:0/16:0	551.5	239.2
16:0/17:1	563.5	251.2
15:0/18:1	563.5	265.2
16:0/18:3	573.5	261.2
16:1/18:2	573.5	263.3
16:1/18:1	575.5	265.2
16:0/18:2	575.5	263.2
18:1/16:1	575.5	237.2
18:0/16:0	579.5	239.2
17:1/18:1	589.5	265.2
17:0/18:2	589.5	263.2

DAGs SPECIES	[M+H]+ Q1	[M+H]+ Q3
16:0/20:4	599.5	287.3
18:1/18:3	599.5	261.2
18:2/18:2	599.5	263.3
18:3/18:1	599.5	265.2
18:2/18:1	601.5	265.2
18:0/18:3	601.5	261.2
16:0/20:3	601.5	289.2
16:0/20:2	603.5	291.2
18:1/18:1	603.5	265.2
18:0/18:2	603.5	263.2
18:0/18:1	605.5	265.2
16:0/20:1	605.5	293.3
20:4/18:1	625.5	265.2
20:2/18:1	629.5	265.2

9.2.4. HPLC Mass Spectrometry Analysis of Phospholipids.

The phospholipid fraction was dissolved in 1000 µl of methanol/acetonitrile/ chloroform/water (46:20:17:17) and 10 µl was injected on a Halo C18 (150 × 3.0 mm, particle size of 2.7 µm) HPLC column (Advanced Material Technology, Inc., Wilmington, DE) maintained at 55°C. Lipids were eluted by a linear gradient from acetonitrile/methanol/H_2O (30:45:25, v/v) to acetone/methanol (40:60, v/v) in 7 min, all at a flow rate of 0.3 ml/min. 2.5 mM ammonium acetate and 1 µM serine were used in all solvents as additives. The column effluent was introduced by an atmospheric pressure chemical ionization (APCI) interface (AB Sciex Instruments, Toronto, ON, Canada) into a 4000 QTRAP mass spectrometer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Source temperature was set to 450°C, and nitrogen was used as curtain gas.

Peaks were identified by comparison of retention time and mass spectrum with authentic standards of the phosphatidic acid, phosphatidylethanolamine, phosphatidylcholine, phosphatidilinositol and phosphatidilserine (Avanti Polar Lipids, Allabaster, AL, USA)

For the determination of phosphatidic acid, the system was operated in negative multi reaction mode (MRM), and the declustering potential was set to -140 V.

The transitions for the phosphatidic acid species were the followed:

TOTAL PA	[M-H]- Q1	[M-H]- Q3
32:2	643.5	153.1
32:1	645.5	153.1
32:0	647.5	153.1
34:4	667.5	153.1
34:3	669.5	153.1
34:2	671.5	153.1
34:1	673.5	153.1
34:0	675.5	153.1
36:5	693.5	153.1
36:4	695.5	153.1
36:3	697.5	153.1

Time (msec)	[M-H]- Q1	[M-H]- Q3
36:2	699.5	153.1
36:1	701.5	153.1
36:0	703.5	153.1
38:6	719.5	153.1
38:5	721.5	153.1
38:4	723.5	153.1
38:3	725.5	153.1
38:2	727.5	153.1
38:1	729.5	153.1
38:0	731.5	153.1

Other transitions of phospholipids used were

PL	[M-H]- Q1	[M-H]- Q3
PI (38:4)	885.5	241.1
PI (36:2)	861.5	241.1
PI (36:1)	863.5	241.1
PE (36:2)	742.5	196.1

For the rest of phospholipids, system was operated in positive ion mode using the following settings: source temperature 420ºC, nebulizer gas (GS1) 5, nebulizer current 3 mA, curtain gas 10, collision gas High and declustering potential 100 V. The optimal collision energy was set to +10 V (full scan mode).

In all full scan runs spectra were obtained from m/z 350–900. For quantitative analysis, relative response factors of lipid classes were determined using the authentic standards described above.

9.3. Data Processing.

Lipid data were recorded with Analyst version 1.4.2 software (MDS Sciex, Concord, ON, Canada) and exported to an mzXML format. Peak detection, integration and alignment were performed using the open source software package XCMS running on R software *(Smith et al., 2006)*. A correlation matrix of the combined data sets was calculated in R. Principal component discriminant analysis was performed with Markerview version 1.1 software (MDS Sciex), using Pareto scaling, where each value is subtracted by its average in all samples and divided by the square root of the standard deviation. In this way, data maintains a dimension (units), and peaks with a good signal noise ratio will gain importance, without having intense peaks dominating the analysis.

CHAPTER III

Phosphatidylinositol 4,5-bisphosphate decreases the concentration of Ca^{2+}, phosphatidylserine and diacylglycerol required for protein kinase C α to reach maximum activity.

ABSTRACT

The C2 domain of PKCα possesses two different binding sites, one for Ca^{2+} and phosphatidylserine and a second one that binds PIP_2 with very high affinity. The enzymatic activity of PKCα was studied by activating it with large unilamellar lipid vesicles, varying the concentration of Ca^{2+} and the contents of dioleylglycerol (DOG), phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphadidylserine (POPS) in these model membranes. The results showed that PIP_2 increased the V_{max} of PKCα and, when the PIP_2 concentration was 5 mol% of the total lipid in the membrane, the addition of 2 mol% of DOG did not increase the activity. In addition PIP_2 decreases $K_{0.5}$ of Ca^{2+} more than 3-fold, that of DOG almost 5-fold and that of POPS by a half. The $K_{0.5}$ values of PIP_2 amounted to only 0.11 μM in the presence of DOG and 0.39 in its absence, which is within the expected physiological range for the inner monolayer of a mammalian plasma membrane. As a consequence, PKCα may be expected to operate near its maximum capacity even in the absence of a cell signal producing diacylglycerol. Nevertheless, we have shown that the presence of DOG may also help, since the $K_{0.5}$ for PIP_2 notably decreases in its presence. Taken together, these results underline the great importance of PIP_2 in the activation of PKCα and demonstrate that in its presence, the most important cell signal for triggering the activity of this enzyme is the increase in the concentration of cytoplasmic Ca^{2+}.

1. INTRODUCTION

PKCα is a classical PKC isoenzyme that is activated by second messengers, namely the increase in Ca^{2+} concentration in the cytoplasm of the cell and the appearance of diacylglycerol in the membrane, where it establishes specific interactions with phosphatidylserine and PIP_2 *(Corbalan-Garcia and Gomez-Fernandez, 2006)*.

The translocation of classical PKCs (cPKCs) to the plasma membrane is mediated by the C1 and C2 domains, and it has been shown that initial membrane affinity is mainly determined by C2 domain–membrane interactions, followed by C1 domain–diacylglycerol interactions *(Corbalan-Garcia and Gomez-Fernandez, 2006)*. One of the main sources of diacylglycerol in the plasma membrane following cell stimulation is PIP_2 which is hydrolyzed by phospholipase C to produce diacylglycerol and IP_3, which together activate protein kinase C for sustained cellular responses *(Nishizuka, 1995)*. However, it has been shown that PIP_2 may also activate PKCα by direct binding to a polylysine motif located in strands β3 and β4 *(Corbalan-Garcia et al., 2003a; Guerrero-Valero et al., 2007b; Marin-Vicente et al., 2005a; Marin-Vicente et al.,*

2008b; Sanchez-Bautista et al., 2006a) and that can be considered a specific site for PIP_2 *(Guerrero-Valero et al., 2009a)* (see Fig. III.1). Other molecules like phosphatidylserine or phosphatidic acid *(Ochoa et al., 2002)* or even retinoic acid *(Ochoa et al., 2003)* may also bind with lower affinity to this site. It has been clearly shown that PIP_2 is important for PKCα translocation to the membrane and for prolonging this translocation . Rapid *(Evans et al., 2006; Marin-Vicente et al., 2008a; Perez-Lara et al., 2012*) kinetics studies on the binding of this enzyme to model membranes suggested that the interaction of PKCα with membranes occurs via two steps: a rapid weak recruitment to the membrane due to non-specific interactions with (primarily) anionic lipids and the formation of a high affinity complex due to stereospecific interactions of each PKCα domain with its specific ligands *(Perez-Lara et al., 2012)*.

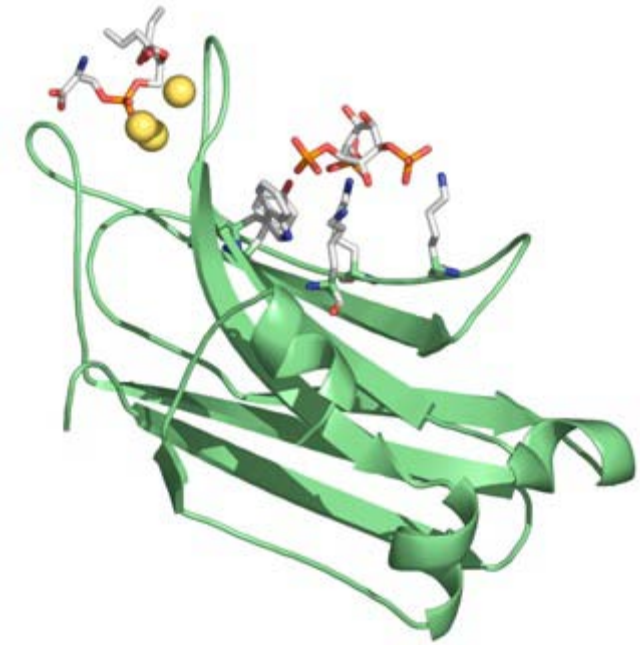

Figure III.1. Structure of PKCα C2 domain bound to Ca^{2+}-POPS-PIP_2 in a quaternary complex. The C2 molecule is shown in green. The three calcium ions are shown in yellow spheres, one of them bridging the protein with phosphatidylserine (PS) at the tip of the domain. The PIP_2 molecule is bound to the β3-β4 chains *(Guerrero-Valero et al., 2009a; Guerrero-Valero et al., 2009b)*. PDB accession number 3GPE.

PKCα enzyme is a paradigmatic example for bearing a C2 domain which may simultaneously bind three different activators, in this case Ca^{2+}, phosphatidylserine and PIP2. Fig. III.1 shows this C2 domain in which Ca^{2+} binds to its site, acting as a bridge for phosphatidylserine, although this phospholipid also directly interacts with several protein residues *(Conesa-Zamora et al., 2001; Verdaguer et al., 1999)*. In another site located in a β-groove, PIP_2 binds with great affinity.

Previous work has shown that PKCα exhibits high cooperativity in its activity by phosphatidylserine *(Hannun et al., 1985; Newton and Koshland, 1989)* and that the two second messengers of the kinase, diacylglycerol and Ca^{2+},

markedly increase the affinity of the kinase for phosphatidylserine *(Orr and Newton, 1992)*. In this paper, we use highly purified full-length PKCα to perform a kinetic study of the activation of PKCα by model membranes, in which the concentrations of POPS, DOG, PIP_2 and Ca^{2+} are varied. Our results indicate that PIP_2 enhances PKCα activity and decreases the required concentrations of the other activators, to reach maximum activities.

2. RESULTS

2.1 Enzyme kinetic studies.

The important contribution of PIP_2 to PKCα enzymatic activity was clearly observed when it was studied as a function of Ca^{2+} concentration. A POPC/POPS molar ratio of about 4 was used in these assays since the concentration of POPS in the inner monolayer of eukaryotic plasma membranes, such as in erythrocyte or platelet cells, is roughly this *(Chap et al., 1977; Leventis and Grinstein, 2010; Verkleij et al., 1973)*. The physiological concentration of PIP_2 has been described to be around 1 mol% of the total lipid of plasma membranes *(Di Paolo and De Camilli, 2006; McLaughlin et al., 2002)* and it is likely to be concentrated in the inner monolayer at 2 mol%, which increase locally if it forms clusters or patches *(Huang et al., 2004)*. As regards diacylglycerol, the physiological levels of this lipid in biomembranes were reviewed in *(Gomez-Fernandez and Corbalan-Garcia, 2007)*. For example, quantitative measurements of diacylglycerols present in stimulated cells have shown that they may reach 1.45 mol% of the total lipid concentration *(Preiss et al., 1986)* or about 2 mol% *(Takuwa et al., 1987)*. So the concentrations of diacylglycerol used in this work can be considered physiological and well within the range of diacylglycerol concentrations used in standard procedures for PKC activation assays, which use values similar to those used here *(Sanchez-Pinera et al., 1999)* or even as high as 11.5 mol% with respect to total lipid *(Ogita et al., 1991)* or as 19 mol% *(Wooten et al., 1987)* or 25 mol% *(Bolen and Sando, 1992)*. In enzymatic studies where the effect of lipid concentrations were studied, 200 μM Ca^{2+} was used in order to ensure that this cation was not a limiting factor.

When the dependence of PKCα activity on Ca^{2+} concentration was studied (Fig. III.2) the sigmoidal curves obtained in all cases, pointed to

cooperativity. In the presence of POPC/POPS (80:20 molar ratio) alone, increasing concentrations of Ca^{2+} led to a cooperative increase in activity, with a $K_{0.5}$ of 1.30 μM in Ca^{2+} (see Table III.1), rising from 107.6 nmol Pi/min/mg at 0.1 μM in Ca^{2+} to a V_{max} of 898.4 nmol Pi/min/mg and a Hill coefficient of 2.28. If DOG was added to the membrane to give a composition of POPC/POPS/DOG (78:20:2 molar ratio) the cooperative behavior was again present, but now the $K_{0.5}$ was 0.84 μM in Ca^{2+}, with the activity raising from 70.5 nmol Pi/min/mg at 0.1 μM Ca^{2+} to a V_{max} of 1192.7 nmol Pi/min/mg and a Hill coefficient of 2.42. It is clear that in the presence of DOG, the $K_{0.5}$ for Ca^{2+} decreases, and there is an increase in V_{max}.

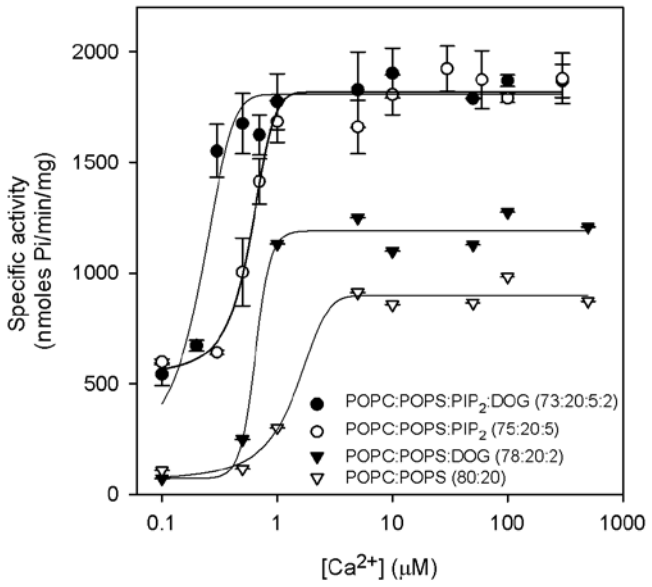

Figure III.2. The dependence of PKCα activity on Ca^{2+} concentration. The molar ratios of the lipid components of the vesicles used to activate the enzyme are shown.
SD calculated from 3 independent experiments.

When PIP_2 was added to the membrane to give a composition of POPC/POPS/PIP_2 (75:20:5 molar ratio), $K_{0.5}$ was now 0.59 μM of Ca^{2+} (Table III.1), the activity was 597.4 nmol Pi/min/mg at 0.1 μM of Ca^{2+} and V_{max} was 1790.7 nmol Pi/min/mg at 10 μM of Ca^{2+}, the higher cooperativity giving a Hill coefficient of 4.10. Even higher cooperativity (Hill coefficient of 8.61) was observed for a membrane which incorporated also DOG, namely PC/PS/PIP_2/DOG (73:20:5:2 molar ratio), although the activity levels did not change with respect to the membrane without DOG, being now 512.9 nmol Pi/min/mg at 0.1 μM of Ca^{2+} while V_{max} was 1804.9 nmol Pi/min/mg at 10 μM of Ca^{2+}. It is interesting that at very low concentration of Ca^{2+}, e.g. 0.1 μM, the activity in the presence of PIP_2 was higher than in the absence of this phosphoinositide, both in the presence and in the absence of DOG. However, the addition of 2% DOG did not increase the activity levels when 5 mol% PIP_2 was present.

Lipid mixture	$K_{0.5}$	V_{max} (nmol Pi/min/mg)	*n*
POPC:POPS (80:20)	1.30 ± 0.08	898.2 ± 27.8	2.28 ± 0.89
POPC:POPS:DOG (78:20:2)	0.84 ± 0.27	1192.7 ± 96.3	2.42 ± 0.69
POPC:POPS:PIP_2 (75:20:5)	0.58 ± 0.09	1790.7 ± 64.4	4.10 ± 0.92
POPC:POPS:PIP_2:DOG (73:20:5:2)	0.26 ± 0.01	1804.9 ± 104.4	8.61 ± 0.27

Table III.1. Dependence of PKCα activation on Ca^{2+}. $K_{0.5}$, *n* and V_{max} were obtained by nonlinear least square fit of the data in the equation described in the Chapter II, Material and Methods.

The effect of increasing POPS concentration was also studied (Fig. III.3). When PIP_2 was absent in a POPC/POPS/DOG mixture (98-x:x:2 molar ratio), the effect observed was of positive cooperativity (very high Hill coefficient of 13.18), with a $K_{0.5}$=15.12 mol% of POPS (Table III.2), which is similar to the effect observed previously for membrane activation of this enzyme [14]. V_{max} was 1260.2 nmol Pi/min/mg.

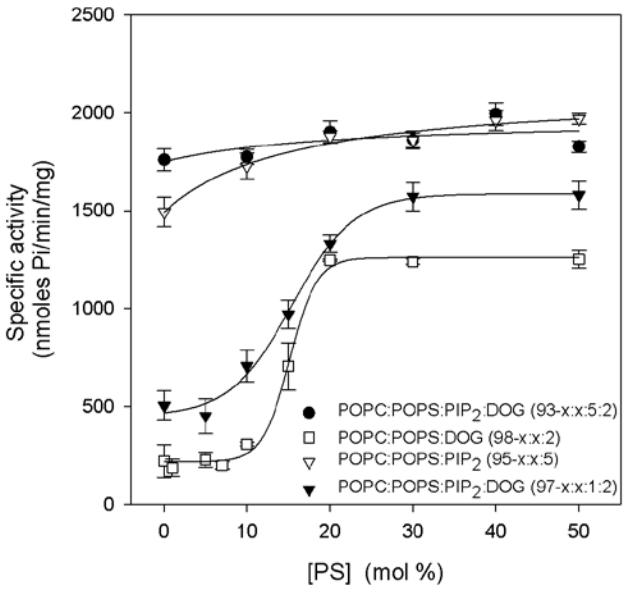

Figure III.3. The dependence of PKCα activity on POPS molar percentage in vesicles. The molar ratios of the lipid components of the vesicles used to activate the enzyme are shown. Ca^{2+} concentration was 200 µmol.
SD calculated from 3 independent experiments.

If PIP_2 was also present, at just 1 mol%, in a mixture containing POPC/POPS/PIP_2/DOG (97-x:x:1:2 molar ratio),positive cooperativity was still

observed, with a Hill coefficient of 3.71, an initial activity of 490 nmol Pi/min/mg in the absence of POPS and a V_{max} of 1586.2 nmol Pi/min/mg. $K_{0.5}$ was 15.4 mol% of POPS.

Lipid mixture	$K_{0.5}$	V_{max} (nmol Pi/min/mg)	n
POPC:POPS:DOG (98-x:x:2)	15.12 ± 0.24	1260.2 ± 48.4	13.18 ± 1.9
POPC:POPS:PIP2:DOG (97-x:x:1:2)	15.40 ± 0.71	1586.2 ± 71.1	3.71 ± 0.77
POPC:POPS:PIP_2 (95-x:x:5)	13.94 ± 1.45	2084.0 ± 73.6	1.10 ± 0.39
POPC:POPS:PIP_2:DOG (93-x:x:5:2)	8.20 ± 0.84	1895.8 ± 101.3	1.50 ± 0.56

Table III.2. Dependence of PKCα activation on POPS. $K_{0.5}$, n and V_{max} were obtained by nonlinear least square fit of the data in the equation described in the Chapter II, Material and Methods.

If PIP_2 was present in the lipid mixture, but with no DOG, POPC/POPS/PIP_2 (95-x:x:5), the initial activity, even in the absence of POPS, was already high, with a value of 1492 nmol Pi/min/mg. V_{max} reached a value of 2084.1 nmol Pi/min/mg and $K_{0.5}$ was 13.94 mol% of POPS. Thus the addition of PIP2 decreased $K_{0.5}$ even if DOG was not present, and the activity was almost saturated and no apparent cooperativity was observed (n=1.10).

Fig. III.3 also shows that when PIP_2 was increased to 5 mol%, to give a lipid mixture of POPC/POPS/PIP_2/DOG (93-x:x:5:2 molar ratio), a very small increase in activity was already observed when POPS was increased since nearly maximum activity was observed in the absence of POPS (1760 nmol Pi/min/mg) and V_{max} was 1895.8 nmol Pi/min/mg, with $K_{0.5}$ of 8.20 mol% POPS and a Hill coefficient of 1.50.

Fig.III.4 shows the activity studied as function of DOG concentration. When the membrane was composed of POPC/POPS/DOG (75-x:25:x), $K_{0.5}$ was 0.82 mol% of DOG (Table III.3).

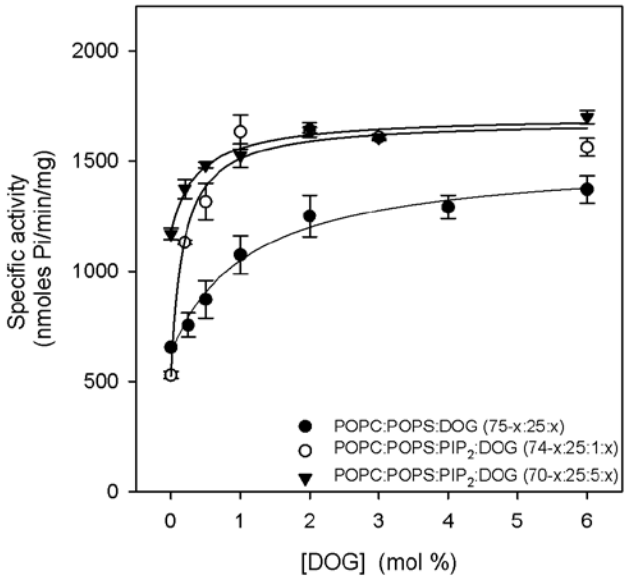

Figure III.4. The dependence of PKCα activity on DOG molar percentage in vesicles. The molar ratios of the lipid components of the vesicles used to activate the enzyme are shown. Ca^{2+} concentration was 200 µmol.
SD calculated from 3 independent experiments.

Lipid mixture	$K_{0.5}$	V_{max} (nmol Pi/min/mg)	*n*
POPC:POPS:DOG (75-x:25:x)	0.82 ± 0.08	1307.9 ± 52.7	1.59 ± 0.22
POPC:POPS:PIP_2:DOG (74-x:25:1:x)	0.38 ± 0.10	1681.7 ± 85.9	1.39 ± 0.01
POPC:POPS:PIP_2:DOG (70-x:25:5:x)	0.17 ± 0.05	1701.9 ± 69.2	0.47 ± 0.15

Table III.3. Dependence of PKCα activation on DOG. $K_{0.5}$, *n* and V_{max} were obtained by nonlinear least square fit of the data in the equation described in the Chapter II, Material and Methods.

The activity was 666 nmol Pi/min/mg at 0 mol% of DOG and rose to give a Vmax of 1307.9 nmol Pi/min/mg and a Hill coefficient of 1.59, indicating low positive cooperativity. When PIP_2 was incorporated into this assay at just 1 mol% in a POPC/POPS/PIP_2/DOG (74-x:25:1:x molar ratio) mixture, $K_{0.5}$ was 0.38 mol% of DOG, which was notably lower than the 1.10 mol% observed in the absence of PIP_2, while the Hill coefficient showed little change (1.39). When the PIP_2 concentration was raised to 5 mol%, to give a POPC/POPS/PIP_2/DOG mixture (70-x:25:5:x molar ratio), $K_{0.5}$ decreased to 0.17 mol% DOG, although V_{max} maintained a similar value of 1701.9 nmol Pi/min/mg and the Hill coefficient (0.47) indicated an apparent negative cooperativity.

In another set of experiments, the concentration of PIP_2 was varied in the presence and in the absence of DOG, keeping the Ca^{2+} concentration constant at 200 µM. Fig. III.5 depicts the results obtained when the molar percentage in the membrane of PIP_2 was increased in the absence of DOG, POPC/POPS/DOG (75-x:25:x).

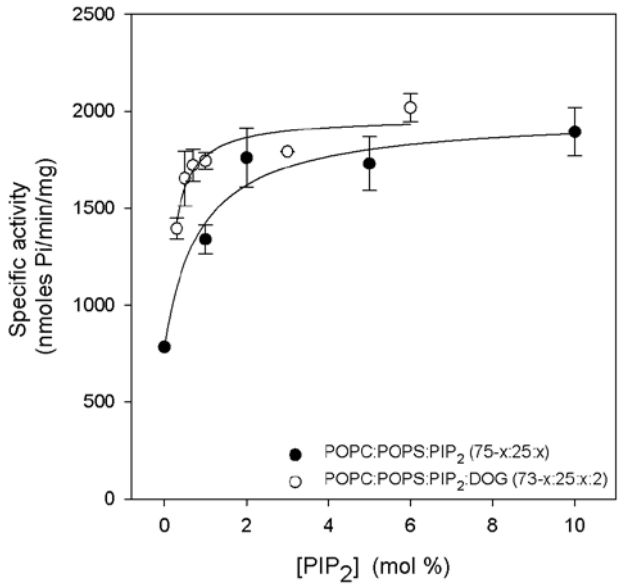

Figure III.5. The dependence of PKCα activity on PIP_2 molar percentage in vesicles. The molar ratios of the lipid components of the vesicles used to activate the enzyme are shown. Ca^{2+} concentration was 200 µmol.
SD calculated from 3 independent experiments.

Lipid mixture	$K_{0.5}$	V_{max} (nmol Pi/min/mg)	*n*
POPC:POPS:PIP_2 (75-x:25:x)	0.39 ± 0.61	1816.2 ± 143.5	1.60 ± 0.03
POPC:POPS:PIP_2:DOG (73-x:25:x:2)	0.11 ± 0.02	1857.6 ± 73.9	0.78 ± 0.15

Table III.4. Dependence of PKCα activation on PIP_2. $K_{0.5}$, *n* and V_{max} were obtained by nonlinear least square fit of the data in the equation described in the Chapter II, Material and Methods.

As can be seen, the $K_{0.5}$ was 0.39 (Table III.4) and the V_{max} 1816.2 nmol Pi/min/mg, with low positive cooperativity (n=1.60). In the presence of 2 mol% with a POPC/POPS/PIP_2/DOG membrane (73-x:25:x:2, molar ratio) $K_{0.5}$ decreased to 0.11 mol% PIP_2. This is interesting since it clearly demonstrates that very low concentrations (well below physiological concentrations) are sufficient to significantly enhance the activity of PKCα. V_{max} was 1857.6 nmol Pi/min/mg in this case, which is not significantly differrent with respect to the mixture without DOG, but the Hill coefficient was now 0.78, indicating apparent negative cooperativity during PIP_2 activation.

3. DISCUSSION

This study was designed to show the importance of PIP_2 for the catalytic activity of PKCα. Previous studies have shown that PIP_2 binds to a site located in the β3- and β4-sheets of the C2 domain, which is a conserved site found also in other C2 domains (Fig. III.1) *(Guerrero-Valero et al., 2009a)*. It was also shown that the presence of PIP_2 considerably increased the kinase activity of PKCα *(Corbalan-Garcia et al., 2003a)*. More recently the binding of PKCα to model membranes was studied by monitoring rapid kinetics *(Perez-Lara et al., 2012)*. It was observed that, in the absence of PIP_2, the protein rapid rate of binding was especially dependent on the POPS concentration and a high affinity complex evolved more slowly, which implies specific interactions with POPS and DOG. Both association and dissociation constants were decreased by the presence of PIP_2, but the association equilibrium constant was increased, i.e. the species of PKCα bound to the membrane was favored. A model was proposed in which PKCα binds to the membranes via a two-step mechanism consisting of a rapid initial recruitment of PKCα to the membrane driven by interactions with POPS and/or PIP_2, although interactions with DOG are also involved. PKCα carries out a 2-D search on the lipid bilayer to establish specific interactions with its specific ligands. In this way the longer time of residence of the enzyme in the bound state induced by PIP_2 could explain its activating effect.

In this work we have assayed in detail the enzymatic activity of PKCα, changing the concentrations of the different activators in order to obtain a comprehensive picture of the way in which PIP_2 may affect the catalytic action of this enzyme.

With respect to the dependence on Ca^{2+}, it was observed that, as it has long been known *(Takai et al., 1979)*, the addition of DOG to POPC/POPS increased to activity, the V_{max} going from 898.2 to 1192.7 nmol Pi/min.mg (Table III.1), and decreased $K_{0.5}$ *(Medkova and Cho, 1998)*, but the Hill coefficient was not significantly altered, showing positive cooperativity. Therefore, the binding of DOG to the C1 domain will influence Ca^{2+} binding to the C2 domain, facilitating the binding of the C2 domain to the membrane at lower Ca^{2+} concentrations, an increase in activity that can only be due to its capacity to retain the C1 domain bound to the membrane, impeding return to the inactive structure of PKC.

The addition of PIP_2 in the absence of DOG increased V_{max} with respect to a membrane containing POPC/POPS/DOG (1790.7 compared to 1192.7 nmol Pi/min.mg) and the further addition of DOG led to the maximum activation (V_{max} reaching 1804.9 nmol Pi/min.mg). However, the effect of DOG in this case was not great since the activity was already close to the maximum in the absence of DOG. Note that when PIP_2 is introduced and POPC/POPS/DOG and POPC/POPS/DOG/PIP_2 are compared, the $K_{0.5}$ for Ca^{2+} is reduced in the presence of PIP_2 more than three-fold (from 0.84 to 0.26 μM) and the Hill coefficient increases from 2.42 to 8.61. Since it is known that the presence of PIP_2 collaborates in the anchoring of the C2 domain to the membrane *(Corbalan-Garcia et al., 2003a; Corbin et al., 2007; Guerrero-Valero et al., 2009a; Perez-Lara et al., 2012)* and that Ca^{2+} is necessary to allow the binding of the protein to the membrane, it is clear that there is an interplay between these activators. It seems that the presence of PIP_2 lowers the amount of Ca^{2+} required for binding and activity and, at the same time, increases the cooperativity for the binding of Ca^{2+}. Indeed, it has been shown that PIP_2 markedly reduces the concentration of Ca^{2+} required for the binding of isolated C2 domain *(Corbin et al., 2007; Guerrero-Valero et al., 2007b)*.

It is known that up to 3 Ca^{2+} ions may bind at the calcium binding site of the C2 domain of PKCα *(Ochoa et al., 2002; Torrecillas et al., 2004a)*. A sequential model for classical PKC membrane binding and activation has been proposed *(Corbalan-Garcia et al., 2003a; Corbalan-Garcia and Gomez-Fernandez, 2006; Marin-Vicente et al., 2008a)*, whereby the increase in intracellular Ca^{2+} produces the binding of Ca1 and Ca2 when the protein is still in the cytosol, leading to the membrane being targeted by the enzyme through the C2 domain. Ca1 is responsible for bridging the protein to the phospholipid molecules (Fig. III.1), which are also recognized with the help of Asn189 and Arg216, whereas Ca2 is responsible for keeping Ca1 in its proper location and for inducing a conformational change in PKC, which partially penetrates and docks in the phospholipid bilayer by means of CBR3 (Arg249 and Thr251). Once recruited to the membrane, a third Ca^{2+} binds, stabilizing the C2 domain-membrane complex. This enables PKC to reside in the membrane for a longer time, allowing the C1 domain to find the diacylglycerol generated upon receptor stimulation *(Bolsover et al., 2003; Conesa-Zamora et al., 2000; Conesa-Zamora et al., 2001; Corbalan-Garcia et al., 1999; Feng et al., 2000; Garcia-Garcia et al., 1999; Medkova and Cho, 1998; Nalefski and Newton, 2003; Oancea and Meyer, 1998)*.

In the case of PKCα dependence on POPS, V_{max}, was clearly increased by the addition of 1 mol% of PIP_2, and the addition of 5 mol% in the absence of DOG led to very high activities, which did not increase even when DOG was added. This is an interesting result and confirms the great activation capacity of PIP_2, and shows that fixing the C2 domain of PKCα to the membrane through the calcium binding site and the PIP_2 site decreases the importance of the C1 domain respect to activity. However, the addition of DOG to the membrane containing 5 mol% PIP_2 reduced $K_{0.5}$ from 13.94 to 8.20 μM, showing that binding of the C1 domain may also play a role.

It has been described that POPS binds cooperatively to PKC, with a stoichiometry of 4 *(Ganong et al., 1986)*, ≥12 *(Newton and Koshland, 1989)* or approximately 8 *(Mosior and Newton, 1998)* lipid molecules per molecule of protein. A number of authors have observed apparent cooperativity for the activation in mixed micelles with Triton X-100, leading to high Hill coefficients (higher than 8 *(Hannun and Bell, 1988; Hannun et al., 1986a; Hannun et al., 1986b)* or about 5 *(Bazzi and Nelsestuen, 1987)* but when the activation was carried out with lipid vesicles, the Hill coefficients were about 2.6 (Orellana et al., 1990) or close to 1 *(Bazzi and Nelsestuen, 1987)*. In our case, a high degree of positive cooperativity was observed in the absence of PIP_2, with a Hill coefficient of 13.18. The use of different types of vesicles in the studies mentioned above may be the reason for the disparity of the results. However, the addition of just 1 mol% of PIP_2 reduced n to 3.71, and at 5 mol% PIP_2 no cooperativity was evident. Just one POPS molecule is known to bind to the C2 domain of PKCα *(Guerrero-Valero et al., 2009a)*, although more POPS molecules may bind to the C1 domain *(Bittova et al., 2001)*. It is interesting in this respect that Hill coefficients close to 1 were observed for the binding of the isolated C2 domain to phospholipid vesicles (A. Torrecillas, Ph.D. Thesis, University of Murcia, 2003).

