UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

FACULTAD DE VETERINARIA

DEPARTAMENTO DE FARMACOLOGÍA

ESTUDIO DEL PAPEL MODULADOR DE LA PROTEÍNA QUINASA C Y LA AURORA QUINASA SOBRE LAS LÍNEAS CELULARES HMC-1^{560} Y HMC-1^{560,816}.

Tesis doctoral

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INFORMA

Que la tesis doctoral titulada "Estudio del papel modulador de la Proteína Quinasa C y la Aurora Quinasa sobre las líneas celulares HMC-1^{560} y HMC-$1^{560,816}$", recogida en la presente memoria, de la que es autora la Licenciada en Veterinaria por la Universidad de Santiago de Compostela Doña Araceli Tobío Ageitos, ha sido realizada bajo su codirección y cumple las condiciones exigidas para que su autora pueda optar al grado de Doctora por la Universidad de Santiago de Compostela, otorgando su aprobación para la lectura y defensa de la misma.

Para que así conste a los efectos oportunos, firma la presente en Lugo, a 1 de octubre de 2013.

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Dña. Mª Amparo Alfonso Rancaño
Doctora en Farmacia
Codirectora de la presente tesis doctoral

Dña. Araceli Tobío Ageitos

Non te rendas que a vida é iso,
continua-la viaxe,
persegui-los teus soños,
destraba-lo tempo,
move-los escombros
e destapa-lo ceo.

Mario Benedetti
(1920-2009)

Agradecementos

Grazas.

Fermosa verba,

mais semella non supor dabondo.

Adicada aos persoeiros,

todos eles,

que outorgaron senso a este traballo.

Sinceiro agradecemento

ao doutor Luis Botana,

polo seu apoio, pola súa amizade.

Á doutora Amparo Alfonso,

sabedora, amiga e compañeira.

Ás miñas compañeiras e compañeiros

neste camiño, ledo e solleiro.

Aos meus pais,

miña orixe,

miña cerna,

Á miña irmá, aos meus avós,

cariño, ledicia e tenrura.

A ti, compañeiro eterno,

piar e sustento,

fonte de lus,

alento neste camiño

perdurábel e infindo.

Ronsel, áncora e vida.

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1. Abreviaturas y acrónimos

A	Alanina
Abl	Gen Abelson
ADN	Ácido desoxirribonucleico
ADP	Adenosín difosfato
AKAP	Proteína de anclaje de la quinasa A
AMPc	Adenosín monofosfato cíclico
APAF-1	Factor 1 activador de la proteasa apoptótica
ARNm	Ácido ribonucleico mensajero
ASM	Mastocitosis sistémica agresiva
ATP	Adenosín trifosfato
Bcr	Gen de región de fractura
BMM	Mastocitosis aislada en médula ósea
Btk	Tirosina quinasa de Bruton
C	Cisteína
Ca^{2+}	Ión calcio
C-fms	Receptor para el factor estimulador de macrófagos
CM	Mastocitosis cutánea
CML	Leucemia mieloide crónica
CR	Receptores del complemento
CRH	Hormona liberadora de corticotropina
D	Aspartato
DAG	Diacilglicerol
DAGKζ	Diacilglicerol quinasa ζ
E	Glutamato
F	Fenilalanina
FA	Ácidos grasos
FCCP	Carbonil-cianida-p-trifluorometoxifenilhidrazona

FCεRI	Receptor de alta afinidad para IgE
FCγRI	Receptor de alta afinidad para IgG
FLD1	Fosfolipasa D1
FLD2	Fosfolipasa D2
Flk-2	Receptor tirosina quinasa de hígado fetal-2
Flt-3	Receptor tirosina quinasa-3 similar a fms
G	Glicina
GAP43	Proteína activadora de GTPasa 43
GM-CSF	Factor de crecimiento de granulocitos y monocitos
GnRH	Hormona liberadora de gonadotropinas
Grb2	Proteína de unión a receptores de factores de crecimiento-2
GRK2	Quinasa receptora de proteína G doble
GSK3β	Glicógeno sintasa quinasa-3β
GTPasa	Guanosina trifosfatasa
H	Histidina
HMC-1	Línea celular de mastocitos humanos-1
HRF	Factor liberador de histamina
I	Isoleucina
Ig	Inmunoglobulina
IκB	Inhibidor de kappaB
IL	Interleucina
IMDM	Medio Iscove modificado por Dulbecco
INCENP	Proteína interna del centrómero
INF	Interferón
IP_3	Inositol 1,4,5-trifosfato
ITAMs	Inmunoreceptores basados en tirosinas transmembrana
JAK2	Quinasa Janus 2

K	Lisina
KDa	Kilodalton
L	Leucina
M	Metionina
MAPK	Quinasa activada por mitógeno
MCAS	Síndrome de activación mastocitaria
MC_C	Subpoblación de mastocitos positiva a quimasa
MCL	Leucemia de mastocitos
MCP	Proteína quimioatrayente de monocitos
MCS	Sarcoma de mastocitos
MC_T	Subpoblación de mastocitos positiva a triptasa
MC_{TC}	Subpoblación de mastocitos positiva a triptasa y quimasa
µg	Microgramo
MIF	Factor inhibidor de la migración de macrófagos
MIRRs	Receptores multicadena de reconocimiento múltiple
mL	Mililitro
µm	Micrómetro
µM	Micromolar
MML	Leucemia mielomastocitaria
N	Asparagina
NF-κB	Factor nuclear potenciador de las cadenas ligeras κ de células B activadas
ng	Nanogramo
NGF	Factor de crecimiento nervioso
NK	Agresoras naturales
PA	Ácido fosfatídico
PAF	Factor activador de plaquetas
PARP	Poli-(ADP-ribosa) polimerasa

PC	Fosfatidilcolina
PCR	Reacción en cadena de la polimerasa
PDGF	Factor de crecimiento derivado de plaquetas
PDGFR	Receptor del factor de crecimiento derivado de plaquetas
PDK1	Quinasa-1 dependiente de fosfoinosítidos
pg	Picogramo
PGD_2	Prostaglandina D_2
pH_i	pH intracelular
PI	Fosfatidilinositol
PI3K	Quinasa de fosfatidilinositol-3
PICK1	Proteína de interacción con la quinasa C1
PIP_2	Fosfatidilinositol 4,5-bifosfato
PIP_3	Fosfatidilinositol 3,4,5-trifosfato
PKA	Proteína quinasa A
PKC	Proteína quinasa C
cPKC	Isoformas clásicas de la proteína quinasa C
nPKC	Isoformas nuevas de la proteína quinasa C
aPKC	Isoformas atípicas de la proteína quinasa C
PLC	Fosfolipasa C
PM	Peso molecular
PMA	Forbol 12-miristato 13-acetato
PP1	Proteína fosfatasa 1
PS	Fosfatidilserina
PTK	Proteína tirosina quinasa
RACK	Receptor de quinasa C activada
RANTES	Ligando quimiocina 5
S	Serina

SCF	Ligando del receptor de c-kit (*Stem cell factor*)
Sdr	Proteínas de repetición serina-aspartato
SERCA	ATPasa de Ca^{2+} del retículo endoplasmático
SH2	Dominio de homología src tipo 2
Shc	Proteína adaptadora con dominio de unión SH2
SHIP	Fosfatasa del inositol en posición 5' que contiene un dominio SH2
SM	Mastocitosis sistémica
SM-AHNMD	Mastocitosis sistémica asociada a otras enfermedades hematológicas clonales con células no-mastocitos
SM-AML	Mastocitosis sistémica con leucemia mieloide aguda
SM-CMML	Mastocitosis sistémica con leucemia mielomonocítica crónica
SM-HES	Mastocitosis sistémica con síndrome hipereosinofílico
SM-MDS	Mastocitosis sistémica con síndrome mielodisplásico
SM-MPD	Mastocitosis sistémica con enfermedad mieloproliferativa
SM-NHL	Mastocitosis sistémica con linfoma no Hodking
Src	Oncogén homólogo al gen src del virus de sarcoma aviar
STAT3	Transductor de señal y activador de transcripción 3
TGF	Factor transformador de tejidos
TNF	Factor de necrosis tumoral
TRAIL	Ligando inductor de la apoptosis relacionado con TNF
TyrK	Tirosina quinasa
V	Valina
VEGF	Factor de crecimiento del endotelio vascular
VHL	Proteína de Von Hippel-Lindau
VIH	Virus de inmunodeficiencia humana
VIP	Péptido intestinal vasoactivo
Y	Tirosina

YTX	Yesotoxina
ZIP3	Proteína de interacción con PKCζ

2. Introducción

2. 1. Origen de los mastocitos. Maduración y proliferación.

"Mast zellen", de este modo denominó por vez primera Paul Ehrlich (1854-1915) a los mastocitos en el año 1878. La característica más relevante de estas células fue la presencia de numerosos gránulos metacromáticos de color rojo y violeta tras una tinción con un colorante básico, la anilina, en el citosol de un tipo celular no descrito hasta aquel entonces [1]. De este modo, al creer que se trataban de gránulos que habían sido fagocitados por las células les adjudicó el término de "células cebadas".

Los mastocitos, al igual que las restantes células sanguíneas, se forman en la hematopoyesis [2]. En este proceso participan dos linajes celulares sanguíneos diferentes: linfoide y mieloide. Ambos linajes proceden de una única célula pluripotente. La línea linfoide está formada por tres tipos celulares diferentes responsables de la respuesta inmune específica: linfocitos T, linfocitos B y las células agresoras naturales (NK). Por otro lado la línea mieloide se caracteriza por presentar una mayor variabilidad en cuanto a morfología, fenotipo y funcionalidad [3]. El progenitor mieloide da lugar a los siguientes grupos celulares (esquema 1):

- Granulocitos: este grupo está integrado por neutrófilos, eosinófilos y basófilos. De todos ellos, los neutrófilos son los que presentan una mayor capacidad fagocítica. Los eosinófilos poseen un papel importante en la defensa específica frente a parásitos y en cuanto a los basófilos, estos carecen de función fagocítica y son esenciales en la hipersensibilidad inmediata liberando al medio mediadores inflamatorios.

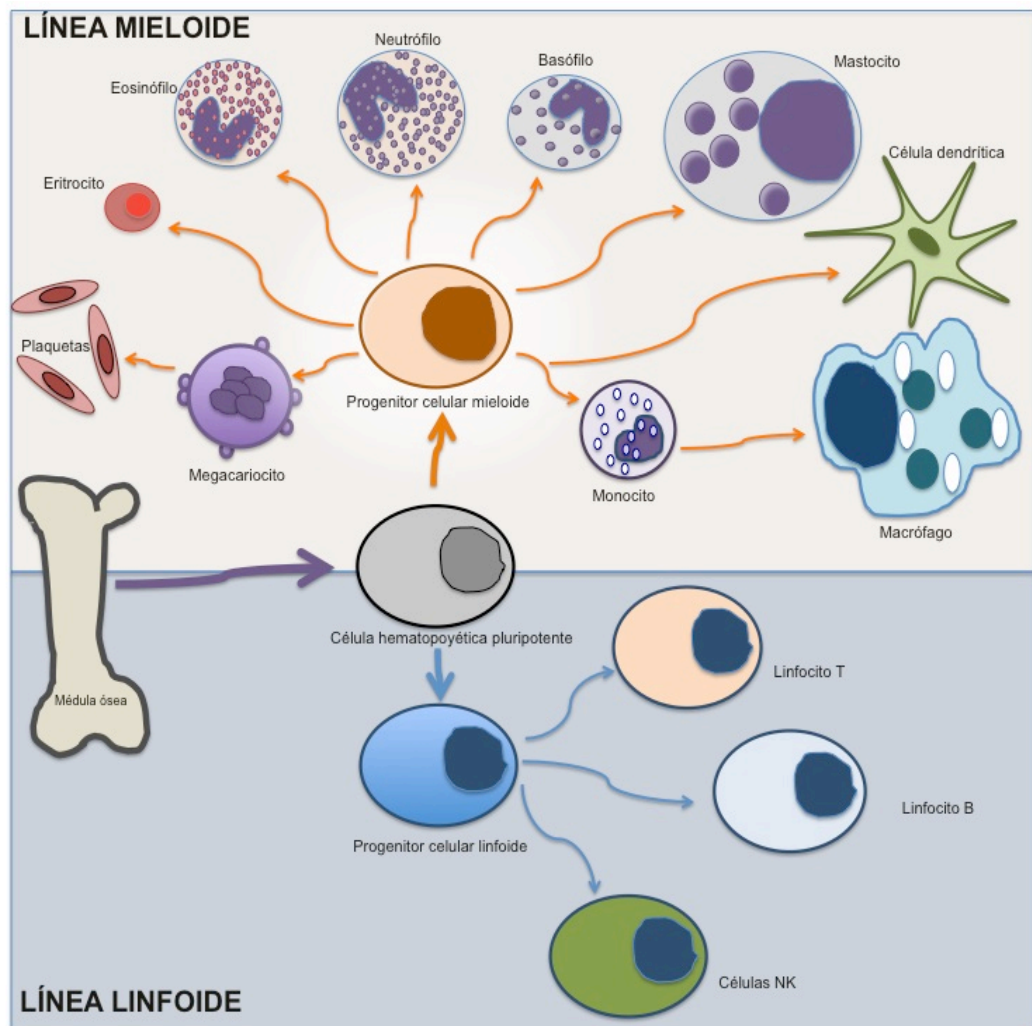
- Células dendríticas: no poseen una actividad fagocítica importante. Son las encargadas de presentar el material antigénico a células más especializadas, los linfocitos.

- Macrófagos: proceden de sus progenitores los monocitos. Pueden permanecer en los tejidos realizando funciones específicas o bien libres y estratégicamente situados para atrapar material extraño en órganos linfoides secundarios.

- Eritrocitos: son las células sanguíneas más abundantes, también denominados hematíes o glóbulos rojos. Carecen de núcleo y se encargan de transportar el oxígeno a los tejidos.

- Plaquetas: son células anucleadas formadas a partir de los megacariocitos. En su papel inmune son las encargadas de reparar el tejido endotelial lesionado y desencadenar la respuesta inmunitaria.

- Mastocitos: poseen un papel fundamental en las reacciones alérgicas, liberando al medio mediadores inflamatorios como la histamina, responsable principal de los síntomas alérgicos.



Esquema 1: Hematopoyesis. Formación de las distintas células sanguíneas.

Los mastocitos humanos proceden de una única célula progenitora común que expresa en su superficie los receptores antigénicos CD13, CD34 y el CD117 (c-kit) [4,5]. Las células progenitoras de los mastocitos se generan en la médula ósea y se dirigen al torrente sanguíneo como leucocitos agranulares mononucleares [6]. Todavía en este estado inmaduro son reclutadas hacia los tejidos debido a la presencia de citocinas secretadas por las células del estroma. Una vez en los tejidos la maduración de los mastocitos es estimulada por factores de crecimiento y citocinas presentes, dando lugar a una célula madura de aproximadamente 20 µm [7]. Los mastocitos se encuentran en las inmediaciones de vasos sanguíneos, nervios, piel y en la mucosa del sistema respiratorio e intestinal [8,9]. El proceso de proliferación y maduración mastocitaria es estimulado fundamentalmente por un factor de crecimiento que se une al receptor c-kit de la superfice celular. Este factor se denomina “stem cell factor” (SCF) o ligando del receptor c-kit y es producido por células del estroma, endoteliales, fibroblastos y queratinocitos [10,11]. Además, junto con la interleucina-3 (IL-3) son los dos factores de crecimiento mastocitarios más importantes [12]. El número de mastocitos en el interior de los tejidos se mantiene constante debido a un equilibrio entre proliferación y muerte celular programada o apoptosis [13,14]. En concreto, se ha comprobado que el SCF es capaz de bloquear la muerte celular por apoptosis debido a la ausencia de IL-3. Este rescate se debe exclusivamente al SCF y no a la acción de otras citocinas, lo que demuestra la alta dependencia de estas células por este factor de crecimiento [15]. El SCF es una citocina hematopoyética esencial para la supervivencia del organismo, tanto es así que la ausencia de este factor o bien de su receptor c-kit causan anemia macrocítica, lo que provoca muerte uterina o perinatal [16]. En cuanto a los factores que regulan negativamente el crecimiento de los mastocitos cabe destacar el interferón (IFN)-γ y la IL-5.