However, it is nowadays recognized that a number of mechanisms may lead to kinetic cooperativity in the absence of true cooperative interactions, and kinetic models have been suggested to explain the apparent cooperativity observed for the binding of lipid to PKC, for example, proposing ligand trapping *(Sandermann and Duncan, 1991)* or effects specific to the interaction with multiple membrane associated ligands *(Mosior and Newton, 1998)* have been suggested, the last authors observing that the apparent cooperativity may be abolished in conditions where membrane binding involves a non-

phosphatidylserine mechanism, as in the presence of activators such as phorbol esters. This explains why PIP_2 reduces the apparent cooperativity so drastically.

Therefore, Hill indexes obtained for the binding of proteins, such as PKC to lipids in vesicles or in micelles, may be informative as regards threshold-binding and how this type of binding may be regulated by different ligands.

Diacylglycerol also plays a role, especially in the absence of PIP_2, but in the presence of the phosphoinositide its role is reduced. The reason for that may be related to the tighter anchoring of the enzyme as seen by stopped flow experiments using the full-length enzyme *(Perez-Lara et al., 2012)* and the different orientation of the C2 domain with respect to the membrane, as seen by studying the membrane docking of this domain *(Ausili et al., 2011)*. These effects occasioned by the interaction with PIP_2 may prolong the activated state. The interplay between DOG and PIP_2 was also evident when DOG was changed. Even at 1 mol% of PIP_2, $K_{0.5}$ decreased from 1.10 mol% of DOG in its absence to 0.38 in its presence. If PIP_2 was 5 mol%, then $K_{0.5}$ further decreased to 0.17 mol% of DOG, a substantial decrease compared with the total absence of the phosphoinositide. This illustrates that in the presence of PIP_2 the enzyme is tightly bound to the membrane and small concentrations of DOG are sufficient to maintain the activity. It is interesting that small apparent positive cooperativity was detected in the absence of PIP_2 (n=1.59), which was reduced following the addition of 1 mol% PIP_2. This apparent cooperativity in the binding of DOG may not necessarily reflect that the two C1 subdomains bind to DOG when its concentration is sufficiently high, but may be explained by the apparent cooperativity effect described above for POPS, while PIP_2 will reduce the apparent cooperativity due to its increasing of the membrane affinity of the protein. It is remarkable, that at 5 mol% of PIP_2 the Hill coefficient (0.47) indicated apparent negative cooperativity for diacylglycerol, which might be explained by a change in membrane structure at relatively high DOG concentrations *(Gomez-Fernandez and Corbalan-Garcia, 2007)*.

It is interesting that very low $K_{0.5}$ values were observed for PIP_2 even in the absence of DOG, the value (0.39 μM) being within the physiological range of concentrations. In the presence of DOG a very reduced $K_{0.5}$ value of 0.11 μM was observed for PIP_2, although V_{max} increased very slightly as a result of the addition of DOG, confirming that in the presence of PIP_2, diacylglycerol is playing a relatively secondary role in the activation of PKCα. Low K_D values have

been reported for the binding of PIP_2 to the isolated C2 domain of PKCα *(Guerrero-Valero et al., 2007b; Sanchez-Bautista et al., 2006a)* with about 1.9 μM for POPC-POPS-PIP2 vesicles, a value which is compatible with our observations for $K_{0.5}$.

Taken together, the results show that PIP_2 increases the V_{max} of PKCα and that when its concentration is 5 mol%, the addition of 2 mol% of DOG does not further increase the activity. Moreover, this concentration decreases $K_{0.5}$ for Ca^{2+} more than 3-fold, almost 5-fold that of DOG and by a half that of POPS. It is also noteworthy that $K_{0.5}$ values for PIP_2 amounted to only 0.11 μM in the presence of DOG and 0.39 in its absence, therefore well below the maximum physiological concentration for the inner monolayer of a mammalian plasma membrane. As a consequence, PKCα may be expected to operate near its maximum capacity even in the absence of a cell signal producing diacylglycerol. Nevertheless, we have shown that the presence of DOG may also help, since $K_{0.5}$ for PIP_2 notably fell in its presence. On the other hand, since Ca^{2+} has been shown to be essential for the binding of PIP_2 to the C2 domain of PKCα *(Guerrero-Valero et al., 2007b; Sanchez-Bautista et al., 2006a)*, this enzyme may be triggered simply by an increase in the cytoplasm concentration of this cation. Since it has been shown that the other classical isoenzymes of PKC are similar to PKCα as regards to the affinity of their C2 domains for PIP_2 *(Guerrero-Valero et al., 2007b)*, the above observations may well be extended to them.

CONCLUSION

The results obtained in this work are compatible with the sequential mechanism previously proposed (Corbalan-Garcia et al., 2003a) and further confirmed in vivo (Marin-Vicente et al., 2008a). Basically, intracytosolic Ca^{2+} elevations are the trigger to translocate PKCα to the plasma membrane. Once there, two situations can be found: in microdomains enriched only with phosphatidylserine, the docking of the C2 domain is not enough to liberate the catalytic domain for substrate access, and as seen in the 3D structure recently solved (Leonard et al., 2011), the C1B domain might still keep blocking the catalytic domain. Due to this, the presence of DAG in the lipid vesicles by docking at least the C1A domain enables the enzyme to gain its full activation (Ananthanarayanan et al., 2003b). A second situation can be found when the microdomains are enriched in phosphatidylserine and PIP_2 at the plasma membrane. In this case, the C2 domain docks in a different orientation since it has to anchor through two different points, i.e. the CBR (Ca^{2+}/PS) and the lysine rich cluster (PIP_2), this might induce a conformational change that unleash the C1 domain from the blocking conformation and enables the catalytic domain to access the substrate and consequently full activation of the enzyme. Whether the C1 domains can interact with the membrane independently of DAG is not known but there are previous reports indicating that the C1 domains can interact unspecifically with negatively charged phospholipids through the Arg and Lys residues located in its surface (Sanchez-Bautista et al., 2009).

CHAPTER IV

The C1B domains of novel PKCε and PKCη have a higher membrane binding affinity than those of the also novel PKCδ and PKCθ.

ABSTRACT

The C1 domains of novel PKCs mediate the diacylglycerol-dependent translocation of these enzymes. The four different C1B domains of novel PKCs (δ, ε, ϑ and η) were studied, together with different lipid mixtures containing acidic phospholipids and diacylglycerol or phorbol ester. The results show that in the presence of 5 mol% of diacylglycerol, C1Bε and C1Bη exhibit a substantially higher propensity to bind to vesicles containing negatively charged phospholipids than C1Bδ and C1Bϑ. The observed differences between the C1B domains of novel PKCs (in two groups of two each) were also evident in RBL-2H3 cells and it was found that, as with model membranes, in which C1Bε and C1Bη could be translocated to membranes by the addition of a soluble phosphatidic acid without diacylglycerol or phorbol ester, C1Bδ and C1Bϑ were not translocated when soluble phosphatidic acid was added, and diacylglycerol was required to achieve a detectable binding to cell membranes. It is concluded that two different subfamilies of novel PKCs can be established with respect to their propensity to bind to the cell membrane and that these peculiarities in recognizing lipids may explain why these isoenzymes are specialized in responding to different triggering signals and bind to different cell membranes.

1. INTRODUCTION

PKC (protein kinase C) is a large family of phospholipid-dependent serine/threonine kinases, which are activated by many extracellular signals, and which play a critical role in several signalling pathways in the cell. Mammalian isoenzymes have been grouped into three subfamilies according to their enzymatic properties. The first group, called classical or conventional isoenzymes (cPKCs), includes PKCα, βI, βII and γ, all of which contain the conserved C1 and C2 domains in the regulatory region.

These isoenzymes are regulated by Ca^{2+} and acidic phospholipids, which interact with the C2 domain, and by DAG (diacylglycerol), which interacts with the C1 domain *(Corbalan-Garcia and Gomez-Fernandez, 2006; Newton, 2001)*. Classical PKC isoenzymes are translocated to the plasma membrane of mammalian cells when the Ca^{2+} concentration is increased in the cytoplasm through the bridging made by this cation between the C2 domain and negatively charged phospholipids in the membrane (with preference phosphatidylserine) *(Verdaguer et al., 1999)*, and also by interaction with PIP_2 in the membrane through a second site *(Guerrero-Valero et al., 2009a; Guerrero-Valero et al., 2007b; Marin-Vicente et al., 2008a; Sanchez-Bautista et al., 2006a)*, followed by C1 domain–diacylglycerol interactions *(Corbalan-Garcia and Gomez-Fernandez, 2006; Leonard et al., 2011)*. In contrast, the enzymes of the

novel PKC subfamily (δ, ε, θ and η) do not bind Ca^{2+} and are activated through diacylglycerol or phorbol esters binding to the C1 domain *(Newton, 2001)*, although the C2 domain also plays a role through interactions with ligands like phosphatidic acid *(Corbalan-Garcia et al., 2003c; Lopez-Andreo et al., 2003)*.

Both classical and novel PKCs possess two different C1 domains, which form a tandem in the regulatory domain, C1A and C1B. A large number of experimental observations suggest that both domains are functionally dissimilar, as is evident for example, from their different affinity for diacylglycerols or phorbol esters *(Corbalan-Garcia and Gomez-Fernandez, 2006; Irie et al., 2002a; Irie et al., 1997; Shindo et al., 2001)*.

The affinity of C1A and C1B for these ligands changes depending on the isoenzyme in question. For example, it has been described that whereas the C1A domain of PKCα shows a much higher DAG affinity than the C1B domain, furthermore, the C1A has also a higher affinity for phorbol esters than the C1B but affinities are not so different in this case *(Ananthanarayanan et al., 2003b)*. In the case of other classical isoenzymes, like β *(Irie et al., 2002a)* and γ *(Ananthanarayanan et al., 2003b; Burns and Bell, 1991b; Irie et al., 2002a; Quest and Bell, 1994b)* differences are not big for diacylglycerols or phorbol esters affinities between the C1A and C1B domain affinities.

For PKCδ, conflicting results have been published, one group *(Irie et al., 2002a)* claiming that C1B has a much higher binding affinity for diacylglycerol than C1A, while another group *(Stahelin et al., 2004b)* maintains that the opposite is true. PKCε is similar to PKCγ in that both C1A and C1B domains are involved in membrane binding and activation, although the C1A domain has about a 3-fold higher DAG affinity than the C1B domain *(Stahelin et al., 2005a)*. With respect to the other new isoenzymes, fewer studies have been carried out, but it seems that in both θ and η the C1B domain has a higher affinity than the C1A domain for phorbol esters *(Irie et al., 2002a; Quest and Bell, 1994b)*.

It has long been known that phosphatidylserine enhances the membrane affinity and activity of PKCs *(Newton, 1993b)*, although it has also been claimed that the phosphatidylserine dependence may vary significantly among PKC isoforms *(Ananthanarayanan et al., 2003b; Medkova and Cho, 1998)*. Among conventional PKCs, PKCα *(Medkova and Cho, 1998)* and PKCβII *(Newton and Keranen, 1994)* prefer PS to phosphatidylglycerol, whereas PKCγ shows little preference between phosphatidylserine and phosphatidylglycerol

(Ananthanarayanan et al., 2003b). Among novel PKCs, PKCδ shows a certain degree of phosphatidylserine selectivity *(Stahelin et al., 2005a)*, whereas PKCε does not *(Medkova and Cho, 1998; Stahelin et al., 2005a)*.

Nevertheless, it should be taken into account that both the C1 and C2 domains bind anionic phospholipids, and so it is difficult to use whole-enzyme activity to detect from which domain the observed specificity arises. For this reason, it is important to study the isolated domains. In the case of the C1 domain, only a limited number of studies have been carried out for this purpose, and, among the findings, it may be mentioned that the C1B domain of PKCβII (in the presence of diacylglycerol) has a dissociation constant of of 780 μM in the presence of phosphatidylserine and 1690 μM in the presence of phosphatidylglycerol *(Dries et al., 2007)*. However, when a Tyr residue was mutated to Trp, the C1B domain showed a dissociation constant of 24 μM for a membrane containing phosphatidylserine and 130 μM for a membrane containing phosphatidylglycerol, i.e., although the constant was considerably reduced, the preference was not pronounced preference for phosphatidylserine *(Dries et al., 2007)*. In the case of C1Bδ, it was clearly shown that the preference was for phosphatidylserine (K_D of 35 μM) rather than for phosphatidylglycerol (K_D of 700 μM) *(Dries et al., 2007)*.

A study was carried out using isolated C1B domains of three different PKCs (the classical γ, and the novel δ and ε) binding to liposomes of different compositions, using three different DAGs (DOG, SAG and DPG) and three different anionic phospholipids (phosphatidylserine, phosphatidic acid and phosphatidylglycerol) to prepare the model vesicles. C1Bε was found to have the highest binding affinity to vesicles containing phosphatidic acid as acidic phospholipid, and DOG or SAG as diacylglycerol. In general, DOG and SAG led to a higher membrane binding affinity than DPG in all the C1B domains *(Sanchez-Bautista et al., 2009)*. In this paper we amplify this study by comparing, side by side, the four C1B domains from novel PKCs, and their affinities for membranes containing different negatively charged phospholipids. Two groups could be established, one with a lower K_D for model membranes, constituted by C1Bε and C1Bη, and another formed of C1Bδ and C1Bθ which show substantially higher K_D. These differences in membrane affinities were confirmed by expressing the four domains in RBL-2H3 cells and triggering the translocation to the plasma membrane by adding a soluble phosphatidic acid or a soluble diacylglycerol.

2. RESULTS

The aim of this work was to study the membrane binding affinity of C1 domains of novel PKCs for different anionic phospholipids in the presence or absence of DOG or PMA. C1B domains from PKCδ, PKCε, PKCη and PKCθ were used.

Since these C1B domains lack suitable residues to induce fluorescence energy transfer between a membrane probe and Trp residues, a FRET method was used, in which the acceptor was phosphatidylethanolamine labelled with Oregon Green-488 (OG-PE) located in the membrane, and the donor was the C1 domain fused to enhanced cyan fluorescent protein (ECFP). In a second phase the membrane translocation of these C1B domains fused to a fluorescent protein was examined in RBL-2H3.

2.1. The influence of acidic phospholipids on the binding of C1B domains to membrane in the presence of DOG.

Using the FRET method mentioned above, the binding of C1B domains to small unilamellar vesicles containing different lipid compositions was studied. Fig. IV.1 shows the binding of the C1Bδ domain to increasing concentrations of POPC/POPX/DOG vesicles, where POPX stands for POPS (Fig. IV.1A), POPG (Fig. IV.1B) or POPA (Fig. IV.1C).

In this assay, the effect of increasing the relative percentage of acidic phospholipid on C1B membrane binding was also studied. The data were analyzed and best fitted to the Hill model. The diacylglycerol (DOG) concentration was kept constant at 5 mol% in all the following cases.

C1Bδ (Fig. IV.1 (A, B and C), Table IV.1) showed the highest binding affinity for membranes when POPS was present although all the values were within the same order of magnitude (at 40 mol% of anionic phospholipids, the K_D was 11.57 μM for POPS compared to 19.55μM for POPA and 37.54 μM for POPG). In the case of C1Bθ (Fig. IV.1 (D, E and F), Table IV.1), the lowest K_D corresponded to membranes incorporating POPA (Fig. IV.1.F), followed by those with POPS (Fig. IV.1.D) and finally those with POPG (Fig. IV.1.E), although all of them were in the same order of magnitude (at 40 mol% of the anionic phospholipids, the K_D was 6.78 μM for POPA, 11.99 for POPS and 22.64 μM for POPG).

When the binding of C1Bε was studied, it was observed (Fig. IV.2 and Table IV.1) that the extent of binding was much higher than with the two previously mentioned isoenzymes. In this case, the highest binding affinity was very clearly for POPA (Fig. IV.2.C), followed by POPS (Fig. IV.2.A) and POPG (Fig. IV.2.B and Table IV.1) (at 40 mol% of the anionic phospholipids, the K_D was 0.27 μM for POPA, 1.01 μM for POPS and 3.01 μM for POPG).

In the case of C1Bη, binding affinities were more similar to those observed for the ε isoenzyme than to those observed for the δ and θ isoenzymes. As with the C1Bε, the highest binding affinity was observed for POPA (Fig. IV.2F), followed by POPS (Fig. IV.2D) and POPG (Fig. IV.2E) (at 40 mol% of the anionic phospholipids, the K_D was 0.40 μM for POPA, 0.98 for POPS and 1.08 μM for POPG) (Table IV.1).

An interesting finding was the important role played by anionic phospholipids in the binding of C1B domains to the membranes (Table IV.1). As the contents of the anionic phospholipids increased from 5 to 40 mol%, the membrane binding affinity increased up to one order of magnitude at a constant DOG concentration (5 mol%). See, for example, the case of C1Bε with POPA, with K_D 2.74 μM at 5 mol% and 0.27 μM at 40 mol%; or that of C1Bη with POPA, when the increase was from K_D of 3.63 μM at 5mol% and 0.40 μM at 40 mol% (Table IV.1). These results confirm that not only is diacylglycerol important for the binding of C1 domains to the membrane, but that anionic phospholipids are also important.

Another interesting result was that there were substantial differences in the binding affinity of the domains studied. The highest binding affinities were observed in C1Bε (Fig. IV.2 and Table IV.1) for the three anionic phospholipids, the increase being about 25-fold for 40 mol% of POPA (K_D values of 0.27 μM for C1Bε compared with 19.55 μM for C1Bδ and 6.78 μM for C1Bθ). More similar were the membrane binding affinities of isoenzyme δ compared with θ and that of ε compared with η. Increases of up to 10-fold were also observed in the presence of 40 mol% POPS (K_D values of 1.01 μM for C1Bε compared to 11.99 μM for C1Bθ and 11.57 μM for C1Bδ) and up to 37-fold when 40 mol % POPG was present in the membrane (K_D values of 1.08 μM for C1Bη and 3.01 for C1Bε compared with 22.64 μM for C1Bθ and 37.54 μM for C1Bδ).

Thus, in general, similar membrane binding affinities were observed for C1Bθ and C1Bδ and, on the other hand, for C1Bε and C1Bη.

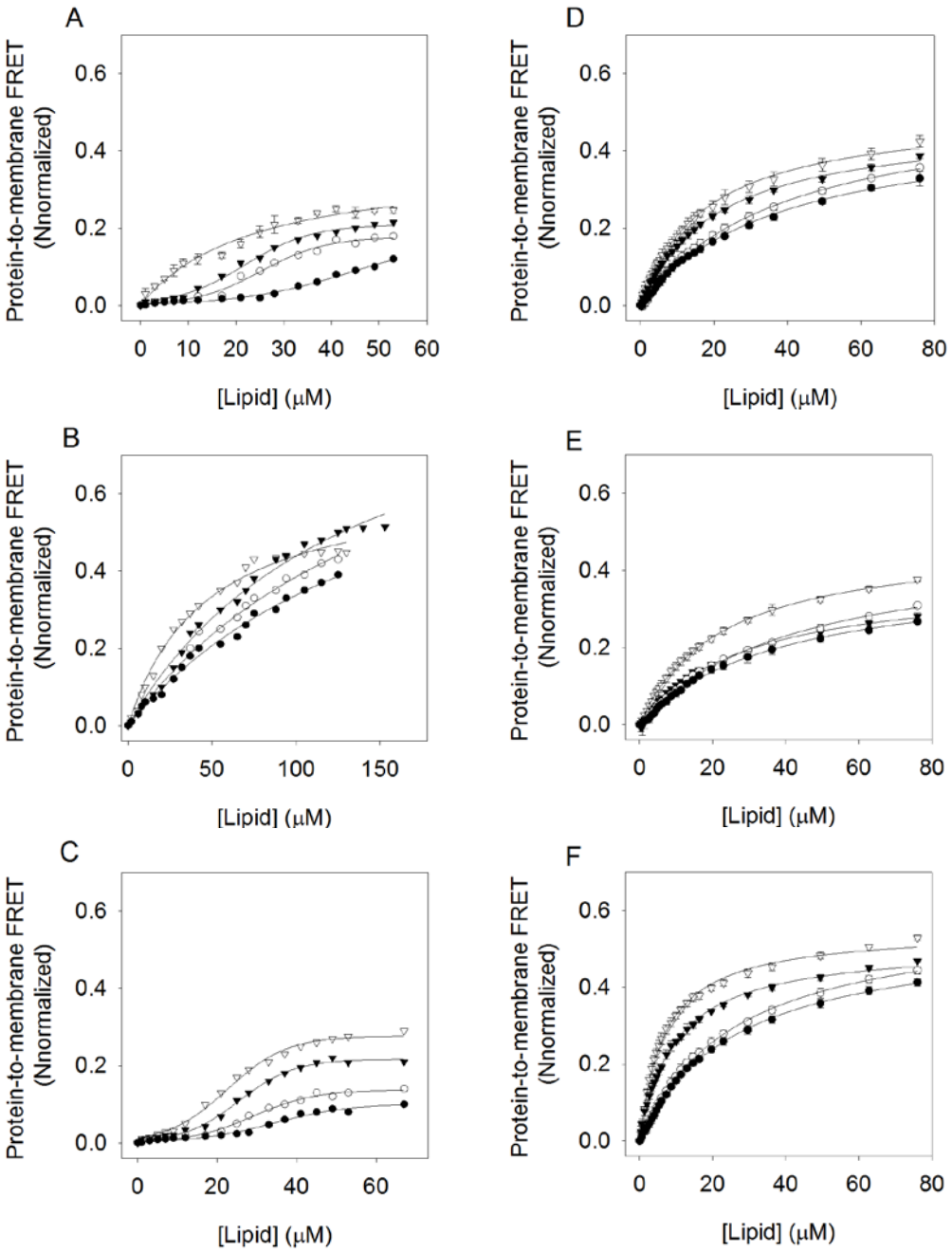

Figure IV.1. Binding of C1Bδ and C1Bθ to lipid vesicles in the presence of DOG. Binding of C1Bδ-ECFP domain (A, B and C) and C1Bδ-ECFP domain (D, E and F) to lipid vesicles, which contained different types of anionic phospholipids. Vesicles contained POPC/POPX/OGPE in molar ratios of 90:5:5 (•), 85:10:5 (o), 75:20:5 (▼), and 55:40:5 (▽). POPX was POPS (A and D), POPG (B and E) or POPA (C and F). DOG was present in all cases at 5 mol %. Normalized FRET values are depicted versus total lipid concentration.

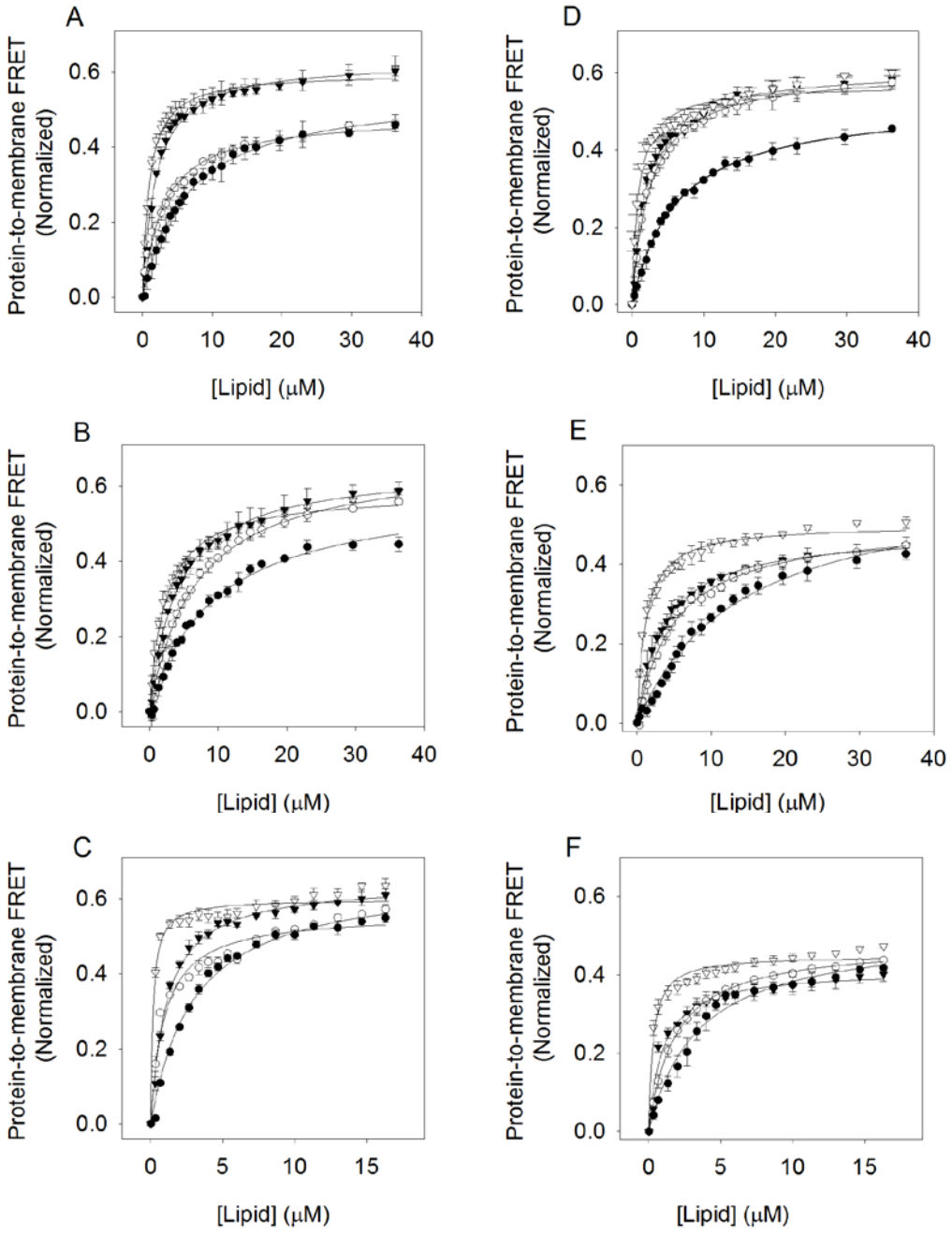

Figure IV.2. Binding of C1Bε and C1Bη to lipid vesicles in the presence of DOG. Binding of C1Bε-ECFP domain (A, B and C) and C1Bη-ECFP domain (D, E and F) to lipid vesicles, which contained different types of anionic phospholipids. Vesicles contained POPC/POPX/OGPE in molar ratios of 90:5:5 (•), 85:10:5 (o), 75:20:5 (▼), and 55:40:5 (▽). POPX was POPS (A and D), POPG (B and E) or POPA (C and F). DOG was present in all cases at 5 mol %. Normalized FRET values are depicted versus total lipid concentration.

	Mol (%)	C1Bδ		C1Bθ		C1Bε		C1Bη	
		ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)
POPS	5	0.12 ± 0.02	40.15 ± 0.4	0.32 ± 0.02	40.25 ± 0.78	0.46 ± 0.01	6.76 ± 0.66	0.45 ± 0.01	6.28 ± 0.63
	10	0.20 ± 0.01	28.22 ± 0.2	0.35 ± 0.01	39.36 ± 0.53	0.46 ± 0.02	3.23 ± 0.62	0.59 ± 0.03	2.55 ± 0.15
	20	0.24 ± 0.02	22.60 ± 0.2	0.38 ± 0.02	20.92 ± 0.49	0.60 ± 0.01	1.91 ± 0.12	0.58 ± 0.02	2.02 ± 0.04
	40	0.30 ± 0.01	11.57 ± 0.2	0.42 ± 0.01	11.99 ± 1.79	0.61 ± 0.03	1.01 ± 0.19	0.61 ± 0.03	0.98 ± 0.81
POPA	5	0.11 ± 0.03	35.33 ± 0.4	0.44 ± 0.01	30.39 ± 1.46	0.54 ± 0.01	2.74 ± 0.48	0.45 ± 0.01	3.63 ± 1.15
	10	0.16 ± 0.02	30.29 ± 0.4	0.44 ± 0.01	26.02 ± 0.93	0.57 ± 0.01	1.69 ± 0.06	0.47 ± 0.01	1.89 ± 0.21
	20	0.21 ± 0.05	20.40 ± 0.8	0.46 ± 0.01	9.66 ± 1.13	0.62 ± 0.01	1.18 ± 0.08	0.43 ± 0.01	1.11 ± 0.03
	40	0.30 ± 0.01	19.55 ± 0.4	0.52 ± 0.01	6.78 ± 1.19	0.65 ± 0.01	0.27 ± 0.05	0.52 ± 0.01	0.40 ± 0.08
POPG	5	0.43 ± 0.01	64.78 ± 0.2	0.26 ± 0.01	42.49 ± 2.89	0.50 ± 0.01	8.03 ± 0.55	0.42 ± 0.01	12.6 ± 0.64
	10	0.43 ± 0.02	52.67 ± 0.2	0.30 ± 0.01	37.74 ± 2.61	0.61 ± 0.01	6.32 ± 0.42	0.44 ± 0.01	5.01 ± 0.54
	20	0.52 ± 0.01	48.29 ± 0.2	0.29 ± 0.01	28.88 ± 2.56	0.64 ± 0.02	4.18 ± 0.45	0.44 ± 0.01	3.71 ± 0.65
	40	0.53 ± 0.01	37.54 ± 0.7	0.37 ± 0.01	22.64 ± 1.01	0.67 ± 0.01	3.01 ± 0.45	0.53 ± 0.03	1.08 ± 0.15

Table IV.1. Binding parameters of C1B-ECFP domains to lipid vesicles. Phospholipid vesicle contained POPC/DOG/POPX/OG-PE (90-X:5:X:5 mol %), whereas anionic and POPC phospholipids varied as indicated in the table. FRET data were fitted to a Hill equation. Values given as mean ± SE of three different experiments.

2.2. The influence of acidic phospholipids on the binding of C1B domain to membrane in the absence of diacylglycerol or in the presence of PMA.

The binding dependency of the C1B domains to lipid vesicles was also studied in the total absence of diacylglycerol. Fig. IV.3 shows a comparison of the four C1B domains studied, in the presence of 20 mol% of each anionic phospholipid. The existence of two groups of C1B domains delimited by their membrane binding affinity is clearly revealed by these results, especially in the presence POPA (Fig. IV.3C) and POPS (Fig. IV.3A). The same pattern was found in the presence of POPG (Fig. IV.3B) although with a less pronounced separation between the two pairs. Table IV.2 shows that K_D values had a similar order of magnitude for membranes containing any of the anionic phospholipids tested in the case of C1Bδ and C1Bθ, ranging from 32.06 for C1Bδ with POPS to 52.21 μM for the same C1Bδ with POPG. These figures were much lower in the case of C1Bε (K_D values of 25.43 μM for POPS, 3.02 for POPA and 21.61 for POPG) and for C1Bη (K_D values of 9.48 μM for POPS, 1.82 for POPA and 15.98 for POPG). Note the strongly decreased value in the presence of POPA, indicating that in these cases membrane translocation may already be quite high even without diacylglycerol.

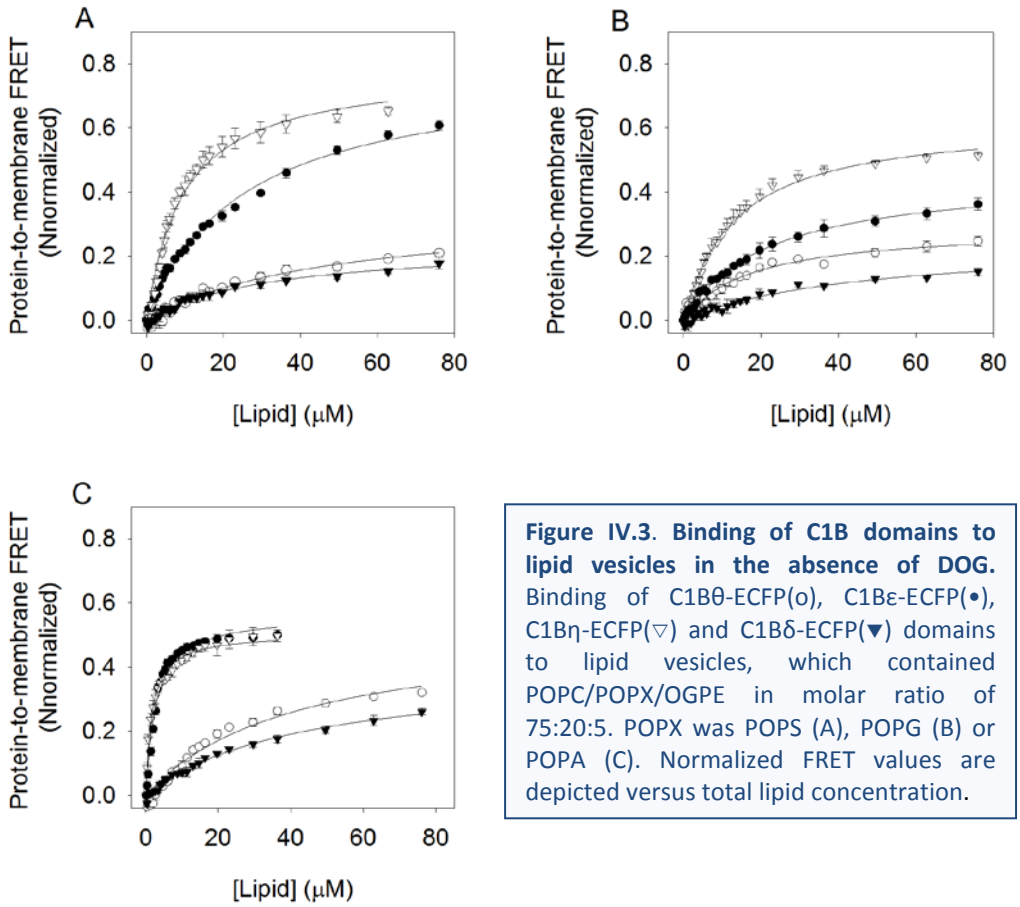

Figure IV.3. Binding of C1B domains to lipid vesicles in the absence of DOG. Binding of C1Bθ-ECFP(o), C1Bε-ECFP(•), C1Bη-ECFP(▽) and C1Bδ-ECFP(▼) domains to lipid vesicles, which contained POPC/POPX/OGPE in molar ratio of 75:20:5. POPX was POPS (A), POPG (B) or POPA (C). Normalized FRET values are depicted versus total lipid concentration.

The addition of 5 mol% DOG increases membrane affinity in all cases compared with membranes without any diacylglycerol, but especially with C1Bε and C1Bη, revealing once again the higher affinity for membranes of these two domains.

Such was the case of C1Bε with POPS (K_D 25.43 in the absence and 1.91 μM in the presence of DOG) and with POPG (K_D 21.61 in the absence and 4.18 μM in the presence of DOG) and also of C1Bη with POPS (K_D 9.48 in the absence and 2.02 μM in the presence of DOG) and POPG (K_D 15.98 in the absence and 3.71 μM in the presence of DOG). The decrease in K_D values was less marked in the presence of POPA in the case of C1Bε (from 1.18 μM in the presence of DOG to 3.02 in its absence) and for C1Bη (1.11 μM in the presence and 1.82 in the absence of DOG).

	Mol (%) DOG	C1Bδ	C1Bθ	C1Bε	C1Bη
		K_D (μM)	K_D (μM)	K_D (μM)	K_D (μM)
POPS	0	32.06 ± 0.46	47.02 ± 0.68	25.43 ± 0.19	9.48 ± 0.80
	5	22.60 ± 0.20	20.92 ± 0.49	1.91 ± 0.12	2.02 ± 0.04
POPA	0	43.65 ± 0.79	41.35 ± 0.88	3.02 ± 0.34	1.82 ± 0.50
	5	20.40 ± 0.83	9.66 ± 1.13	1.18 ± 0.08	1.11 ± 0.03
POPG	0	52.21 ± 0.84	44.94 ± 0.65	21.61 ± 0.39	15.98 ± 0.76
	5	48.29 ± 0.25	28.88 ± 2.56	4.18 ± 0.45	3.71 ± 0.65

Table IV.2. Binding parameters of C1B-ECFP domains to lipid vesicles. Phospholipid vesicle contained 75 mol % POPC, X mol % of DOG, 5 mol % OG-PE, and 20 mol % of the indicated anionic phospholipid. Values given as mean ± SE of three different experiments.

In the presence of 1 mol% of PMA with respect to the total lipid, however (Fig. IV.4) membrane binding affinities were similar for all C1B domains studied and for all anionic phospholipids, indicating that this activator was at saturating concentration and that all these domains are able to translocate to the membrane if the concentration of activating molecules is sufficiently high (see also Table IV.3). Note that the K_D values reached in this case for C1Bε and C1Bη were not much lower than those reached with 5 mol% DOG and even in the presence of POPA (in the total absence of DOG or PMA), as it occurred for C1Bη with POPA (K_D was 1.82 in the absence of DOG and PMA, but 1.07 μM in the presence of 5mol% DOG and 1.01 in the presence of 1 mol% PMA).

This indicates that these C1B domains may reach nearly maximum membrane binding in the presence of POPA alone.

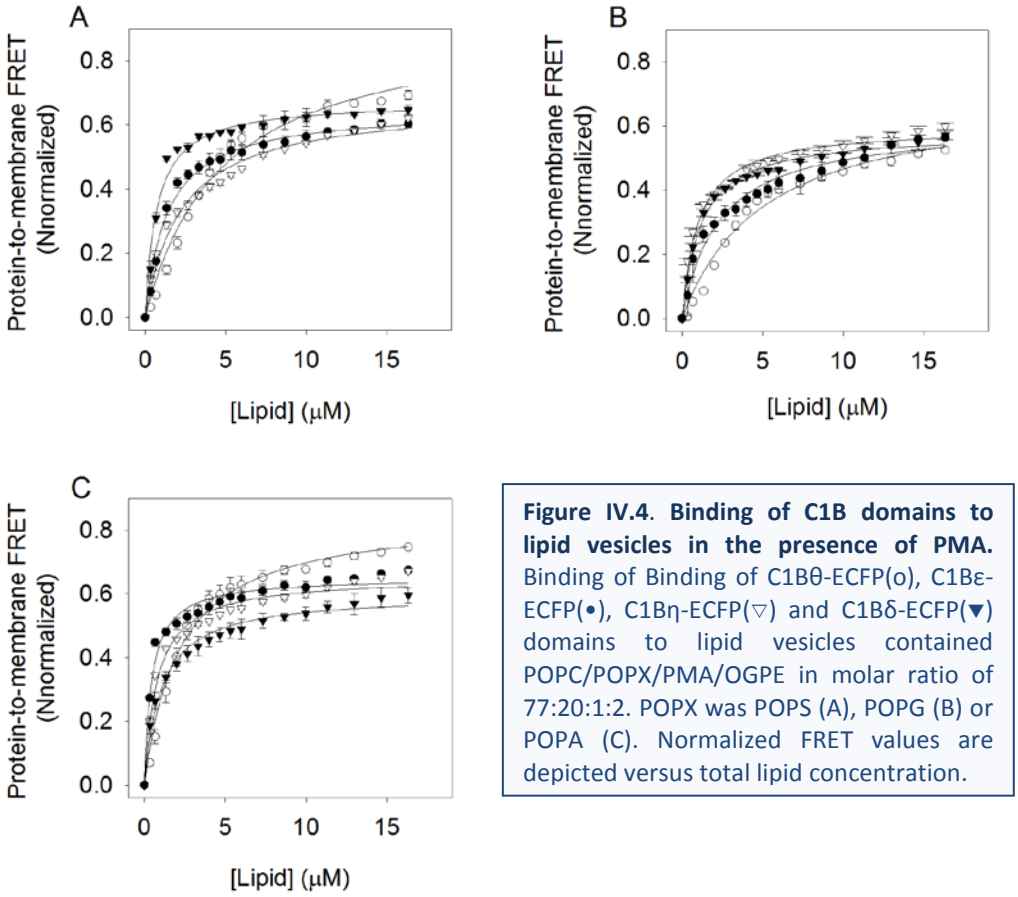

Figure IV.4. Binding of C1B domains to lipid vesicles in the presence of PMA. Binding of Binding of C1Bθ-ECFP(o), C1Bε-ECFP(•), C1Bη-ECFP(▽) and C1Bδ-ECFP(▼) domains to lipid vesicles contained POPC/POPX/PMA/OGPE in molar ratio of 77:20:1:2. POPX was POPS (A), POPG (B) or POPA (C). Normalized FRET values are depicted versus total lipid concentration.

	Mol (%) PMA	C1Bδ	C1Bθ	C1Bε	C1Bη
		K_D (μM)	K_D (μM)	K_D (μM)	K_D (μM)
POPS	1	0.74 ± 0.06	3.71 ± 0.17	1.39 ± 0.06	0.86 ± 0.44
POPA	1	1.32 ± 0.20	2.14 ± 0.12	0.49 ± 0.07	1.01 ± 0.12
POPG	1	1.46 ± 0.15	5.15 ± 0.12	2.67 ± 0.12	1.14 ± 0.19

Table IV.3. Binding parameters of C1B-ECFP domains to lipid vesicles. Phospholipid vesicle contained 74 mol % POPC, 1mol % of PMA, 5 mol % OG-PE, and 20 mol % of the indicated anionic phospholipid. Values given as mean ± SE of three different experiments.

2.3. Membrane translocation of C1B domains in RBL-2H3 cells induced by cell activation and endogenous production of activators

To investigate membrane translocation of the four domains *in vivo* we have used RBL-2H3 cells transfected with the different protein C1B-ECFP constructs. These cells may be activated by their stimulation of the RBL-2H3 cells by an antigen as DNP-HSA.

The cross-linking of the high-affinity IgE receptor (FcεRI) with antigen in mast or basophilic cells (as the RBL-2H3 ones) stimulates a number of lipid-signalling events, which include the activation of phospholipase Cγ phospho-inositide 3-kinase, and phospholipase D (PLD) *(Brown et al., 1998; Schneider et al., 1992)* and many serine/threonine kinases *(Park et al., 1991)*, including the PKC family among others *(Ozawa et al., 1993)*. Diacylglycerols are generated through this stimulation and also phosphatidic acid *(Lopez-Andreo et al., 2003)*.

The results obtained (Fig. IV.5 and Table IV.4) indicate that the four domains may be translocated to the plasma membrane after the stimulation of the cells but some differences were observed between them. With respect to R_{max} C1Bε and C1Bη present slightly higher values (0.62 and 0.59, respectively) than C1Bδ and C1Bθ (0.52 and 0.56, respectively) but substantial differences were observed for $t_{½}$ with values remarkably lower for C1Bε and C1Bη (19 and 23 *s*, respectively) in comparison to C1Bδ and C1Bθ (37 and 40 *s*, respectively). This means that higher concentrations of activating factors are needed for the second couple of domains and thus a longer time for the membrane translocation is needed, although they are able of doing so, as it was also shown with the model system when PMA was added (Table IV.3).

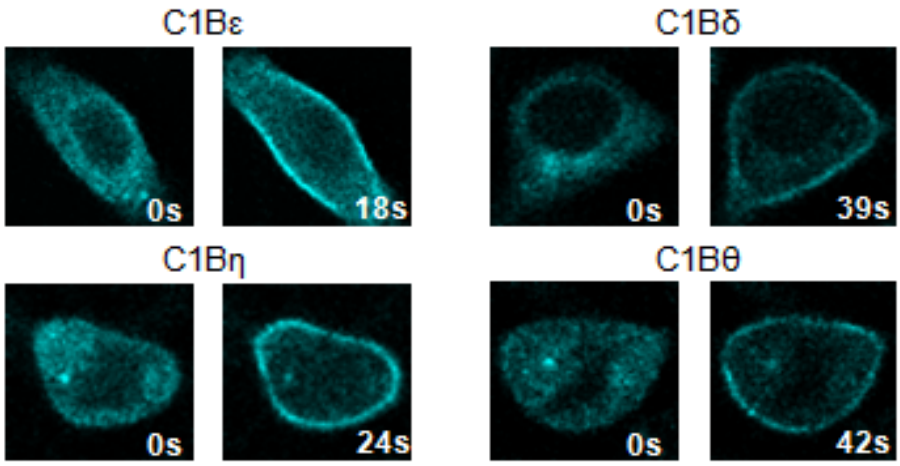

Figure IV.5. Membrane translocation of C1B domains to the plasma membrane of RBL-2H3 cells. Cells were transfected with ECFP- C1Bδ, ECFP-C1Bθ, ECFP-C1Bε and ECFP-C1Bη constructs, as indicated. The pictures show the effect of the addition of antigen (DNP-HSA).

Stimulation 2µg/ml DNP-HSA	*N* cells	M.L., %	R_{max}	$t_{½}$, s
C1Bε-ECFP	62	80	0.62 ± 0.01	19 ± 2
C1Bη-ECFP	77	76	0.59 ± 0.02	23 ± 2
C1Bδ-ECFP	63	61	0.52 ± 0.03	37 ± 2
C1Bθ-ECFP	50	66	0.56 ± 0.02	40 ± 6

Table IV.4. Binding parameters of C1B-ECFP domains to lipid vesicles. RBL-2H3 cells were primed with 0.5µg/ml antiIgE antibody for 16 h and then stimulated with 2µg/ml DNP-HSA. *M.L.* is membrane localization and indicates the percentage of cells responding to DNP-HSA stimulation with plasma membrane translocation. *R*max is the maximum relative increase in plasma membrane localization of the domain. $t_{1/2}$ is the half time of translocation.

2.4. Membrane translocation of C1B domains in RBL-2H3 cells induced by permeable activators.

To check the importance of phosphatidic acid and diacylglycerol on the membrane translocation of the four domains and if the retard shown by C1Bδ and C1Bθ is really due to their need of higher concentrations of activators, water soluble forms of the lipids were used that are known to permeate the membranes, namely phosphatidic acid (DOcPA) and soluble diacylglycerol (DOcG).

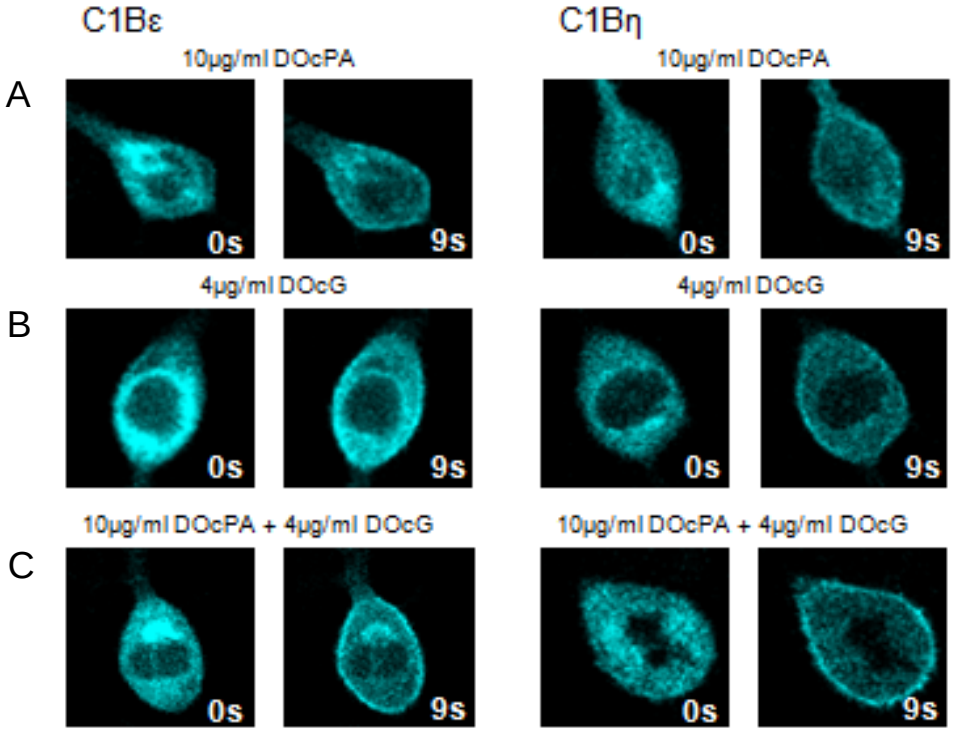

Figure IV.6. Membrane translocation of C1B domains to the plasma membrane of RBL-2H3 cells. Cells were transfected with ECFP- C1Bδ, ECFP-C1Bθ, ECFP-C1Bε and ECFP-C1Bη constructs, as indicated. The pictures show the effect of the addition of the indicated amounts of soluble diacylglycerol (DOcG) and/or soluble phosphatidic acid (DOcPA).

Table IV.5 show that when a concentration of 20 µg/ml of DOcPA was added to RBL-2H3 cells membrane translocation was induced in the case of C1Bε and C1Bη (R_{max} 0.44 and 0.41, respectively). The addition of also low concentrations of DOcG (10 µg/ml) (Fig. IV.6.A and Table IV.5) led to still detectable membrane translocation (R_{max} 0.33 and 0.21, respectively).

On the other hand, the addition of 12 µg/ml of DOcG without DOcPA (Table IV.5) induced considerable membrane translocation (R_{max} 0.46 for C1Bε and 0.41 for C1Bη).

Stimulation C1Bε-ECFP	***N* cells**	**M.L., %**	**R_{max}**	**$T_{½}$, s**
20 µg/ml DocPA	22	96	0.44 ± 0.02	3 ± 1
15 µg/ml DocPA	31	83	0.40 ± 0.02	3 ± 1
10 µg/ml DocPA	24	79	0.33 ± 0.03	3 ± 1
5 µg/ml DocPA	28	46	0.25 ± 0.03	6 ± 1
2 µg/ml DocPA		0		
12 µg/ml DocG	29	93	0.46 ± 0.02	3 ± 1
8 µg/ml DocG	23	78	0.39 ± 0.01	3 ± 1
4 µg/ml DocG	46	34	0.29 ± 0.03	4 ± 1
2 µg/ml DocG				
10 µg/ml DocPA + 4 µg/ml DocG	21	95	0.51 ± 0.01	3 ± 1
5 µg/ml DocPA + 4 µg/ml DocG	18	94	0.41 ± 0.03	3 ± 1
2 µg/ml DocPA + 2 µg/ml DocG		0		
Stimulation C1Bη-ECFP	***N* cells**	**M.L., %**	**R_{max}**	**$T_{½}$, s**
20 µg/ml DocPA	24	96	0.41 ± 0.02	3 ± 1
15 µg/ml DocPA	22	90	0.37 ± 0.01	3 ± 1
10 µg/ml DocPA	22	59	0.21 ± 0.02	3 ± 1
5 µg/ml DocPA		0		
12 µg/ml DocG	27	92	0.41 ± 0.01	4 ± 1
8 µg/ml DocG	22	36	0.43 ± 0.01	3 ± 1
4 µg/ml DocG	31	19	0.26 ± 0.01	6 ± 1
2 µg/ml DocG		0		
10 µg/ml DocPA + 4 µg/ml DocG	27	100	0.53 ± 0.03	2 ± 1
5 µg/ml DocPA + 4 µg/ml DocG	31	45	0.34 ± 0.01	3 ± 1
2 µg/ml DocPA + 2 µg/ml DocG		0		

Table IV.5. Plasma membrane translocation parameters of C1Bε-ECFP and C1Bη-ECFP domains in RBL-2H3 cells stimulated with DOcPA and/or DOcG. *M.L.* is membrane localization and indicates the percentage of cells responding to DOcPA and/or DOcG stimulation with plasma membrane translocation. R_{max} is the maximum relative increase in plasma membrane localization of the domain. $t_{1/2}$ is the half time of translocation.

Considerable less translocation was observed (Fig. IV.6.B and Table IV.5) when only 4 µg/ml of DOcG was added (R_{max} 0.29 for C1Bε and 0.26 for C1Bη). An additive effect was observed when DOcPA (10 µg/ml) and DOcG (4 µg/ml)

were added together (Fig. IV.6.C and Table IV.5) producing, in this case, a good level of translocation (R_{max} 0.51 for C1Bε and 0.53 for C1Bη).

In contrast with the results shown above in RBL-2H3 cells for C1Bε and C1Bη, no translocation was observed for C1Bδ and C1Bθ when the same concentrations of DOcPA were used (Fig. IV.7.D and Table IV.6) and some degree of translocation was only observed for these domains when high concentrations of DOcG were used (e.g. 12 µg/ml) (Fig. IV.7.E and Table IV.6). In this case the R_{max} was 0.43 for C1Bδ (but note that Membrane Localization was observed in 53% of the cells) and 0.37 for C1Bθ (Membrane Localization in 48% of the cells). No membrane translocation was observed for these two domains when 20 µg/ml of DOcPA and 4 µg/ml of DOcG were added together (Table IV.6). However, when the addition was 20 µg/ml of DOcPA plus 12 µg/ml of DOcG, i.e. high concentrations of both activators, some translocation was observed with *Rmax* being 0.44 for C1Bδ and 0.47 for C1Bθ (Fig. IV.7.F and Table IV.6).

Stimulation C1Bδ-ECFP	***N* cells**	**M.L., %**	**R_{max}**	**$T_{½}$, s**
50 µg/ml DOcPA		0		
30 µg/ml DOcPA		0		
20 µg/ml DOcPA		0		
12 µg/ml DOcG	21	53	0.43 ± 0.01	8 ± 1
8 µg/ml DOcG	25	44	0.28 ± 0.02	11 ± 3
4 µg/ml DOcG		0		
20 µg/ml DOcPA + 4 µg/ml DOcG		0		
20 µg/ml DOcPA + 12 µg/ml DOcG	32	81	0.44 ± 0.01	8 ± 1
Stimulation C1Bθ-ECFP	***N* cells**	**M.L., %**	**R_{max}**	**$T_{½}$, s**
50 µg/ml DOcPA		0		
30 µg/ml DOcPA		0		
20 µg/ml DOcPA		0		
12 µg/ml DOcG	21	48	0.37 ± 0.03	3 ± 1
8 µg/ml DOcG	21	28	0.29 ± 0.02	4 ± 1
4 µg/ml DOcG		0		
20 µg/ml DOcPA + 4 µg/ml DOcG		0		
20 µg/ml DOcPA + 12 µg/ml DOcG	41	75	0.47 ± 0.01	5 ± 1

Table IV.6. Plasma membrane translocation parameters of C1Bδ-ECFP and C1Bθ-ECFP domains in RBL-2H3 cells stimulated with DOcPA and/or DOcG. *M.L.* is membrane localization and indicates the percentage of cells responding to DOcPA and/or DOcG stimulation with plasma membrane translocation. R_{max} is the maximum relative increase in plasma membrane localization of the domain. $t_{1/2}$ is the half time of translocation.

These results confirm that C1Bδ and C1Bθ need higher concentrations of activators for their membrane translocation. Furthermore, C1Bε and C1Bη may respond to DOcPA without the need for DOcG and that a combination of low concentrations of both activators triggers a bigger membrane translocation of these two domains. In contrast, in the case of C1Bδ and C1Bθ they do not respond to DOcPA and high combined concentrations of DOcG (12 μg/ml) and DOcPA (20 μg/ml) are required for their membrane translocations.

3. DISCUSSION

Structural studies have established that all C1 domains have a similar fold *(Hommel et al., 1994; Ichikawa et al., 1995; Rahman et al., 2013; Shen et al., 2005; Xu et al., 1997; Zhang et al., 1995a; Zhou et al., 2002)* based on a compact α/β structural unit, which tightly binds two zinc ions. This fold contains an *unzipped* β-sheet that forms a single ligand-binding site for diacylglycerol or phorbol esters (Thr12, Gly23 and Leu21) in its top, surrounded by hydrophobic residues that are involved in membrane insertion *(Zhang et al., 1995a)*.

Conventional and novel PKCs contain two C1 domains, C1A and C1B, although the exact function of each of these domains and the reason for the existence of this double domain are not fully clear. Much research effort has focused on trying to understand the specific function of these domains, which we have reviewed in detail elsewhere *(Corbalan-Garcia and Gomez-Fernandez, 2006)*.

Studying C1A domains is hampered by the difficulty involved in obtaining them in soluble form from transfected cell cultures. C1B, however, can be obtained in soluble form and in sufficient amounts to carry out studies like the one reported here, whose objective was to compare the membrane binding affinity of the C1B domains of the four novel PKCs, studying them side by side and using both model membranes and live cells.

As regards the C1B domain, we compared the sequence homology of the four domains from novel PKCs, using the program ClustalW2 *(http://www.ebi.ac.uk/Tools/msa/clustalw2)* and the sequences for the C1B domains deduced from Uniprot *(http://www.uniprot.org/)* for PKCε (code P16054), PKCη (code P23298), PKCδ (code P28867) and PKCθ (Q02111), all of them from mouse. It was seen that whereas ε and η have 84% homology

between them, both of them only have 62% homology with δ and 66% with θ. On the other hand, δ and θ present 80% homology.

In the first part we studied the binding of the C1B domains to membranes using a FRET assay. From the binding affinity results (the reciprocal of the apparent K_D values) for the four domains, it can be concluded that C1Bε and C1Bη have a much higher binding affinity than C1Bδ and C1Bθ. This pattern of two well differentiated groups with respect to C1B domains from novel PKCs was confirmed by studies both in the presence and absence of diacylglycerol, when all of them showed similar membrane affinity in the presence of saturating PMA concentration (1 mol% of the total lipid). The two groups were also clearly distinguished when the experiments were conducted using living cells and stimulating membrane binding with either an antigen or permable forms of phosphatidic acid and/or diacylglycerol. Note that this division of novel C1B domains into two groups correlates well with the sequence homology mentioned above.

Several observations deserve comment. One is related with the specificity for the different anionic phospholipids studied. C1Bε, C1Bη and C1Bθ were seen to have a higher specificity for POPA, judging from the K_D values. Only C1Bδ had a higher specificity for POPS. If the concentration of anionic phospholipids in the membrane was increased from 5 to 40 mol%, the membrane binding affinity increased by up to one order of magnitude, illustrating the importance of anionic phospholipids for enabling the binding of C1B domains to the membrane. This was also shown by the experiments-in which DOG was omitted from the model membranes, especially in the case of C1Bε and C1Bη in the presence of POPA, when the effect of DOG was very modest (in the case of C1Bη the K_D fell from 1.82 μM in the absence to 1.07 in the presence of 5 mol% DOG). However, in the presence of PMA at 1 mol%, which is a very high concentration, the membrane binding differences between domains and their specificity for phospholipids, were minimized.

It should be remarked that the influence of the interaction with anionic phospholipids on the membrane binding of C1Bs domains of novel PKCs was clearly demonstrated by the experiments in the absence of DOG, since in this type of experiment it was very clear that C1Bε and C1Bη had a much higher membrane binding affinity than C1Bδ and C1Bθ, as was the case for all the anionic phospholipids tested. It is to be expected that this would also be the

case in physiological conditions for translocation to the cell plasmatic membrane. As regards diacylglycerol, the physiological levels of this lipid in biomembranes were reviewed in *(Gomez-Fernandez and Corbalan-Garcia, 2007)*. For example, quantitative measurements of diacylglycerol present in stimulated cells have showed that it may reach 1.45 mol% of the total lipid concentration *(Preiss et al., 1986)* or about 2 mol% *(Takuwa et al., 1987)*.

So the concentrations of diacylglycerol used in this work can be considered physiological and well within the range of diacylglycerol concentrations used in standard procedures for PKC activation assays, which use values similar to those used here *(Sanchez-Pinera et al., 1999)* or even as high as 11.5 mol% with respect to total lipid *(Ogita et al., 1991)* or 19 mol%- *(Wooten et al., 1987)* or 25 mol% *(Bolen and Sando, 1992)*. With respect to the physiological concentration of phosphatidylserine in the inner monolayer of eukaryotic plasma membranes, such as in erythrocyte or platelet cells, this reaches roughly 20 mol% *(Chap et al., 1977; Leventis and Grinstein, 2010; Verkleij et al., 1973)*, so that the concentration used in this work is within the physiological range.

It is also interesting that PKCη showed a higher membrane binding affinity than PKCε in the absence of DOG and when POPS and POPG were the anionic phospholipids present. Nevertheless, POPA was the anionic phospholipid for which the lowest K_D was observed. Phosphatidic acid is produced in membranes by enzymatic activity through two routes, the action of phospholipase D and that of diacylglycerol kinases. Phosphatidic acid constitutes approximately 1–5% of total cellular lipids *(Athenstaedt and Daum, 1999; Stace and Ktistakis, 2006)* and is found both in plasma and inner membranes like the Golgi network *(Fernandez-Ulibarri et al., 2007; Merida et al., 2008; Yang et al., 2008)*.

Nevertheless, it should be taken into account that local concentrations will be considerably higher in the immediate vicinity of the points where phosphatidic acid is being synthesized. It is interesting that phosphatidic acid has been seen to be important in the activation of PKCε *(Corbalan-Garcia et al., 2003c; Lopez-Andreo et al., 2003)* and, although it has been shown that this phospholipid binds to the C2 domain of PKCε *(Corbalan-Garcia et al., 2003c; Garcia-Garcia et al., 2001; Lopez-Andreo et al., 2003; Sanchez-Bautista et al., 2007)*, the findings described here suggest that lipid microdomains containing

both PA and diacylglycerol also play an important role in inducing the anchorage of C1 domain in membranes.

The experiments carried out with RBL-2H3 cells confirmed that two groups can be defined within the C1Bs domains of novel PKCs. This was observed when cells were stimulated with DNP-HSA, although differences were found in the time of translocation rather than in the maximum translocation rate. It seems that high concentrations of activators are generated when using this concentration of antigen and thus this experiment has a similarity with the one in which we used PMA (Table IV.3).

When using permeable activators, it was found that whereas high percentages of C1Bη and C1Bε may be translocated to the cell membrane following the addition of phosphatidic acid, C1Bδ and C1Bθ need the addition of diacylglycerol before their translocation. According to our observations made in model membranes, if enough phosphatidic acid can be incorporated in the cell membranes the four domains will be translocated, but when soluble phosphatidic acid is added only a limited concentration is expected to be reached in the cell membrane, which will not be sufficient for the translocation of C1Bδ and C1Bθ in the absence of diacylglycerol.

It would be interesting to ascertain whether if the differences observed between the C1Bs of novel PKCs are related with their physiological specializations. It has been described, for example, that the activation of PKCε and PKCδ has opposing consequences in ischemic myocardium *(Murriel and Mochly-Rosen, 2003)*. Whereas PKCε activation has a cardioprotective effect *(Inagaki et al., 2006)*, PKCδ activation mediates much of the acute injury induced after transient myocardial ischemia *(Chen et al., 2001; Churchill et al., 2005)*; also PKCθ and PKCη play different roles in signal transductions in T cells. When an immunological synapse is formed between a T cell and an antigen-presenting cell, PKCθ concentrated in the central region of the synapse, while PKCη diffuses over the whole area of the synapse, suggesting that each may bind to a different activator in the membrane *(Fu and Gascoigne, 2012)*. In addition, the deficiency of PKCη or PKCθ in T cells had opposing effects in knockout mice, since PKCη knockout had a higher CD4 to CD8 ratio *(Fu and Gascoigne, 2012)*. Given these differences in functions and effects within the same cells, it seems plausible that PKCε and PKCη may have different degrees of sensitivity to activators from PKCδ and PKCθ (as we have shown in this paper): each of these

isoenzymes would be translocated to the area of the plasma membrane or to the organelle where there is a more favourable combination of diacylglycerol and anionic phospholipid, even though some of them may be more responsive to low concentrations of one of these activators.

It is widely known that the C1 domain binds to the membrane by interacting with DAGs (or phorbol esters). It has been observed that each C1 domain may have preference for a certain subcellular membrane and these differences help to explain the divergent localization and distinct functional roles of the full-length proteins, which contains them. This selectivity has been explained by their capacity to discriminate between DAG pools [59]. But here we may demonstrate that electrostatic interactions provided by anionic phospholipids also make an important contribution to the specific targeting of C1 domains to cell membranes. These protein-phospholipid interactions have been found to be quite important for the binding of many extrinsic proteins, such as cytochrome *c (Gorbenko et al., 2006; Smith et al., 1983)*, myelin basic protein *(Jo and Boggs, 1995)*, phospholipases *(Rebecchi et al., 1992)*, K-Ras *(Hancock et al., 1990)*, charybdotoxin *(BenTal et al., 1997)*, cardiotoxin *(Carbone and Macdonald, 1996)* and Aβ peptides *(Martinez-Senac et al., 1999)*.

CONCLUSSION

Taken together, our results show that (i) C1B domains from novel PKCs have different binding affinities for model membranes depending on the presence or not of diacylglycerol and of specific anionic phospholipids, (ii) this is also observed in RBL-2H3 cells in which the different C1B domains were expressed (iii) two groups of C1B domains from novel PKCs can be established as a function of their membrane binding affinity: those from PKCε and PKCη with a higher membrane binding affinity and those with PKCδ and PKCθ with a low affinity.

CHAPTER V

Role of positive charged amino acid residues in the C1B domain of PKCε on membrane translocation and enzymatic activity.

ABSTRACT

In this work we have studied the role of positively charged amino acids residues located on top of the C1Bε domain and their involvement in the interaction membrane-protein. More specifically, K251, R268, R282 and R283 were replaced by alanine and we characterized the effect of single, double, triple and quadruple mutations in isolated domains and in the full-length PKCε.

To carry out this study the different C1Bε mutants were expressed fused to ECFP in HEK293 cells. Fluorescent constructs were obtained from cell lysates and binding to lipid vesicles (SUV) labelled with Oregon Green was monitored through FRET. Furthermore, each mutant in isolated domains and in the full-length PKCε was expressed as well in RBL-2H3 cells and their subcellular localization studied after stimulation with different concentrations of 1,2-dioctanoyl-sn-glycero (DOcG), 1,2-dioctanoyl-sn-glycerol 3-phosphate (DOcPA) and with the endogenous activators generated through the stimulation of RBL-2H3 cells with the antigen. These endogenous activators have been characterized by a lipidomic analysis, confirming the appearance of DAG and PA upon cell stimulation.

Results show that membrane binding decreases when te number of residues mutated increases, an effect that is overcome by diacylglycerol. In conclusion, the electrostatic interactions derived of these positively amino acids residues is important to give place to membrane translocation which is further stabilized by interaction with the diacylglycerol.