Tradicionalmente los mastocitos reciben el nombre de “células de la alergia”, puesto que están relacionadas con los síntomas de la respuesta inmune de tipo inmediata y se encuentran en los puntos de entrada de alérgenos, toxinas y microorganismos patógenos, tales como la dermis, el tracto respiratorio o la mucosa intestinal [17]. Además de en los procesos alérgicos, los mastocitos participan en el inicio, desarrollo, expresión y regulación de las respuestas asociadas a la inmunidad adquirida. El papel de los mastocitos en dichas respuestas está mediado por dos importantes receptores: el c-kit y el receptor de alta afinidad para inmunoglobulina (Ig) E (FCεRI) [18]. Las funciones en las que participan los

mastocitos son clasificadas en dos grandes grupos: con efecto protector o con efecto patológico (tabla 1). En el primer grupo de funciones destaca su papel en la angiogénesis, curación de heridas y formación de tejido mientras que poseen efectos patológicos entre los que se incluye su actividad en los procesos asmáticos o alérgicos y como promotores del crecimiento tumoral.

Efectos protectores	Efectos patológicos
Tolerancia inmunitaria	**Inflamación inducida por estrés**
Inmunidad frente a bacterias, virus y parásitos	**Asma y alergia**
Degradación de toxinas y homeostasis	**Desarrollo de enfermedades autoinmunes: artritis reumatoide y esclerosis múltiple**
Angiogénesis	**Reservorio de VIH**
Regeneración ósea	**Promotor del crecimiento tumoral**
Transporte iónico	**Enfermedad cardiovascular**
Crecimiento folículo piloso	
Curación de heridas y formación de tejido	

Tabla 1: Efectos protectores y patológicos de los mastocitos [9]. VIH: virus de inmunodeficiencia humana.

Las células eucariotas poseen la característica de liberar al exterior numerosas sustancias que pasan a formar parte de la matriz extracelular a través de un proceso de exocitosis constitutiva. Sin embargo, existe otra variante de exocitosis denominada regulada presente en células más especializadas, como son los mastocitos y las neuronas. El origen de este proceso radica en la presencia de un potente estímulo que desencadena la activación celular. A continuación, los gránulos situados en el citoplasma se dirigen hacia la membrana plasmática y una vez allí se fusionan a ella y liberan su contenido al exterior. A diferencia de las neuronas, los mastocitos tienen la capacidad de liberar el contenido de todos los

gránulos en un único acto. Por el contrario, el proceso de exocitosis en neuronas tiene lugar en varias fases separadas por un corto periodo de tiempo en el que se produce la síntesis de los mediadores. En el año 1991 fue descrita una variante de la exocitosis en mastocitos, denominada "exocitosis piecemal" [19]. Este tipo de exocitosis, causada por estímulos distintos a la convencional, se caracteriza por la pérdida progresiva del contenido de los gránulos citoplasmáticos sin aparente fusión con la membrana plasmática [20]. Posteriores estudios han descrito una íntima relación de los gránulos con la membrana plasmática, sin embargo no se ha observado ningún proceso de fusión [20].

2.2. Papel de los mastocitos en la respuesta inmune. Activación.

El sistema inmunitario es el encargado de reconocer sustancias ajenas o bien propias denominadas antígenos. Los antígenos, normalmente de naturaleza proteica, son reconocidos por anticuerpos desencadenando en la mayoría de los casos una respuesta inmune. El sistema inmunitario ha evolucionado con el individuo, de tal forma que le permite responder de manera eficaz a una amplia variedad de antígenos. En el mecanismo por el cual el sistema inmune protege al individuo participan dos componentes que colaboran mutuamente: la inmunidad innata, presente en el momento del nacimiento, y la inmunidad adquirida. Los mastocitos participan activamente tanto en la respuesta inmunitaria innata como adquirida [21]. La función que poseen los mastocitos en la repuesta inmune comenzó a describirse hacia 1931, año en el que Webb observó que las células peritoneales de rata daban lugar a una desgranulación tras el contacto con agentes como la clara de huevo (causante de una reacción semejante a la urticaria cuando se inyecta intraperitonealmente) [22]. Más tarde, en los años 1939 y 1953, se describió que los mastocitos liberaban los mediadores inflamatorios heparina e histamina [23,24].

A nivel inmunológico, los mastocitos se caracterizan por liberar mediadores inflamatorios al torrente sanguíneo tras su activación, condición indispensable para que se desencadene el proceso de desgranulación. Dicha activación tiene lugar tras la múltiple interacción de un antígeno específico a su receptor FCεRI, denominada

activación dependiente de IgE o de FCεRI, o bien ser desencadenada por la acción de neuropéptidos, opiáceos, compuestos básicos, péptidos o citocinas entre otros. Esta segunda vía de activación se denomina no dependiente de IgE [25]. El proceso de activación desencadena numerosos desórdenes caracterizados por la acumulación de mastocitos en uno o varios tejidos y también por la aparición de una gran variedad de mediadores inflamatorios producidos por esta línea celular [26].

Algunas respuestas inmunitarias son inadecuadas o excesivas, de ahí que reciban el nombre de reacciones de hipersensibilidad. Estas reacciones se clasifican en cuatro grupos:

- Grupo I: reacciones de hipersensibilidad inmediata de tipo I, caracterizadas por estar mediadas por los mastocitos. Están desencadenadas por la unión de un alérgeno (habitualmente polen o ácaros domésticos) a la IgE, provocando un entrecruzamiento de dichos anticuerpos, lo que conlleva a la activación del receptor FCεRI.

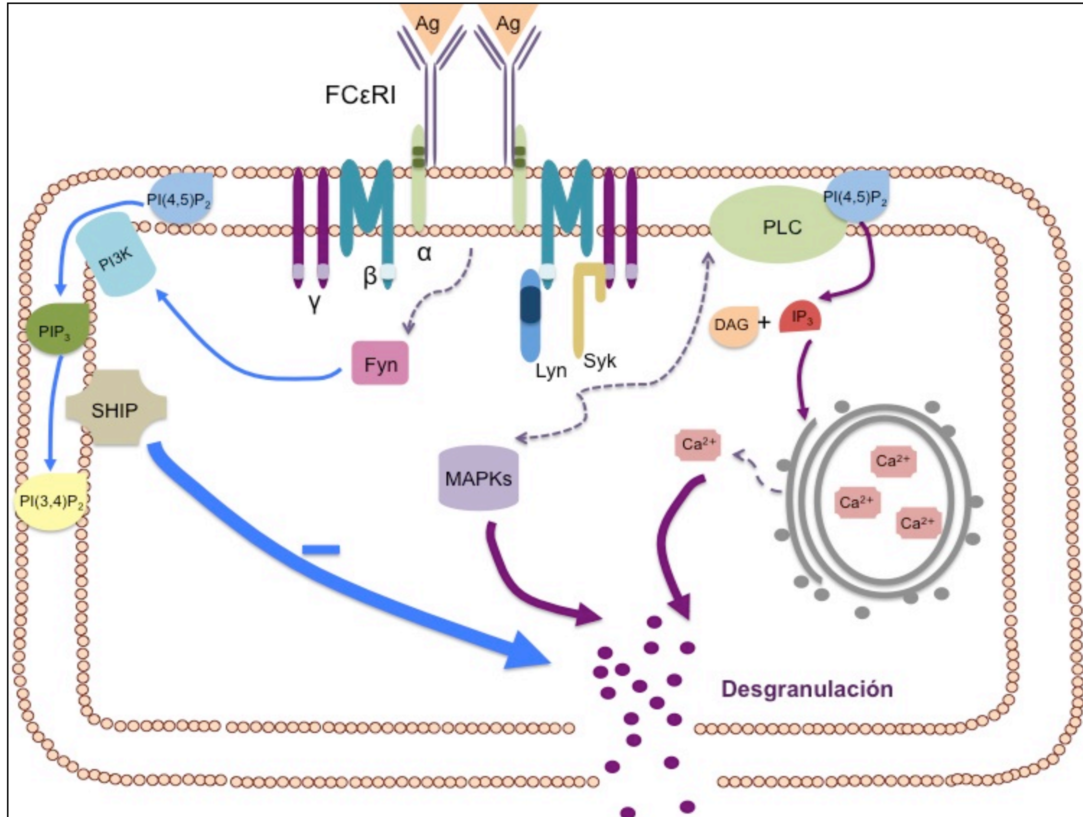
- Grupo II: también se denominan reacciones mediadas por anticuerpos (IgG o IgM) que pueden activar el sistema del complemento. El complemento, parte fundamental del sistema inmunitario, está compuesto por numerosas proteínas intracelulares que estimulan la respuesta inflamatoria, la fagocitosis y la muerte celular programada.

- Grupo III: se caracteriza por la formación de inmunocomplejos en circulación. Tiene lugar en enfermedades como el lupus eritematoso sistémico o la glomerulonefritis crónica.

- Grupo IV: en este caso las células efectoras primarias son las células T, encargadas de activar posteriormente a los macrófagos, dando lugar a una desgranulación. Uno de los casos en los que aparece esta reacción es en el rechazo a injertos.

2.2.1. Activación dependiente de IgE.

El receptor de superficie celular FCεRI no es exclusivo de los mastocitos, pues se encuentra también en otros tipos celulares, tales como basófilos, células de Langerhans y monocitos activados [25]. La agregación del receptor FCεRI tras su exposición a un antígeno provoca la activación de los mastocitos, fundamento del proceso anafiláctico así como de otras enfermedades alérgicas [7]. La densidad del receptor FCεRI sobre la superficie celular está regulada por los niveles de IgE así como por la IL-4 [7]. El FCεRI pertenece a la familia de receptores multicadena de reconocimiento múltiple (MIRRs) y se caracteriza por poseer una cadena extracelular α, una cadena β y dos cadenas γ, siendo estas dos últimas subunidades las que poseen un mayor papel en las señales de transducción [27,28]. El lugar de anclaje de la IgE es la subunidad α, sin embargo esta unión no conlleva la activación de la célula, puesto que para que la desgranulación tenga lugar es necesario que varios receptores FCεRI se unan a IgE, produciéndose a continuación la agregación de estos receptores [25,29]. La activación de la proteína Lyn, perteneciente a la familia del gen homólogo al virus de sarcoma aviar (Scr) de proteínas tirosina quinasas (PTKs) y asociada a la subunidad β del receptor, tiene lugar como consecuencia de esta agregación. Lyn es la encargada de fosforilar los residuos de tirosina situados en los inmunoreceptores basados en tirosinas transmembrana (ITAMs), localizados en la región citoplasmática de las subunidades β y γ [25,30]. Una vez activado el receptor, se activa también la proteína Syk (otra PTK) por parte de la subunidad γ. En la activación de mastocitos dependiente de IgE tienen un papel fundamental las PTKs, participando en numerosas señales intracelulares relacionadas con el proceso de desgranulación celular, tales como la ruta de la fosfolipasa C (PLC) /calcio (Ca^{2+}), o la de las quinasas activadas por mitógeno (MAPKs). El receptor FCεRI activa una tercera PTK denominada Fyn, responsable de la activación de la quinasa de fosfatidilinositol-3 (PI3K), dando lugar a la producción de fosfatidilinositol 3,4,5-trifosfato (PIP_3) [31]. A su vez, el PIP_3 es hidrolizado por la fosfatasa del inositol en posición 5’ que contiene un dominio de homología src tipo 2 (SH2) denominada SHIP, encargada de inhibir la desgranulación bloqueando la actividad de la PLC (esquema 2) [30,32].



Esquema 2: Receptor FCεRI y señales de transducción desencadenadas tras su activación [33,34].

2.2.2. Activación no dependiente de IgE.

La activación de los mastocitos no solo se desencadena por la unión de anticuerpos IgE a la superficie celular, puesto que la célula puede ser activada de forma IgE-independente. La presencia de esta otra vía alternativa de activación se describió tras observar síntomas anafilácticos en ratones deficientes para IgE. En este caso participan otro tipo de receptores, tales como el c-kit, receptores tipo Toll, receptores de adenosina, receptores del complemento (CR), receptores CD200, proteínas G y los receptores de alta afinidad para IgG (FCγRI) [29,35,36]. Este último receptor FCγRI se une al complejo formado por IgG-antígeno, a diferencia

del receptor para IgE (FCεRI), que se une primero al anticuerpo y el antígeno es reconocido posteriormente [9].

En el caso del receptor c-kit, una vez activado tras su unión al SCF, participa en numerosas señales de transducción (esquema 3). Estas señales pueden dividirse en cinco grandes bloques atendiendo a las proteínas implicadas y al efecto final producido:

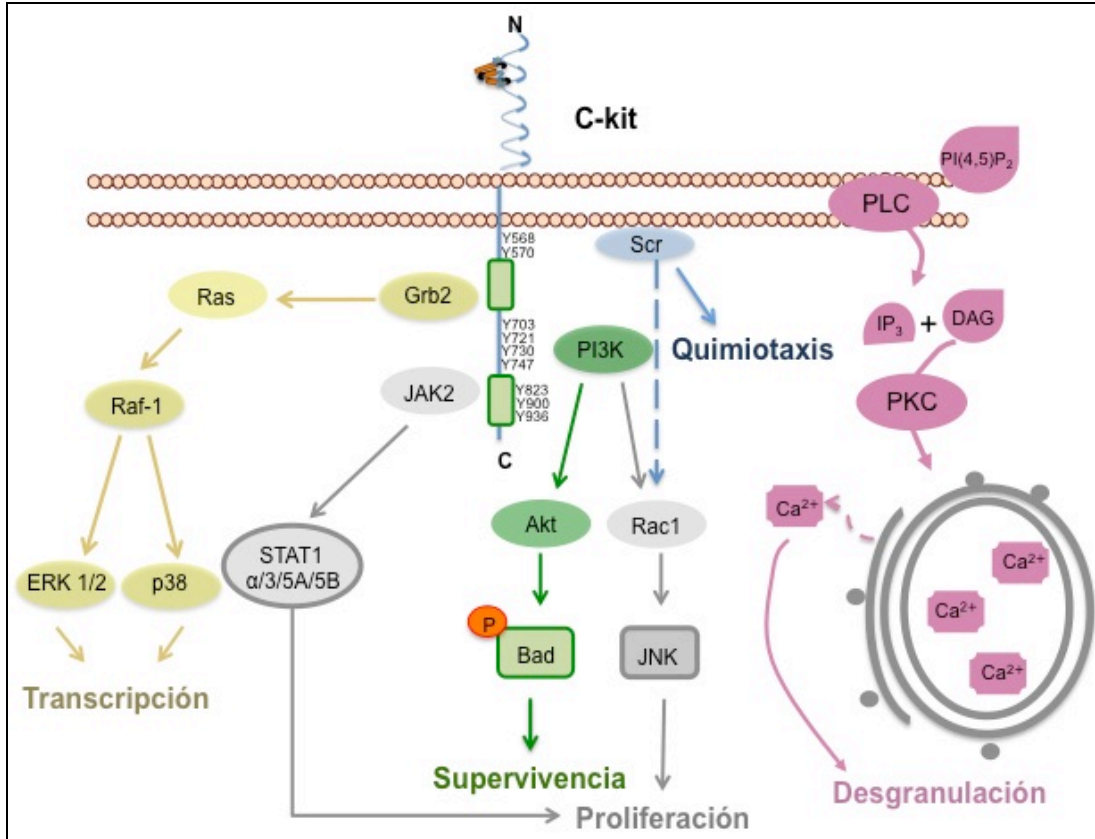
Ruta de la adhesión celular y desgranulación: en ella participa la proteína PLCγ y la proteína quinasa C (PKC), activada a su vez por el diacilglicerol (DAG). La PKC provoca la salida de Ca^{2+} de los reservorios intracelulares, estímulo para la desgranulación celular.