1. INTRODUCTION

Protein kinases C (PKC) are a family of serine/threonine kinases that mediate numerous cellular processes *(Corbalan-Garcia and Gomez-Fernandez, 2006; Newton, 1995b; Nishizuka, 1988)*. Based on structural differences in the regulatory domain of PKCs are classified into three groups: classical or conventional PKCs (α, βI, βII, and γ), novel PKCs (ε, η, δ and θ) and atypical PKCs (ζ and ι/λ) *(Corbalan-Garcia and Gomez-Fernandez, 2006; Corbalan-Garcia et al., 2003c; Hofmann, 1997; Mellor and Parker, 1998)*. Classical and novel isoenzymes bear C2 and C1 domains. In conventional PKCs, C1A and C1B domains are connected to a calcium-binding C2 domain at the carboxyl-terminal side, whereas in novel PKCs a non-calcium binding C2 domain is located at the amino-terminal side of the C1 domains. The C2 domain of classical PKCs

responds to the increase in Ca^{2+} concentration in the cytoplasm through the bridging made by this cation between the C2 domain and negatively charged phospholipids in the membrane (with preference phosphatidylserine) *(Verdaguer et al., 1999)*, and also by interaction with PIP_2 in the membrane through a second site *(Corbalan-Garcia et al., 2003b; Guerrero-Valero et al., 2009b; Guerrero-Valero et al., 2007a; Marin-Vicente et al., 2005b; Marin-Vicente et al., 2008a; Sanchez-Bautista et al., 2006b)*, followed by C1 domain–diacylglycerol interactions *(Corbalan-Garcia and Gomez-Fernandez, 2006; Gallegos and Newton, 2008; Leonard et al., 2011)*.

In contrast, the enzymes of the novel PKC subfamily do not bind Ca^{2+} and are activated through diacylglycerol or phorbol esters binding to the C1 domain *(Newton, 2001)*, although the C2 domain also plays a role through interactions with ligands like phosphatidic acid *(Corbalan-Garcia et al., 2003c; Lopez-Andreo et al., 2003)*. It is still not yet understood why classical and novel PKCs contain two highly homologous C1 domains and what specific roles the two C1 domains play in the activation of the PKCs. It has been described that each C1 domain posses a different affinity for diacylglycerol and phorbol ester *(Ananthanarayanan et al., 2003b; Irie et al., 2002a; Irie et al., 1997; Quest and Bell, 1994a; Shindo et al., 2001; Stahelin et al., 2005b; Stahelin et al., 2004b)*

The affinities of C1A and C1B for these lipids change depending on the isoenzyme. For example, it has been described that whereas the C1A domain of PKCα shows a much higher DAG affinity than the C1B domain, furthermore, the C1A has also a higher affinity for phorbol esters than the C1B but affinities are not so different in this case *(Abdo et al., 2001; Ananthanarayanan et al., 2003a; Irie et al., 2002b)*. In the case of other classical isoenzymes, like β *(Irie et al., 2002b)* and γ *(Ananthanarayanan et al., 2003a; Burns and Bell, 1991a; Irie et al., 2002b; Quest and Bell, 1994a)* the affinities of C1A and C1B domains for these lipids are very similar.

For PKCδ conflicting results have been published, one group *(Irie et al., 2002b)* claims that C1B has a much higher binding affinity for diacylglycerol than C1A, while another group *(Stahelin et al., 2004a)* maintains that the opposite is true. PKCε is similar to PKCγ in that both C1A and C1B domains are involved in membrane binding and activation, although the C1A domain has been described as having about a 3-fold higher DAG affinity than the C1B domain *(Stahelin et al., 2005b)*. With respect to the other new isoenzymes, fewer studies have been carried out, but it seems that in both θ and η the C1B domain has a

higher affinity than the C1A domain for phorbol esters *(Irie et al., 2002b; Quest and Bell, 1994a)*.

It has been previously shown that the C1B domains of PKCγ, PKCε and PKCδ have a notorious affinity for negatively charged phospholipids *(Sanchez-Bautista et al., 2009)* evidencing that membrane binding may depend not only on the interaction of C1 domains with diacylglycerol or phorbol esters but also with other phospholipids residing at the plasma membrane. The studies of the binding of the C1B domains of novel PKCs to model membranes and to the plasma membrane of RBL-2H3 indicate that those of ε and η have a considerably higher affinity than those of δ and θ (Chapter IV).

In this paper we have studied the importance of some positively charged residues for the translocation of the C1B domain of PKCε and how some of these residues are changed in PKCδ explaining its lower affinity for membrane binding. These residues were also shown to be important for the membrane translocation of full enzyme PKCε when expressed in RBL-2H3 and their mutation decreased their membrane translocation when cells were stimulated. It was also shown, using lipidomics techniques, that stimulation of these cells led to an increase in diacylglycerol and phosphatidic acid and these activating lipids explain the translocation of PKCε to the membrane.

2. RESULTS

Given the differences observed for the affinity of the C1B domains of novel PKCs for negatively charged phospholipids, the amino acid sequence and model structure of C1Bε was studied deeply (Figure V.1 and Figure V.2). We identified several amino acidic residues that could be involved in the interaction with acidic phospholipids: lysine 251 and arginiges 268, 282 and 283.

Figure V.2. shows the C1Bε domain in complex with PMA and the localization of the aminoacidic residues in the 3D structure.

C1Bε-PHKFGIHNYKVPTFCDHCGSLLWGLLRQGLQCKVCKMNVHRRCETNVAPNC

Figure V.1. Sequence alignment of C1Bε domain. In total 51 amino acids. The red letters in yellow boxes represent the amino acids residues that have been selected as possible candidates in the binding of the C1Bε domain to membranes. Blue letters represent other positive charged amino acid residues that were ruled out.

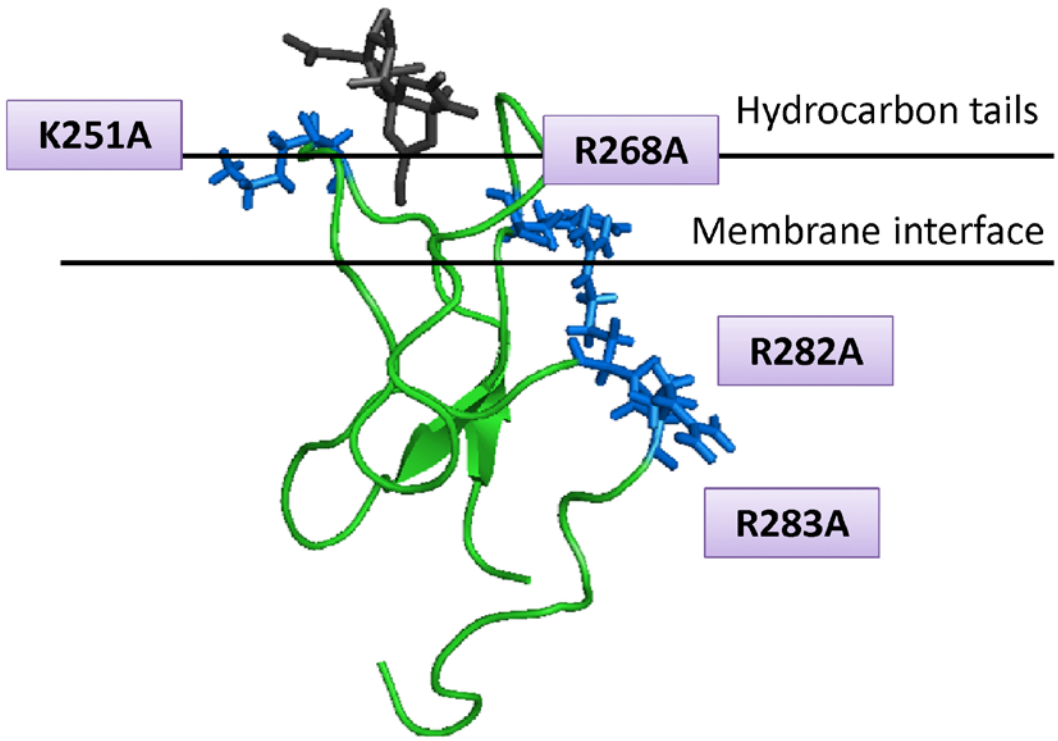

Figure V.12. Secondary structure prediction of C1Bε domain in complex with PMA. C1Bε domain is anchored to the membrane through the polar part of a PMA molecule. The ribbon drawing is represented in green. The PMA molecule is represented as grey sticks and the positively charged residues are blue sticks. These are labeled with their type and position in the sequence in purple boxes. Molecular figures were prepared by using PyMOL (Delano WL (2002) The PyMOL Molecular Graphics System).

The aim of this work was to confirm that these amino acid residues located on the C1B domain play an important role in the translocation of the full-length PKCε to the plasma membrane. To that end, we will do site-directed point mutations to make singles, doubles, triples and quadruple mutants of isolated domains and full-length protein. FRET studies will be carried out using membrane models containing two different negatively charged phospholipids (POPA and POPS) in the presence or absence of DOG. In addition, RBL-2H3 cells will be transfected with these constructs and their translocation from the cytosol

to the plasma membrane will be induced by permeable activators or cell activation through antigen-receptor binding. Finally, assays of kinase activity will be performed to elucidate if the substitutions by alanine could inhibit the activity of the enzyme.

2.1. Binding to membranes of C1Bε and the effect of mutating key positively charged amino acid residues.

Using the FRET method, the binding of C1B domains to small unilamellar vesicles containing different lipid compositions was studied. Figure V.3 and V.4 show the binding of the C1Bε domain in vesicles containing 20 mol% of POPS or POPA respectively. In this assay, the effect of the presence or absence of DOG on C1B membrane binding was also studied. The effect of introducing site-directed point mutations that targeted key positively charged residues, which were inferred to be important for the electrostatic interaction protein-membrane, according to the molecular model discussed above was also studied. The data were analyzed and best fitted to the Hill model.

When the composition was POPC/POPS/DOG/OGPE (70:20:5:5 molar ratio), single mutations decreased the binding affinity of the protein for the membrane. Whereas wild type C1Bε showed a K_D of 1.91 μM, C1BεK251A-ECFP has 2.37 μM, C1BεR283A-ECFP 3.44 μM, C1BεR268A-ECFP 7.39 μM and C1BεR282A-ECFP 12.11 μM (Table V.1). The mutations have a greater effect when diacylglycerol was omitted from the membrane (Figure V.3.D), and in this case K_D values were 25.43 μM for the wild type C1Bε-ECFP domain, C1BεK251A-ECFP has 43.56 μM, C1BεR283A-ECFP 55.60 μM, C1BεR268A-ECFP 35.05 μM and C1BεR282A-ECFP 38.46 μM (Table V.1). It is clear that the single mutation that produced the highest effect was R282A in the presence of DOG, while in the absence of DOG, all the single mutations had a similar effect, it could be explained if the presence of DOG could not compensate the lack of the positive charge arginine located in the position 282, this residue being necessary for the docking of the domain to the phosphatidylserine containing membrane. Also ΔF_{max} indicated that the construct with a single mutation giving the maximum effect was C1BεR282A-ECFP in the presence of DOG.

Since single mutations have a very limited change capacity on the binding of C1Bε to membranes, double mutations on the domain were

performed producing considerably higher effects. In the presence of DOG (Figure V.3.B), the three double mutations obtained increased the K_D from 1.91 μM in the wild type to about 20 μM, i.e. about one order of magnitude as seen in Table V.1 (C1BεK251A/R268A-ECFP, 23.47 μM, C1BεR268A/R283A-ECFP 17.23 μM, C1BεR282A/R283A-ECFP and 21.43 μM). In the absence of DOG (Figure V.3.E) and after the double mutations, K_D increased with respect to the wild type domain (25.43 μM) to about 50-60 μM (C1BεK251A/R268A-ECFP , 62.78 μM, C1BεR268A/R283A-ECFP, 60.44 μM and C1BεR282A/R283A-ECFP, 56.83 μM) (Table V.1).

The effect of introducing triple mutations was even bigger than with single or double mutations and in the presence of DOG, going up to near 30 times higher than with the wild type protein (C1BεK251A/R268A/R282A-ECFP, 36.06 μM; C1BεK251A/R282A/R283A-ECFP, 38.56 μM; C1BεR268A/R282A/R283A-ECFP 59.64 μM). In the absence of diacylglycerol the differences were not very important with respect to the double mutants (C1BεK251A/R268A/R282A-ECFP, 64.37 μM C1BεK251A/R282A/R283A-ECFP, 59.07 μM and C1BεR268A/R282A/R283A-ECFP, 76.67 μM). Again as with single and double mutants, ΔF_{max} decreased considerably with respect to the wild type protein (Table V.1), from 0.63 in the wild type to about 0.25 in the triple mutants.

As a summary, it appears that, in the presence of DOG, mutation of residue R282 causes the most damaging effect for the binding, followed by R268 and R283, K251 being the least effective one. In the case of double mutants only small differences were found between them. In the triple mutants, however, C1BεR268A/R282A/R283A-ECFP was clearly the most damaged one, confirming that R282 and R268 are the most important residues for the binding. It is interesting that in the absence of DOG, however, only small differences between them were observed for the single and double mutants and only in the case of the triple mutants C1BεK251A/R282A/R283A-ECFP clearly stands out as the most damaged one of the three triple mutants studied that could suggest us that there is no residue more important than others in the absence of DOG.

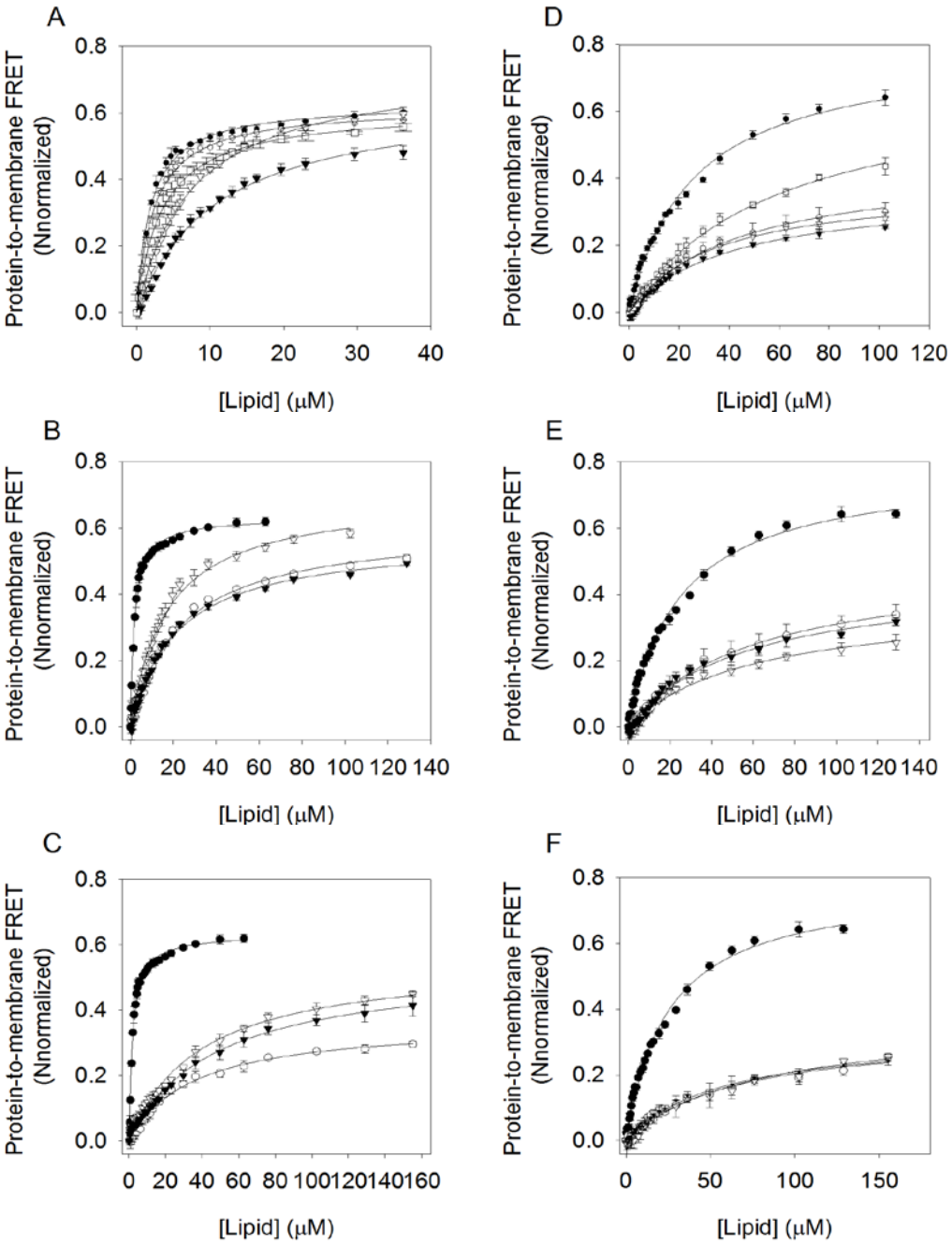

Figure V.3. Binding of C1Bε and its mutants to lipid vesicles containing POPS in the presence or absence of DOG. Binding of C1Bε-ECFP domain (•) and its simple mutants K251A (o), R268A (▽), R282A (▼) and R283A (□) (A and D); double mutants K251A/R268A (o), R268A/R283A (▽), R282A/R283A (▼) (B and E); and triple mutants K251A/R268A/R282A (o), K251A/R282A/R283A (▽), R268A/R282A/R283A (▼) (C and F) to lipid vesicles, which contained POPC/POPS/OGPE in molar ratios of 75:20:5 in the presence of DOG at 5 mol% (A, B and C) or its absence (D, E and F). Normalized FRET values are depicted versus total lipid concentration.

	POPC/POPS/DOG/OGPE (70:20:5:5)		POPC/POPS/OGPE (75:20:5)	
C1Bε-ECFP	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)
Wild-type	0.62 ± 0.03	1.91 ± 0.12	0.63 ± 0.04	25.43 ± 0.2
K251A	0.58 ± 0.01	2.37 ± 0.2	0.30 ± 0.03	43.56 ± 1.2
R268A	0.60 ± 0.02	7.39 ± 0.2	0.27 ± 0.02	35.05 ± 1.0
R282A	0.56 ± 0.02	12.11 ± 1.1	0.25 ± 0.01	38.46 ± 0.2
R283A	0.56 ± 0.01	3.44 ± 0.3	0.44 ± 0.03	55.60 ± 0.5
K251A/R268A	0.51 ± 0.01	23.47 ± 0.9	0.37 ± 0.03	62.78 ± 0.2
R268A/R283A	0.58 ± 0.01	17.23 ± 0.6	0.25 ± 0.02	60.44 ± 0.8
R282A/R283A	0.49 ± 0.01	21.43 ± 0.8	0.34 ± 0.02	56.83 ± 0.9
K251A/R268A/R282A	0.27 ± 0.01	36.06 ± 0.5	0.24 ± 0.01	64.37 ± 0.7
K251A/R282A/R283A	0.45 ± 0.01	38.56 ± 0.5	0.26 ± 0.01	59.07 ± 0.3
R268A/R282A/R283A	0.32 ± 0.02	59.64 ± 1.1	0.25 ± 0.01	76.67 ± 0.8

Table V.1. Binding parameters of C1Bε-ECFP mutant domains to phospholipid vesicles.
Phospholipid vesicle contained 75 mol % POPC, 20 mol % POPS, X mol % of DOG, 5 mol % OG-PE. FRET data were fitted to a Hill equation. Values given as mean ± SE of three different experiments.

When the composition was POPC/POPA/DOG/OGPE (70:20:5:5 molar ratio), single mutations had a moderate impact of the binding affinity drop of the protein for the membrane (Figure V.4-A). In this case wild-type C1Bε-ECFP showed a K_D of 1.18 μM (Table V.2), and the mutation that gave the maximum effect was C1BεR268A-ECFP for which K_D increased to 7.98 μM (Table V.2). Other single mutations were less effective (K_D for C1BεK251A-ECP, 2.05μM; C1BεR282A-ECFP, 2.32 μM; C1BεR283A-ECFP, 2.14 μM). The double mutations had a bigger effect (Figure V.4-B), especially in the C1BεK251A/R268A-ECFP construct with a K_D of 16.29 μM. Very high was the effect on the binding of the triple mutations (Figure V.4-C), especially in the case of the construct C1BεK251A/R282A/R283A-ECFP, for which K_D increased to 49.08 μM and also significant increases in the other two constructs (C1BεR268A/R282A/R283A-ECFP, 34.89 μM and C1BεK251A/R268A/R282A-ECFP, 31.57μM). The case of the triple mutants ΔF_{max} was also remarkably affected decreasing in about 50% in the three mutants studied.

More damaging resulted these single mutations when DOG was absent from the membrane (POPC/POPA/OGPE, molar ratio 75:20:5), wild-type C1Bε-ECFP showed a K_D of 3.03 μM and except C1BεK251A-ECFP with a K_D of 7.98 μM, the other three have a similar effect of increasing K_D in about 4-5 times (C1BεR268A-ECFP, 13.17; C1BεR282A-ECFP, 11.57; C1BεR283A-ECFP, 15.43 μM)

(Figure V.4-D). Especially inhibitory with respect to the binding were the double mutations as seen both through K_D (C1BεK251A/R268A-ECFP, 45.12 μM; C1BεR268A/R283A-ECFP, 45.59 μM; C1BεR282A/R283A-ECFP, 31.86 μM) (Figure V.4-E and Table V.2). The inhibitory effect of these double mutations is also clearly seen through the decrease in ΔF_{max}, with a decrease of up to 50% with respect to the wild type (Figure V.4-E and Table V.2). The highest inhibitory effects were observed in the case of the triple mutations with very important increases in K_D (C1BεK251A/R268A/R282A-ECFP, 57.1 μM; C1BεK251A/R282A/R283A-ECFP, 77.89 μM and C1BεR268A/R282A/R283A-ECFP, 53.21μM) and also in ΔF_{max} decreasing to 0.18-0.19 from 0.58 in the wild type.

These results confirm that in spite of the fact that POPA is a bigger activator than POPS, when the positive charges of the domain are neutralized when they are changed to alanines, there is no difference between both phospholipids, not only when the membranes contain DOG, but also in their absence.

	POPC/POPA/DOG/OGPE (70:20:5:5)		**POPC/POPA/OGPE (75:20:5)**	
C1Bε-ECFP	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)
Wild-type	0.67 ± 0.02	1.18 ± 0.1	0.58 ± 0.04	3.03 ± 0.4
K251A	0.57 ± 0.03	2.05 ± 0.2	0.52 ± 0.04	7.98 ± 0.8
R268A	0.54 ± 0.02	7.34 ± 0.9	0.39 ± 0.01	13.17 ± 0.1
R282A	0.54 ± 0.02	2.32 ± 0.1	0.53 ± 0.04	11.57 ± 0.7
R283A	0.43 ± 0.02	2.14 ± 0.8	0.04 ± 0.01	15.43 ± 0.9
K251A/R268A	0.55 ± 0.02	16.29 ± 0.4	0.33 ± 0.01	45.12 ± 1.0
R268A/R283A	0.68 ± 0.01	10.57 ± 0.9	0.27 ± 0.04	45.59 ± 0.7
R282A/R283A	0.63 ± 0.02	10.10 ± 0.5	0.31 ± 0.03	31.86 ± 0.2
K251A/R268A/R282A	0.31 ± 0.01	31.57 ± 0.5	0.19 ± 0.02	57.10 ± 0.7
K251A/R282A/R283A	0.40 ± 0.01	49.08 ± 0.1	0.18 ± 0.01	77.89 ± 0.7
R268A/R282A/R283A	0.38 ± 0.01	34.89 ± 0.5	0.19 ± 0.01	53.21 ± 0.7

Table V.2. Binding parameters of C1Bε-ECFP mutant domains to phospholipid vesicles.
Phospholipid vesicle contained 75 mol % POPC, 20 mol % POPA, X mol % of DOG, 5 mol % OG-PE. FRET data were fitted to a Hill equation. Values given as mean ± SE of three different experiments.

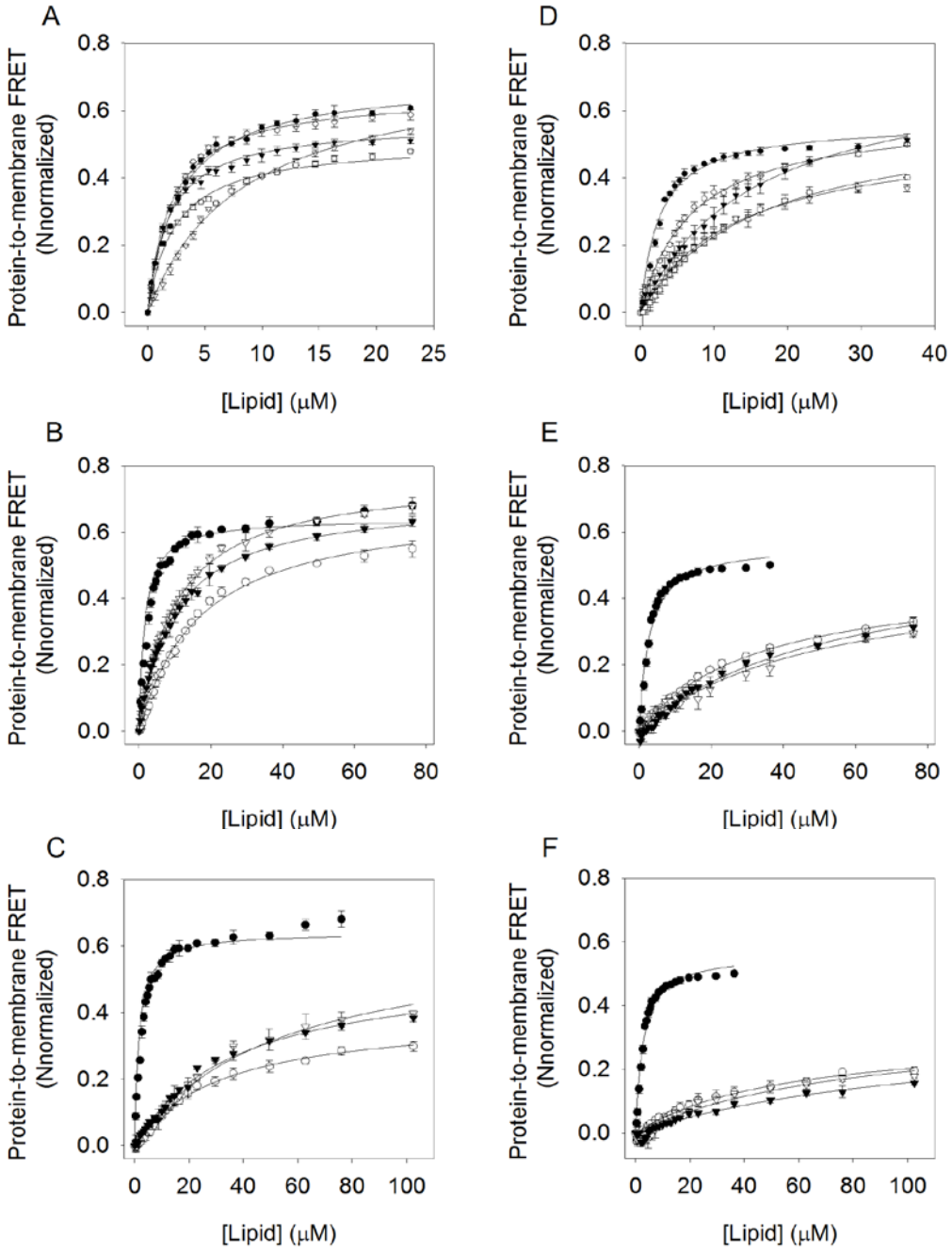

Figure V.3. Binding of C1Bε and its mutants to lipid vesicles containing POPA in the presence or absence of DOG. Binding of C1Bε-ECFP domain (•) and its simple mutants K251A (o), R268A (▽), R282A (▼) and R283A (□) (A and D); double mutants K251A/R268A (o), R268A/R283A (▽), R282A/R283A (▼) (B and E); and triple mutants K251A/R268A/R282A (o), K251A/R282A/R283A (▽), R268A/R282A/R283A (▼) (C and F) to lipid vesicles, which contained POPC/POPS/OGPE in molar ratios of 75:20:5 in the presence of DOG at 5 mol% (A, B and C) or its absence (D, E and F). Normalized FRET values are depicted versus total lipid concentration.

2.2. Transforming C1Bε in C1Bδ-like.

It has been confirmed in the experiments commented above that the mutated residues are important for the binding of the C1Bε domain to negatively charged membranes. According to Figure V.2 there are a few important residues that could be the base of the differences observed in the binding between isoenzymes where C1Bε showed the highest binding affinity for negatively charged phospholipids and C1Bδ was the lowest.

It can be thought on these bases that it is possible to convert the binding properties of C1Bε in something similar to those shown by C1Bδ. It can be observed in Figure V.5 that of the 4 residues mutated in C1Bε, 2 of them are positively charged in C1Bδ (K256 and K271) but the two other residues correspond to M239 and H270. Therefore the construct C1BεK251M/R282H–ECFP would be a C1Bδ-like domain.

```
C1Bε 242-PHKFGIHNYKVPTFCDHCGSLLWGLLRQGLQCKVCKMNVHRRCETNVAPNC
C1Bδ 230-PHRFKVHNYMSPTFCDHCGSLLWGLVKQGLKCEDCGMNVHHKCREKVANLC
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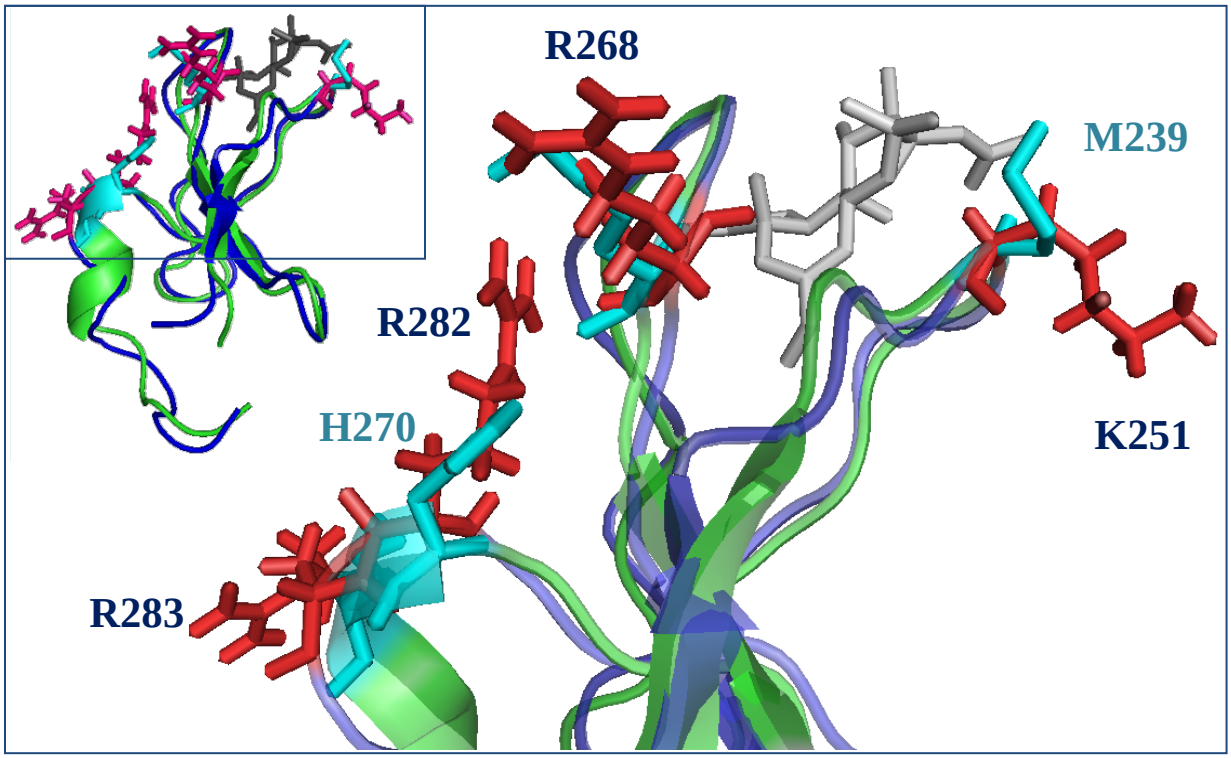

Figure V.5. Sequence alignment of C1Bε domain and C1Bδ domains and an overlay of C1Bε (cartoon blue) and C1Bδ (cartoon green). The residues that could be the base of the differences between C1Bε (sticks red) and C1Bδ (cyan) are represented. Molecular models were prepared by using PyMOL (Delano WL (2002) The PyMOL Molecular Graphics System).

The spatial orientation of these selected amino acid residues are different between both domains, as can be seen in Figure V.5, being more accessible to the interaction of membranes those residues belonging to the C1Bε domain.

The binding of this construct to membranes was studied side by side with the wild-type domain. Figure V.6-A shows that these mutations decreased ΔF_{max} and increased K_D with respect to the wild-type C1Bε domain for the binding to POPC/POPS/DOG/OGPE (70:20:5:5 molar ratio) and whereas wild-type C1Bε-ECFP shows a K_D 1.91 μM the K_D of the construct C1BεK251M/R282H-ECFP increased to 11.30 μM although still lower than the K_D value of the wild-type C1Bδ-ECFP that is 22.60 μM. ΔF_{max} value decreased with the mutations and was considerably lower for C1BεK251M/R282H-ECFP compared to wild type C1Bε-ECFP and very similar for the obtained in C1Bδ (Table V.3).

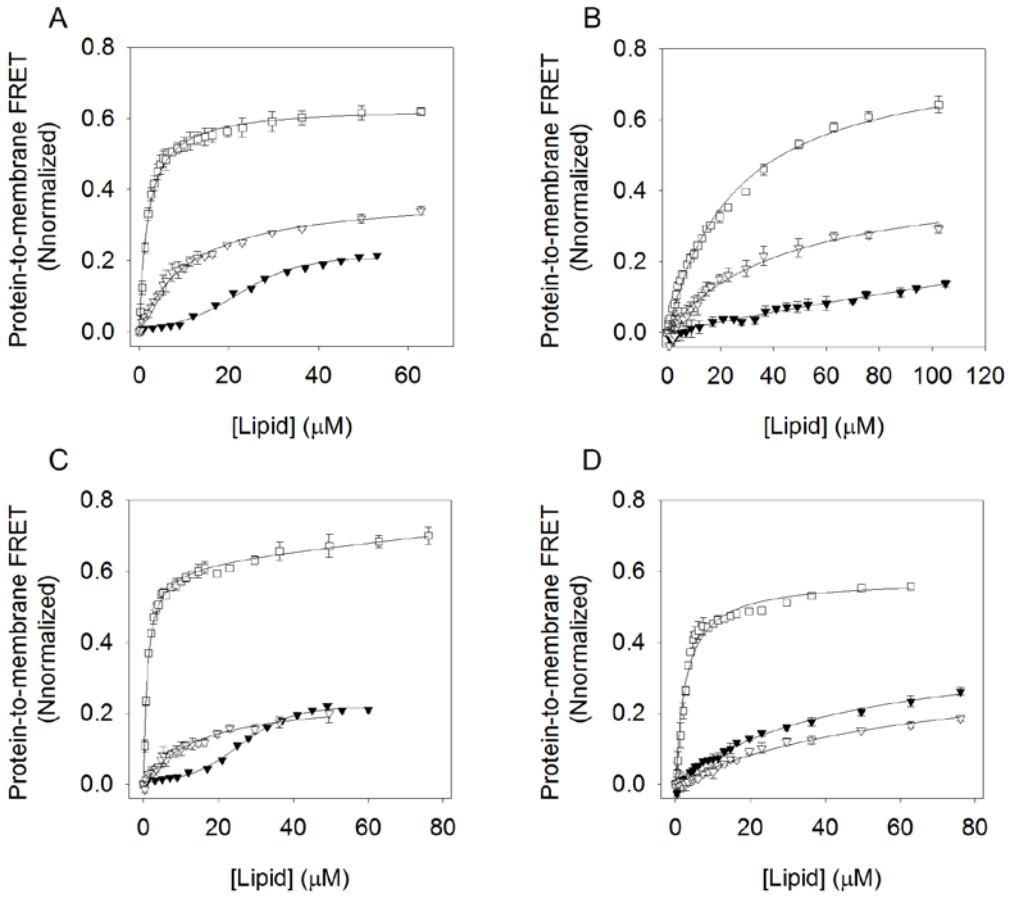

Figure V.6. Binding of C1B domains to lipid vesicles in the presence or absence of DOG. Binding of C1Bε-ECFP (□), C1Bδ-ECFP (▼) and C1BεK251M/R282H-ECFP (▽) domains to lipid vesicles, which contained POPC/POPX/OGPE in molar ratio of 75:20:5. POPX was POPS (A and B) and POPA (C and D). DOG was present at 5 mol% (A and C). Normalized FRET values are depicted versus total lipid concentration.

In the absence of diacylglycerol (Figure V.6.B), with a membrane composition POPC/POPS/OGPE (75:20:5, molar ratio), the differences between wild-type C1Bδ-ECFP and construct C1BεK251M/R282H-ECFP were smaller than in the presence of diacylglycerol (Table V.3).

When the membrane vesicles contained POPC/POPA/DOG/OGPE (70:20:5:5, molar ratio), the results were similar to the values observed with POPS, in particular, the K_D value of the wild type C1Bδ-ECFP was a bit higher compared to the mutant C1BεK251M/R282H-ECFP in the presence of DOG (Figure V.6.C and Table V.3) (C1BεK251M/R282H-ECFP, 14.53 μM; C1Bδ-ECFP, 20.40 μM). On the other hand, wild type C1Bδ-ECFP had a lower K_D than the mutant in the absence of DOG (Figure V.6.D and Table V.3) (C1Bδ-ECFP, 32.06 μM; C1BεK251M/R282H-ECFP, 47.89 μM). Nevertheless, wild type C1Bε-ECFP showed the lowest K_D values in both cases (1.18 and 3.03 μM in the presence or in the absence of DOG respectively).

The results obtained show that transforming C1Bε in C1Bδ-like is possible, according to this FRET data, with the substitutions of the positively charged residues K251 and R282 by a methionine and a histidine in that order.

	PC/PS/DOG/OGPE (70:20:5:5)		**PC/PS/OGPE (75:20:5)**	
	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)
Wild-type C1Bε-ECFP	0.62 ± 0.03	1.91 ± 0.1	0.63 ± 0.04	25.43 ± 0.2
C1BεK251M/R282H -ECFP	0.32 ± 0.01	11.30 ± 1.9	0.26 ± 0.03	47.89 ± 1.3
Wild-type C1Bδ-ECFP	0.24 ± 0.02	22.60 ± 0.2	0.17 ± 0.02	32.06 ± 0.5

	PC/PA/DOG/OGPE (70:20:5:5)		**PC/PA/OGPE (75:20:5)**	
	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)
Wild-type C1Bε-ECFP	0.62 ± 0.03	1.18 ± 0.1	0.58 ± 0.04	3.03 ± 0.3
C1BεK251M/R282H -ECFP	0.20 ± 0.03	14.53 ± 1.2	0.17 ± 0.03	46.47 ± 1.5
Wild-type C1Bδ-ECFP	0.21 ± 0.05	20.40 ± 0.8	0.26 ± 0.02	43.65 ± 0.8

Table V.3. Phospholipid vesicle contained 75 mol % POPC, X mol % of DOG, 5 mol % OG-PE, and 20 mol % of the indicated anionic phospholipid. FRET data were fitted to a Hill equation. Values given as mean ± SE of three different experiments.

2.3. Membrane translocation of C1Aε, C1Bε and C1ABε domains in RBL-2H3 cells induced by permeable activators or cell activation and endogenous production of activators.

As a previous step of the expression and stimulation of the C1Bε-ECFP domains and full-length PKCε-EGFP mutants, RBL-2H3 cells were transfected with the constructs C1Aε-ECFP, C1Bε-ECFP and the tandem C1ABε-ECFP, to know, how important would be the level of translocation to the plasma membrane for these mutants, compared to wild type C1Bε domain, and on the other hand, the involvement that could have the C1A domain in the translocation of the full-length protein, because the mutations could be being covered or compensated by this domain.

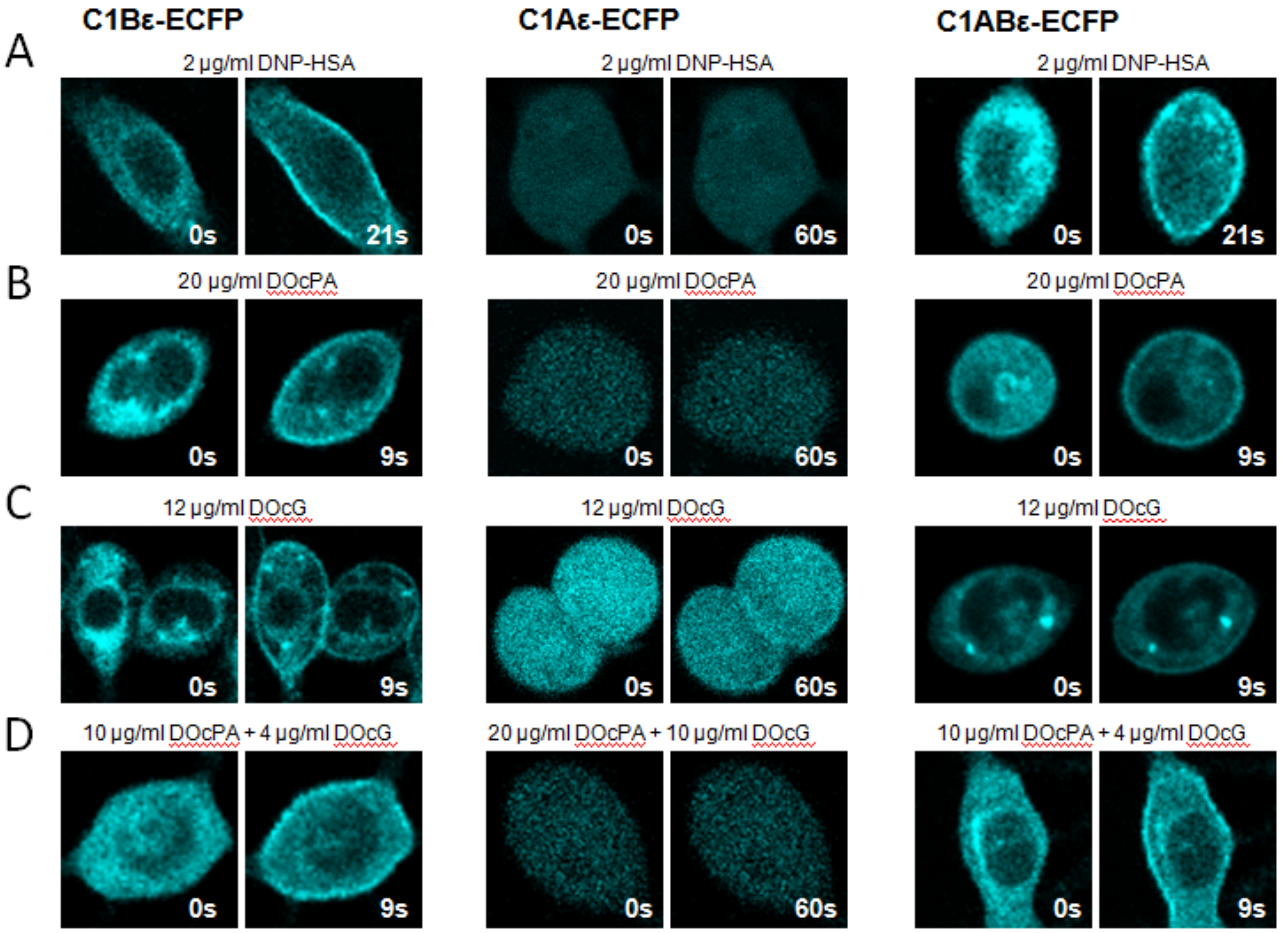

Figure. V.7. Membrane translocation of C1 domains to the plasma membrane of RBL-2H3 cells. Cells were transfected with C1Aε-ECFP, C1Bε-ECFP and C1ABε-ECFP constructs. The pictures show the effect of the addition of antigen (DNP-HSA) or the indicated amounts of soluble diacylglycerol (DOcG) and/or soluble phosphatidic acid (DOcPA).

The first row (A) of the Figure V.7 shows the RBL-2H3 cells stimulated by an antigen as DNP-HSA. Figure V.7-A and Table V.4 indicate that the C1Bε-ECFP and C1ABε-ECFP constructs were able to localize at the plasma membrane after the stimulation of the cells with hardly any difference in R_{max} or $t_{½}$ values between them (C1Bε-ECFP, 0.59 and 19 seconds; C1ABε-ECFP 0.57 and 21 seconds). With respect to C1Aε-ECFP no translocation was observed after being stimulated by the antigen.

Rows B, C and D in the Figure V.7 show the response of the cells after the stimulation of higher concentrations of water soluble forms of activators, in particular, 20 µg/ml of DOcPA (Figure V.7-B); 12 µg/ml of DOcG (Figure V.7-C); and a combination of both (10 µg/ml of DOcPA and 4 µg/ml of DOcG) (Figure V.7-D). As in the previous case, C1Bε-ECFP and C1ABε-ECFP were the constructs that reached membrane translocation under all conditions described. The addition of 20 µg/ml of DOcPA (Table V.4) induced a considerable membrane translocation in C1Bε-ECFP and C1ABε-ECFP (R_{max} 0.44 and 0.40 respectively); the same for the addition of 12 µg/ml of DOcG (R_{max} 0.46 and 0.39 respectively); and the addition of both activators in a low concentration produced the same effects than high concentrations of isolated activators (R_{max} 0.51 and 0.47 respectively). $t_{½}$ were 3 s in all the cases.

Taken together, these results may be concluded that the C1Aε domain does not respond either endogenous activator generated through antigen stimulation nor to permeable activators in this *in*-vivo system. This could be the reason why C1ABε-ECFP and C1Bε-ECFP had the same behavior, because in both cases the domain C1B would be involved in the anchoring of both constructs to the plasma membrane.

The integrity or folding of C1Aε-ECFP construct was checked by stimulation with PMA, responding to the phorbol ester in a same way as C1Bε-ECFP.

Stimulation C1ABε-ECFP	*N* cells	M.L., %	R_{max}	$t_{½}$, s
20 µg/ml DOcPA	16	93	0.40 ± 0.01	3 ± 1
12 µg/ml DOcG	15	93	0.39 ± 0.01	5 ± 1
10 µg/ml DOcPA + 4 µg/ml DOcG	18	100	0.47 ± 0.01	3 ± 1
2 µg/ml DNP-HSA	39	77	0.57 ± 0.01	21 ± 1
Stimulation C1Bε-ECFP	***N* cells**	**M.L., %**	**R_{max}**	**$t_{½}$, s**
20 µg/ml DOcPA	22	96	0.44 ± 0.02	3 ± 1
15 µg/ml DOcPA	31	83	0.40 ± 0.02	3 ± 1
10 µg/ml DOcPA	24	79	0.33 ± 0.03	3 ± 1
5 µg/ml DOcPA	28	46	0.25 ± 0.03	6 ± 1
2 µg/ml DOcPA		0		
12 µg/ml DOcG	29	93	0.46 ± 0.02	3 ± 1
8 µg/ml DOcG	23	78	0.39 ± 0.01	3 ± 1
4 µg/ml DOcG	46	34	0.29 ± 0.03	4 ± 1
2 µg/ml DOcG				
10 µg/ml DOcPA + 4 µg/ml DOcG	21	95	0.51 ± 0.01	3 ± 1
5 µg/ml DOcPA + 4 µg/ml DOcG	18	94	0.41 ± 0.03	3 ± 1
2 µg/ml DOcPA + 2 µg/ml DOcG		0		
2 µg/ml DNP-HSA	62	80	0.59 ± 0.02	19 ± 2
Stimulation C1Aε-ECFP	***N* cells**	**M.L., %**	**R_{max}**	**$t_{½}$, s**
20 µg/ml DOcPA		0		
12 µg/ml DOcG		0		
10 µg/ml DOcPA + 4 µg/ml DOcG		0		
2 µg/ml DNP-HSA		0		

Table V.4. Plasma membrane translocation parameters of C1ABε-ECFP, C1Bε-ECFP and C1Aε-ECFP domains in RBL-2H3 cells stimulated with DNP-HSA, DOcPA and/or DOcG. RBL2H3 cells were primed with 0.5µg/ml antiIgE antibody for 16 h and then stimulated with 2µg/ml DNP-HSA *M.L.* is membrane localization and indicates the percentage of cells responding to DNP-HSA, DOcPA and/or DOcG stimulation with plasma membrane translocation. R_{max} is the maximum relative increase in plasma membrane localization of the domain. $t_{1/2}$ is the half time of translocation.

2.4. Membrane translocation of mutants C1Bε-ECFP in RBL-2H3 cells induced by permeable activators or cell activation and endogenous production of activators.

To investigate membrane translocation of the C1Bε-ECFP mutants, i.e. the involvement of each specific positively amino acidic residue, RBL-2H3 cells were transfected with single mutants constructs (Figure V.8); double mutants constructs (Figure V.9); and triple mutant construct (Figure V.10).

The stimulation with the antigen to start the lipid signaling events as a result of the cross linking of the high-affinity IgE receptor with antigen was

investigated first (Figure V.8-A). All the simple mutants constructs get translocation to the membrane, but they showed different values than the observed for the wild type C1Bε-ECFP (Table V.4 and Table V.5), the membrane localization being ~50% in all the cases (C1BεK251A-ECFP, 58%; C1BεR268A-ECFP, 50%; C1BεR282A-ECFP, 58%; C1BεR283A-ECFP, 47%), i.e. near 1.5 times lower than the obtained for wild type C1Bε-ECFP (80%). Substantial differences were also observed for the t½ with values quite higher for the mutants (C1BεK251A-ECFP, 33 s; C1BεR268A-ECFP, 38 s; C1BεR282A-ECFP, 36 s; C1BεR283A-ECFP, 34 s) in comparison to wild type C1Bε-ECFP (19 s). This means that higher concentrations of activators are required and a longer time is needed for the mutant constructs membrane translocation. We had observed previously the same effect with C1Bδ-ECFP and C1Bθ-ECFP domains (Chapter IV). Nevertheless, in spite of the fact that they needed more time for translocation to the plasma membrane and membrane localization is around a 50% (which means that there is another half of the population that it is not stimulated), R_{max} values are quite similar to that obtained for the wild type in the cases of C1BεK251A-ECFP and C1BεR282A-ECFP (0.54 and 0.52, respectively), followed by C1BεR268A-ECFP (0.47). The mutant C1BεR283A-ECFP showed the highest binding differences with respect to the wild type, with a R_{max} of 0.39.

When we added 20 µg/ml of the soluble form of phosphatidic acid (DOcPA) (Figure V.8-B) to RBL-2H3 cells, only a weak response was observed in mutants C1BεR268A-ECFP and C1BεR282A-ECFP with a membrane localization (M.L.) of ~15% (Table V.5) and a R_{max} of ~13, falling far short from the wild type C1Bε-ECFP with a M.L. of 96% and a R_{max} of 0.44 (Table V.4).

Figure V.8-C and Table V.5 show that 8 µg/ml of DOcG were needed to get membrane translocation of any of the single mutants, whereas when lower concentrations of DOcG (as 4 µg/ml) were no sufficient. C1BεK251A-ECFP and C1BεR283A-ECFP exhibited the same pattern observed for the wild type, with a M.L close to 80% and a R_{max} of ~0.35. However, the other 2 mutants obtained lower values compared to the wild type (Table V.4 and Table V.5) (C1BεR268A-ECFP with a M.L. of 35% and R_{max} of 0.34; C1BεR282A-ECFP, with a M.L. of 35% and R_{max} of 0.24).

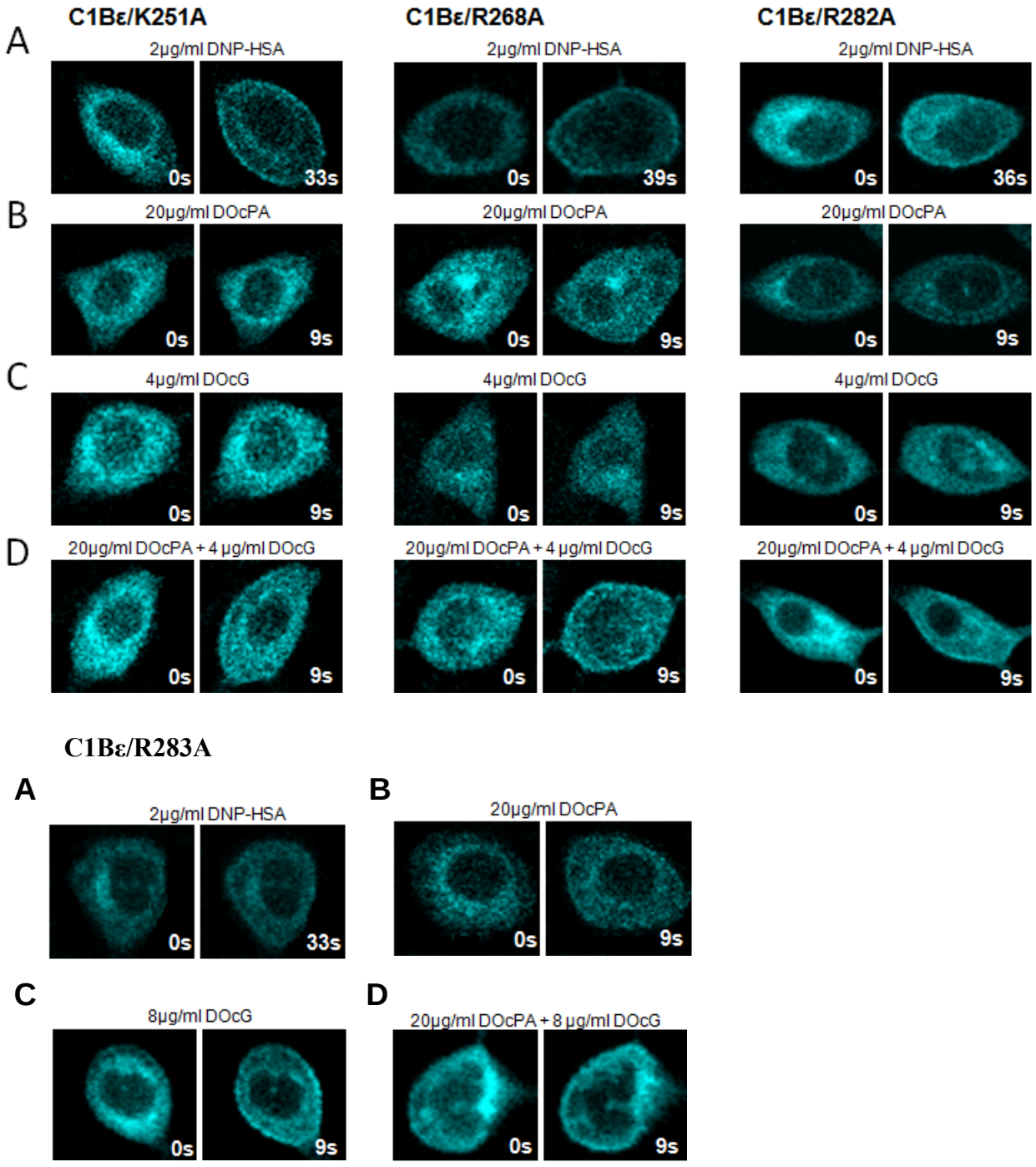

Figure. V.8 Membrane translocation of C1 domains to the plasma membrane of RBL-2H3 cells. Cells were transfected with C1Aε-ECFP, C1Bε-ECFP and C1ABε-ECFP constructs. The pictures show the effect of the addition of antigen (DNP-HSA) or the indicated amounts of soluble diacylglycerol (DOcG) and/or soluble phosphatidic acid (DOcPA).

As we mentioned above, all the studied mutants responded when 4 μg/ml of DOcG in combination with 20 μg/ml of DOcPA were added (Figure V.8-D) showing a remarkable membrane translocation in C1BεK251A-ECFP and C1BεR268A-ECFP (C1BεK251A-ECFP with a M.L. of 76% and R_{max} of 0.43;

C1BεR282A-ECFP, with a M.L. of 64% and R_{max} of 0.36). However, it was necessary to increase DOcG concentration to the double in order to achieve the same response in the cases of C1BεR282A-ECFP and C1BεR283A-ECFP. It is important to remark that wild type C1Bε-ECFP just needed a combination of 5 µg/ml of DOcPA and 4 µg/ml of DOcG to show a M.L. of 94 % and a R_{max} of 0.41 (Table V.4).

1BεK251A-ECFP	***N* cells**	**M.L., %**	R_{max}	$t_{½}$, s
20 µg/ml DOcPA		0		
8 µg/ml DOcG	23	73	0.34 ± 0.01	5 ± 1
4 µg/ml DOcG		0		
20 µg/ml DOcPA + 4 µg/ml DOcG	26	76	0.43 ± 0.02	3 ± 1
2 µg/ml DNP-HSA	34	58	0.54 ± 0.01	33 ± 3
C1BεR268A-ECFP	***N* cells**	**M.L., %**	R_{max}	$t_{½}$, s
20 µg/ml DOcPA	22	18	0.12 ± 0.01	14 ± 1
8 µg/ml DOcG	17	35	0.34 ± 0.01	2 ± 1
4 µg/ml DOcG		0		
20 µg/ml DOcPA + 4 µg/ml DOcG	17	64	0.36 ± 0.02	3 ± 1
2 µg/ml DNP-HSA	22	50	0.47 ± 0.01	38 ± 4
C1BεR282A-ECFP	***N* cells**	**M.L., %**	R_{max}	$t_{½}$, s
20 µg/ml DOcPA	24	16	0.14 ± 0.01	6 ± 1
8 µg/ml DOcG	25	35	0.24 ± 0.03	7 ± 1
4 µg/ml DOcG		0		
20 µg/ml DOcPA + 8 µg/ml DOcG	48	60	0.42 ± 0.01	3 ± 1
20 µg/ml DOcPA + 4 µg/ml DOcG	26	19	0.21 ± 0.01	5 ± 1
2 µg/ml DNP-HSA	26	58	0.52 ± 0.01	36 ± 3
C1BεR283A-ECFP	***N* cells**	**M.L., %**	R_{max}	$t_{½}$, s
20 µg/ml DOcPA		0		
8 µg/ml DOcG	24	79	0.36 ± 0.01	3 ± 1
4 µg/ml DOcG		0		
20 µg/ml DOcPA + 8 µg/ml DOcG	28	82	0.47 ± 0.01	3 ± 1
20 µg/ml DOcPA + 4 µg/ml DOcG		0		
2 µg/ml DNP-HSA	36	47	0.39 ± 0.01	34 ± 2

Table V.5. Plasma membrane translocation parameters of different simple mutants constructs of C1Bε-ECFP domains in RBL-2H3 cells stimulated with DNP-HSA, DOcPA and/or DOcG. RBL2H3 cells were primed with 0.5µg/ml antiIgE antibody for 16 h and then stimulated with 2µg/ml DNP-HSA *M.L.* is membrane localization and indicates the percentage of cells responding to DNP-HSA, DOcPA and/or DOcG stimulation with plasma membrane translocation. R_{max} is the maximum relative increase in plasma membrane localization of the domain. $t_{1/2}$ is the half time of translocation.

When RBL-2H3 cells transfected with double mutants were subjected to the stimulation with the antigen (Figure V.9-A and Table V.6), only C1BεK251M/R282H-ECFP showed a membrane translocation with a M.L. of 32% (three times less than wild type C1Bε-ECFP) and a R_{max} of 0.29 (two times less than wild type C1Bε-ECFP). It should be remembered that this double mutant is the C1Bδ-ECFP-like, and wild type C1Bδ-ECFP showed a R_{max} of 0.52 and a M.L. of 61% (see Chapter IV).

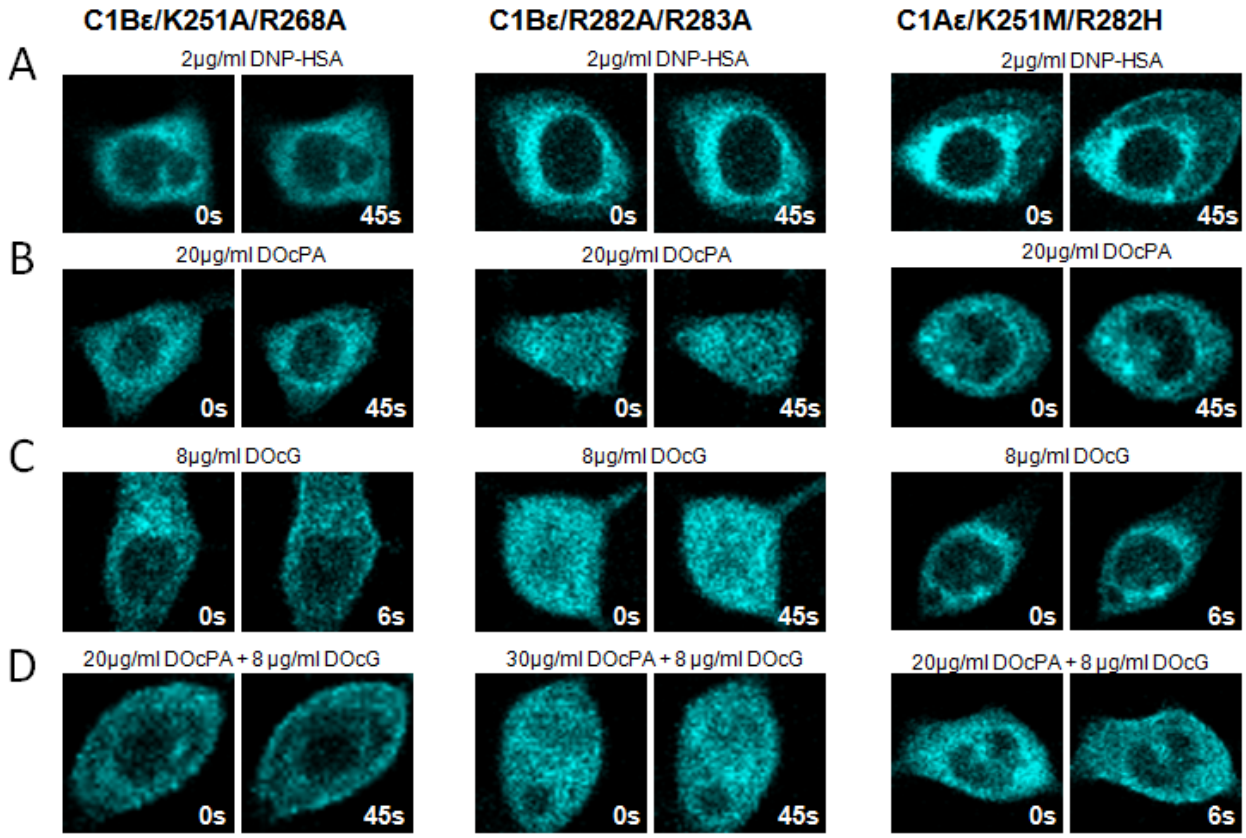

Figure V.9 Membrane translocation of C1 domains to the plasma membrane of RBL-2H3 cells. Cells were transfected with C1Aε-ECFP, C1Bε-ECFP and C1ABε-ECFP constructs. The pictures show the effect of the addition of antigen (DNP-HSA) or the indicated amounts of soluble diacylglycerol (DOcG) and/or soluble phosphatidic acid (DOcPA).

The addition of 20 µg/ml of DOcPA to the RBL-2H3 cells (Figure V.9-B and Table V.6) did not produce any translocation to the membrane of the double mutants and just a weak degree of translocation was observed when 8 µg/ml were added (Figure V.9-C) for mutants C1BεK251A/R26A-ECFP and C1BεK251M/R282H-ECFP, obtaining a M.L. and a R_{max} below 20% and 0.15 respectively (Table V.6). The combination of 20 µg/ml of DOcPA and 8 µg/ml of DOcG did not improve those translocation values (Figure V.9-D) in contrast to the observed for single mutants.

C1BεK251A/R268A-ECFP	*N* cells	M.L., %	R_{max}	$t_{½}$, s
20 µg/ml DOcPA	24	0		
8 µg/ml DOcG	25	12	0.14 ± 0.01	4 ± 1
20 µg/ml DOcPA + 8 µg/ml DOcG	28	0		
2 µg/ml DNP-HSA	19	0		
C1BεK251M/R282H-ECFP	***N* cells**	**M.L., %**	R_{max}	$t_{½}$, s
20 µg/ml DOcPA	26	0		
8 µg/ml DOcG	34	18	0.15 ± 0.01	3 ± 1
20 µg/ml DOcPA + 8 µg/ml DOcG	28	35	0.13 ± 0.01	3 ± 1
2 µg/ml DNP-HSA	28	32	0.29 ± 0.01	46 ± 3
C1BεR282A/R283A-ECFP	***N* cells**	**M.L., %**	R_{max}	$t_{½}$, s
20 µg/ml DOcPA	26	0		
8 µg/ml DOcG	22	0		
20 µg/ml DOcPA + 8 µg/ml DOcG	20	0		
2 µg/ml DNP-HSA	38	0		

Table V.6. Plasma membrane translocation parameters of different mutants constructs of C1Bε-ECFP domains in RBL-2H3 cells stimulated with DNP-HSA, DOcPA and/or DOcG. RBL2H3 cells were primed with 0.5µg/ml antiIgE antibody for 16 h and then stimulated with 2µg/ml DNP-HSA *M.L.* is membrane localization and indicates the percentage of cells responding to DNP-HSA, DOcPA and/or DOcG stimulation with plasma membrane translocation. R_{max} is the maximum relative increase in plasma membrane localization of the domain. $t_{1/2}$ is the half time of translocation.

The stimulation of one triple mutant in RBL-2H3 cells was also performed (Figure V.10) and no response was observed under any condition as it could be expected. It was described above that the double mutant C1BεR282A/R283A-ECFP already showed inability to translocate.

C1Bε/R268A/R282A/R283A

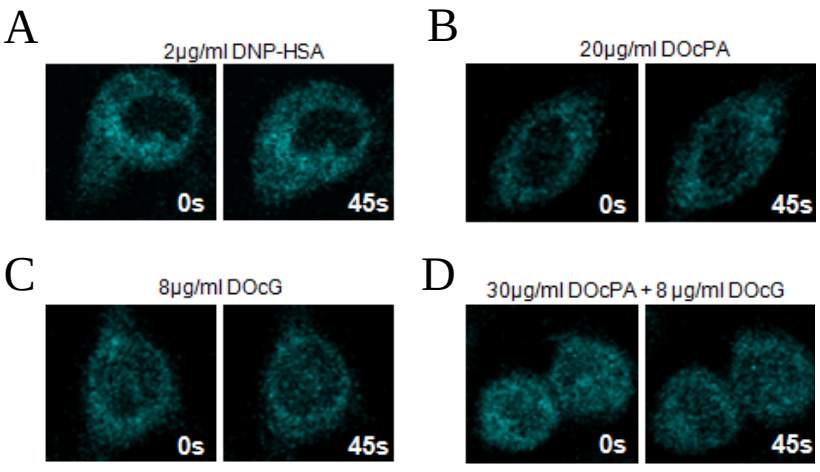

Figure V.10 Membrane translocation of C1BεR268A/R282A/R283A-ECFP mutant to the plasma membrane of RBL-2H3 cells. Cells were transfected with C1BεR268A/R282A/R283A-ECFP mutant construct. The pictures show the effect of the addition of antigen (DNP-HSA) or the indicated amounts of soluble diacylglycerol (DOcG) and/or soluble phosphatidic acid (DOcPA).

Nevertheless it should be underlined that both of them translocated to the membrane when a low concentration of PMA (1 µg/ml) was added, and an extra mutation (C1BεR268AR282A/R283A-ECFP) is not expected to affect to the hydrophobic binding site for phorbol esters. This shows that whereas phorbol esters may drive the translocation of C1B domains without the help of phospholipids, this is not the case for diacylglycerols.

C1BεR268A/R282A/R283A-ECFP	*N* cells	M.L., %	R_{max}	$t_{½}$, s
20 µg/ml DOcPA	18	0		
8 µg/ml DOcG	18	0		
20 µg/ml DOcPA + 8 µg/ml DOcG	15	0		
2 µg/ml DNP-HSA	20	0		

Table V.7. Plasma membrane translocation parameters of C1BεR268A/R282A/R2283A-ECFP construct in RBL-2H3 cells stimulated with DNP-HSA, DOcPA and/or DOcG. RBL2H3 cells were primed with 0.5µg/ml antiIgE antibody for 16 h and then stimulated with 2µg/ml DNP-HSA *M.L.* is membrane localization and indicates the percentage of cells responding to DNP-HSA, DOcPA and/or DOcG stimulation with plasma membrane translocation. R_{max} is the maximum relative increase in plasma membrane localization of the domain. $t_{1/2}$ is the half time of translocation.

2.5. The C1B domain of PKCε plays an important role in the phosphatidic acid-dependent membrane localization of the enzyme.

To investigate how those mutations could affect to the translocation of the full-length PKCε, we transfected two simple mutants in RBL-2H3 cells (PKCεK251A-EGFP and PKCεr268A-EGFP); a double mutant with the two other amino acidic residues mutated previously (PKCεR282A/R283A-EGFP); and a construct with the four residues mutated (PKCεK251A/R268A/R282A/R283A-EGFP).

The purpose of the experiments with the single mutants was to know if these specific mutations in the full-length protein would cause the same drastic effect that we observed previously in the C1Bε mutants with the binding of the domain to the plasma membrane, or if, on the other hand, the two other intact domains (C1A and C2) could compensate these punctual mutations.

Figure V.11 shows the response profiles of RBL-2H3 cells transfected with wild type PKCε-EGFP and two single mutants. Once the cells were stimulated by the antigen (Figure V.11-A and Table V.8), the observed M.L. for the wild type

PKCε-EGFP (92%) was similar to that obtained for PKCεK251A-EGFP (81%) and PKCεR268A-EGFP (92%). However, the R_{max} fell down from 0.80 for wild type PKCε-EGFP to 0.69 for PKCεR268A-EGFP and 0.56 for PKCεK251A-EGFP. This could mean that the docking of the full length protein to the membrane is not as efficient as the observed in the wild type but the protein remained able to detect their signaling lipids in the plasma membrane.

When 20 µg/ml of DOcPA were added to the RBL-2H3 cells, the membrane localization of the wild type was 100%, closely followed by PKCεK251A-EGFP (87%) and PKCεR268A-EGFP (85%). However, as it was commented previously when the antigen was used for stimulation, R_{max} values were different between wild type and mutants, in particular, near two times less for the mutants (Table V.8). A large effect was observed with 10 µg/ml of DOcPA, because the wild type showed a R_{max} of 0.77 whereas for the PKCεK251A-EGFP and PKCεR268A-EGFP values were 0.28 and 0.20, respectively. No effects were observed in the mutants when a concentration of 5 µg/ml of DOcPA was used (Figure V.11-B and Table V.8). Nevertheless a R_{max} of 0.67 and a M.L. of 34% were observed for the wild type. If t½ values are compared, it can be seen that they were 10 seconds for the wild type with 20 µg/ml and 10 µg/ml of DOcPA. These results are in contrast to the situation observed for the mutants, because t½ values for PKCεK251A-EGFP were 38 and 45 seconds for 20 µg/ml and 10 µg/ml of DOcPA, and in the case of PKCεR268A-EGFP 25 and 29 seconds respectively. These data could indicate that a longer time to interact with DOcPA in the plasma membrane is needed for the single mutants.

When DOcG was used in a high concentration such as 25 µg/ml it was observed that the same effect described with high concentrations of DOcPA (Table V.8) was taking place and M.L. values were similar for wild type and mutants (~100% in all the cases) but the R_{max} is higher in wild type (0.77) than in PKCεK251A-EGFP and PKCεR268A-EGFP (0.58 and 0.61 respectively) and t½ values are very different as well (PKCε-EGFP, 3 seconds; PKCεK251A-EGFP, 22 seconds; and PKCεR268A-EGFP, 20 seconds). The biggest differences appeared at low concentrations of DOcG, because R_{max} was two times lower for the mutants than for the wild type when we added 10 µg/ml of DOcG, or 5 times less when 5 µg/ml were added (Figure V.11-C and Table V.8).

The combination of 5 µg/ml of DOcPA, which alone had not been enough to achieve the translocation of the single mutants to the plasma

membrane and 4 µg/ml of DOcG led to an important translocation to the plasma membrane (Figure V.11-D and Table V.8) and their values were comparable to those obtained for 25 µg/ml of DOcG, and even higher than for 20 µg/ml of DOcPA. Nevertheless, R_{max} values were 0.65 and 0.66 for PKCεK251A-EGFP and PKCεR268A-EGFP respectively, and therefore lower than the R_{max} value for PKCε-EGFP, 0.86.

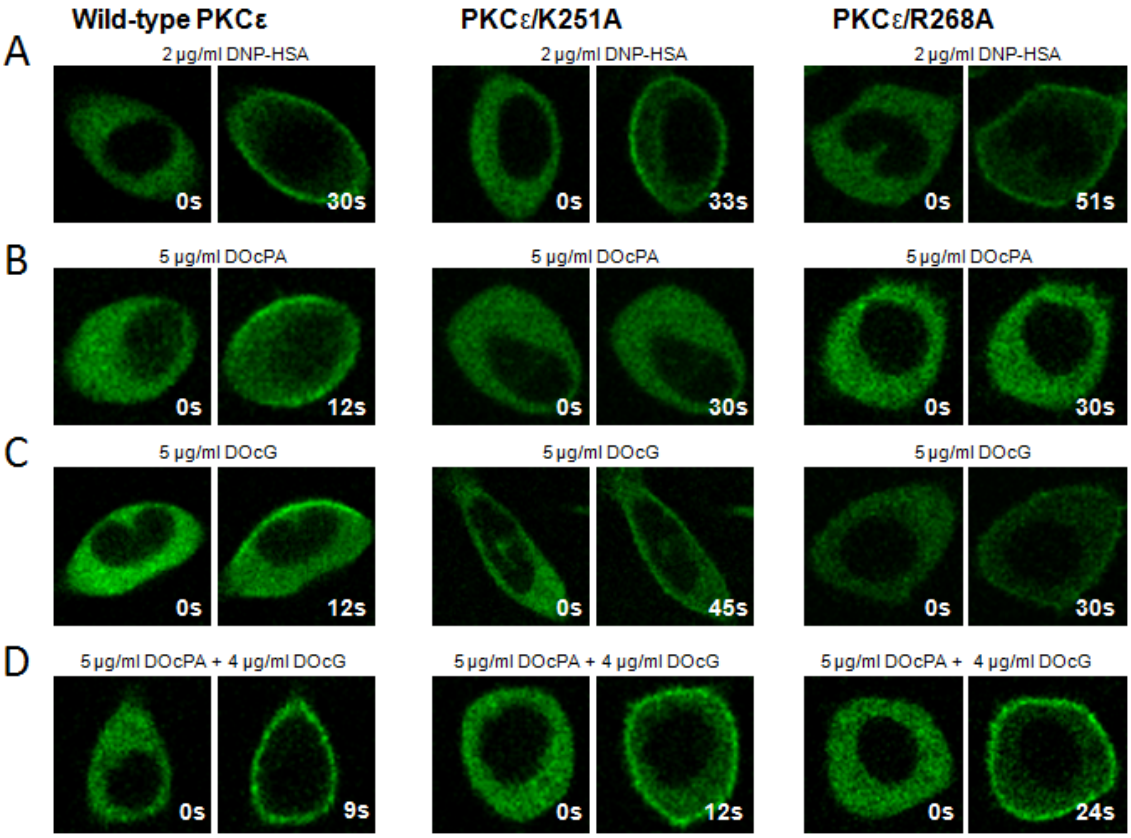

Figure V.11 Membrane translocation of the full length PKCε-EGFP and two simple mutants to the plasma membrane of RBL-2H3 cells. Cells were transfected with the full length PKCε-EGFP and its mutant, as indicated. The pictures show the effect of the addition of antigen (DNP-HSA) or the indicated amounts of soluble diacylglycerol (DOcG) and/or soluble phosphatidic acid (DOcPA).

Taken all these data together, we could suggest that in spite of the fact that the single mutations caused an effect in the normal translocation of the enzyme, these would not be enough to totally block this translocation. If the values obtained for the isolated C1Bε mutants are compared to those of the full-length PKCε mutants, it could be assumed that the higher R_{max} values obtained for the full-length protein than for the isolated domains would be due to the involvement of the C2 domain of the protein, that would increase the anchoring of the protein to the plasma membrane, and whose importance was