Ruta de la proliferación celular: se produce a través de la activación de dos proteínas, la proteína quinasa Janus 2 (JAK2), encargada de fosforilar los transductores de señales y activadores de transcripción (STAT 1, α, 3, 5A y 5B). La otra proteína implicada es la PI3K, encargada de activar a la proteína Rac1 y a la JNK.

Ruta de la supervivencia celular: tras la activación de la PI3K se activa la proteína Akt, lo que conlleva la fosforilación del factor proapoptótico Bad. Esta fosforilación implica el bloqueo de Bad, promoviendo la supervivencia celular [37].

Ruta de la transcripción génica: la proteína de unión a factores de crecimiento 2 (Grb2), tras fosforilar los residuos Y703 e Y936 del receptor c-kit activa la proteína G Ras. A continuación tiene lugar la activación de la proteína Raf-1, ERK-1/2 y la MAPK p38, encargadas de la transcripción génica.

Ruta de la quimiotaxis: la proteína Src es la encargada de fosforilar los residuos Y568 e Y570 del receptor c-kit. Además de la quimiotaxis la Src estimula la ruta de la proliferación celular a través de la proteína Rac1.



Esquema 3: Rutas de transducción activadas por el c-kit [38].

En la activación no dependiente de IgE los compuestos básicos poseen un papel fundamental ya que tienen capacidad de estimularla y provocar la liberación de mediadores inflamatorios [39-41]. Los compuestos que inducen exocitosis no dependiente de FCεRI se clasifican en los siguientes grupos: compuestos básicos, péptidos, citocinas, anafilatoxinas, dextranos y lectinas [25].

- Compuestos básicos: el compuesto 48/80, el mastoparan y la polimixina B son compuestos básicos que tienen la capacidad de inducir liberación de histamina en los mastocitos, a través de la interacción con proteínas G [42]. El mastoparan fue el primero en ser caracterizado como activador de las proteínas G en el año 1988, dos años más tarde, el mismo efecto fue descrito para el compuesto 48/80 [43].

- Péptidos: los péptidos que estimulan la exocitosis pueden dividirse en tres grupos: péptidos con parte del dominio C_H4 de IgE (por ejemplo la hormona adrenocorticotropa), neuropéptidos y péptidos Rab3A. En mastocitos humanos, el grupo que presenta una mayor actividad es el de los neuropéptidos, entre los que destacan la sustancia P, el factor de crecimiento nervioso (NGF) y el péptido intestinal vasoactivo (VIP) [44,45].

- Citocinas: la capacidad de inducir desgranulación es una característica presente en varios tipos de citocinas tales como el anteriormente citado SCF, así como la IL-3, IL-1 y el factor de crecimiento de granulocitos y monocitos (GM-CSF) [46,47]. Debido al papel que juegan en la exocitosis, a este grupo se los denominó factores liberadores de histamina (HRFs) [25].

- Anafilatoxinas: reciben esta denominación los fragmentos del complemento C3a, C4a y C5a (procedentes de las fracciones C'3, C'4 y C'5 respectivamente). Estimulan la desgranulación en mastocitos potenciando el proceso anafiláctico, de ahí su nombre [48].

- Dextranos y lectinas: los dextranos son compuestos formados por múltiples moléculas de glucosa. Esta característica explica que la ruta de actuación para estimular la exocitosis tiene lugar a través de los receptores de glucosa situados en la membrana [25,49]. Por el contario, las lectinas producen una activación de los mastocitos dependiente de IgE [50,51].

2.3. Mediadores inflamatorios.

Tras su activación, los gránulos presentes en el citoplasma se fusionan con la membrana plasmática liberando su contenido hacia el exterior celular. Los mastocitos producen numerosos mediadores inflamatorios, dando lugar a un grupo de gran heterogeneicidad en cuanto a potencia y actividad biológica se refiere [25]. Los mediadores inflamatorios se caracterizan por poseer más de una función, a veces compartida por varios mediadores y se clasifican en dos grupos: mediadores asociados a los gránulos y mediadores sintetizados *de novo* [52]. Sin embargo en

esta clasificación no se consideran algunas excepciones como es el caso del factor de necrosis tumoral (TNF) α, que aunque es una citocina puede encontrarse almacenado en los gránulos o bien ser sintetizado *de novo* [53].

2.3.1. Mediadores asociados a los gránulos.

Dichos mediadores se liberan al torrente sanguíneo en un tiempo muy corto tras la activación celular. Entre ellos se encuentran la histamina, las proteasas neutras, los proteoglicanos y la carboxipeptidasa A [53].

- Histamina: es una amina generada a partir del aminoácido histidina, cuya reacción está catalizada por la enzima histidina descarboxilasa. El contenido en histamina en mastocitos presentes en pulmón, piel, tejido linfoide e intestino delgado está en torno a 3-8 pg/célula [54,55]. La histamina tiene una vida media en el exterior celular muy corta, aproximadamente 1 minuto, siendo degradada por la histamina N-metiltransferasa [7]. Este mediador posee un papel fundamental en el proceso anafiláctico provocando un aumento de la secreción mucosa. Otros efectos de la histamina son la contracción del músculo liso, vasodilatación e incremento de la excitabilidad de las neuronas del sistema nervioso central y aumento de la permeabilidad vascular [53]. Hay cuatro tipos distintos de receptores de histamina en el organismo, el H_1, H_2, H_3 y el H_4. En cuanto a su distribución, los H_1 y H_2, se encuentran dispersados por todo el organismo mientras que el H_3 está localizado en el células del cerebro y por último el H_4 en células hematopoyéticas e intestinales [56,57]. Además de las diferencias en cuanto a su distribución, los receptores histaminérgicos poseen mediadores intracelulares diferentes (tabla 2). Tras la activación de los receptores H_1 se producen cambios en los niveles de Ca^{2+} intracelulares, mientras que la activación de los H_2 causa un incremento en los niveles de adenosín monofosfato cíclico (AMPc) y la de los H_3 y H_4 modifica los niveles de AMPc y Ca^{2+}.

TIPO DE RECEPTOR	SEGUNDO MENSAJERO
H_1	Ca^{2+}
H_2	AMPc
H_3	AMPc /Ca^{2+}
H_4	Ca^{2+}

Tabla 2: Tipos de receptores de histamina y segundo mensajero asociado a cada tipo [58].

Otra amina liberada por los mastocitos humanos es la serotonina, aunque en mucha menor cantidad que en mastocitos de rata o ratón [59]. La serotonina, entre otras muchas funciones, está relacionada con el crecimiento celular, adhesión, quimiotaxis, tumorigénesis y regeneración tisular [59-61].

- Proteoglicanos: los proteoglicanos están formados por un núcleo central proteico rico en aminoácidos glicina y serina, unidos covalentemente a un tipo de polisacáridos, denominados glucosaminoglicanos. Una característica importante de este grupo es la presencia de grupos sulfato y ácido urónico en su estructura, permitiéndole formar parte de la matriz fibrosa junto con el colágeno [25]. Los proteoglicanos asociados a los mastocitos son la heparina y el E condroitín sulfato [62,63]. La heparina posee una importante acción anticoagulante y ambos mediadores son asimilados por células como los fibroblastos, células endoteliales y por los integrantes de la matriz de tejido conectivo [64,65]. El contenido en heparina de los mastocitos es de 2,4-7,8 μg/10^6 células [62]. Por lo tanto, el alto contenido en heparina de los gránulos mastocíticos, junto con el hecho de que sea una molécula cargada negativamente, explica la predilección de estas células por las tinciones catiónicas [21]. De este modo, su carga negativa les permite formar complejos junto con la histamina, proteasa neutras y otros mediadores [7]. Además existe también una gran diversidad en cuanto a la proporción heparina/E condroitín sulfato en el interior celular. Mientras que en mastocitos de origen pulmonar el contenido de ambos mediadores es semejante, los de la mucosa gástrica poseen una mayor cantidad de E

condroitín sulfato, lo que conlleva a una diferencia de tinción entre las distintas poblaciones [66].

- Proteasas neutras: Se caracterizan por poseer su capacidad proteolítica a pH neutro [25]. Las proteasas neutras triptasa, quimasa, carboxipeptidasa A y catepsina G componen el grupo más abundante de mediadores inflamatorios alojados en el interior de los gránulos [21]. La importancia de estas proteasas radica en su función en la degradación de fibrinógeno, proteínas de la matriz extracelular o péptidos y en el proceso de broncoconstricción [21]. El contenido en proteasas neutras difiere entre los mastocitos atendiendo a su localización en el organismo, de tal forma que se han definido varias subpoblaciones atendiendo a este criterio. Los gránulos intracelulares contienen numerosos mediadores inflamatorios que comparten junto con otros tipos celulares. Sin embargo, las proteasas neutras, triptasa y quimasa se alojan única y exclusivamente en el interior de los mastocitos. Esto convierte a estos mediadores en un marcador útil para diferenciar las distintas subpoblaciones celulares [25]. Un estudio realizado en el año 1989, en el que se emplearon anticuerpos frente a ambas proteínas, triptasa y quimasa, definió dos subpoblaciones distintas de mastocitos: (1) MC_T; caracterizados por poseer únicamente triptasa y (2) MC_{TC}; con contenido en triptasa y quimasa [67,68]. Además de diferenciarse por su contenido en proteasas neutras, las dos subpoblaciones se caracterizan por poseer diferente localización. Por un lado, los MC_T se encuentran en el tejido alveolar y mucosa del intestino delgado, mientras que las MC_{TC} están presentes en la piel y en la submucosa del intestino delgado. Otras localizaciones tales como la mucosa nasal y tonsilas tienen un contenido semejante de ambas subpoblaciones [25]. Otro estudio definió una nueva población: MC_C, con contenido en quimasa y carboxipeptidasa y negativas a triptasa [69]. Más recientemente, se ha descubierto que el marcaje de triptasa identifica todos los mastocitos, por lo que es la técnica empleada para detectar estas células en tejidos [21]. Sin embargo, en un estudio posterior se ha demostrado que todos los mastocitos son capaces de producir quimasa, por tanto esto implica que no se trata de una proteasa específica de las subpoblaciones MC_{TC} y MC_C [70]. En definitiva, la controversia en cuanto a la heterogeneidad de los mastocitos está vigente y

la incógnita en cuanto a número y características de las diferentes subpoblaciones sigue de momento sin resolver.

- Quimiocinas: las quimiocinas son mediadores inflamatorios con función quimiotáctica. Estas se caracterizan porque una vez liberadas quedan ancladas en la matriz extracelular, formando un gradiente sólido que sostiene el transporte de leucocitos. El compuesto más importante de este grupo es la IL-8.

2.3.2. Mediadores sintetizados *de novo*.

Este grupo de mediadores, al no estar sintetizados previamente, como es el caso de los mediadores asociados a los gránulos, tardan más en liberarse a la circulación sanguínea. Uno de los grupos más importantes es el de los derivados del ácido araquidónido. Tras su activación, los mastocitos comienzan a sintetizar mediadores procedentes del metabolismo de este ácido [25]. El ácido araquidónico puede ser metabolizado por la enzima ciclooxigenasa, lipooxigenasa o bien epoxigenasa, dando lugar a tres grupos de compuestos distintos.

Los productos obtenidos tras la reacción catalizada por la ciclooxigenasa son las prostagladinas, prostaciclinas y tromboxanos. Dentro de este grupo destaca un mediador: la prostaglandina D_2 (PGD_2). La PGD_2 produce hipotensión sistémica, síntoma que coexiste junto con un aumento de PGD_2 en la orina de pacientes con mastocitosis [25]. Además, tiene la capacidad de inducir liberación de histamina inducida por varios factores como por ejemplo antígenos. La PGD_2 posee además muchas otras funciones como la de inhibir la agregación plaquetaria [71].

En cuando a los derivados del ácido araquidónico tras la reacción mediada por la lipooxigenasa caben destacar dos grupos: leucotrienos y lipoxinas. Los leucotrienos poseen funciones muy importantes a nivel de las vías respiratorias, ya que estimulan la broncoconstricción (son entre 10-1.000 veces más potentes que la histamina), incrementan la producción de secreción bronquial, aumentan la permeabilidad vascular e inducen constricción arterial [72]. Los mastocitos son el mayor productor de un tipo concreto de leucotrieno: el leucotrieno B4, descrito

como mediador fundamental en el proceso del asma [73,74]. En concreto, el leucotrieno B4 actúa como atrayente de varios tipos celulares a nivel pulmonar: neutrófilos, macrófagos, monocitos y eosinófilos [75].

Además de los compuestos derivados del ácido araquidónico, los mastocitos liberan otros mediadores de origen lipídico como es el factor activador de plaquetas (PAF), producido también por otros tipos celulares [76]. La síntesis de este mediador puede tener lugar a través de dos rutas, dependiendo de la disponibilidad de sustratos: "*de novo*" o "*de remodelación*" [76]. El PAF es liberado al torrente sanguíneo dando lugar a la agregación y desgranulación plaquetaria, liberando mediadores como la serotonina o la histamina [25]. Una de sus principales características es la de producir hipotensión, al igual que la PGD_2 [77]. Además, este mediador está íntimamente ligado con el proceso anafiláctico [76-80]. Tanto es así que la cantidad de PAF está directamente relacionada con la severidad de la anafilaxis, al igual que otros mediadores como la histamina o la triptasa [81]. Junto con la anafilaxis, el PAF provoca un aumento de la permeabilidad vascular, mostrando una potencia entre 1.000 y 10.000 veces superior a la histamina [82].

Las citocinas son mediadores de bajo peso molecular (PM), entre 15-30 KDa, originariamente descritas en leucocitos y que sirven para enviar numerosas señales de comunicación, de ahí que se les otorgara el nombre de ILs. Más tarde, al comprobarse que eran producidas por diversos tipos celulares recibieron el nombre de citocinas. Algunas de ellas fueron descubieras *in vitro* durante estudios funcionales, por lo que aún conservan su nombre original, tal es el caso del TNF o del factor transformador de tejidos (TGF). La citocina producida en mayor cantidad por los mastocitos es el TNFα, el cual participa en la adhesión de moléculas epiteliales y tiene efecto antitumoral [7]. Otras citocinas procedentes de este tipo de células son la IL-4 (producida en menor medida que en basófilos) [83], IL-3, IL-5, IL-6, IL-9, IL-10, IL-13 y el GM-CSF, participando entre otras funciones en el desarrollo eosinofílico y supervivencia celular [7,21]. Cabe destacar el papel de la IL-5 ya que promueve la proliferación mastocitaria en presencia de SCF [7]. Aunque la activación vía IgE es la más frecuente en mastocitos, la activación de otro tipo de receptores, como los tipo Toll, por parte de productos bacterianos, desencadena la liberación de otras citocinas, como es el caso del IFNα [21].

Por tanto, los mastocitos son una fuente importante de mediadores inflamatorios, ya sean almacenados, entre los que destacan la histamina, la serotonina, la triptasa, la PGD_2 y el PAF o bien sintetizados *de novo* como las citocinas y los mediadores derivados del metabolismo de fosfolípidos (tabla 3).

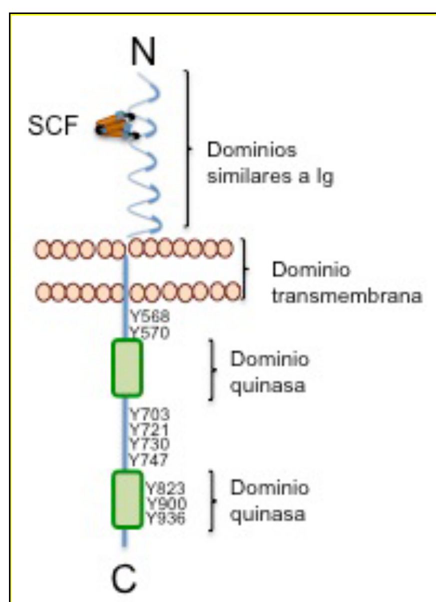
Mediadores asociados a los gránulos				
Aminas biógenas: Histamina y serotonina.	Polipéptidos: CRH, endorfinas, sustancia P, somatostatina y péptido intestinal vasoactivo.	Quimiocinas: IL-8, MCP-3, MCP-4 y RANTES.	Enzimas: Carboxipeptidasa A, pro-caspasas 3 y 4, β-hexosaminidasa, fosfolipasa y triptasa	Proteoglicanos: Condroitín sulfato, heparina y acido hialurónico

Mediadores sintetizados *de novo*			
Citocinas: ILs-3,4,5,6,9,10,13 INF-γ, TNF-α y MIF	Factores de crecimiento: SCF, GM-CSF, GnRH-I y NGF	Metabolitos de los fosfolípidos: Leucotrieno B_4, leucotrienoT_4, PAF, PGD_2 y VEGF	Óxido nítrico

Tabla 3: Clasificación de los mediadores inflamatorios [52]. Proteína quimioatrayente de monocitos (MCP), ligando quimiocina 5 (RANTES), hormona liberadora de corticotropina (CRH), factor inhibidor de la migración de macrófagos (MIF), hormona liberadora de gonadotropinas (GnRH) y factor de crecimiento del entotelio vascular (VEGF).

2.4. El receptor c-kit y su ligando SCF.

El c-kit (CD117) es un receptor de tirosina quinasa tipo III, grupo al que pertenecen otros receptores como el receptor del factor de crecimiento derivado de plaquetas (PDGFR), el receptor para el factor estimulador de macrófagos (c-fms), el receptor tirosina quinasa de hígado fetal-2 (Flk-2) y el receptor tirosina quinasa-3 similar a fms (Flt-3) [84,85]. El receptor c-kit no se encuentra exclusivamente en la superficie de los mastocitos, sino que también está presente en otros tipos celulares como progenitores hematopoyéticos, melanocitos, células germinales, células NK y células intersticiales Cajal [86,87]. Sin embargo, la presencia del receptor c-kit en la superficie celular de estas células disminuye drásticamente en el proceso de maduración, a excepción de los mastocitos, en donde los niveles de expresión siguen siendo elevados en células maduras [21]. El gen que codifica la información para este receptor se encuentra en el cromosoma 4 en humanos (q11-12). En cuanto a su estructura, estos receptores se caracterizan por tener un dominio extracelular con cinco regiones de unión a su ligando, una región transmembrana y finalmente una región citoplasmática con actividad tirosina quinasa (esquema 4). Esta última región citoplasmática está a su vez dividida en dos dominios: el de unión al trifosfato de adenosina (ATP) y el dominio fosfotransferasa [16]. En su conjunto, todas estas regiones otorgan al c-kit un PM de 145 KDa.



Esquema 4: Estructura del receptor c-kit [16].

El SCF presenta dos formas: soluble y transmembrana, ambas biológicamente activas [88]. La existencia de este factor permite la diferenciación de mastocitos a partir de células de la médula ósea $CD34^{+}$, células mononucleares de la sangre periférica o células de hígado fetal [89-91]. Además de promover la proliferación y maduración de los mastocitos, el SCF estimula la función secretora de estas células, la quimiotaxis y la adhesión celular [92-95]. La unión del receptor c-kit a su ligando SCF, presente en la circulación sanguínea a una concentración de 3.3 ng/mL, desencadena la homodimerización y fosforilación del mismo [96]. Concretamente, la quinasa PI3K y la MAPK son las dos proteínas implicadas en el proceso de fosforilación del c-kit [88]. La aparición de una o varias mutaciones en este receptor implica la presencia de numerosas variantes del mismo. En concreto, dichas mutaciones tienen lugar en la región citoplasmática y han sido descritas en mastocitos procedentes de pacientes con mastocitosis, definida como la proliferación incontrolada de mastocitos clonales de manera SCF-independiente. La substitución del aminoácido aspartato por valina en el codón 816, situado en el exón 17, es la mutación más frecuente en pacientes con mastocitosis sistémica. Dicha mutación, Asp-816->Val, está asociada a la fosforilación permanente del receptor c-kit, y por consiguiente, a su permanente estado de activación [88]. Aún así, se ha comprobado que la estimulación con SCF incrementa el nivel basal de fosforilación del c-kit en las células que presentan esta mutación [88]. Los mastocitos que presentan el c-kit permanentemente activado siguen dependiendo de la proteína PI3K para su activación, al igual que en células donde el c-kit no está mutado. De esta forma, se ha observado que el tratamiento con Ly 294002, inhibidor de la PI3K, provoca apoptosis e inhibe la proliferación de las células con las mutaciones en el codón 816 y también en la posición 560 [88]. Los primeros estudios realizados situaban la mutación Asp-816->Val en los pacientes con mastocitosis de edad adulta [97], mientras que su frecuencia en pacientes pediátricos era muy baja. Sin embargo, en estudios recientes se ha observado que la mutación Asp-816->Val, al contrario de lo que se pensaba, tiene una alta prevalencia (36%) en niños con mastocitosis [98].

A pesar de que la mutación Asp-816->Val es la más frecuente, existen otras múltiples mutaciones presentes en este receptor (tabla 4).

Mutación	Cambios en la secuencia de aminoácidos	Referencia
D52N	Aspartato -> Asparagina	[97]
Δ417-419insY	Substitución de treonina y aspartato por tirosina	[98]
Δ419	Eliminación de aspartato	[98]
InsFF419	Inserción de dos aminoácidos fenilalanina	[98]
K509I	Lisina -> Isoleucina	[99]
F522C	Fenilalanina -> Cisteína	[99]
V530I	Valina -> Isoleucina	[97]
V560G	Valina -> Glicina	[99]
D816Y	Aspartato -> Tirosina	[98]
D816I	Aspartato -> Isoleucina	[98]
D816F	Aspartato -> Fenilalanina	[99]
D816H	Aspartato -> Histidina	[99]
D820G	Aspartato -> Glicina	[99]
V825A	Valina -> Alanina	[97]
E839K	Glutamato -> Lisina	[97]

Tabla 4: Mutaciones presentes en el receptor c-kit [97-99].

Las mutaciones que se describen en la tabla 4, al igual que la Asp-816->Val, causan la activación permanente del receptor [98]. Hay que destacar que de todas las mutaciones presentes en el c-kit solo la mutación Asp-816->Val posee una alta frecuencia (80%) en pacientes con mastocitosis sistémica, mientras que para el resto de mutaciones la frecuencia es del 5%.

2.5. Mastocitosis.

En el término mastocitosis se integran una serie de patologías caracterizadas por un crecimiento incontrolado de mastocitos clonales de manera indenpendiente del SCF y su consiguiente expansión y acumulación en la piel y/o órganos viscerales [99-106]. La mastocitosis se considera una enfermedad no hereditaria y rara [99,107], con una prevalencia del 0,005-0,01%, si bien es cierto que en las últimas dos décadas ha aumentado la incidencia de esta dolencia, debido en parte a las mejoras en el diagnóstico.

La primera clasificación de las mastocitosis data del año 1949, tras el diagnóstico del primer caso de afección de un órgano interno ya que hasta entonces esta enfermedad era reconocida como cutánea y se denominaba urticaria pigmentosa [108]. Actualmente las mastocitosis se clasifican dependiendo de la localización de las lesiones en mastocitosis cutáneas (CM), frecuentes en niños y con buen pronóstico, mastocitosis sistémicas (SM), frecuentes en adultos y con peor pronóstico y tumores mastocitarios localizados [99,109].

Mastocitosis cutánea: el criterio de diagnóstico de este grupo es la presencia de lesiones macroscópicas en la piel y la ausencia de criterios suficientes para el diagnóstico de SM [99]. Los diferentes tipos de CM son:

- Urticaria pigmentosa: es la forma más frecuente de mastocitosis cutánea. Aparece en niños de corta edad.
- Mastocitosis cutánea difusa.
- Mastocitoma en piel.

Mastocitosis sistémica: la presencia de mastocitos clonales en uno o varios órganos viscerales se considera criterio mayor para el diagnóstico de esta enfermedad. De esta forma para el diagnóstico definitivo de la misma debe cumplirse este criterio mayor y uno de los cuatro criterios menores, o bien la coexistencia de al menos tres criterios menores. Los criterios considerados menores son:

1. Presencia de mastocitos morfológicamente anormales.

2. Expresión de los receptores CD2 y CD25.
3. Presencia de la mutación Asp-816->Val en el c-kit.
4. Niveles superiores a 20 ng/mL de triptasa en suero. Es importante medir los niveles de triptasa al menos 48 horas después del periodo de anafilaxis para tener una medida fiable [99,110].

Dentro de este grupo de SM están presentes varios tipos:

- Mastocitosis sistémica indolente.
  - Mastocitosis aislada en médula ósea (BMM).
- Mastocitosis sistémica latente.
- Mastocitosis sistémica asociada a otras enfermedades hematológicas clonales con células no-mastocitos (SM-AHNMD).
  - Mastocitosis sistémica con leucemia mieloide aguda (SM-AML).
  - Mastocitosis sistémica con síndrome mielodisplásico (SM-MDS).
  - Mastocitosis sistémica con enfermedad mieloproliferativa (SM-MPD).
  - Mastocitosis sistémica con leucemia mielomonocítica crónica (SM-CMML).
  - Mastocitosis sistémica con linfoma no Hodgkin (SM-NHL).
  - Mastocitosis sistémica con síndrome hipereosinofílico (SM-HES).
- Mastocitosis sistémica agresiva (ASM).
  - Mastocitosis sistémica linfadenopática con eosinofilia.

El tercer y último grupo de mastocitosis son los tumores mastocitarios localizados, entre los que se encuentran:

- Leucemia de mastocitos (MCL).
- Sarcoma de mastocitos (MCS).
- Mastocitoma extracutáneo.
- Leucemia mielomastocitaria (MML).

La presencia de mutaciones que activan constitutivamente el receptor c-kit es muy frecuente en pacientes con mastocitosis. Dentro del gran número de

mutaciones descritas en este receptor, la Asp-816->Val es la de mayor prevalencia en los pacientes con SM, incluso en la variante indolente y en niños con CM [97-99,107,111]. Para la detección de esta mutación se extrae una muestra de sangre periférica y a continuación se determina su presencia por reacción en cadena de la polimerasa (PCR) [99]. En la mayoría de los pacientes se biopsia la médula ósea para el aislamiento de mastocitos siendo los marcadores usados con mayor frecuencia el receptor c-kit, los receptores CD2 y/o CD25 y los niveles séricos de triptasa [99,112].

Además de la CM, SM y los tumores mastocitarios localizados hay otras dos patologías que se incluyen dentro del grupo de las mastocitosis, como son el síndrome de activación mastocitaria (MCAS) y la hiperplasia mastocitaria. El MCAS tiene lugar en procesos alérgicos y otras reacciones inflamatorias así como en la mastocitosis. El término de MCAS se aplica cuando coexisten estos tres criterios [113]:

- Presencia de signos clínicos de manera recurrente: urticaria, rubor, prurito, angioedema, congestión nasal, prurito nasal, sibilancias, dolor de cabeza, hipotensión y diarrea.
- Incremento de mediadores derivados de mastocitos en suero: triptasa (20% de incremento), histamina, PGD_2 y metabolitos urinarios.
- Respuesta al tratamiento con bloqueantes de los receptores de histamina o agentes cuya diana sean los mastocitos (por ejemplo el cromoglicato).

El MCAS se clasifica en tres grupos diferentes [113]:

- Primario: es el MCAS en el que deben cumplirse todos los criterios de diagnóstico de la activación mastocitaria y los mastocitos son monoclonales.
- Secundario: se cumplen todos los criterios de activación mastocitaria junto con el diagnóstico de alergias u otro trastorno subyacente y no aparecen mastocitos clonales.
- Idiopático: los criterios de activación mastocitaria se cumplen pero no se encuentra una enfermedad subyacente. Tampoco aparecen mastocitos clonales.

Por su parte la hiperplasia mastocitaria hace referencia al crecimiento incontrolado de mastocitos no clonales. Habitualmente coexiste una enfermedad de fondo y no se encuentran signos de activación mastocitaria. También puede estar presente en trastornos de origen linfoproliferativo, infecciones crónicas y tras la administración del SCF [113].

2.5.1. Mastocitos humanos HMC-1.

La línea celular de mastocitos humanos-1 (HMC-1) se encuentra presente en pacientes con mastocitosis sistémica. La primera referencia que se tiene de esta línea celular data del año 1988, procedente de una paciente con leucemia de mastocitos de 52 años [114]. Los autores del estudio resaltaron la capacidad de las células para teñirse metacromáticamente con azul de toluidina y la carencia de receptores FCεRI en su superficie [114]. La línea celular HMC-1 se caracteriza por crecer en suspensión en un medio Iscove modificado por Dulbecco (IMDM) suplementado con suero fetal bovino. Además, poseen un tiempo de duplicación de 80 horas aproximadamente y numerosos marcadores de superficie, tales como [115]:

- Marcador de células T: CD2.
- Marcador de células B: CD37.
- Marcador mielomonocítico: CD88.
- Marcadores eritroide-megacariocíticos: CD9 y CD63.
- Marcadores de adhesión: CD11a, CD11c, CD18, CD43, CD44 y CD54.
- Receptores de citocinas y quimiocinas: CD25, CD116, CD117, CD121b, CD122 y CD131.

Las células HMC-1 se clasifican en dos sublíneas: HMC-1^{560} y HMC-1^{560,816} [115]. La primera de ellas se caracteriza por poseer una mutación en la posición 560 de la región citoplasmática del receptor c-kit en la que se substituye el aminoácido glicina por valina. La segunda línea celular posee además una segunda mutación en la posición 816 de este mismo receptor en la que se cambia el

aminoácido aspartato por valina [88]. Morfológicamente las células HMC-1^{560} se caracterizan por ser más heterogéneas y de mayor tamaño que las HMC-1^{560,816}, sin embargo estas crecen más rápidamente [88]. Ambas mutaciones provocan un estado de fosforilación permanente del receptor, por lo que se encuentra constantemente activado. Por esta razón, ambas líneas celulares crecen de manera independiente del SCF, aunque se ha descrito que la estimulación con este factor aumenta los niveles basales de fosforilación del receptor [88]. Como consecuencia de la permanente activación del c-kit se activan diversas rutas celulares, descritas anteriormente, en las que intervienen enzimas como la PI3K o las MAPKs relacionadas con la supervivencia y proliferación celular [88].