described by *(Lopez-Andreo et al., 2003)*, and not due to the C1A domain, as it was demonstrated previously in this Chapter.

In the next step of one more mutation was added to the full-length PKCε-EGFP protein to make the double mutant PKCεR282A/R283A-EGFP. After the activation of the RBL-2H3 through the antigen receptor (Figure V.12-A and Table V.8), double mutant showed a partial translocation to the plasma membrane, but the most significant result was the elevated t½ (87 seconds) reaching a R_{max} of 0.36, which fell far from the t½ of 33 seconds and the R_{max} of 0.8 obtained for the wild type (Table V.3).

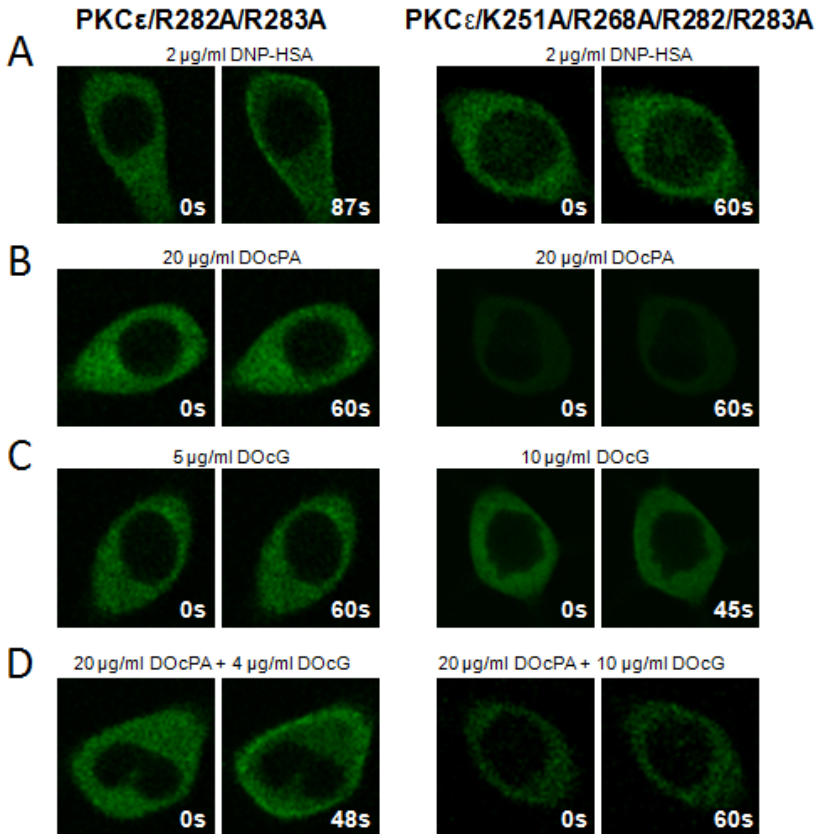

Figure V.12. Membrane translocation of the full length PKCε-EGFP and a doble and quadruple mutant to the plasma membrane of RBL-2H3 cells. Cells were transfected with the full length PKCε-EGFP and its mutant as indicated. The pictures show the effect of the addition of antigen (DNP-HSA) or the different amounts of soluble diacylglycerol (DOcG) and/or soluble phosphatidic acid (DOcPA).

No translocation was observed when 20 µg/ml of DOcPA was added (Figure V.12-B). The addition of 25 µg/ml of DOcG only induced a R_{max} of 0.35, which is near 2-fold less than for the single mutants. The combination of 20 µg/ml of DOcPA with 4 µg/ml of DOcG (Figure V.12-D) produced an incomplete

translocation of the protein, thus the addition of four times more of DOcPA than that used for the single mutants led to a R_{max} of 0.32, 2-fold less than the values obtained for PKCεK251A-EGFP and PKCεR268A-EGFP (0.65 and 0.66 respectively).

To further block the interaction between positively charged amino acidic residues in the C1Bε domain and the plasma membrane, a quadruple mutant construct (PKCεK251A/R268A/R282A/R283A-EGFP) was prepared (Figure V.12). In contrast to the results shown above in RBL-2H3 cells for wild type, single or double mutants, no detectable translocation was observed for quadruple mutants when the same concentrations of DOcPA, DOcG and their combination were used. Nevertheless, this mutant was able to translocate to the plasma membrane when we added PMA, suggesting that it was perfectly folded (Figure V.13).

PKCεK251A/R268A/R282A/R283A

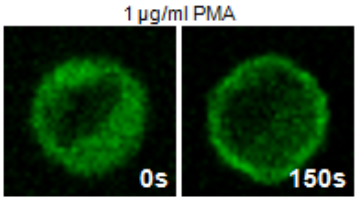

Figure V.13 Membrane translocation of PKCεK251A/R268A/R282A/R283A-EGFP mutant to the plasma membrane of RBL-2H3 cells. Cells were transfected with PKCεK251A/R268A/R282A/R283A-EGFP mutant construct. The pictures show the effect of the addition of 1 µg/ml of PMA.

One conclusion that could be drawn from these results is the importance of the C1B domain for the traslocation to the membrane of the PKCε and corroborate our previous results *(Lopez-Andreo et al., 2003)* in which mutations of residues involved in the membrane binding of the C2 domain were also damaging upong IgE receptor stimulation, but completely recovered when DOcG was used as stimulant.

Stimulation wild type PKCε-EGFP	*N* cells	M.L., %	R_{max}	$t_{½}$, s
20 µg/ml DOcPA	28	100	0.80 ± 0.01	10 ± 2
10 µg/ml DOcPA	24	83	0.77 ± 0.04	10 ± 2
5 µg/ml DOcPA	32	34	0.67 ± 0.03	14 ± 1
25 µg/ml DOcG	32	94	0.77 ± 0.02	3 ± 1
10 µg/ml DOcG	26	84	0.71 ± 0.01	5 ± 1
5 µg/ml DOcG	17	41	0.69 ± 0.01	13 ± 2
5 µg/ml DOcPA + 4 µg/ml DOcG	26	100	0.86 ± 0.03	8 ± 2
2 µg/ml DNP-HSA	65	92	0.80 ± 0.01	33 ± 2

Stimulation PKCεK251A-EGFP	*N* cells	M.L., %	R_{max}	$t_{½}$, s
20 µg/ml DOcPA	16	87	0.43 ± 0.03	38 ± 4
10 µg/ml DOcPA	18	77	0.28 ± 0.03	45 ± 5
5 µg/ml DOcPA		0		
25 µg/ml DOcG	16	100	0.58 ± 0.02	22 ± 3
10 µg/ml DOcG	34	67	0.32 ± 0.01	24 ± 2
5 µg/ml DOcG	16	37	0.17 ± 0.02	46 ± 4
5 µg/ml DOcPA + 4 µg/ml DOcG	19	94	0.65 ± 0.04	11 ± 2
2 µg/ml DNP-HSA	21	81	0.56 ± 0.01	36 ± 4
Stimulation PKCεR268A-EGFP	***N* cells**	**M.L., %**	**R_{max}**	**$t_{½}$, s**
20 µg/ml DOcPA	20	85	0.49 ± 0.01	25 ± 3
10 µg/ml DOcPA	19	52	0.20 ± 0.02	29 ± 4
5 µg/ml DOcPA		0		
25 µg/ml DOcG	14	100	0.61 ± 0.02	20 ± 2
10 µg/ml DOcG	22	63	0.39 ± 0.02	28 ± 4
5 µg/ml DOcG	19	26	0.13 ± 0.02	35 ± 2
5 µg/ml DOcPA + 4 µg/ml DOcG	23	100	0.66 ± 0.03	26 ± 2
2 µg/ml DNP-HSA	27	92	0.69 ± 0.02	53 ± 7
Stimulation PKCεR282A/R283A-EGFP	***N* cells**	**M.L., %**	**R_{max}**	**$t_{½}$, s**
20 µg/ml DOcPA		0		
25 µg/ml DOcPA	17	70	0.35 ± 0.01	34 ± 2
10 µg/ml DOcG	21	23	0.18 ± 0.02	37 ± 2
5 µg/ml DOcG		0		
10 µg/ml DOcPA + 4 µg/ml DOcG		0		
20 µg/ml DOcPA + 4 µg/ml DOcG	29	65	0.32 ± 0.04	47 ± 6
2 µg/ml DNP-HSA	25	68	0.36 ± 0.03	87 ± 7
Stimulation PKCε*K251A/R268A/R282A/R283A-EGFP*	***N* cells**	**M.L., %**	**R_{max}**	**$t_{½}$, s**
20 µg/ml DOcPA		0		
12 µg/ml DOcG		0		
10 µg/ml DOcPA + 4 µg/ml DOcG		0		
2 µg/ml DNP-HSA		0		

Table V.8. Plasma membrane translocation parameters of PKCε-EGFP and PKCε/K251A/R268A/R282A/R283A-EGFP domains in RBL-2H3 cells stimulated with DNP-HSA, DOcPA and/or DOcG. RBL2H3 cells were primed with 0.5µg/ml antiIgE antibody for 16 h and then stimulated with 2µg/ml DNP-HSA *M.L.* is membrane localization and indicates the percentage of cells responding to DNP-HSA, DOcPA and/or DOcG stimulation with plasma membrane translocation. R_{max} is the maximum relative increase in plasma membrane localization of the domain. $t_{1/2}$ is the half time of translocation.

2.6. Role of the C1B domain in the phosphatidic acid-dependent activation of PKCε.

The effect of phosphatidic acid on wild type PKCε-EGFP and the mutant PKCεK251A/R268A/R282A/R283A-EGFP was investigated by observing the activity of the enzyme at different percentages of POPA in the membranes in the absence or presence of DOG (Figure V.14). It is interesting to note that no basal specific activity was detected when the phospholipid vesicles contained 5 mol% DOG in the absence of POPA. Besides, it was necessary the addition of DOG to the lipid vesicles to observe a POPA-dependent activation, thus POPA and DOG interactions would be necessary for the activation of the enzyme in this *in* vitro system.

When we studied the effect of the substitutions of the amino acidic residues in C1B domain on the POPA-dependent activation of the enzyme, a drastic effect was seen in comparison with the wild type (Figure V.13), since 60 mol% of POPA in the lipid vesicles only produced 20% of the catalytic activity of the enzyme in the case of the mutated form.

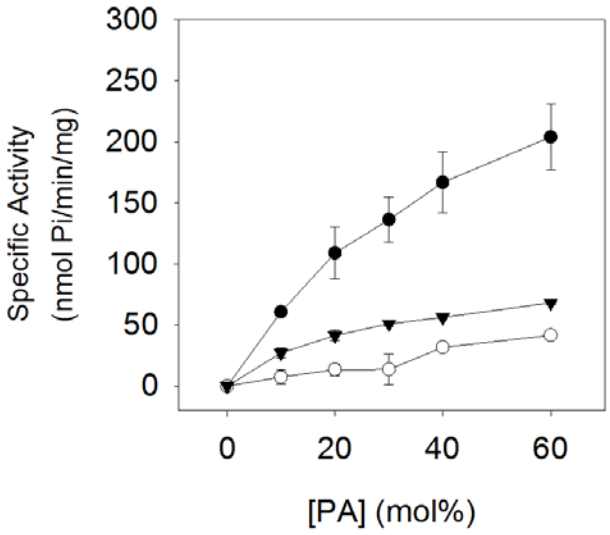

Figure V.14. Specific activity of full length PKCε and its mutant by using large unilamellar vesicles. PtdOH-dependent activation of wild-type PKCε and its mutant by using large unilamellar vesicles containing POPC/POPA (99-X:X, with X being the molar fraction of POPA in each case) for wild type PKCε (o) and POPC/DOG/POPA (95-X:5:X, with X being the molar fraction of POPA in each case) for wild type PKCε (•) or PKCεK251A/R268A/R282A/R283 (▼). The total lipid concentration was 0.2 mM and Histone III-S was used as a substrate. Error bars indicate the SEM for triplicate determinations.

2.7. Lipidomic analysis of variation in response to antigen stimulation in RBL-2H3 cells.

RBL-2H3 cells have been used along this work as an endogenous production of PKC activators such as diacylglycerols (DAGs) and phosphatidic acid (PA) in order to quantify the differences in the translocation profiles of isolated C1B domains and full-length proteins. Nevertheless it is uncertain what is the exact mechanism by which the generation of DAGs or PA, and if this last activator is really produced.

The cross-linking of the high-affinity IgE receptor (FcεRI) with antigen in these cells stimulates a number of lipid-signaling events (Figure V.15), which includes the activation of phospholipase Cγ, phospho-inositide 3-kinase, and phospholipase D (PLD) *(Brown et al., 1998; Djouder et al., 2001; Schneider et al., 1992)* and many serine/threonine kinases *(Gruchalla et al., 1990; Millard et al., 1989; Park et al., 1991)*, including the PKC family among others *(Ozawa et al., 1993)*. However, it is not well established how PLC and PLD, or other enzymes, participate in the generation of the different activators of the variety of PKCs or how they contribute to the localization and activation of each isoenzyme *(Lopez-Andreo et al., 2003)*.

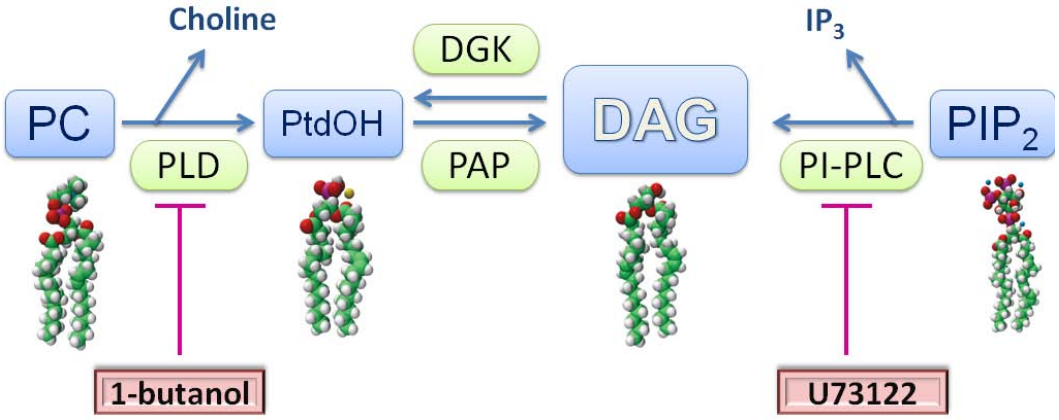

Figure V.15. Mechanism of activation of PKCε in RBL-2H3 cells upon activation of the IgE receptor. The scheme shows the lipid intermediate generated upon receptor stimulation. In a first step, phosphatidylinositol-4.5-bisphosphate is hydrolyzed to produce DAG and inositol triphosphate by PI-PLC; simultaneously PC is hydrolyzed by PLD to produce PtdOH and choline. In a second step, DAG and PtdOH generated are transformed to PtdOH and DAG, respectively, by the action of DGK and PtdOH-phosphohydrolase. The inhibitors of each reaction are marked with red boxes. Taken from *Lopez-Andreo et al. 2003*.

In order to shed light on the membrane of cell activation by the antigen, a lipidomic study was carried out in collaboration with the Department of Biochemistry and Cell Biology in the Faculty of Veterinary at the University of Utrecht. Briefly, RBL-2H3 cells were stimulated with the antigen, which had been subjected previously, or not, to the phospholipase inhibitors described above. Neutral lipids and phospholipids were extracted from complete pellet or from the isolated plasma membrane and loaded in a LC/MS/MS system as is indicated in Chapter II.

2.7.1. Lipidomic changes in DAGs.

For the analysis of the neutral lipids we assume that all species of 1,2-*sn*-diacylglycerols that have been measured in this experiment (fatty acyl chains between 14 and 20 carbons) are able to be recognized by PKCs *(Sanchez-Pinera et al., 1999)*, thus the data have been represented as the sum of them.

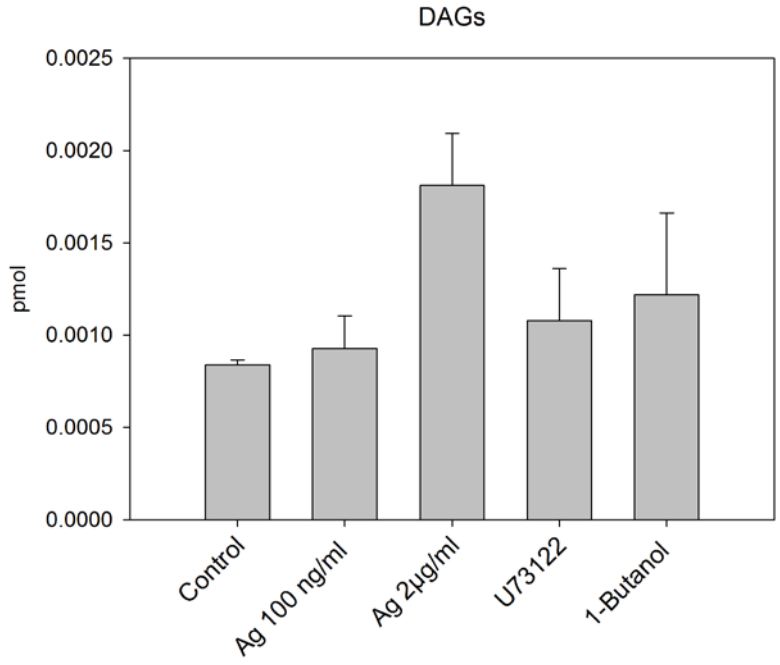

Figure V.16. Amount (pmol) of DAGs in each condition of study. Control (Cells were growing during three days before being harvested); once the cells were primed overnight with Ig-E-anti-dinitrophenyl, they were stimulated with the antigen (DNP-HSA) during 1 minute and at different concentrations of DNP-HSA **2 µg/ml** and **100 ng/ml;** The various phospholipase inhibitors were present 10 min before stimulation at the following concentrations: 30 µM **U73122** and 50 mM **1-butanol**. Each bar represents mean ± SE from 3 independent samples.

Figure V.16 shows the concentration of DAGs obtained in each condition. It is interesting to note the effect on the stimulation of two different

concentrations of antigen, 100 ng/ml and 2 µg/ml during 1 min of exposure. The biggest differences were observed at the highest concentration comparing with the control cells. These results corroborate the data described by *Lopez-Andreo et al., 2003* and subsequently corroborated in Chapters IV and V, where a minimum concentration of antigen is needed to reach the translocation of the domains or the full-length protein to the plasma membrane.

When the inhibitor U73122 was added before the stimulation with the antigen, the total concentration of DAGs fell down to the baseline level. This fact would mean that PI-PLC was blocked by U73122 and DAGs could not be generated through PIP_2 hydrolisis.

These data confirm the importance of PI-PLC in the generation of DAGs in RBL-2H3 cells after stimulation with antigen.

2.7.2. Lipidomic study of phophatidic acids.

As with the previous analysis of DAGs, all the species of phosphatidic acids, that were generated by stimulation with the antigen-receptor binding, have been added up to plot the data, because it has not been described that differences in the chain length of fatty acids could affect to their affinity with PKCs.

Some unexpected findings were observed about phosphatidic acids coming from RBL-2H3 cells samples after priming overnight with 500 ng/ml IgE anti-dinitrophenyl, because their composition was different than that of the control cells Figure V.17. Thus, the simple incubation with the antibody would cause a partial activation of the PLD among others, with a low production of PA, which could not lead to the translocation of the C1B domains of novel PKCs (Chapter IV) or full-length PKCε (Chapter V), the antigen stimulation being required, as it has been previously described in Chapter V.

The concentration of Ag used for the stimulation of the cells was also an important factor in the generation of PAs. When RBL-2H3 were stimulated with 100 ng/ml of DNP-HSA no differences were observed compared with the cells which had been just primed by the antibody. On the other hand, when the

concentration was increased up to 2 µg/ml, the level of phosphatidic acids increased more than 3 times compared to the control cells.

When the cells were subjected to the inhibitor 1-butanol before stimulating with 2 µg/ml of DNP-HSA, no effects were observed on the concentration of phosphatidic acids and their concentration was similar to those obtained in the cells stimulated with a low proportion of DNP-HSA. This observation can be explained as the inhibition of PLD by the inhibitor 1-butanol and it sheds light on the importance of PLD in the generation of phosphatidic acids in these cells.

No differences were observed when inhibitor U73122 was used because the inhibition of PI-PLC does not have any effect over the production of PA.

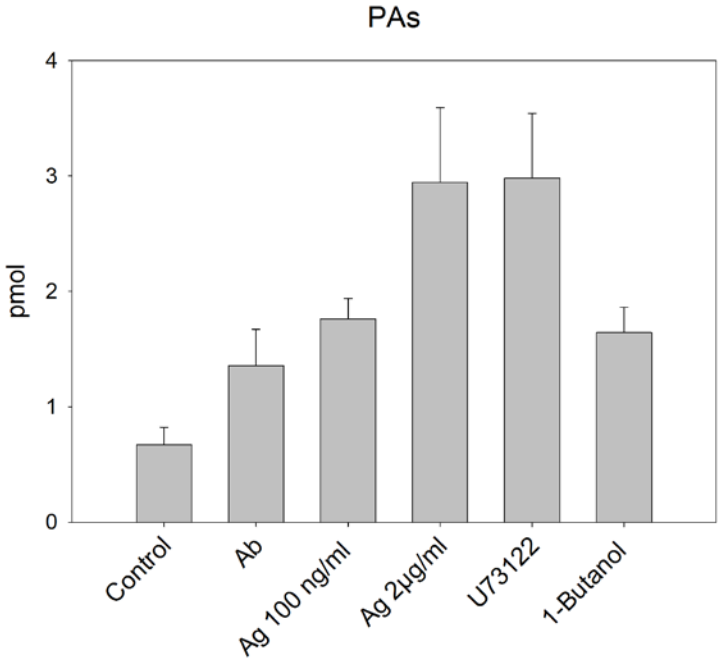

Figure V.16. Bar diagram shows the amount (pmol) of PA levels in each condition of study. Control (Cells were growing during three days before being harvested); **Ab** (After being one day plated, cells were primed overnight with 500 ng/ml IgE-anti-dinitrophenyl, growing for another 24 hours); Once the cells were primed overnight with Ig-E-anti-dinitrophenyl, they were stimulated with the antigen (DNP-HSA) during 1 minute and at different concentrations of DNP-HSA **2 µg/ml** and **100 ng/ml;** The various phospholipase inhibitors were present 10 min before stimulation at the following concentrations: 30 µM **U73122** and 50 mM **1-butanol**.
Each Bar represents mean ± SE from 3 independent samples.

3. DISCUSSION

The first part of this work was devoted to elucidate the reasons why, as observed in Chapter IV, the C1Bε domain showed the highest binding affinity among other C1B domains of novel PKCs, and in particular, in comparison with C1Bδ. An alignment of its sequence was carried out (Figure V.1) to investigate whether there would be some amino acid residues in their structure that allows it to interact with higher affinity with membranes than the rest of domains.

Some positively charged amino acids were selected as possible candidates. This model revealed that 4 of them could be involved in the interaction with membranes (Figure V.2), in particular, lysine 251 and arginines 268, 282 and 283. Hence, it was decided to carry out site-directed punctual mutations over these residues, substituting alanines for the positive amino acids, not only in isolated C1Bε domains, but also in the full-length PKCε protein.

In the first part of this work a FRET study was carried out using isolated C1Bε-ECFP mutants and model membranes that contained POPC:POPX:OGPE, where POPX was POPS or POPA in 20 mol%, in the presence or absence of DOG. Results showed that single mutants in the presence of DOG led to an increase of the K_D values to around 3-4 times higher than that found for the wild type. These differences were more important in the absence of DOG, which could be interpreted as if the presence of DOG might compensate the decrease in membrane binding after the substitution of one residue.

Despite the differences of affinity obtained for POPS and POPA when C1Bε domain binding was studied (Chapter IV), the consequences of these mutations were similar independently of using either of these negatively charged phospholipids.

More dramatical were the inhibitions of the domain binding when double mutations were introduced and membrane binding was almost totally abolished in the case of triple mutants independently of the presence or the absence of DOG. We can conclude that there were no just single indispensable residues, and it would be the combination between these four positively charged ones what determines the binding to membranes.

With the goal of corroborating if these positively charged residues would be involved in the high binding affinity values observed in C1Bε domain, its sequence was compared to the sequence of C1Bδ, which was identified as the novel C1B domain with the lowest binding affinity to membranes in the novel PKCs group (Chapter IV). Once their sequences were compared by using a structural model (Figure V.5) it was observed that residues K251 and R282 in C1Bε corresponded to M239 and H270 in C1Bδ. Therefore, C1Bε was transformed into a C1Bδ-like and the FRET studies revealed that the binding properties of C1BεK251M/R282H-ECFP were very similar to wild type C1Bδ-ECFP than to wild type C1Bε-ECFP. These results illustrated the importance of the existence of these positively charged residues in the C1Bε domain to achieve high binding affinities to membranes.

Another aspect studied was the membrane translocation patterns followed by these mutants when expressed in RBL-2H3 cells after different stimulation treatments. Before doing that, the behavior of each C1 domain present in the PKCε was studied, specifically C1Aε, C1Bε and the tandem C1ABε, because it would facilitate the understanding of the consequences of the mutations carried on in the C1Bε domain had for the translocation of full-length PKCε to the plasma membrane.

When we stimulated RBL-2H3 cells transfected with either C1Aε-ECFP, C1Bε-ECFP or C1ABε-ECFP constructs were stimulated by the antigen-receptor binding, a membrane localization response was only observed for C1Bε-ECFP and C1ABε-ECFP constructs, with R_{max} and t½ very similar between them but not for C1Aε-ECFP. The same happened when different concentrations of DOcPA and DOcG were added to these transfected with translocation of the two constructs that include the C1B sequence but no response either of C1Aε-ECFP, which means that it would be the C1Bε domain the only responsible for the recognition of the activators used in this experiment and similar values for the isolated C1Bε domain and for C1ABε were obtained.

No big differences in R_{max} values could be observed when stimulating with the antigen. The most important differences were obtained for the membrane localization (M.L.) of the cells, being ~50% of the total for the single mutants, when the M.L. of the wild type was 80%, and in the t½ values, being ~15 seconds bigger in the single mutants than in the wild type. This result could be interpreted as if all the residues studied make a contribution to membrane

binding. And thus, a mutation in any of them would be enough to affect the extension and kinetics of binding. When 20 µg/ml of DOcPA were added, the effect was bigger, with an insignificant translocation for C1BεR268A-ECFP and C1BεR282A-ECFP as indicated by M.L. 5 times lower than the value for the wild type and a R_{max} 3 times lower than the observed for the wild type. However when stimulation was performed using the combination of 20 µg/ml of DOcPA with 4 µg/ml of DOcG in C1BεRK251A-ECFP and C1BεR268A-ECFP or 8 µg/ml of DOcG in C1BεR282A-ECFP and C1BεR283A-ECFP, the drastic effect observed when the activator was just DOcPA was aminorated. These results indicated that the 4 residues studied had a contribution on the membrane binding of this domain and that two different interactions are established, one through the diacylglycerol-binding site and the other through electrostatic interactions with negatively charged phospholipids.

Whereas mutating one single residue was not decisive when stimulation was done through the antigen, which is known to generate different activators as diacylglycerols and phosphatidic acid *(Lopez-Andreo et al., 2003)*, the effect observed for the double mutants, were bigger, specially for C1BεR282A/R283A-ECFP that was not able to translocate to the plasma membrane under any condition, followed by C1BεK251A/R268A-ECFP that showed a very limited translocation when stimulated by 8 µg/ml DOcG (M.L. and R_{max} ~6 and ~3times lower than wild type respectively), and C1BK251M/R268M-ECFP that was the C1Bδ-like domain. This one showed a stimulation closer to that obtained for wild type C1Bδ-ECFP, allowing us to confirm that the residues mutated in domain to make it C1Bε-ECFP-like are really important for membrane binding.

In another set of experiments the full enzyme, wild type and mutated in the residues studies before in the isolated C1B domain, were studied after being expressed in RBL-2H3 cells. When the stimulation was done with antigen in RBL-2H3 no big effects were seen in the case of single mutants with respect to M.L.

However, the R_{max} was ~1.5 times lower than the wild type, reflecting that one thing is the percentage of cells that respond to the stimuli and another thing is the efficiency in the response. More significant and interesting was the result obtained when the cells were stimulated with 20 µg/ml of DOcPA. The M.L. of the mutants continued to be very similar to that observed for the wild type in contrast to the R_{max} that was ~2 times lower than for the wild type. In this case, the R_{max} differences were more important than when the stimulation

was done by the antigen, because in this case DAG was also produced. Similarly when the combination of 5 μg/ml DOcPA and 4 μg/ml DOcG were added to RBL-2H3 cells, the values obtained for M.L. and R_{max} were similar to those observed when the stimulation was carried out by the antigen.

Taken all these findings together, it could be interpreted as the if the single mutants of PKCε-EGFP are still able to translocate to the membrane due to their high membrane localization (nearly 100%) but in a less efficient way according to the low R_{max}.

To further confirm that these residues could inactivate the enzyme, we generated a double mutant of the C1Bε domain in the full-length PKCε-EGFP, in particular, PKCεR282A/R283A-EGFP, mutations that were shown above were observed to abolish the binding to the membrane of isolated C1Bε-ECFP and the translocation that was observed for the full-length protein was a consequence of the participation of the other regulatory domains.

Specifically, this double mutant showed a M.L. of ~65% and a Rmax ~0.33 when it was stimulated by the antigen, a high concentration of DOcPA (25 μg/ml DOcG) and with a combination of DOcPA and DOcG of 20 μg/ml and 4 μg/ml DOcG respectively. However, no effect was seen when a 20 μg/ml of DOcPA were added.

Taken together, these data suggest that the addition of high concentrations of DOcPA in RBL-2H3 cells transfected with single mutants would not affect to the percentage of cell response, but however, the localization of the protein to the plasma membrane would be undermined. Nevertheless, in the case of the double mutants the presence of a low amount of DOcG together the high concentration of DOcPA would be needed in order to achieve some membrane translocation, due to the fact that DOcPA per se would not be able to translocate this double mutant.

And finally, a quadruple mutant (PKCεK251A/R268A/R282A/R283A-EGFP) was not able to translocate in any condition described above, but it continued to be able to localize in the membrane under the stimulation with PMA, showing as we mentioned in Chapter IV, that the response of the C1Bε with PMA is independent of the presence of negatively charged phospholipids or

not. Therefore, these mutations would only affect to the binding affinity to acidic phospholipids and diacylglycerol.

The lipidomic study carried out in the Department of Biochemistry and Cell Biology in the Faculty of Veterinary at the University of Utrecht allowed us to conclude that the translocations to the plasma membrane of RBL-2H3 cells that were observed in isolated domains and in the full-length PKCε is the consequence of the production of DAGs and phosphatidic acids through the activation of phosphatidylinositol-specific phospholipase C (PI-PLC) and phospholipase D (PLD) respectively.