En las células con dos mutaciones en el c-kit se ha descrito la fosforilación permanente de la proteína glicógeno sintasa quinasa-3β (GSK3β), lo que conlleva a una activación constitutiva de la misma [116]. Dicha proteína se encuentra también permanentemente activada en mastocitos humanos primarios derivados de sangre periférica y se caracteriza por ser un potente factor antiapoptótico y participar en la producción de citocinas y quimiotaxis [116,117]. En este sentido se ha comprobado que en células HMC-1^{560,816} la silenciación de esta proteína provoca una mayor mortalidad celular [116]. Sin embargo, se desconoce el efecto de esta silenciación en la línea celular HMC-1^{560}.

Además de la GSK3β, el factor nuclear potenciador de las cadenas ligeras κ de células B (NF-κB) se encuentra también permanentemente activado en la línea celular HMC-1^{560,816}. El inhibidor de NF-κB, denominado inhibidor de kappaB (IκB), hace que este factor permanezca en estado inactivo en el citosol. Tras la degradación del IκB por parte de lipopolisacáridos bacterianos, ésteres de forbol o bien citocinas inflamatorias el factor de transcripción se encuentra libre para dirigirse al núcleo [118,119]. El NF-κB es un factor íntimamente ligado al sistema inmunitario, ya que una vez en el núcleo activa la transcripción de genes que codifican para proteínas relacionadas con la respuesta inmune y el estrés [118,120]. Sin embargo, el papel del NF-κB en las células HMC-1^{560,816} no se conoce al completo [119]. Se sabe que la inhibición de la ruta del NF-κB implica la supresión del crecimiento de estas células [121], hecho de vital importancia pues supone una posible ruta de modulación sobre la que actuar en el tratamiento de la mastocitosis.

La línea celular HMC-1 no se encuentra en pacientes con CM, sin embargo el 80% de los casos de mastocitosis sistémica son positivos a la línea celular HMC-1^{560,816} [122]. Por esta razón se ha establecido la presencia de la mutación Asp-816->Val como criterio para el diagnóstico de esta patología [99].

2.5.2 Tratamiento de la mastocitosis.

Debido a su mal pronóstico, la SM es el tipo de mastocitosis que ha requerido mayores estudios a la hora de diseñar un tratamiento eficaz y que provocase efectos secundarios mínimos al paciente. Para el caso de la mastocitosis sistémica indolente el tratamiento se basa en la administración de antagonistas de receptores H_1 y H_2 de histamina y no se requiere un tratamiento citorreductor, a diferencia del resto de tipos de SM.

En los casos de mastocitosis agresivas el tratamiento no se simplifica a un solo fármaco, sino que se lleva a cabo una combinación de varios compuestos, siendo los más empleados el IFNα, la cladribina y el grupo de los inhibidores de tirosina quinasa (TyrK). A continuación se describen cada uno de estos fármacos:

- IFNα: fue considerado fármaco de primera elección para el tratamiento citorreductivo en los casos de SM sintomática a principios de la década de los noventa. Sin embargo el tratamiento con IFNα da lugar a una respuesta muy variable dependiendo del paciente. Se combina con frecuencia con corticoesteroides como la prednisolona, aumentando su eficacia en un 10% aproximadamente [123-125]. Sin embargo posee numerosos efectos secundarios como la fiebre, el hipotiroidismo o citopenias [126,127]. Su efecto en algunos pacientes ha sido cuestionado tras observarse que al abandonar el tratamiento durante un periodo corto de tiempo los síntomas reaparecían, sugiriendo un efecto citostático del fármaco y no citolítico [126].

- Cladribina: se trata de un análogo de nucleósido indicado en el tratamiento de pacientes con SM intolerantes al IFNα. Este compuesto inhibe la proliferación de las células HMC-1^{560} y HMC-1^{560,816}, sin embargo puede dar

lugar a efectos segundarios como mielosupresión e infecciones oportunistas.

- Inhibidores de TyrK: se trata del grupo más importante de fármacos utilizados en el tratamiento de la SM. Este grupo de fármacos se caracteriza por su capacidad de unión a receptores TyrK como el c-kit en el caso de mastocitos HMC-1.

    - Primera generación de inhibidores de TyrK:

      El primer inhibidor de TyrK utilizado en el tratamiento de la SM es el imatinib mesilato (STI571 o Gleevec®), comercializado a partir de mayo de 2001 [128]. Se trata de un fármaco que actúa inhibiendo varios receptores TyrK: c-kit, gen de región de fractura (Bcr)/gen Abelson (Abl) y el PDGFR [129]. En concreto su punto de unión en células HMC-1 son los dominios quinasa del receptor c-kit, impidiendo así la unión del ATP y bloqueando el receptor [130]. Este fármaco se ha considerado tratamiento de primera elección en pacientes con leucemia mieloide crónica (CML) hasta la aparición de la segunda generación de inhibidores de TyrK. La CML es una enfermedad mieloproliferativa que se caracteriza por la fusión de los genes Bcr/Abl situados en el cromosoma 22 y 9 dando lugar a un cromosoma 22 de menor tamaño denominado *philadelphia* descubierto en 1960 [131]. La CML es una enfermedad que se divide en dos fases: una primera crónica caracterizada por la expansión de granulocitos y células progenitoras de macrófagos y una segunda fase en la que se produce la aparición de formas mutantes de Bcr/Abl [132]. El imatinib no es eficaz en pacientes en fase avanzada de la enfermedad, debido probablemente a la presencia de células quiescentes, causadas por mutaciones en el receptor Bcr/Abl, resistentes al fármaco. En cuanto a su eficacia frente a la línea celular HMC-1, cabe destacar que la sublínea HMC-1^{560,816} es resistente al fármaco [129,133]. Por esta razón se han llevado a cabo numerosos estudios con el objetivo de identificar nuevos compuestos que tengan actividad frente a la mutación Asp-816->Val, lo que dió lugar a la aparición de una segunda generación de inhibidores TyrK.

- Segunda generación de inhibidores de TyrK:

Dentro de este grupo destacan varios fármacos: dasatinib (BMS-354825), midostaurina (PKC412) y nilotinib (AMN107). Todos ellos se caracterizan por poseer efecto citotóxico frente a células HMC-1 con mutación Asp-816->Val además de frente a la línea celular HMC-1^{560}.

El dasatinib es un inhibidor de múltiples TyrKs: c-kit, Bcr/Abl, Src, receptor de efedrina, p38 MAPK y PDGFR [128,132,134]. Se ha convertido en el tratamiento de elección de los pacientes con CML desde el año 2010, siendo tres veces más potente que el imatinib frente al complejo proteico Bcr-Abl [128,135,136]. A pesar de tener múltiples dianas, la proteína Lyn y la tirosina quinasa de Bruton (Btk) han sido descritas como las dos más importantes [137-139]. Ambas están en estado activo y colaboran en el crecimiento de las líneas celulares HMC-1^{560} y HMC-1^{560,816} [140]. Por tanto, el dasatinib, al bloquear la actividad de Lyn y Btk, anula el efecto estimulador de dichas proteínas sobre la proliferación de las dos líneas celulares HMC-1.

La midostaurina es un derivado alcaloide de la estaurosporina e inhibe también varias TyrK: c-kit, Flt-3 y PDGFR [130]. Además de su acción sobre las TyrK, la midostaurina inhibe también las isoformas clásicas (α, βI, βII y γ) de la PKC, mostrando escasa actividad frente a las restantes isoformas de la PKC [141]. Este compuesto da lugar a dos metabolitos: CGP52421 y CGP6221, cinco veces más potentes que la midostaurina [142]. La actividad de este fármaco ha sido estudiada *in vitro*, describiéndose un efecto de ralentización del crecimiento tumoral, afectando especialmente a las células en fase de mitosis. Por otro lado, estudios *in vivo* demostraron que la midostaurina potencia el efecto de otros fármacos antitumorales como el paclitaxel o la doxorrubicina [143,144]. En estudios realizados en ambas líneas celulares HMC-1 se ha descrito que la actividad antitumoral de la midostaurina es dependiente de la proteína proapoptótica Bim. Los niveles de Bim están regulados por el SCF, encargado de disminuír sus niveles en mastocitos normales [145]. Sin embargo, las células HMC-1, al crecer de manera SCF-independiente, no sufren esta regulación, mientras que la

activación permanente del c-kit causa una regulación negativa de los niveles de Bim [146]. Se ha comprobado que la actividad apoptótica de la midostaurina es Bim-dependiente tras observar la supresión del efecto citotóxico del fármaco al silenciar esta proteína [146].

El nilotinib, al igual que los dos anteriores compuestos, actúa sobre diversos receptores TyrK: c-kit, Bcr/Abl y PDGFR [147]. A diferencia del dasatinib y midostaurina, que presentan una citotoxicidad semejante en células HMC-1^{560} y HMC-1^{560,816}, el nilotinib es mucho más eficaz en la línea celular HMC-1^{560} [130].

A continuación se muestra el resultado de la combinación de los distintos fármacos empleados en el tratamiento de la SM (tabla 5). Cabe destacar la ausencia de efectos sumatorios en la línea celular HMC-1^{560}, al contrario de lo que ocurre en la línea celular HMC-1^{560,816}. Además en estas células el nilotinib es el fármaco que comparte un efecto sumatorio con la mayor parte de los fámacos probados, excepto con la cladribina.

	Imatinib	**Dasatinib**	**Midostaurina**	**Nilotinib**	**Cladribina**
Imatinib		+	+	+	nd
Dasatinib	±		+	nd	nd
Midostaurina	nd	+		+	+
Nilotinib	±	±	±		+
Cladribina	nd	+	±	±	

Tabla 5. Interacciones entre fármacos empleados en el tratamiento de la SM [140]. +: efecto sinérgico, ±: efecto sumatorio, nd: efecto no descrito.

Una característica muy importante de los inhibidores de Tyrk es que dan lugar numerosos efectos adversos cutáneos y sistémicos. Dentro de los efectos cutáneos destacan la alopecia, alteraciones en la

pigmentación y prurito. En cuanto los efectos sistémicos pueden aparecer alteraciones como trombocitopenia, alteraciones cardíacas o anemia. En la tabla 6 se describen los asociados a cada fármaco [148]:

CUTÁNEOS	IMATINIB	DASATINIB	NILOTINIB
Erupciones	✔	✔	✔
Prurito	✔		✔
Edema superficial	✔	✔	
Despigmentación	✔		
Hiperpigmentación	✔		
Piel seca			✔
Alopecia			✔
SISTÉMICOS			
Anemia	✔	✔	✔
Trombocitopenia	✔	✔	✔
Neutropenia	✔	✔	✔
Efusión pleural		✔	
Alteraciones cardíacas		✔	✔
Hipofosfatemia		✔	✔
Pancreatitis			✔
Hipoglucemia			✔

Tabla 6: Efectos adversos asociados al tratamiento con inhibidores de TyrK [130].

2.6. Proteína quinasa C.

La PKC fue descubierta en el año 1977 al identificar una quinasa activada por el tratamiento con calpaína (proteasa neutra dependiente de Ca^{2+}) [149-151]. Puesto que la activación de la enzima dependía de Ca^{2+}, recibió el nombre de PKC [152]. La importancia de la PKC se puso de manifiesto al descubrirse que era la receptora de un grupo de promotores tumorales, los ésteres de forbol [153], diterpenos procedentes de las familias de plantas *Euphorbiaceae* (con 170 especies) y *Thymelaeaceae* [154]. Dentro de la familia *Euphorbiaceae* el género

Jatropha (nombre procedente de dos términos griegos: *jatros* que significa doctor y *trophe* comida) ha sido objeto de numerosos estudios, ya que las plantas de este género se han utilizado de manera generalizada en la medicina tradicional de los países tropicales y subtropicales [155]. Sin embargo, más tarde se describió que no todas las isoformas de esta enzima se unían a ésteres de forbol y que estos poseían otras dianas aparte de la PKC [156]. Aunque los ésteres de forbol son utilizados para la activación de esta proteína, realmente su ligando fisiológico es el DAG. La generación de esta molécula, segundo mensajero lipídico y agonista natural del dominio C1 de dicha proteína, provoca la translocación de la PKC desde el citosol hasta la membrana, lo que recibe el nombre de activación alostérica. De este modo, la actividad de la PKC en la célula está regulada por diferentes mecanismos entre los que destacan: fosforilación, unión a cofactor (activación alostérica) e interacción con proteínas que dirigen la PKC hacia sus reguladores y sustratos.

2.6.1. Isoformas y estructura.

En mamíferos, la familia de PKCs está integrada por diez isoformas clasificadas en tres grupos atendiendo a su estructura y a los cofactores que requieren para su activación:

- PKCs clásicas (cPKC): activadas por Ca^{2+}, DAG y fosfatidilserina (PS). En este grupo se incluyen las isoformas: α, β1, β2 y γ.
- PKCs nuevas (nPKC): activadas por DAG y PS e independientes de Ca^{2+}. Este grupo está integrado por las isoformas: δ, ε, η y θ.
- PKCs atípicas (aPKC): activadas por PS pero independientes de Ca^{2+} y DAG. En este grupo hay dos isofomas: ζ y ι/λ.

En un principio se incluyó un cuarto grupo de PKCs, formado por las isoformas μ y ν, que sin embargo pasaron a conformar un grupo diferente denominado proteína quinasa D al presentar grandes diferencias en el dominio quinasa, muy conservado en todas las PKCs [157].

Todas las isoformas de la PKC se caracterizan por estar codificadas por genes diferentes, excepto las clásicas β1 y β2. Además, poseen un PM en torno a los 80kDa, aunque éste difiere ligeramente según la isoforma. La distribución en tejidos de las distintas isoformas se presenta en la tabla 7. Como puede observarse existe una amplia variabilidad entre unas isoformas y otras, siendo las isoformas α, β, δ, ζ y ι/λ las más ampliamente extendidas por todo el organismo [158].

	Isoforma	PM	Activadores	Proteínas de interacción	Distribución
cPKC	α	76.8	Ca^{2+},DAG,PS, PMA, FA, PC.	DAGKζ, fascina,laminina A y B1, PLD2, syndecan-4, caveolina, tropomodulina, PICK1, Sdr.	Universal
	β1	76.8	Ca^{2+},DAG,PS, PMA, FA, PC.	GRK2	Algunos tejidos
	β2	76.9	Ca^{2+},DAG,PS, PMA, FA, PC.	Gravina, receptor de insulina, pericentrina.	Muchos tejidos
	γ	77.5	Ca^{2+},DAG,PS, PMA, FA, PC.		Tejido nervioso
nPKC	δ	77.5	DAG, PS, PMA, FA, PI.	Actina, PLD1, Fyn, PDGF-β, Src, receptor TNF-α, STAT3, GAP43, syndecan.	Universal
	ε	83.5	DAG, PS, PMA, PI.	RACK1, RACK2, Btk, caveolina, MAPK, miosina.	Tejido nervioso, sistema inmune, epitelio y corazón.
	η	78	DAG, PS, PMA, PI, PIP_3.		Tejidos nervioso y epitelial.
	θ	81.6	DAG, PS, PMA, PI.	Fyn, Btk	Ovario, músculo esquelético, plaquetas y monocitos
aPKC	ζ	67.7	PS, FA, PIP_3, PA, ceramida.	Tubulina, caveolina, ZIP3, VHL.	Universal
	ι/λ	67.2			Muchos tejidos

Tabla 7: Isoformas de PKC en tejidos de mamífero [159-161]. Forbol 12-miristato 13-acetato (PMA), ácidos grasos (FA), fosfatidilcolina (PC), fosfatidilinositol (PI),

diacilglicerol quinasa ζ (DAGK ζ), fosfolipasa D1 (FLD1), fosfolipasa D2 (FLD2), proteína de interacción con la quinasa C1 (PICK1), proteínas de repetición serina-aspartato (Sdr), quinasa receptora de proteína G doble (GRK2), transductor de señal y activador de transcripción 3 (STAT3), proteína activadora de guanosina trifosfatasa (GTPasa) 43 (GAP43), receptor de quinasa C activada (RACK) 1, ácido fosfatídico (PA), proteína de interacción con PKCζ (ZIP3) y proteína de Von Hippel-Lindau (VHL).

Los tres grupos de la familia PKC están formados por una cadena polipeptídica con una región amino-terminal reguladora y una región carboxi-terminal catalítica, representadas en el esquema 5. La PKC consta de un dominio regulador y uno catalítico formados por el dominio pseudosusbrato, cuatro regiones conservadas (C1-C4) y cinco regiones variables (V1-V5). El dominio pseudosustrato tiene una secuencia similar a la del sustrato salvo la presencia de una alanina en lugar de serina, de tal manera que la proteína no puede ser fosforilada [152,156,161,162]. La PKC es regulada por este dominio, que ocupa el sitio de unión al sustrato, otorgándole a la enzima una conformación inactiva e impidiendo la actividad enzimática [156,163].

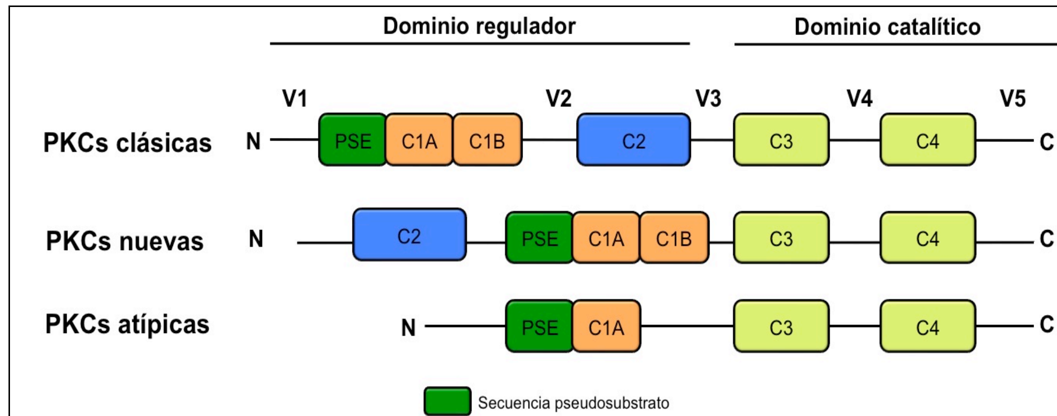
La mitad amino-terminal reguladora posee las regiones conservadas C1 y C2, lugares de unión al DAG y Ca^{2+} respectivamente. El dominio C1 se caracteriza por ser rico en cisteína, con aproximadamente 50 residuos, y presente en todos los grupos de PKCs [156]. Este dominio, formado por dos partes C1A y C1B, es el lugar de unión a los sustratos PS, DAG y ésteres de forbol. Dicha unión ligando-proteína tiene lugar a través de una única región C1A ó C1B. Ambas partes del dominio poseen la misma relevancia, sin embargo en ciertas isoformas la región C1B parece ser más importante [164]. En el caso de las PKC atípicas se denomina dominio C1 atípico puesto que no se une a DAG ó ésteres de forbol [165], sino que está relacionado con la interacción con ceramida y con la formación de complejos proteína-proteína [166].

El dominio C2 es el lugar de unión al Ca^{2+} en el caso de las cPKC, ya que en el caso de las isoformas nuevas hay una pérdida de residuos de ácido aspártico que

evita la unión a este segundo mensajero [167]. El dominio C2 está presente no solo en la PKC sino también en otras proteínas como las fosfolipasas y las proteínas activadoras de GTPasa [168]. La región V3 es la que separa el dominio catalítico del regulador. Denominada también “bisagra”, es una secuencia sensible a la tripsina y a proteasas neutras dependientes de Ca^{2+}, las cuales provocan una ruptura proteolítica y la formación de una quinasa constitutivamente activa.

La mitad catalítica, de aproximadamente 45kDa [169], contiene los dominios C3, lugar de unión del ATP, y el dominio C4 de unión al sustrato. La secuencia de los dominios C3 y C4 presenta una homología del 40% con respecto a la proteína quinasa A (PKA) [170]. La región C3 contiene una zona de unión a ATP mientras que la C4 posee los sitios de unión al sustrato y de transferencia del grupo fosfato [171]. A diferencia de la PKA, cuyo dominio catalítico termina con el aminoácido fenilalanina, la PKC posee una región hidrofóbica seguida de la secuencia carboxilo-terminal. Dicha secuencia juega un papel fundamental en la regulación de la PKC ya que supone el sitio de unión de la quinasa que activa a la PKC, la quinasa-1 dependiente de fosfoinosítidos (PDK-1). Además, esta región determina la distribución celular de la enzima [156].

Finalmente, la región V5, al igual que el dominio catalítico está relacionada con la localización de la proteína. Prueba de esto es que las isoformas PKCβ1 y PKCβ2, cuya única diferencia radica en esta región, presentan diferente localización celular. Además, está implicada en la localización, translocación y función de las isoformas PKCδ y PKCε, así como en el anclaje de la PKCα durante el proceso de maduración [172,173].



Esquema 5: Estructura de los tres grupos de PKCs [156,161].

2.6.2. Regulación de la PKC.

La actividad de la PKC está regulada a través de diferentes mecanismos: regulación por fosforilación y adquisión de la capacidad catalítica, regulación alostérica por unión a cofactores, regulación intraestérica por autoinhibición en el sitio pseudosustrato, interacción con proteínas de anclaje que la sitúan cerca de sus reguladores y sustratos y finalmente degradación proteolítica [156]. A continuación se describen cada una de ellas:

- Regulación por fosforilación: todas las isoformas de la PKC se regulan a través de una serie de fosforilaciones en aminoácidos, fundamentalmente serina y treonina, aunque también por medio de fosforilaciones en varias tirosinas en la región catalítica y reguladora [174,175].

- Regulación alostérica:

    - PS: se trata de un aminofosfolípido situado únicamente en la cara citoplasmática de la membrana. El dominio C1 es el que le confiere especificidad a la proteína para unirse a este cofactor [156].

  - DAG y ésteres de forbol: provocan un gran aumento de la afinidad de la PKC por la membrana, en concreto por la PS. A diferencia del DAG, los ésteres de forbol son metabolizados más lentamente que el DAG por lo que dan lugar a una activación más prolongada [156].
  - Ca^{2+}: el inositol 1,4,5-trifosfato (IP_{3},) generado a partir de la hidrólisis del fosfatidilinositol 4,5-bifosfato (PIP_2) en una reacción catalizada por la PLC, causa la movilización del ión Ca^{2+} a su receptor y provoca su liberación de los reservorios intracelulares, induciendo la translocación de la PKC. Además, en el caso de las isoformas clásicas, existe un efecto sinérgico entre el Ca^{2+} y el DAG, ya que la presencia de uno reduce la cantidad necesaria del otro para la activación de la enzima [156].

- Regulación intraestérica: se trata de un tipo de autorregulación protagonizada por la región pseudosustrato, ya que evita la unión al sustrato manteniendo a la enzima en una conformación inactiva [163].

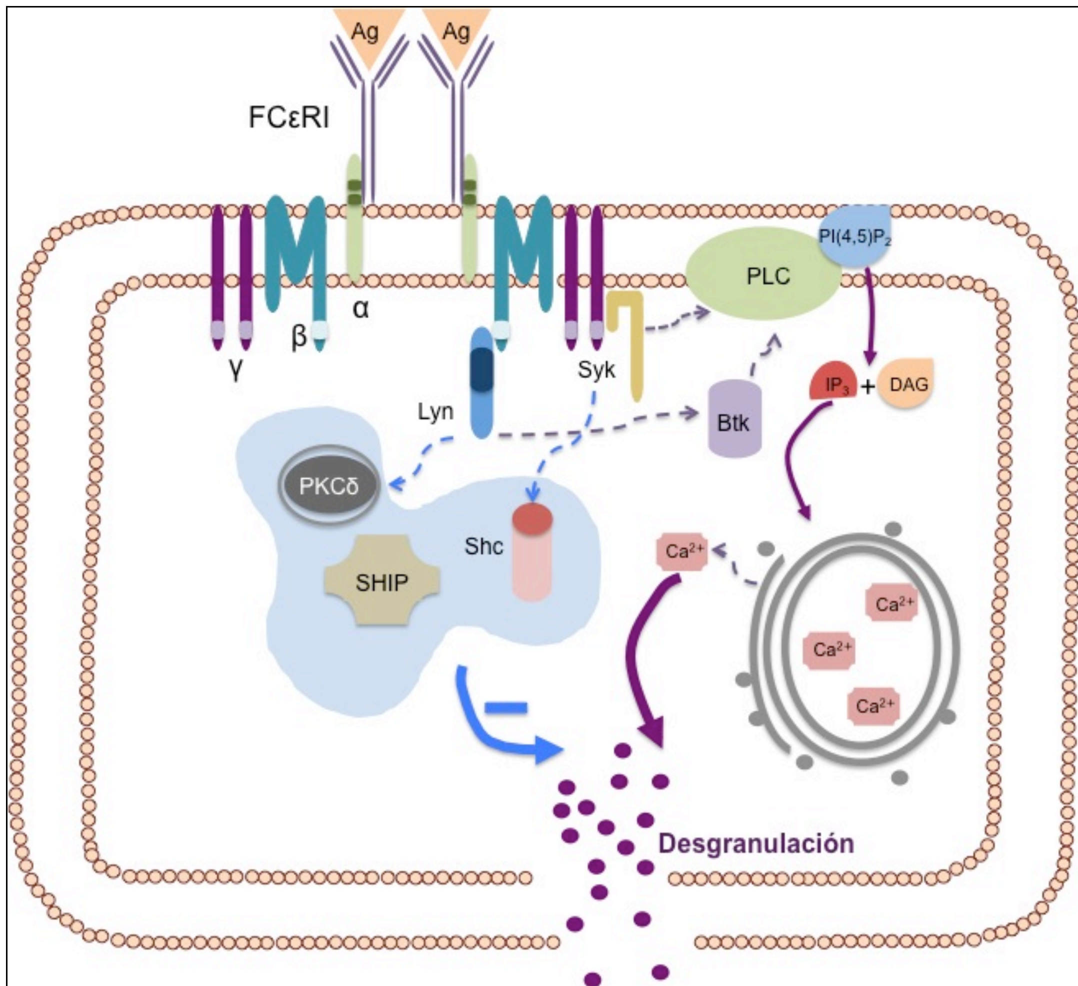
- Regulación por unión a proteínas de anclaje: las proteínas de anclaje, entre las que destacan las proteínas de anclaje de la quinasa A (AKAP), se encargan de dirigir a la PKC hacia distintos lugares de actuación dentro de la célula. Estas proteínas sitúan a la PKC fosforilada pero inactiva cerca de sus sustratos. Otro grupo de proteínas de anclaje son los RACKs, que conducen a la PKC a localizaciones celulares específicas [176]. Las proteínas del citoesqueleto también juegan un papel importante en el anclaje de la PKC. De esta forma se ha establecido una fuerte relación entre esta proteína y los componentes integrantes de los filamentos intermediarios, como vimentinas y citoqueratinas. También se sabe que la interacción de la PKC con los filamentos de actina (F-actina) aumenta la actividad de esta enzima y que la isoforma PKCζ está relacionada con la tubulina [177,178].

- Degradación proteolítica: se trata de un proceso inactivador para la enzima y su existencia se puso de manifiesto al comprobar que el tratamiento prolongado con ésteres de forbol provocaba un descenso de la cantidad de PKC en la célula. A este efecto, originado en parte por un aumento de la

proteolisis, se lo denominó "down-regulation", por lo que se trata de un mecanismo de retroalimentación negativa [179].

2.6.3. PKC en mastocitos.

La PKC tiene un papel importante sobre la exocitosis actuando como reguladora de este proceso fisiológico. Este papel se observó en un principio en células de la pituitaria en las que estaban involucradas las isoformas PKCα y PKCβ [180]. A continuación se realizaron los primeros estudios en mastocitos de rata. La activación de estas células está regulada por varias rutas de señalización intracelular en las que participa el Ca^{2+}, el cAMP, el pH intracelular (pH_i) así como la PKC. De esta forma se ha descrito que la activación de la PKC acciona el intercambiador Na^+/H^+, induciendo de esta forma un aumento del pH_i [181-185]. Otros estudios describieron además una estrecha relación entre la PKC y el Ca^{2+} intracelular, ya que la activación de la enzima estimula la liberación de histamina inducida por la thapsigargina, inhibidor de la ATPasa de Ca^{2+} del retículo endoplasmático (SERCA) [186]. Sin embargo, se ha descrito un efecto contrario en la liberación de histamina inducida por el compuesto 48/80, ya que la activación de la PKC en este caso tiene un efecto inhibitorio [187]. Además la PKC regula la liberación de Ca^{2+} al citosol procedente de los reservorios intracelulares [188-191]. Una mención especial merece la isoforma nueva PKCδ, puesto que ha sido descrita como reguladora negativa de la desgranulación en mastocitos (esquema 6). Tal y como se ha descrito anteriormente, en la exocitosis mediada por IgE, poseen un papel fundamental las proteínas Lyn y Syk encargadas de activar a la PLC. Esta proteína da lugar a la formación de DAG e IP_3 a partir de PIP_2. A continuación el IP_3 estimula la salida de Ca^{2+} de los reservorios intracelulares con una consiguiente desgranulación. A su vez la desgranulación está regulada de manera negativa por la enzima SHIP, encargada de bloquear la ruta de la PLC [192,193]. Otra enzima encargada de regular este proceso es la proteína adaptadora con dominio de unión SH2 (Shc). Hay muy pocas moléculas que interaccionen con SHIP y Shc y la PKCδ es una de ellas, uniéndose a través de su dominio C2. De esta forma el complejo formado por SHIP/Shc/PKCδ es considerado esencial en el proceso de regulación de la desgranulación [34].