Since it has been shown here that the mutation of these positively charged residues impairs membrane binding, it was thought necessary to check if it would also produce an inhibition of the enzymatic activity of the PKCε, the ability of PKCε to phosphorylate a specific substrate in vitro under POPA-dependent conditions was study. The kinase activity was also measured for the mutant of the C1B domain (PKCεK251A/R268A/R282A/R283A-EGFP) in the same conditions as the wild type enzyme in order to compare them. This mutant showed residual catalytic activity, and only at high concentrations of POPA it recovered a little activity, in particular, at 60 mol% of POPA, the inhibition reaching approximately 80%.

Taken together, these results confirm the hypothesis that the C1B domain of PKCε plays an important role in the activation of the enzyme by negatively charged phospholipids. Furthermore, the results also suggest that the C2 domain would also play a role in the activation as was described by *Lopez-Andreo et al),* in contrast to what was claimed for the C2 domain of PKCδ, which does not have a relevant role in the membrane binding. Nevertheless the role of C2ε would be less crucial than that played by the C1B domain because it is not enough to lead to the translocation by itself as described above.

A large body of evidence indicates that C1A and C1B domains have distinct roles in the full-length PKC molecules according to their different binding affinity to membranes *(Medkova and Cho, 1999; Slater et al., 1996; Slater et al., 1994*), and maybe that these differences are related to their penetration behaviors. The presence of more hydrophobic residues in the C1A domain would allow it to penetrate deep into the membrane to bind DAG with a higher affinity than the C1B domain, which lacks membrane penetration capacity

(Bittova et al., 2001). C1B domain might not penetrate as much as C1A domain to the membrane, but the data obtained along this work make us to consider the importance of the possible orientation that is acquired by the C1Bε domain for its docking to the membrane. The importance of 4 amino acid residues in the C1B domain for the localization of the PKCε to the membrane is fully demonstrated in this work, thus we propose a model in which the C1B domain would be orientated in such a way that these four residues (Lysine 251 and arginines 268, 282 and 283) could interact with the membrane, facilitating the protein interactions with the negatively charged phospholipids present in the membrane (Figure V.17).

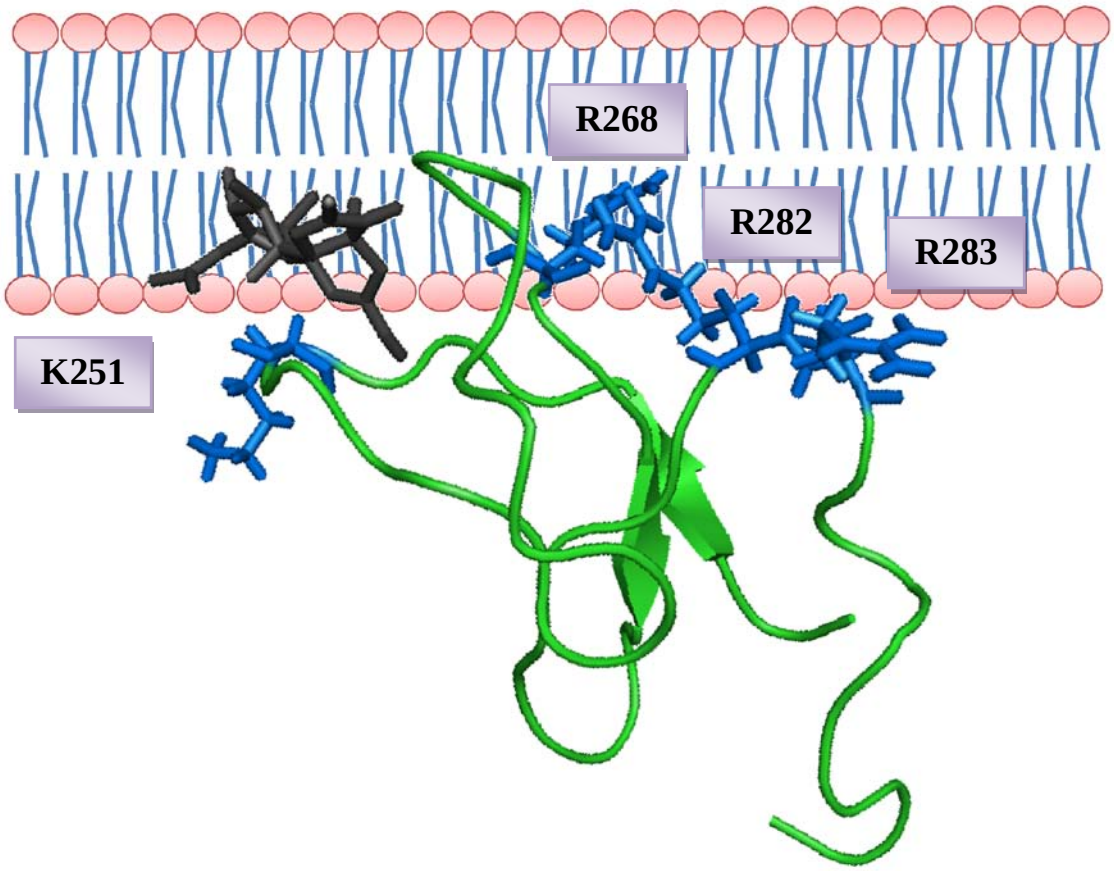

Figure V.17. Secondary structure prediction of C1Bε domain in complex with PMA. The ribbon drawing is represented in green. The PMA molecule is represented as grey sticks and the positively charged residues are blue sticks. These are labeled with their type and position in the sequence in purple boxes. The plasma membrane is emulated by phospholipids (head groups are red and fatty chains are blue). Molecular models were prepared by using PyMOL (Delano WL (2002) The PyMOL Molecular Graphics System).

Taking into account all the results obtained in the FRET studies, *in*-vivo stimulations in RBL-2H3 cells and kinase activity assays, we can conclude that the C1B domain of novel PKCε is necessary for the translocation of the protein to the membrane and the activation of the enzyme.

CONCLUSION

The importance of the C1B domain of PKCε for the translocation to the membrane and activation of the enzyme has been broadly confirmed in this work, and in particular, the presence of 4 positively charged amino acid residues present on the top of the domain. These residues would be able to lead the interaction of the C1B domain to the membranes and the punctual mutations that were generated in them, were important enough to decrease the membrane affinities of the C1B domains in FRET studies and the translocation to plasma membrane of the full-length protein in stimulations of RBL-2H3 cells, as well as its enzymatic activity.

CHAPTER VI

Identification of key residues in the C1B domain involved in its translocation to mitochondria upon an ischemia condition. The C1B domain of PKCε interacts with cardiolipin.

ABSTRACT

The C1B domains of novel PKCs mediate the DAG-dependent translocation of these enzymes and among them, C1Bε and C1Bη presented the highest binding affinity for membranes that contained acidic phospholipids. In this work we will study whether cardiolipin, a phospholipid that occurs exclusively in the mitochondria, is an important ligand for these domains, particularly C1Bε and if this phospholipid is able to activate the enzyme. It has been described that in episodes of ischemia PKCε translocates to mitochondria. We will also study if the interaction between C1B and cardiolipin could be involved in the translocation of PKCε. For that purpose, we will carry out an in-vitro model of ischemia, by treating SH-SY5Y cells to oxygen glucose deprivation after being transfected with the full-length PKCε and mutants of its C1B domain.

1. INTRODUCTION

An interesting effect has been described, which takes place during ischemic and reperfusion injury. That is the translocation of PKCs to the mitochondria, particularly, PKCε and δ in cardiac ischemic injury *(Armstrong, 2004; Budas et al., 2012; Duquesnes et al., 2011)* and PKCε and γ in cerebral ischemic *(Bright and Mochly-Rosen, 2005b)*. The same PKC activities have been observed in ischemic injury in other tissues such as liver *(Piccoletti et al., 1992)* or kidney *(Padanilam, 2001)*, suggesting their involvement in a conserved ischemic response pathway.

Despite the fact that mRNA expression of α, βI, βII, ε, δ, η, θ and ζ, has been reported in human and rat cardiomyocytes *(Palaniyandi et al., 2009)*, the relative content of each isoenzyme in the heart has been a controversial issue since it was found to depend on the species. PKCε was described as the principal, if not the only PKC isoenzyme to be expressed in the rat heart *(Bogoyevitch et al., 1993)*. Later, many other were found in the heart, principally PKCα, PKCδ and PKCζ *(Mackay and Mochly-Rosen, 2001b)*. However, two PKC isoenzymes of the group of novel PKC isoenzymes, PKCε and PKCδ, are among the principally expressed PKCs in cardiomyocytes *(Duquesnes et al., 2011)*. The same happens in the case of brain, mRNA expression of α, βI, βII, γ, ε, δ, η, θ, and ζ, has been identified *(Bright and Mochly-Rosen, 2005b)*, but there are two

PKCs from different subfamilies that have been implicated in the process of ischemia and reperfusion, in particular, PKCε and PKCγ.

The molecular mechanisms of ischemic and reperfusion injury, are a multiple cellular process rapidly activated in response to ischemic/reperfusion-induced stress. The ischemic core is the region of tissue that is immediately distal to an occluded artery, which undergoes rapid, anoxic cell death within minutes of the ischemia onset. Irreversible processes including mitochondrial collapse, rapid energy depletion, and ion pump failure result in large increases in intracellular calcium and in extracellular potassium *(Bright and Mochly-Rosen, 2005b; Dirnagl et al., 1999; Lipton, 1999)*. However, is in the area surrounding the core or penumbra, where the metabolism and intracellular signaling cascades are maintained partly by hypoperfusion from a diminished collateral blood supply *(Phan et al., 2002)*. This tissue is affected by multiple stresses including regional glutamate and potassium diffusion and peri-infarct depolarizations emanating from the ischemic core, and is in that tissue with residual energy levels, where rapid changes occur in the activity of many different signaling paths, alterations in the expression or activity of calcium/calmodulin-dependent protein kinase II (MAPK) family members c-Jun N-terminal kinase and extracellular signal-regulated kinase (ERK), protein kinase B (Akt), and protein kinase C (PKC), suggest that multiple kinases participate in the response of the tissue to ischemia and reperfusion *(Hayashi et al., 2000; Kitagawa et al., 1999; Matsumoto et al., 2004; Noshita et al., 2002)*.

The localization of PKCs in mitochondria suggests a role for them in the ischemia-reperfusion process. Mitochondrial translocation of PKCδ has been shown to cause an alteration in calcium signaling events and to mediate the H_2O_2-induced loss of membrane potential, release of cytochrome c, and activation of caspase-3 *(Sumitomo et al., 2002)*. PKCε was shown to be constitutively present in mitochondria with an increased translocation *(Baines et al., 2003)* but, in contrast with PKCδ, this induced a cardioprotection by both an inhibition of the mitochondrial permeation pore and a K-ATP channel opening *(Costa et al., 2006)*.

We have focused our attention on PKCε among other PKCs in this work, because as we have studied in previous chapters, the interactions established between C1Bε domain and negatively charged phospholipids are the highest in comparison to the rest of C1B domains. Although the mechanism enabling

mitochondrial translocation and import of PKCε to enable phosphorylation of their substrates is not known yet, we have hypothesized that the translocation and anchorage of the PKCε to the mitochondria would be performed not only by the interactions between C2 domain and mitochondrial proteins *(Budas et al., 2010)*, but also by protein-negatively charged phospholipids interactions through its C1B domain as we have shown to take place upon protein translocation to the plasma membrane (see chapter V).

Protein kinase C (PKC) is a family of serine/threonine kinases. PKCs are major mediators of signal transduction pathways and have been shown to regulate sets of biological functions as diverse as cell growth, differentiation, apoptosis, transformation and tumorigenicity *(Nishizuka, 1984; Nishizuka, 1992; Nishizuka and Nakamura, 1995)*. According to differences in the regulatory domain and biochemical characteristics, the enzymes can be divided into three subfamilies: (1) the classical PKCs (α, βI, βII and γ) that contain tandem C1A/C1B motifs that bind diacylglycerol (DAG) or phorbol esters *(Corbalan-Garcia and Gomez-Fernandez, 2006; Leonard and Hurley, 2011; Sharkey et al., 1984; Tanaka et al., 1986)* and a C2 domain that binds anionic phospholipids in a calcium-dependent manner (with preference phosphatidylserine) *(Verdaguer et al., 1999)* and also interact with PIP_2 in the membrane through a second site *(Guerrero-Valero et al., 2009a; Guerrero-Valero et al., 2007b)*; (2) the novel PKCs (ε, δ, θ and η), also contain a twin C1/C1B domains and a C2 domain. However, the positions of the C1A/C1B and C2 domains in nPKCs are switched along the linear sequence of the protein (relative to cPKCs), and nPKC-C2 domains do not bind calcium, though play a role through interactions with ligands like phosphatidic acid *(Corbalan-Garcia et al., 2003c; Lopez-Andreo et al., 2003)*. On the other hand, nPKCs are maximally activated by lipid cofactors (DAG or PMA) without a calcium requirement *(Newton, 2001)*; and (3) the atypical PKCs (λ, ζ and ι), contain an atypical C1 domain that binds PIP_3 and ceramide but not DAG or PMA and a protein-protein interaction PB1 domain.

Both classical and novel PKCs exhibit two different C1 domains allocated in tandem at the regulatory domain. The affinity of these domains for different ligands change depending on the isoenzyme in question as it has been described in many publications *(Ananthanarayanan et al., 2003b; Bell and Burns, 1991; Irie et al., 2002a; Quest et al., 1994; Sanchez-Bautista et al., 2009)*. The affinity of C1B domains of novel PKCs was also a topic of study in this work (see Chapter IV), and in particular, the effect of negatively charged phospholipids

such as phosphatidylserine, phosphatidic acid and phosphatidylglycerol in lipid mixtures in presence, or absence, of DAG. Our results concluded that two different subfamilies of novel PKCs can be established according to their tendency to bind to the cell membrane. In Chapter V it has been shown that there are some positively charged residues in the C1B of PKCε that are very important for the binding of this domain to membranes containing negatively charged phospholipids and the mutation of these residues with Ala substituting for them decreased the membrane binding ability of these domains and also of the full-length PKCε protein in cells.

Our next goal was to determine the affinity of C1B domains of novel and classical PKCs to cardiolipin, another negatively charged phospholipid, which is the predominant negatively charged one in mitochondrial inner membrane.

Cardiolipin (CL), also known as diphosphatidylglycerol (Figure VI.1), is an anionic phospholipid predominantly located in the inner mitochondrial membrane (IMM) *(Krebs et al., 1979)*. It is also found in the outer mitochondrial membrane (OMM) and is enriched at contact sites that are formed between the inner and outer membranes of the mitochondria *(Daum, 1985; deKroon et al., 1997; Schug and Gottlieb, 2009*).

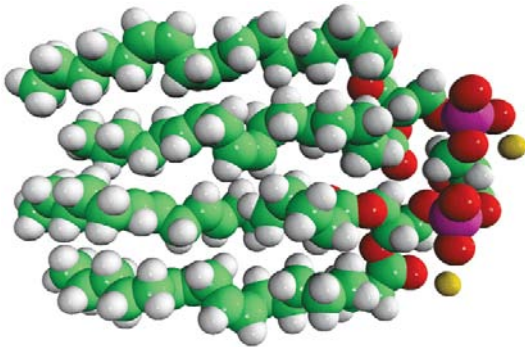

Figure VI.1. Overall structure of the cardiolipin molecule. Cardiolipin is a unique phospholipid which contains four acyl chains, mosts of which are highly unsaturated. CL has two negative charges on the headgroup and is found almost exclusively in mitochondrial membranes.

The importance of CL for mitochondrial topology and function has been mainly described in the context of its association with carriers and the large protein complexes of the inner membrane, where it seems necessary for their proper function and/or formation of super complexes *(Claypool et al., 2008; Hoffmann et al., 1994; Pfeiffer et al., 2003; Schlattner et al., 2009; Zhang et al., 2002)*. The compartment situated at the interface between mitochondria and the cytosol is not only a site of intense metabolite traffic, but is also densely packed with proteins, and CL serves as an anchor for some of this proteins *(Schlattner et al., 2009)*, including cytochrome c *(Rytomaa and Kinnunen, 1995)*, truncated Bid

(t-Bid) *(Liu et al., 2005)*, and metabolic kinases such as the mitochondrial isoforms of creatine kinase (CK) *(Ellington, 2001; Ellington and Suzuki, 2007)* and nucleoside diphosphate kinase (NDPK) *(Boissan et al., 2009)*. It is becoming apparent that CL is a versatile phospholipid which participates not only in determining membrane structure and fluidity and in assuring normal electron transport chain activity, but it also plays a major role in cell signalling through CL-protein interactions *(Schug and Gottlieb, 2009)*.

Cardiolipin could also act as an anchor with PKC in the mitochondria due to the affinity for negatively charged phospholipids which are present in this family of proteins. The activity of some isoenzymes of PKCs was measured in many studies using vesicles containing CL, showing that this lipid can perform as an activator of PKCs, increasing the activity of PKCε 3 to 4 fold compared with phosphatidylserine *(Konno et al., 1989)*. However in the case of classical PKCs, CL did not act similarly, and in the case of PKCγ and PKCα the activity in its presence was two and three fold less than with phosphatidylserine respectively *(Tanaka et al., 1992)*. Another interesting observation was that in the absence of DOG, the activity in PKCα was almost twice as much as in vesicles which contained only phosphatidylserine *(Kochs et al., 1993)*, this being an important point, due to the fact that the presence of DAG in the mitochondrial membrane, is not clear though it has been described the existence of phospholipase D activity in mitochondria from rat myocardium *(Panagia et al., 1991)*.

Our results show that the C1B domain of PKCε has a great affinity for CL and this affinity depends on the same positively charged residues that we have showed to be essential for the binding to membranes containing other negatively charged phospholipids (Chapter V). In addition the mutation of these residues decrease the capacity of PKCε to translocate to mitochondria upon induction of ischemic condition.

2. RESULTS

2.1. The influence of cardiolipin on the binding of C1B domains to membrane in the presence of DOG.

Using the FRET method mentioned above, the binding of C1B domains to small unilamellar vesicles containing different lipid compositions was studied. In this case we used four C1B domains from the novel PKCs (PKCε, PKCη, PKCδ, PKCθ), and one C1B domain that belongs to the classical subfamily, that is the PKCγ isoenzyme.

In this assay, the effect of increasing the relative percentage of CL on C1B membrane binding was studied. The data were analyzed and best fitted to the Hill model. The diacylglycerol (DOG) concentration was kept constant at 5 mol% in all the following cases.

Fig. VI.2 and Table VI.1 shows the binding of the C1B domains to increasing concentrations of POPC/CL/DOG vesicles. C1Bε-ECFP showed the highest binding affinity for membranes when CL was present at 40 mol%, followed by C1Bη-ECFP and C1Bθ-ECFP. The K_D values were 0.54 μM (C1Bε-ECFP), 2.83 μM (C1Bη-ECFP) and 4.06 μM (C1Bθ-ECFP). The largest differences between domains were observed with low concentrations of CL, such as 5 mol%, at which C1Bε-ECFP continued to have the highest binding affinity. At 5 mol% CL the same order of magnitude than at 40 mol% for CL was observed.

An interesting finding was observed at intermediate concentrations of CL. In the case of C1Bε-ECFP, at 20 mol% of CL the K_D was 1.22 μM while at 10 mol% the K_D was 1.96 μM, i.e. there were relatively small differences between K_D values when the phospholipid was reduced from 20 to 10 mol%. In view of this fact, it is important to emphasize that this is the physiological range of cardiolipin that can be found in the inner mitochondrial membrane, around 15 mol% of the total lipid *(van Meer et al., 2008)*.

On the other hand, C1Bη-ECFP had a low K_D value at 40 mol% and increased 2-fold for 20 mol% and 5-fold for 10 mol% (at 40 mol% of anionic phospholipids, the K_D was 2.83 μM compared to 5.38 μM at 20 mol% and 11.59

μM at 10 mol%), these values being quite different from the binding affinities observed for C1Bε-ECFP.

The same happened with C1Bθ-ECFP, raising its K_D value from 4.06 μM at 40 mol% of CL, to 15.62 μM at 10 mol%. The highest K_D values correspond to C1Bδ-ECFP and C1Bγ-ECFP domains when CL was present even at high content of phospholipid (at 40 mol% of CL, the K_D was 24.77 μM for C1Bδ-ECFP and 19.08 μM for C1Bγ-ECFP).

Mol (%)	C1Bη		C1Bε		C1Bθ	
	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)
5	0.57 ± 0.02	15.85 ± 0.3	0.58 ± 0.01	6.28 ± 0.5	0.53 ± 0.02	29.50 ± 0.3
10	0.56 ± 0.02	11.59 ± 1.1	0.59 ± 0.01	1.96 ± 0.3	0.51 ± 0.01	15.62 ± 1.9
20	0.58 ± 0.01	5.38 ± 0.5	0.67 ± 0.02	1.22 ± 0.1	0.57 ± 0.03	11.02 ± 0.7
40	0.60 ± 0.01	2.83 ± 0.2	0.69 ± 0.01	0.54 ± 0.1	0.54 ± 0.01	4.06 ± 0.2

Mol (%)	C1Bδ		C1Bγ	
	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)
5	0.12 ± 0.02	40.15 ± 0.4	0.41 ± 0.02	89.34 ± 1.2
10	0.20 ± 0.01	28.22 ± 0.2	0.56 ± 0.02	50.80 ± 1.1
20	0.24 ± 0.02	22.60 ± 0.2	0.67 ± 0.02	31.31 ± 0.5
40	0.30 ± 0.01	11.57 ± 0.2	0.79 ± 0.01	19.08 ± 1.1

Table VI.1. Binding parameters of C1B-ECFP domains to lipid vesicles. Phospholipid vesicle contained POPC/DOG/CL/OG-PE (90-X:5:X:5 mol %), whereas CL and POPC phospholipids varied as indicated in the table. FRET data were fitted to a Hill equation. Values given as mean ± SE of three different experiments.

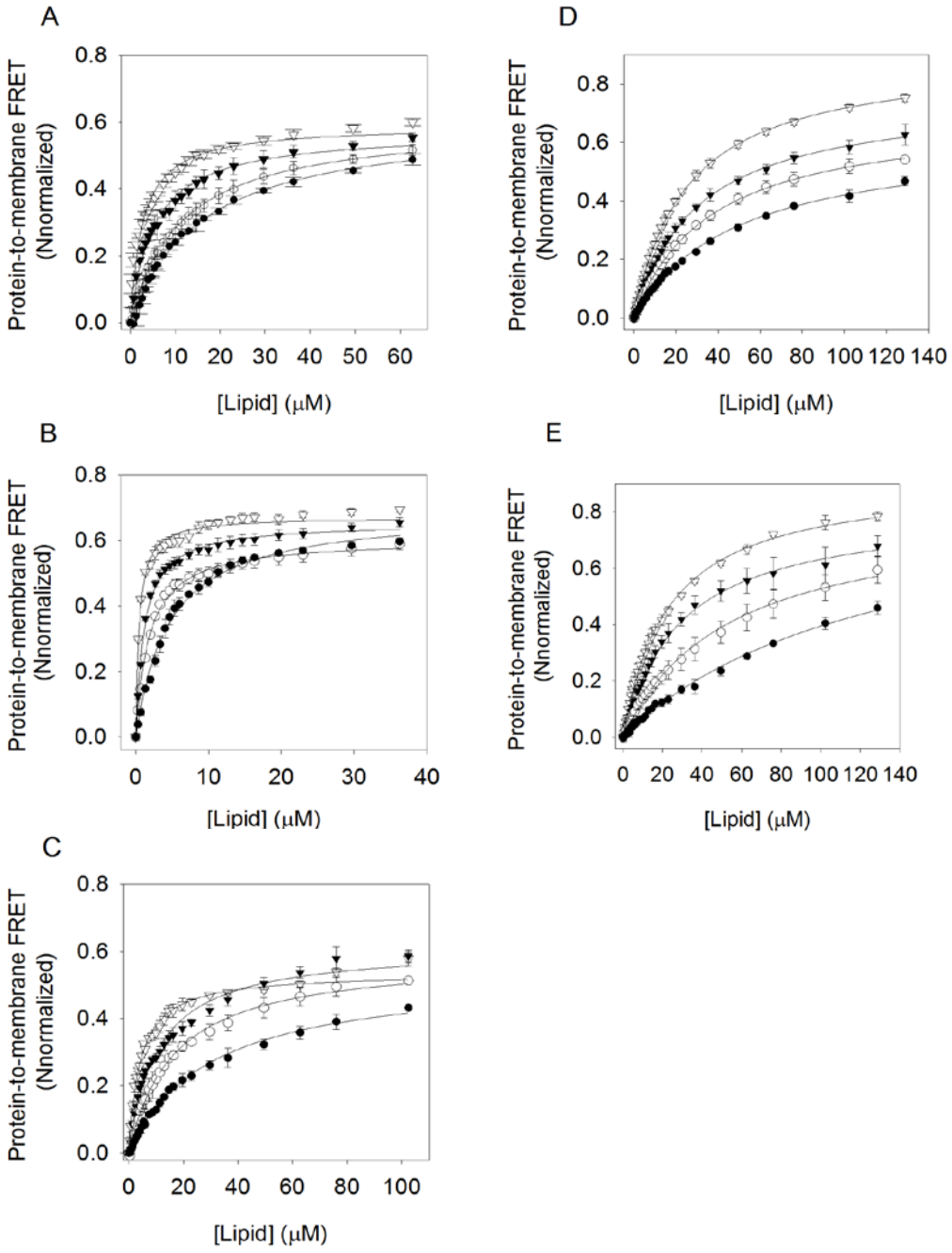

Figure VI.2. Binding of C1B domains to lipid vesicles. Binding of C1Bη-ECFP (A), C1Bε-ECFP (B), C1Bθ-ECFP (C), C1Bδ-ECFP (D) and C1Bγ-ECFP (E) domains to lipid vesicles, which contained POPC/CL/OGPE in molar ratio of 90:5:5 (•), 85:10:5 (o), 75:20:5 (▼), and 55:40:5 (▽). DOG was present in all cases at 5 mol %. Normalized FRET values are depicted versus total lipid concentration.

2.2. The influence of cardiolipin on the binding of C1B domains to membrane simulating the characteristics of lipid composition of the mitochondrial inner membrane.

The mitochondrial inner membrane has a unique composition of proteins and phospholipids, whose interdependence is crucial for mitochondrial function. It is highly enriched in proteins specific to this membrane. And the lipid component contains phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA), as well as phosphatidylglycerol (PG) and cardiolipin (CL), which is located predominantly in the mitochondria *(Gohil and Greenberg, 2009; Zinser et al., 1991)*.

In this assay we emulated the same composition that can be found in the mitochondrial inner membrane. Thus, we prepared liposomes containing POPC/POPE/CL/PI/OG-PE (38:24:19:14:5 mol%).

Figure VI.3 and Table VI.2 show that C1Bε-ECFP remains the domain with the highest binding affinity in these liposomes lacking DOG. The K_D value was 4.67 μM, 3.5-fold higher than the K_D value of 1.22 μM for vesicles which contain 5 mol% of DOG, but still lower than that for membranes composed by 5 mol% of CL and 5 mol% of DOG which was 6.28 μM (Table VI.2). It can be deduced that the presence of CL within a physiological range is enough for this domain to reach a high binding affinity for membranes, even in the absence of DOG.

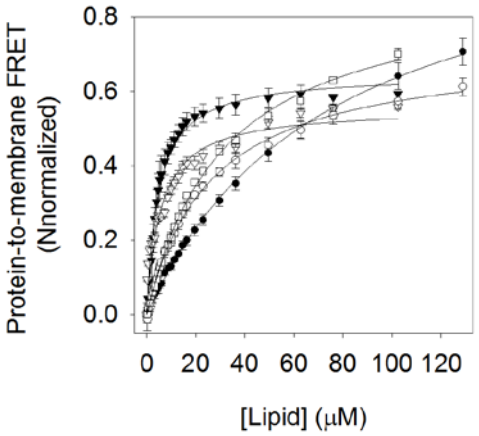

Figure VI.3. Binding of C1B domains to lipid vesicles simulating the characteristics of lipid composition in the inner mitochondrial membrane. Binding of C1Bη-ECFP (▽), C1Bε-ECFP (▼), C1Bθ-ECFP (o), C1Bδ-ECFP (•) and C1Bγ-ECFP (□) domains to lipid vesicles, which contained POPC/PE/CL/PI/OGPE in molar ratio 38:24:19:14:5, in the presence of DOG (A) or its absence (B). Normalized FRET values are depicted versus total lipid concentration.

C1Bη-ECFP showed the same binding affinity for membranes when DOG was present at 5 mol% or not. In the first case, at 20 mol% of CL and 5 mol% of DOG, the K_D value was 5.38 μM (Table VI.1), in the second case, when the

liposomes mimic the mitochondrial inner membrane, the K_D value was 5.50 μM. It might be that the content of PI also played an important role because of the increase in the negative charges around the surface of the membrane. Another possibility is that once the domain has recognized a minimum level of CL, it would not need the existence of DOG to bind to the membrane.

When the binding of C1Bθ-ECFP was studied, it was observed (Figure VI.3 and Table VI.2) that the extent of binding was much lower than for the two previously mentioned C1B isoenzymes, and with the previous assay where the vesicles contained 5 mol% of DOG (at 20 mol% of CL, the K_D was 11.02 μM in presence or DOG compared to 27.15 μM in an emulated mitochondrial inner membrane), illustrating that they have a relatively high binding affinity in the presence of DOG but not due to the anionic phospholipid per se.

C1Bδ-ECFP and C1Bγ-ECFP domains kept up a low binding affinity when this composition of membranes was used, as occurred in the presence of DOG. The K_D values were 30.80 μM for C1Bδ-ECFP and 75.36 μM for C1Bγ-ECFP.

	POPC/PE/CL/PI/OGPE (38:24:19:14:5)
	K_D (μM)
C1Bγ-ECFP	30.80 ± 0.31
C1Bδ-ECFP	75.36 ± 0.25
C1Bθ-ECFP	27.15 ± 0.43
C1Bη-ECFP	5.50 ± 0.43
C1Bε-ECFP	4.67 ± 0.73

Table VI.2. Binding parameters of C1B-ECFP domains to lipid vesicles simulating the characteristics of lipid composition in the inner mitochondrial membrane. Phospholipid vesicle contained POPC/PE/CL/PI/OG-PE (38:24:19:14:5 mol %), FRET data were fitted to a Hill equation. Values given as mean ± SE of three different experiments.

2.3. The influence of cardiolipin on the binding of C1B mutant domains to membrane containing cardiolipin in the presence or absence of DOG.