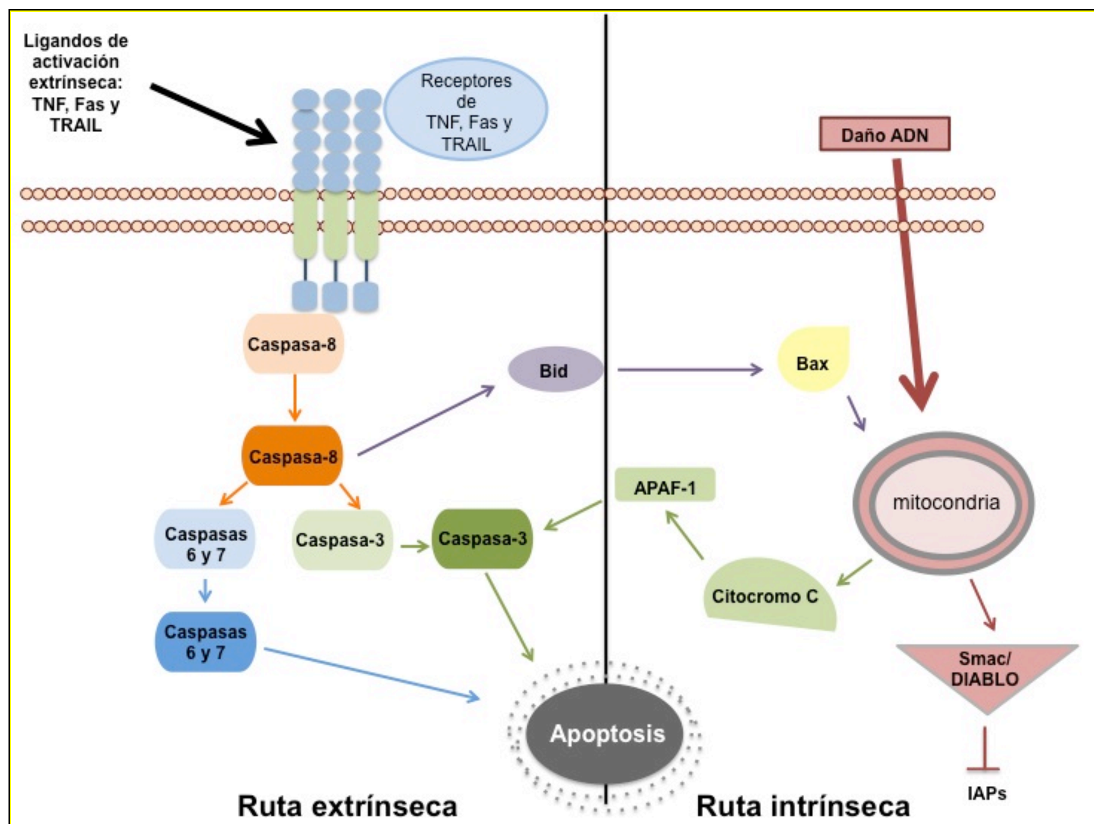
Esquema 6: Inhibición de la desgranulación por parte de la PKCδ. Representación de la ruta intracelular activada [34].

Además del papel regulador en la exocitosis, la PKC participa en el proceso de migración de los mastocitos hacia la región inflamatoria. En concreto la inhibición de las isoformas cPKC reduce la migración inducida bien por SCF o por antígeno [116]. La PKC es también fundamental en la activación de los mastocitos de la línea celular HMC-1. La alcalinización intracelular se ha descrito como un importante estímulo de exocitosis en mastocitos HMC-1 y se ha comprobado que la activación de esta enzima provoca un aumento en la liberación de histamina inducida por alcalinización intracelular en las dos líneas celulares: HMC-1^{560} y HMC-1^{560,816} [194]. Por otro lado, la actividad de la ionomicina, ionóforo del Ca^{2+} y

estímulo de la liberación de histamina [195-198], está regulada por la PKCδ. De esta forma la inhibición de esta isoforma bloquea el efecto del ionóforo en células HMC-1^{560} [194]. En definitiva, la PKC ha sido descrita como una proteína esencial en el proceso de regulación de la exocitosis [199].

2.6.4. PKCδ y apoptosis.

La isoforma PKCδ está íntimamente ligada al proceso de muerte celular programada o apoptosis [200]. Este proceso sigue dos rutas celulares diferentes dependiendo del estímulo apoptótico: extrínseca o intrínseca. Se denomina apoptosis extrínseca o externa a aquella estimulada por ligandos como el TNF, el Fas o el ligando inductor de la apoptosis relacionado con TNF (TRAIL), que tras su unión a sus receptores de membrana activan la caspasa-8 o iniciadora. Las caspasas son enzimas que se encuentran en estado inactivo en la célula y que se activan por proteolisis tras una señal apoptótica. La caspasa-8 es la encargada de activar a la caspasa-3, estimulando a su vez a la poli-(ADP-ribosa) polimerasa (PARP) y desencadenando finalmente la muerte celular. Sin embargo la segunda vía de apoptosis, intrínseca o mitocondrial, también puede ser activada por la caspasa-8 por medio de la activación de la proteína Bid, la cual puede promover la salida del citocromo C de la mitocondria. La vía intrínseca tiene su origen en una alteración mitocondrial causada por estrés. En este caso la proteína proapoptótica iniciadora es la Bax, encargada de reaccionar con el transportador de adenín nucleótido situado en la cara interna de la membrana mitocondrial. Esta unión facilita la apertura del poro mitocondrial con la consiguiente salida de factores apoptóticos al citoplasma como son el citocromo C, el smac/DIABLO y el factor inductor de la apoptosis. Tras su salida, se produce la activación del factor 1 activador de la proteasa apoptótica (APAF-1) y de las caspasas 3, 6, 7 y 9 desencadenando finalmente la apoptosis *per se,* caracterizada por la fragmentación del ácido desoxirribonucleico (ADN), formación de vesículas de membrana, degradación proteica y reducción de volumen celular [201,202].

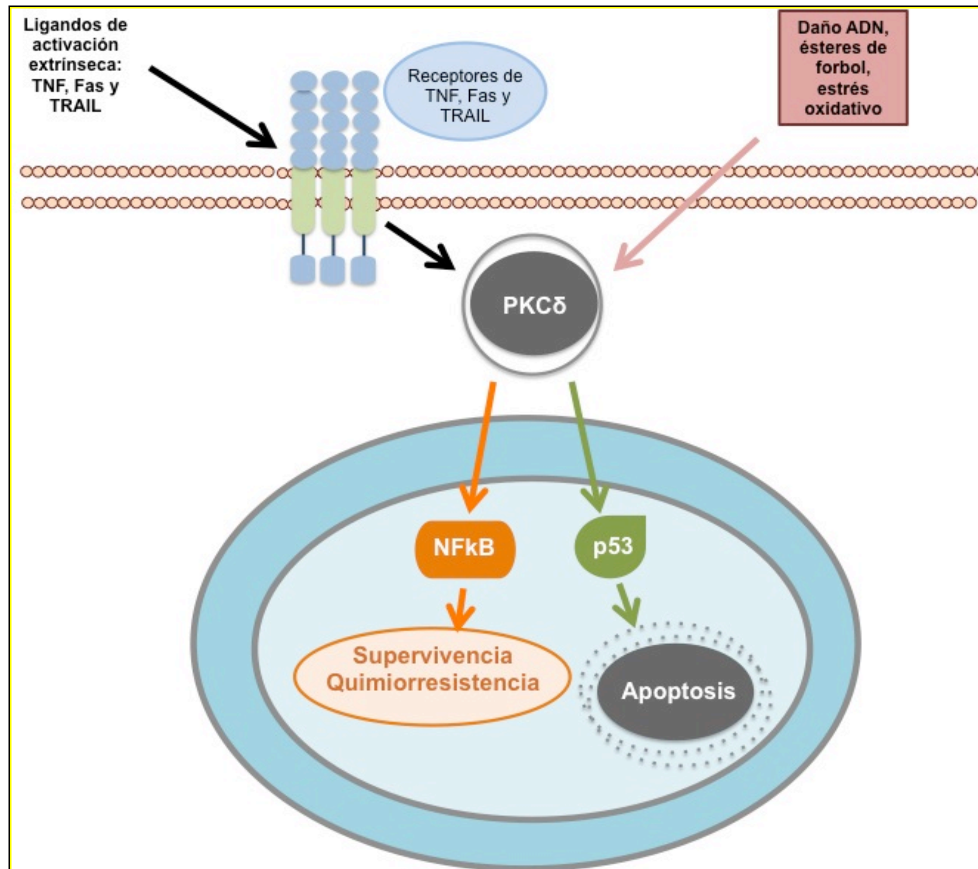


Esquema 7: Ruta intrínseca y extrínseca de la apoptosis.

La PKC posee un importante papel regulador sobre el proceso apoptótico protagonizado por la isoforma PKCδ [200,203-209]. Tanto es así que la activación de la muerte celular programada causada por genotoxinas, estrés oxidativo, ésteres de forbol o ligandos de muerte va acompañada de una traslocación al núcleo de esta isoforma [203,210]. A nivel citosólico la PKCδ desencadena la apoptosis activando la p38 MAPK, inhibiendo a la Akt y disminuyendo los niveles del inhibidor de apoptosis unido a X. Una vez en el núcleo sufre dos fosforilaciones (Y64 e Y155) en el dominio regulador que facilitan la retención de la isoforma en este lugar [204], la cual se encarga de desencadenar el proceso apoptótico vía activación de la JNK [204]. La PKCδ es también sustrato de la caspasa-3, sin embargo esta relación puede estar en algunos casos invertida y que por tanto la caspasa-3 actúe como sustrato de la PKCδ dando lugar a la apoptosis en este caso en monocitos [209,211]. El papel proapoptótico de la PKCδ no es constante, sino que depende

del tipo celular y del estímulo apoptótico. Por esta razón es considerada una isoforma antiapoptótica en numerosos tipos de células cancerígenas, como las presentes en cáncer de páncreas, de hígado o en leucemia crónica [210,212,213]. Se ha descrito que la sobreexpresión de esta isoforma está asociada a la resistencia a fármacos utilizados en quimioterapia en cáncer de cérvix, a la resistencia al cisplatino en cáncer de pulmón [214-216] y que incrementa el potencial metastático de células de melanoma de ratón [217].

En la función de la PKCδ, pro- o antiapoptótica, influyen diversos factores, como son la activación proteolítica de la enzima, la localización intracelular y el estado de otras señales intracelulares y proteínas diana. Esta propiedad de la PKCδ radica en su fragmento catalítico ya que puede desencadenar una estimulación o inhibición de la apoptosis, dependiendo del estímulo. Hay dos factores de transcripción que median también en este doble efecto de la PKCδ: p53 y el factor NF-κB. El primero de ellos, p53, es un supresor tumoral sobre el que actúa la PKCδ dando lugar a un incremento de la apoptosis y supresión tumoral. Por el contrario, la PKCδ actúa también sobre el NF-κB, complejo proteico que regula la transcripción del ADN, facilitando la supervivencia celular y la quimiorresistencia a fármacos (esquema 8) [210].



Esquema 8: Ruta pro- y antiapoptótica de la PKCδ [210].

2.6.5. PKC y cáncer.

El papel de la PKC en el cáncer ha sido descrito desde hace varias décadas al comprobar que promotores tumorales como el éster de forbol PMA se unía al sitio de unión del DAG. A diferencia del DAG, la unión al PMA implica una activación más prolongada a la PKC y su consiguiente retrorregulación [218-220]. La expresión de la PKC en células cancerígenas difiere de la encontrada en células normales. De esta forma las isoformas de la PKC están sobreexpresadas y subexpresadas en numerosos tipos de cáncer, presentando una gran variación entre ellas. En el caso de la isoforma α, su subexpresión está presente en una

amplia variedad de tipos de cáncer, mientras que para las isoformas η e ι no se ha descrito subexpresión en ningún caso (tabla 8).

Isoforma	Sobreexpresión	Subexpresión
α	Cáncer de vejiga, próstata, hígado, mama y endometrial.	Cáncer de piel, riñón, ovarios, cerebro, mama, colon y hepatocelular.
β	Cáncer de colon, páncreas, próstata y linfoma.	Melanoma, cáncer de colon, próstata y vejiga.
δ	Cáncer de colon, esófago, riñón, cerebro y hepatocelular.	Cáncer de piel, colon y vejiga.
ε	Cáncer de vejiga, próstata, mama, cerebro y páncreas.	Cáncer de tiroides y páncreas.
η	Cáncer de riñón.	
ι	Cáncer de colon, pulmón, ovarios y páncreas. CML.	

Tabla 8: Expresión de la PKC en distintos tipos de cáncer [221-231].

La regulación de la expresión de la PKC en los distintos tipos de cáncer no está directamente relacionada con la cantidad de ácido ribonucleico mensajero (ARNm), lo que indica que dicha regulación se produce una vez realizada la transcripción de la enzima [232,233]. Además, la actividad de la PKC en los procesos cancerígenos no está asociada a la presencia de mutaciones de esta proteína, de hecho rara vez se han encontrado mutaciones de genes que codificasen la PKC [234]. A diferencia de lo que cabría esperar del promotor tumoral PMA, se ha descrito que no se trata de un factor importante en el desarrollo de tumores en humanos. Sin embargo, la presencia de sustancias consideradas promotores tumorales como el catecol, presente en el tabaco, es capaz de provocar la activación de la PKC [235,236]. Ocurre lo mismo con el elevado consumo de lípidos, puesto que tienen la capacidad de aumentar la producción de ácidos biliares, lo que incrementa la cantidad de DAG producido por la flora bacteriana desencadenando finalmente la activación de la PKC [237].

El papel que desempeña la PKC en la proliferación celular la convierte en una atractiva diana para el tratamiento de distintos tipos de cáncer. De esta forma los inhibidores de la PKC constituyen un grupo importante en la terapia frente a este tipo de patologías. Los oligonucleótidos, en su papel frente al cáncer y metástasis, han sido el primer grupo de inhibidores de la PKC que dieron lugar a dos patentes con este uso [238,239]. Dentro de este grupo destaca el compuesto denominado ISIS3521, específico frente a la isoforma PKCα [240,241]. Otro de los inhibidores de la PKC que presentan especificidad por ciertas isoformas es el indolocarbazol Gö6976, puesto que actúa frente a las isoformas clásicas PKCα y PKCβ [234,242]. Además, cabe destacar otra familia de compuestos como son los análogos de la estaurosporina y la briostatina-1. El primer grupo está integrado por la enzastaurina, midostaurina, 7-hidroxiestaurosporina, ruboxistaurina y sotrataurina. La enzastaurina es metabolizada por el citocromo 450 y su eficacia frente a pacientes con cáncer en estado avanzado es muy variable [243]. De hecho son muy pocos los estudios que demuestran una actividad antitumoral *in vitro* e *in vivo* significativa [244]. Sin embargo, se ha observado que actúa potenciando la actividad antitumoral *in vitro* de otros fármacos quimioterapéuticos, razón por la cual es frecuente la combinación de enzastaurina con otros compuestos como el bortezomib o la dexametasona [231]. Otro de sus análogos, la midostaurina, mencionado ya anteriormente debido a su actividad como inhibidor de tirosina quinasas, posee actividad frente a las isoformas clásicas de la PKC, al igual que el Gö6976 [234]. Otro grupo de inhibidores de la PKC es el de la lactona macrocíclica briostatina-1 [231]. A diferencia de la enzastaurina, la briostatina-1 es un compuesto natural producido por la proteobacteria *candidatus endobugula sertula* con actividad frente a las isoformas clásicas y nuevas de la PKC [231]. Al igual que ocurre con el grupo de los análogos de la estaurosporina, la actividad de este compuesto por sí solo frente a diferentes tipos de cáncer es mínima, por lo que se encuentra formando parte de diversos protocolos de poliquimioterapia anticancerígena [245]. Ejemplos de esta combinación es el uso conjunto de la briostatina-1 con la vincristina en el tratamiento del linfoma no-Hodgkin o con la fludarabina en linfomas [246,247]. La actividad antitumoral de los inhibidores de la PKC radica en su papel como inhibidores del crecimiento de células cancerígenas, actuando como agentes anti-invasores. Esta actividad tiene lugar bien a través de la estimulación de la vía apoptótica o evitando que las células entren en la fase de mitosis [248,249].