As it was mentioned in Chapter V, the positive charged residues located on the top of the domain, established a positive surface charged which allowed the electrostatic interactions with acidic phospholipids such as phosphatidic acid or phosphatidylserine. In this FRET assay we used the same C1B mutants generated previously to see the effect of these residues on the binding of membranes containing cardiolipin.

Figure VI.4 and Table VI.3 shows the binding of C1Bε-ECFP mutants to lipid vesicles in the presence or absence of DOG and at 20 mol% of CL. In the case of single mutants, the K_D value decreased drastically in the absence of DOG (Figure VI.4-D) (K_D value was 4.83 μM for wild-type C1Bε-ECFP compared to 20.93 μM for C1BεK251A-ECFP or 27.04 μM for C1BεR268A-ECFP or 16.80 μM for C1BεR282A-ECFP or 23.08 μM for C1BεR283A-ECFP). On the other hand, these single mutations did not have the same consequences for vesicles containing 5 mol% of DOG (Figure VI.4-A), keeping the K_D in the same order of magnitude (K_D value was 1.22 μM for wild-type C1Bε-ECFP compared to 3.28 μM for C1BεK251A-ECFP or 7.16 μM for C1BεR268A-ECFP or 3.72 μM for C1BεR282A-ECFP or 5.01 μM for C1BεR283A-ECFP). This result suggests that DOG may compensate the lack of affinity of the domain to CL or alternatively, the domain would need a first contact with DOG.

The results obtained with double mutants showed that in the presence of DOG (Figure VI.4-B), the K_D values increased about 2-fold compared to those corresponding to single mutants but remained lower than those of the single mutants in the absence of DOG. The increase in the K_D value was not as important in double mutants in the absence of DOG (Figure VI.4-E) than in single mutants. This result could be interpreted as the fact that the lack of just one of these residues would be enough to significantly damage the binding affinity of the domain for the membrane.

We also studied the pattern followed by triple mutants. In this case, the differences found in the binding affinity to membranes between membranes containing or not DOG was not as important as seen for single or double mutants (Figure VI.4-C and F and Table VI.3). For instance, the K_D value for the mutant C1BεK251A/R268A/R283A-ECFP was 47.23 μM in the presence of DOG and 55.61 μM in the absence of DOG.

The results obtained show the significance of these mutated residues for the binding of the domain to the membrane and that mutation of these residues can be partially compensated by the presence of DOG. Nevertheless, in its absence, as occurs in the simulated mitochondrial inner membrane, only one amino acid residue mutated would be enough to substantially abolish the docking of C1B.

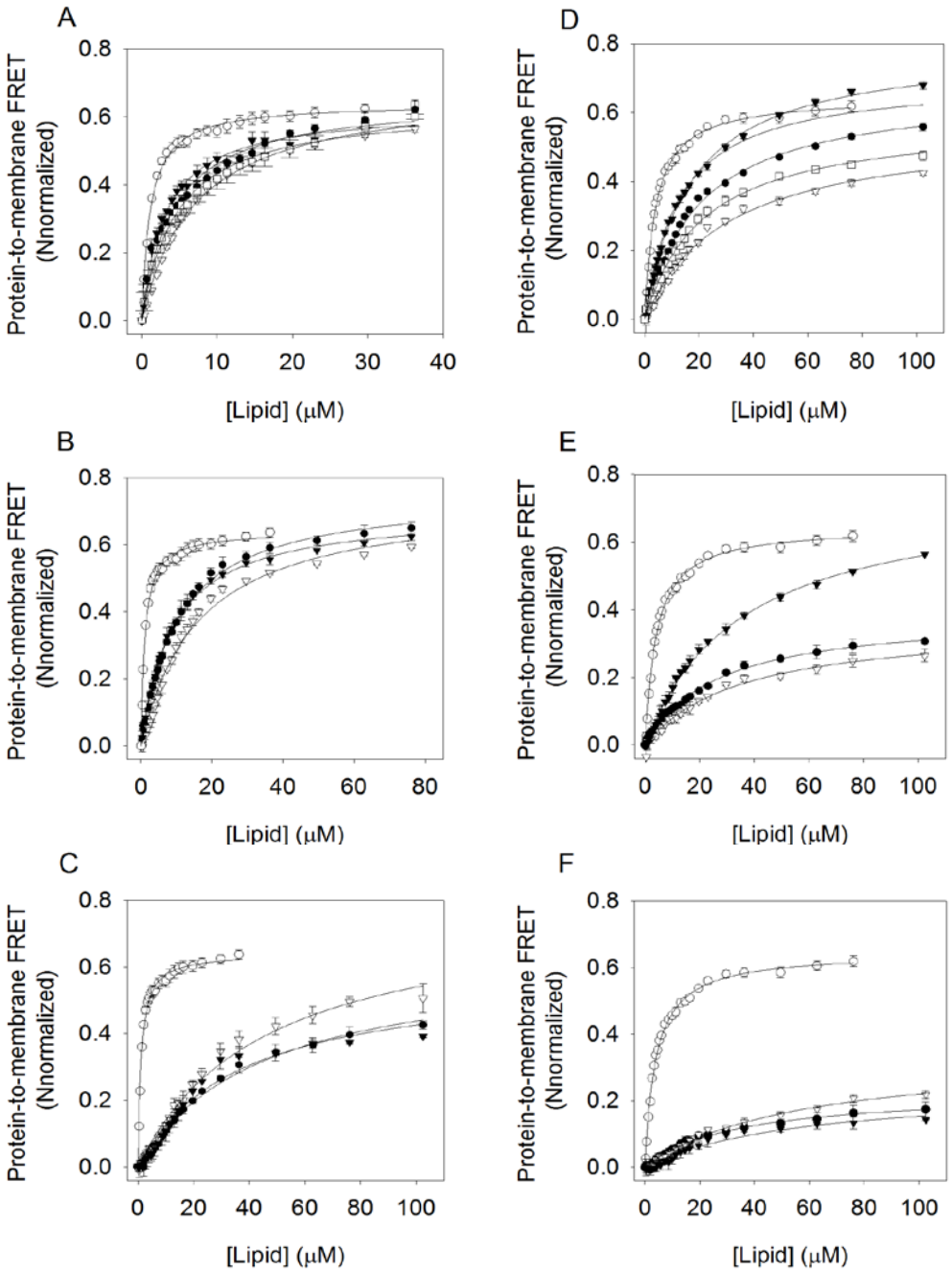

Figure VI.4. **Binding of C1Bε and its mutants to lipid vesicles containing CL in the presence or absence of DOG**. Binding of C1Bε-ECFP domain (o) and its simple mutants K251A (•) , R268A (▽), R282A (▼) and R283A (□) (A and D); double mutants K251A/R268A (•), R268A/R283A (▽), R282A/R283A (▼) (B and E); and triple mutants K251A/R268A/R282A (•), K251A/R282A/R283A (▽), R268A/R282A/R283A (▼) (C and F) to lipid vesicles, which contained POPC/CL/OGPE in molar ratios of 75:20:5 in the presence of DOG at 5 mol % (A, B and C) or absence of DOG (D, E and F). Normalized FRET values are depicted versus total lipid concentration.

	POPC/CL/DOG/OGPE (70:20:5:5)		POPC/CL/OGPE (75:20:5)	
C1Bε-ECFP	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)
Wild-type	0.61 ± 0.01	1.22 ± 0.1	0.59 ± 0.01	4.83 ± 0.9
K251A	0.64 ± 0.03	3.28 ± 0.3	0.56 ± 0.02	20.92 ± 0.7
R268A	0.57 ± 0.02	7.16 ± 0.2	0.40 ± 0.04	27.04 ± 0.4
R282A	0.62 ± 0.03	3.72 ± 0.6	0.69 ± 0.01	16.80 ± 0.6
R283A	0.58 ± 0.03	5.01 ± 0.1	0.45 ± 0.01	23.08 ± 0.9
K251A/R268A	0.65 ± 0.02	10.05 ± 0.3	0.29 ± 0.02	35.64 ± 0.6
R268A/R283A	0.59 ± 0.01	15.52 ± 1.4	0.26 ± 0.02	35.28 ± 0.7
R282A/R283A	0.62 ± 0.03	8.72 ± 0.6	0.55 ± 0.01	26.87 ± 0.7
K251A/R268A/R282A	0.37 ± 0.02	44.80 ± 0.6	0.16 ± 0.02	63.87 ± 1.7
K251A/R282A/R283A	0.50 ± 0.01	47.23 ± 0.9	0.22 ± 0.01	55.61 ± 0.6
R268A/R282A/R283A	0.54 ± 0.01	65.53 ± 1.3	0.13 ± 0.02	119.29 ±1.4

Table VI.3. Binding parameters of C1Bε-ECFP mutant domains to phospholipid vesicles.
Phospholipid vesicle contained 75 mol % POPC, 20 mol % CL, X mol % of DOG, 5 mol % OG-PE. FRET data were fitted to a Hill equation. Values given as mean ± SE of three different experiments.

2.4. PKCε is activated by CL in vitro.

We also studied the ability of PKCε to phosphorylate a specific substrate in vitro under CL-dependent conditions. For this purpose, HEK293 cells were transfected with the same constructs as mentioned above (Chapter II). After purification of the wild-type PKCε-EGFP and the mutant PKCεK251A/R268S/R282A/R283A-EGFP, we studied the CL dependence of enzyme activation in the absence and in the presence of DOG (Figure VI.5 A) by using histone III-S as a substrate. It is interesting to note that under the conditions used in these experiments, the basal specific activity was negligible when the phospholipid vesicles contained 5 mol% DOG in the absence of CL.

Interestingly, Figure VI.5 A also shows that it was necessary to include DOG in the lipid vesicles to observe a CL-dependent activation, suggesting that at least in vitro both CL and DOG interactions with PKCε-EGFP are necessary for the full activation of the enzyme. In addition, we studied the effect of the substitutions of the amino acidic residues in the C1B domain of the enzyme (Figure VI.5 A). Once it was demonstrated that CL can activate PKCε-EGFP, we studied the activation of its mutant, containing modifications of their aminoacidic residues located in the C1B domain, in order to ascertain the role of

C1B domain in the activation of PKCε-EGFP induced by this negative charged phospholipid. A drastic effect was shown where with up to 60 mol% of CL in the lipid vesicles the activity only reached a 15% of the catalytic activity of the wild type enzyme.

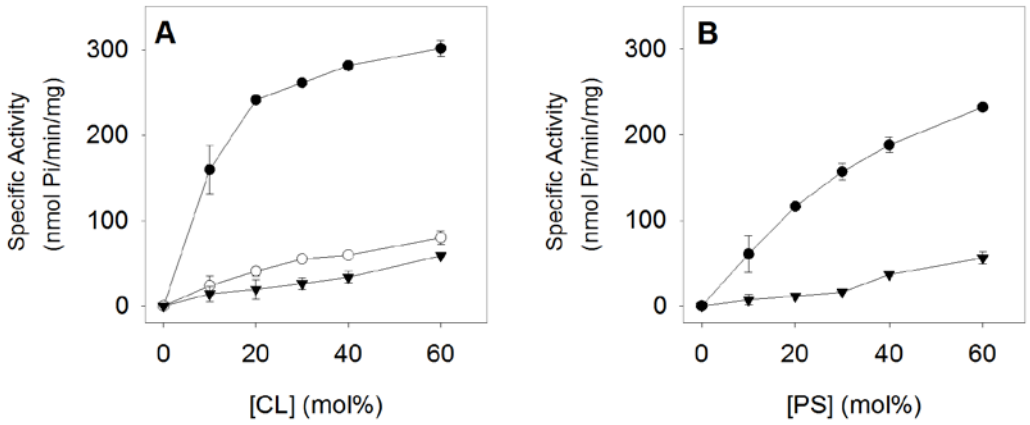

Figure VI.5. Specific activity of full length PKCε and its mutant by using large unilamellar vesicles. **(A)** Cardiolipin-dependent activation of wild-type PKCε and PKCεK251A/R268A/R282A/R283A by using large unilamellar vesicles containing POPC/CL (99-X:X, with X being the molar fraction of CL in each case) for wild type PKCε (o) and POPC/DOG/POPA (95-X:5:X, with X being the molar fraction of CL in each case) for wild type PKCε (•) or PKCεK251A/R268A/R282A/R283 (▼). **(B)** PtdSer-dependent activation of wild-type PKCε and PKCεK251A/R268A/R282A/R283A by using large unilamellar vesicles containing POPC/POPS/DOG (95-X:X:5, with X being the molar fraction of POPS in each case) for wild-type PKCε (•) or PKCεK251A/R268A/R282A/R283 (▼). The total lipid concentration was 0.2 mM and Histone III-S was used as a substrate. Error bars indicate the SEM for triplicate determinations.

To corroborate that the system works correctly, a further experiment was performed by using a different negatively charged phospholipid as a positive control, in this case phosphatidylserine, which has been demonstrated to be able to activate PKCε (Chapter V).

As shown in Figure VI.5-A the CL-dependent activity of the enzyme was 2-folds higher compared to the POPS-dependent activity at 20 mol% of phospholipid (Figure VI.5-B). We should pay an special attention to the 20 mol% of phospholipid experiment in both cases because is the physiological content of POPS in the plasma membrane *(McMurray and Rogers, 1973)* and the CL in the mitochondrial inner membrane *(van Meer et al., 2008)*, suggesting that CL is a more potent activator than phosphatidylserine under these conditions.

2.5. Oxygen glucose deprivation (OGD) model of ischemia.

In the present study, we analyzed the contribution of the specific positive amino acid residues located at the top of the C1Bε domain to the translocation of the full-length protein from the cytosol to the mitochondria by using an in vitro model of ischemia, maintaining SH-SY5Y human neuroblastoma cells under oxygen glucose deprivation (OGD). These cells had been previously transfected with the wild-type PKCε-EGFP or its mutant PKCε/K251A/R268A/R282A/R283A-EGFP. After 2 hours of OGD the cells were collected and kept frozen until the subcellular fractionation was carried out.

In each set of samples we could identify by Western Blot the overexpressed PKCε fused with GFP and the endogenous PKCε (Figure VI.6). This endogenous PKCε would help us to follow the behavior of the native protein and compare it with the overexpressed PKCε. We would also use it as a positive control for the OGD treatment.

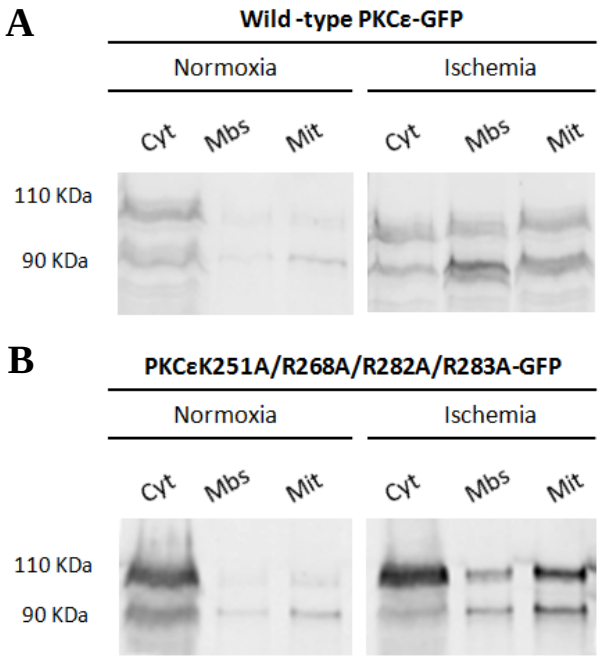

Figure VI.6. Wild-type PKCε-GFP and PKCεK251A/R268A/R282A/Rº283A-GFP translocation from cytosol to membrane and mitochondria under hypoxic-ischemic conditions. SH-SY5Y cells were transfected with wild-type PKCε-GFP **(A)** and its mutant **(B)**. The upper bands of the gel (~110 KDa) are the overexpressed protein, while the ~90 KDa band correspond to the endogenous PKCε. Cytosolic, membrane and mitochondrial fractions were analyzed by Western blot analysis using a specific antibody. The immunoblots are representative of one of six experiments with similar results.

We analyzed the redistribution of PKCε after 2 h of OGD treatment. As it can be seen in Figure VI.6-A and Figure VI.7-A, around ~75% of the overexpressed and endogenous PKCε were detected mainly in the cytosolic fraction under normoxic conditions. The rest was found in mitochondria (~20% of the total), having been suggested that it can be a constitutive protein in this

organelle *(Baines et al., 2003)* and also in the membrane fraction, though with a lower percentage (~5%). When we subjected the cells to OGD during 2 hours without recovery, a change in the distribution of the protein could be observed as is shown in Figure VI.6-A and Figure VI.7-A.

Endogenous and overexpressed PKCε was reduced by half in the cytosol (~75% in normoxic conditions compared to ~40% in OGD), spreading the 40% remaining between membrane and mitochondrial fraction equally. Despite the fact that the results can reveal a certain degree of variability about the percentage of protein which is located in the membrane or mitochondrial fraction during OGD, there is no doubt that the levels of protein in cytosol fell down in this hipoxic condition. It is also important to highlight the same pattern between endogenous PKCε and PKCε fused with GFP, confirming that the fusion with GFP did not disturb the mechanism of translocation of the protein in this *in* vitro model.

When we analyzed the data obtained for the mutant (Figure VI.6-B and Figure VI.7-B) near the 100% of the mutant was found in the cytosol, and there only was observed a very low amount of mutant in the mitochondria under normoxic conditions, compared to the ~20% for the wild-type PKCε-EGFP. We obtained the same results as were observed in the other assay for the endogenous PKCε, ~70% of protein in the cytosol, and ~20% in the mitochondria.

Once the cells were treated to OGD, the presence of the mutant in the cytosol decreased on average by only 15% down to ~70% whilst the endogenous declined from ~65% to ~25%. The presence of PKC mutant in the mitochondria raised to 20% (from ~10% of the total protein in normoxia to ~20% in ischemia), this increase being much lower than that observed for the endogenous PKCε or for the wild-type overexpressed protein translocated to the mitochondria during OGD.

This results suggest that the C1B domain of PKCε is involved in the translocation of the full-length protein to the mitochondria in SH-SY5Y cells in this oxygen glucose deprivation model and that the interaction of this domain with negatively charged phospholipids is important.

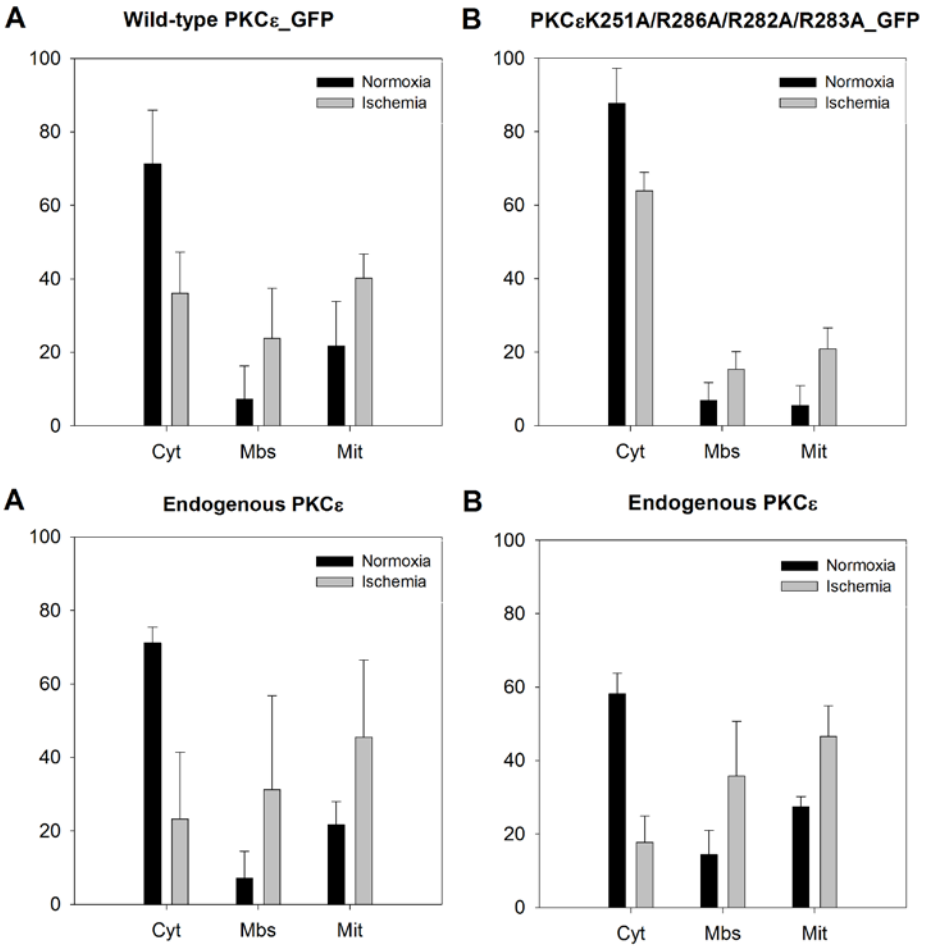

Figure VI.7. Wild-type PKCε-GFP and PKCεK251A/R268A/R282A/R283A-GFP distribution during normoxia or OGD. Representation of the distribution in cytosolic, membrane and mitochondrial fraction of wild-type PKCε-GFP **(A)** or PKCεK251A/R268A/R282A/R283A-GFP **(B)** and the correspondent endogenous PKCε in each case. Cell lysis and subcellular fractionation were carried out as described in Chapter II of this Doctoral Thesis**.**
Quantitation of blottings using ImageJ software. n > 6 /group.

3. DISCUSSION

In the first part of this work, the affinity binding of the C1B domains to membranes which contained different concentrations of CL was studied using a FRET assay. According to the data obtained, C1Bε-ECFP domain showed the highest binding affinity for these membranes, followed very closely by C1Bη-ECFP (at 40 mol% of CL, the K_D value was 0.54 μM for C1Bε-ECFP and 2.83 μM for C1Bη-ECFP). Nevertheless, at concentrations of CL close to physiological C1Bε-ECFP showed a higher membrane binding in comparison to C1Bη-ECFP (at 20 mol% of CL the K_D value was 1.22 μM for C1Bε-ECFP and 5.38 μM for C1Bη-

ECFP). Even bigger differences were found at 10 mol% of CL, (1.96 μM for C1Bε-ECFP compared to 11.59 μM for C1Bη-ECFP).

These data correlate with the results obtained in Chapter IV, where C1Bε-ECFP and C1Bη-ECFP domains showed the highest binding affinity for negatively charged phospholipids, but in this occasion at lower concentrations of acidic phospholipid, C1Bε-ECFP revealed a higher binding affinity than C1Bη-ECFP in the physiological range of CL that is present in the mitochondrial inner membrane.

On the other hand, C1Bδ-ECFP and C1Bθ-ECFP are the domains with the lower binding affinity for negatively charged phospholipids, these being PA, PS, PG (see chapter IV) or CL, together with the classical C1Bγ-ECFP domain, which showed a similar pattern.

It should be remarked that the influence of the interaction with CL on the membrane binding of C1B domains was demonstrated by experiments emulating the mitochondrial inner membrane whose composition lacks DOG. It was very clear that C1Bε-ECFP and C1Bη-ECFP had the highest membrane binding affinity among the C1B domains, which means that these domains would not need the presence of DOG to reach the high binding affinity, and this could happen in the mitochondrial membrane, where the existence of DOG is uncertain.

The last FRET experiments were carried out using C1Bε mutants generated in the Chapter V, and we could test how the binding to membranes containing CL was blocked as we had seen previously with phosphatidic acid and phosphatidylserine.

Our results showed that binding decreased when the number of residues mutated in the domain was increased, and it can be even abolished in the presence of diacylglycerol, meaning that there is not a residue more important than other, and it is their combination what make the domain able of interacting with negatively charged phospholipids.

After demonstrating that C1Bε domain has a high binding affinity for CL, kinase activity assays were run to check whether this lipid was able to activate PKCε-EGFP or its mutant PKCεK251A/R268A/R282A/R283A-EGFP directly.

The results showed that the catalytic activity of the enzyme increased in parallel with the concentration of CL in the lipid vesicles, suggesting that this acidic phospholipid itself is a potential activator of PKCε-EGFP. It was demonstrated that the ability of the PKCεK251A/R268A/R282A/R283A-EGFP mutant to be activated by CL was completely abolished, even at saturating concentrations of CL. Taken together, these results confirm the hypothesis that the C1B domain of PKCε plays an important role in the CL-dependent activation of the enzyme, and the positively charged amino acid residues located on the top of the domain are essential for the activation mechanism.

To investigate whether CL induces the localization of PKCε in the mitochondrial membrane under ischemia conditions, SH-SY5Y cells were transfected with PKCε fused to EGFP and its mutant. In steady-state conditions, the protein was distributed homogeneously through the cytosol in both proteins, although the concentration of wild-type protein in the mitochondria was higher than in the case of the mutant. When the cells were subjected to OGD, no effect was observed on enzyme localization in the mutant, staying most of the protein in the cytosol. In contrast to the wild-type PKCε-EGFP that reduces its presence in cytosol, in favor to the membrane and mitochondrial fraction.

These results coincide with those obtained in the kinase activity assays and FRET, suggesting that these residues which were mutated are essential for the translocation and activation of PKCε to the membrane.

CONCLUSION

Taken together, our results show that (i) C1Bε domain has the highest binding affinity for model membranes containing cardiolipin at the same proportions that are found in the mitochondrial inner membrane (ii) the mutation to alanine of the positively charged amino acid residues located on the top of the domain not only abolish the binding of the domain to membranes containing cardiolipin but also the activity of the full-length enzyme under saturating concentrations of cardiolipin (iii) using an in vitro model of ischemia, maintaining SH-SY5Y human neuroblastoma cells under oxygen glucose deprivation (OGD), the level of translocation of the full-length mutant PKCε to the mitochondria was substantially lower than that of the wild-type, suggesting that the C1B domain of the novel PKCε could interact with the mitochondrial membrane through electrostatic interactions.

CHAPTER VII

General Discussion and Conclusions

1. GENERAL DISCUSSION

Inositol phospholipids play an important regulatory role in cell physiology, controlling a wide variety of cellular processes *(Di Paolo and De Camilli, 2006)*. For example, it has been demonstrated that Ca^{2+} and PtdIns(4,5)P_2 cooperate for PKCα membrane localization and that the *lysine-rich cluster* is essential in this process *(Evans et al., 2006; Marin-Vicente et al., 2005a)* or the specificity of the C2 domains of classical PKCs to bind PtdIns(4,5)P_2 decreases the concentration of Ca^{2+} needed by the domains to bind to phospholipid vesicles *(Guerrero-Valero et al., 2007b)*. The results presented in Chapter III confirm that intracytosolic Ca^{2+} elevations are triggered to translocate PKCα to the plasma membrane and the presence of phosphatidylserine (PS) and PIP_2 at the plasma membrane would make C2 domain to dock in a different orientation due to the fact that it has to anchor through two differrent motif, i.e. the CBR (Ca^{2+}/PS) and the lysine rich cluster (PIP_2). This conformational change would release the C1 domain from blocking conformation and enables the catalytic domain to have access to the substrate and consequently would induce full activation of the enzyme.

Traditionally it has been described that C1 domains of novel PKCs mediate the diacylglycerol-dependent translocation of these enzymes *(Corbalan-Garcia and Gomez-Fernandez, 2006; Newton, 2001)*. We demonstrated that the presence of negatively charged phospholipids in the membrane also play an important role in the translocation of the enzyme (Chapter IV). However, it is important to note that each novel C1B domain show different affinities for model membranes depending on the presence or not of diacylglycerol and specific anionic phospholipids. It should be also remarked that the influence of the interaction with anionic phospholipids on the membrane binding of C1Bε and C1Bη domains was clearly demonstrated by the experiments in the absence of DOG. According with our results, two groups of C1B domains from novel PKCs can be established as a function of their membrane binding affinity: those from PKCε and PKCη with a higher membrane binding affinity and those with PKCθ and PKCδ with a low affinity. These differences may explain the physiological specializations observed between the C1Bs of novel PKCs.

The site-directed mutagenesis approach showed in Chapter V demonstrates the reason why C1Bε possess the highest binding affinities to membranes particularly when compared with C1Bδ. Four positively charged amino acids have been demonstrated to participate in the membrane recruitment of the C1B domain when vesicles containing negatively charged phospholipids were used, independently of the presence or the absence of DOG. This effect was also observed in the C1B subdomain mutant in full-length PKCε, thus C1A and C2 domains in PKCε could not be able to lead the translocation of the enzyme to the plasma membrane when these positively charged amino acids residues located in the C1B domain were replaced by alanines. It was also revealed that the enzymatic activity of the C1B mutant of PKCε decreased significantly compared with the wild type, corroborating the importance of C1B domain in this isoenzyme not only for the binding affinity to membranes but also for its activation.

As regards the biological roles of PKCε, it is known that PKCε translocates to the mitochondria during cardiac ischemic and reperfusion injury *(Armstrong, 2004; Budas et al., 2012)* and in cerebral ischemic *(Bright and Mochly-Rosen, 2005a)*. Although the mechanism enabling mitochondrial translocation and import of PKCε to activating it is not known yet, we have demonstrated that the translocation and anchorage of PKCε to the mitochondria would be performed not only by the interactions between C2 domain and mitochondrial proteins *(Budas et al., 2010)*, but also interactions through its C1B domain, possibly with phospholipids like cardiolipin, are important (Chapter VI).

2. CONCLUSIONS

This study contributes to our knowledge of the molecular mechanism of PKCα activation by PtdIns(4,5)P_2 and the different binding affinities for phospholipids which has been showed by C1B domains of novel PKCs, and in more detail, the role of a group of amino acid residues located in the C1Bε domain. Moreover, the use of an oxygen glucose deprivation model of ischemia revealed the importance of the C1B domain of PKCε for the translocation of the enzyme to the mitochondria. Hence, we can conclude the following:

1. The presence of 5 mol% of PIP_2 in the membrane decreases the requirement of Ca^{2+} more than 3 times, than of DOG almost 5 times and than of POPS by a half the enzymatic activity of PKCα.

2. The C1B domains of PKCε and PKCη bind to negatively charged phospholipids with higher affinity than the C1B domains of novel PKCθ and PKCδ.

3. C1B domains of PKCθ, PKCη and PKCε show the highest binding affinity to membranes that contain POPA whereas the highest binding affinity to membranes of the C1B domain of PKCδ corresponds to membranes containing POPS.

4. Plasma membrane translocation dynamics are also different for each C1B domain of novel PKCs, C1Bε being the most sensitive to phosphatidic acid.

5. The C1B domains of PKCε and PKCη are able to translocate to the plasma membrane in the presence of phosphatidic acid and in the absence of diacylglycerol.

6. The cross-linking of the high-affinity IgE receptor (FcεRI) with antigen in RBL-2H3 cells generates diacylglycerols and phosphatidic acids through the activation of PI-PLC and PLD respectively.

7. The C1B domain of PKCε interacts specifically with acidic phospholipids through the four positively charged amino acid residues located at the top of the domain.

8. PKCε is able to translocate to the mitochondria under an oxygen glucose deprivation ischemic model in SH-SY5Y cells and probably through C1B-lipids interactions, being cardiolipin the most abundant negatively charged phospholipid present in the mitochondria.

9. The enzymatic activity of PKC obtained for membranes that emulated the concentration of cardiolipin in the mitochondrial inner membrane was two times higher than that of membranes containing phosphatydilserine at the physiological composition of the plasma membrane.

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