2.7. Aurora quinasa.

La familia de las aurora quinasas fue descubierta mientras se estudiaba la organización aberrante de los husos mitóticos en *Drosophila melanogaster* en el año 1995 [250]. Se trata de quinasas muy conservadas a lo largo de la evolución y pertenecientes a la familia de quinasas de serina/treonina que participan en la regulación de la fase mitótica del ciclo celular eucariótico [251,252]. Han sido descritos tres miembros dentro de esta familia: aurora quinasa A, B y C [253-255]. La estructura proteica está compuesta por un dominio amino-terminal y un dominio carboxilo-terminal, dando lugar a una secuencia de amioácidos compuesta por 309-403 unidades [254]. Las isoformas A y B poseen una homología muy elevada en su región catalítica, lo que les confiere una cierta similitud en cuanto a sustratos e inhibidores, sin embargo poseen numerosas diferencias funcionales [256]. Las tres isoformas están localizadas en el núcleo donde participan en el proceso mitótico [251] y su sobreexpresión da lugar a la fosforilación de numerosas proteínas relacionas con el ciclo celular así como a la fosforilación aberrante de diversas dianas citoplasmáticas. Este hecho provoca una alteración cromosómica presente en numerosos tipos de cáncer [257].

La isoforma A, también denominada STK15, es esencial para un buen desarrollo de la mitosis. Se encuentra en el centrosoma y huso mitótico y tras su interacción con diversos sustratos entre los que se encuentran la proteína fosfatasa 1 (PP1), la proteína p53, la proteína asociada a los microtúbulos TPX-2, la caderina 1 y la proteína activadora de GTPasa RasGap, interviene en la maduración del centrosoma y en la citocinesis [258]. La sobreexpresión de esta isoforma contribuye a la aparición de una inestabilidad génica que da lugar a la formación anormal de los husos acromáticos y a la poliploidía. Esta sobreexpresión está relacionada con la aparición de tumores en mamas, ovarios, próstata, cérvix, colon, páncreas y pulmón [259-262]. En cuanto a la isoforma B, o STK12, posee un papel íntimamente relacionado con el proceso mitótico, al igual que la isoforma A [253]. Participa en la condensación y segregación de los cromosomas y en la citocinesis, formando un complejo junto con la proteína interna del centrómero (INCENP) [263]. El sustrato más importante de esta forma es la histona H3, componente estructural de la cromatina, aunque también lo son la INCENP y la survivina [253,264]. La sobreexpresión de la aurora quinasa B ha sido también descrita en diversos tipos

de tumores como: tiroideo, oral, carcinoma pulmonar de células grandes, mamario y de colon [254]. Por último, la aurora quinasa C o AIK3, se diferencia de las dos restantes en que su expresión se limita únicamente a células testiculares, participando en el proceso de meiosis de este tipo celular [253,256,265]. Sin embargo su función a nivel celular es muy similar a la de la aurora quinasa B, puesto que interacciona también con la proteína INCENP y participa en la segregación de cromosomas [256]. Dentro de sus sustratos conocidos se encuentran la INCENP y la aurora quinasa B [253] y su sobreexpresión ha sido detectada en cáncer de mama e hígado [254].

Debido a la relación con la aparición de varios tipos de cáncer se han realizado estudios con numerosos inhibidores (en torno a 30) de aurora quinasa en pacientes con diversos tipos de procesos tumorales. La acción principal de los inhibidores de las aurora quinasas es la de inhibir la proliferación celular mediante el bloqueo de la citocinesis y la fosforilación de la histona H3, induciendo finalmente apoptosis celular [258]. Los inhibidores de las aurora quinasas pueden ser específicos de una isoforma en concreto, como lo son el MLN8237 o el AZD1152 para las isoformas A y B respectivamente o bien inhibir las tres isoformas. Además, es importante resaltar la ausencia de un inhibidor específico para la isoforma C (tabla 9).

Inhibidores en ensayos clínicos				
Compuesto	**Aurora A**	**Aurora B**	**Aurora C**	**Actividad antitumoral**
PHA-739358	✔	✔	✔	Tumores sólidos y CML
CYC116	✔	✔	✔	Tumores sólidos
SNS-314	✔	✔	✔	Tumores sólidos
VX-680	✔	✔	✔	Tumores sólidos
AT9283	✔	✔		Tumores sólidos, CML y AML
R763	✔	✔	✔	Tumores sólidos
PF-03814375	✔	✔		Tumores sólidos
GSK1070916		✔	✔	Tumores sólidos
AMG-900	✔	✔	✔	Tumores sólidos
MLN8237	✔			Tumores sólidos y leucemias
ENMD-2076	✔			Tumores sólidos y mieloma múltiple
MK-5108	✔			Tumores sólidos
AZD1152		✔		Tumores sólidos y AML
Inhibidores en ensayos preclínicos avanzados				
PHA-680632	✔	✔	✔	Tumores sólidos y leucemias
VE-465	✔	✔	✔	Tumores sólidos
JNJ-7706621	✔	✔		Tumores sólidos
CCT129202	✔	✔		Tumores sólidos
Inhibidores en ensayos preclínicos preliminares				
AKI-001	✔	✔		Tumores sólidos
Hesperadin		✔		nd
ZM447439	✔	✔		nd

Tabla 9: Inhibidores de la aurora quinasa [251].

De entre todos los inhibidores de aurora quinasa cabe destacar el compuesto CCT129202, ya que pertenece al grupo de los derivados de imidazol de más reciente aparición. El CCT129202 presenta una alta especificidad frente a la aurora quinasa, inhibiendo las auroras quinasas A, B y C. Sin embargo posee la capacidad de inhibir otras 13 quinasas entre las que se encuentran la PKC y el PDGFR [264]. Este compuesto se caracteriza por provocar la acumulación de material génico originando apoptosis celular [264]. Además, estudios realizados en células tumorales humanas demuestran que el CCT129202 disminuye el proceso mitótico [264].

3. Objetivo

La mastocitosis es una enfermedad considerada rara que se diagnostica cada vez con mayor frecuencia debido a los avances en técnicas diagnósticas y a un mejor conocimiento de la misma. La línea de mastocitos humanos HMC-1, aislada de un paciente con mastocitosis, es una herramienta útil en el estudio de esta enfermedad. En esta línea celular se incluyen dos sublíneas que se diferencian en una mutación en el receptor c-kit, HMC-1^{560} y HMC-1^{560,816}. El conocimiento de las señales de transducción implicadas en la activación de estas células es imprescindible para la obtención de nuevos tratamientos contra esta enfermedad. Por lo tanto, el objetivo de la presente tesis doctoral es estudiar las quinasas que modulan la respuesta y la supervivencia de estas células. En particular, el estudio se enfoca a las distintas isofomas de la PKC presentes en los dos modelos celulares, a la aurora quinasa y a la relación con las tirosina quinasas.

4. Publicaciones

4.1. Presentación.

En este apartado se muestran los resultados obtenidos en la presente tesis doctoral organizados en tres secciones con un total de seis artículos. Cada una de las secciones hace referencia a un bloque de resultados. De esta forma en la primera sección se incluye la puesta a punto de varias técnicas utilizadas para estudiar la activación, viabilidad celular y expresión proteica, así como el estudio comparativo de la expresión y traslocación de la PKC en las líneas celulares de mastocitos tumorales HMC-1^{560} y HMC-1^{560,816}. La segunda sección se centra en el estudio de la relación de la PKC con la inhibición de tirosina quinasas en las células HMC-1^{560} y HMC-1^{560,816} y por último, la tercera sección incluye el estudio de la inhibición de la proteína aurora quinasa como mecanismo eficaz frente a la proliferación de la línea celular HMC-1^{560,816}. A continuación se detallan cada una de las secciones:

4.2. Sección I: Estudio de los efectos de la modulación de la PKC sobre diversas señales intracelulares esenciales para la activación de los mastocitos HMC-1^{560} y HMC-1^{560,816}.

En esta sección se incluye la puesta a punto de varias técnicas de laboratorio empleadas en el estudio de la activación celular, de la viabilidad y de la expresión proteica. La yesotoxina (YTX) es un compuesto marino producido por dinoflagelados de los géneros *Gonyaulax*, *Protoceratium* y *Lingulodinium* con un gran potencial terapéutico como antiasmático y antialérgico [266,267]. Este compuesto modula distintas rutas de transducción en células inmunitarias y por lo tanto es una herramienta muy útil para poner a punto técnicas de investigación. Estas técnicas son necesarias para el estudio de los mecanismos moleculares relacionados con la activación de las células HMC-1^{560} y HMC-1^{560,816}. Los mastocitos poseen un papel fundamental en el sistema inmunitario ya que son las células encargadas de liberar al medio extracelular los mediadores inflamatorios, función que comparten con otro tipo celular: los basófilos. Para que este proceso de liberación tenga lugar es necesaria la activación previa de la célula, en la que juega un papel esencial la proteína PKC, además del ión Ca^{2+}, el cAMP y el pH_i. La modulación del pH_i y sus efectos en la activación mastocitaria han sido ampliamente estudiados en diversos trabajos científicos [181,268,269]. De esta forma, se ha descrito que la alcalinización del medio intracelular, añadiendo cloruro amónico a la célula, estimula la activación celular y la liberación de mediadores inflamatorios

como la histamina. La PKC está relacionada con este proceso de exocitosis, ya que la activación de esta proteína (mediante el tratamiento con PMA) provoca un incremento del pH_i [185]. Por el contrario, la inhibición de la PKC causa un descenso de la liberación de histamina inducida por cloruro amónico [270]. El ión Ca^{2+} posee un papel fundamental en el proceso de exocitosis mastocitaria, ya que un aumento de los niveles citosólicos de Ca^{2+} provoca la activación de la célula y liberación de histamina. Este efecto pudo comprobarse con el ionóforo del Ca^{2+}, ionomicina, y con la inhibidora de la ATPasa de Ca^{2+} del retículo endoplasmático, thapsigargina, ya que ambos compuestos provocan un aumento de Ca^{2+} citosólico y desencadenan la salida de histamina hacia el medio extracelular [186]. Al igual que ocurría en la exocitosis inducida por alcalinización, la PKC modula también la desgranulación estimulada por el Ca^{2+}. De esta forma se ha descrito que la activación de la PKC aumenta la liberación de histamina inducida por thapsigargina [186]. Además, la PKC regula la salida de Ca^{2+} de los reservorios intracelulares así como la entrada de Ca^{2+} desde el exterior, por lo que la relación PKC-Ca^{2+} se considera esencial en el proceso de exocitosis mastocitaria [188-191]. En lo que se refiere a los mastocitos HMC-1, estudios anteriores han descrito que la activación de la PKC aumenta la liberación de histamina inducida por cloruro amónico en ambas líneas celulares $HMC\text{-}1^{560}$ y $HMC\text{-}1^{560,816}$. Sin embargo, a diferencia de los mastocitos no tumorales, en células HMC-1 la inhibición de la PKC no afecta negativamente a la liberación de histamina [194]. Además, se ha comprobado que la activación de la PKC provoca un aumento de la liberación de histamina inducida por ionomicina en células $HMC\text{-}1^{560}$. A fin de obtener un estudio comparativo entre las dos líneas celulares, en el presente trabajo se ha estudiado el efecto de la modulación de la PKC sobre $HMC\text{-}1^{560}$ y $HMC\text{-}1^{560,816}$.

A esta sección corresponden las publicaciones:

I.1. *Role of yessotoxin in calcium and cAMP-crosstalks in primary and K-562 human lymphocytes: The effect is mediated by anchor kinase A mitocondrial proteins.*

I.2. *C-kit mutations and PKC crosstalks: PKC translocates to the nucleous only in cells* $HMC\text{-}1^{560,816}$.

I.1. Papel de la yesotoxina en la interacción del calcio y AMPc en linfocitos primarios y K-562: el efecto de la yesotoxina está mediado por las proteínas de anclaje de la quinasa A.

Resumen

La YTX es una toxina marina poliéter descrita como activadora de las fosfodiesterasas, proteínas encargadas de la hidrólisis del AMPc, en linfocitos primarios humanos [271]. El efecto de la YTX sobre los niveles de AMPc citosólicos es dependiente de Ca^{2+}, de tal forma que disminuye la liberación de AMPc en condiciones normales, mientras que posee el efecto contrario en un medio sin Ca^{2+} [271]. En el presente trabajo se ha estudiado el efecto de la YTX sobre linfocitos normales y sobre la línea celular de linfocitos tumorales K-562. Sorprendentemente, los resultados obtenidos en la línea celular K-562 difieren completamente con los descritos en linfocitos normales, ya que la YTX provoca un incremento de los niveles de AMPc. Por otro lado también se ha realizado el estudio del efecto de la YTX sobre la viabilidad celular de linfocitos normales y tumorales. Los resultados en ambos modelos celulares son diferentes, ya que la YTX no posee efecto citotóxico sobre linfocitos primarios, mientras que tiene la capacidad de producir muerte celular en linfocitos K-562. Además, en el presente trabajo se observa que la YTX induce el vaciamiento de los reservorios intracelulares de Ca^{2+} acompañado de la entrada de Ca^{2+} desde el medio extracelular en células K-562, efecto contrario al observado en linfocitos normales [272]. El vaciamiento de los reservorios intracelulares de Ca^{2+} causado por la YTX está inhibido por el análogo del AMPc, dibutiril cAMP, por el rolipram, inhibidor de la fosfodiesterasa-4, así como por el inhibidor de la proteína quinasa A, H89, y por la carbonil-cianida-p-trifluorometoxifenilhidrazona (FCCP), compuesto que desacopla la fosforilación oxidativa mitocondrial. Sin embargo, la entrada de Ca^{2+} procedente del medio extracelular tan solo es inhibida por el FCCP. Este hecho evidencia una clara relación entre el mecanismo de acción de la YTX y la mitocondria, confirmada tras el estudio de los niveles de la AKAP149, proteína encargada de vehiculizar la PKA y las fosfodiesterasas hacia la mitocondria. Una vez más efecto de la YTX sobre la AKAP149 en linfocitos normales y tumorales es diferente. Por un lado, en linfocitos normales, la YTX aumenta los niveles de AKAP149, que acompañado de un descenso de los niveles de AMPc y por lo tanto de la activación de las fosfodiesterasas conduce a la supervivencia celular. Sin embargo, en linfocitos tumorales, se produce un descenso de los niveles de AKAP149 tras la adición de

YTX, lo que junto con un aumento de los niveles de AMPc conduce a la muerte celular. Este estudio muestra por tanto por vez primera que el mecanismo de acción de la YTX está relacionado con la mitocondria y que difiere significativamente entre linfocitos normales y tumorales.

ARTICLE
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Role of Yessotoxin in Calcium and cAMP-Crosstalks in Primary and K-562 Human Lymphocytes: The Effect Is Mediated by Anchor Kinase A Mitochondrial Proteins

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ABSTRACT

Yessotoxin (YTX) is a marine polyether toxin previously described as a phosphodiesterase (PDE) activator in fresh human lymphocytes. This toxin induces a decrease of adenosine 3′,5′-cyclic monophosphate (cAMP) levels in fresh human lymphocytes in a medium with calcium (Ca^{2+}), whereas the contrary effect has been observed in a Ca^{2+}-free medium. In the present article, the effect of YTX in K-562 lymphocytes cell line has been analysed. Surprisingly, results obtained in K-562 cell line are completely opposite than in fresh human lymphocytes, since in K-562 cells YTX induces an increase of cAMP levels. YTX cytotoxicity was also studied in both K-562 cell line and fresh human lymphocytes. Results demonstrate that YTX does not modify fresh human lymphocytes viability, whereas in K-562 cells, YTX has a highly cytotoxic effect. It has been described in a previous study that YTX induces a small cytosolic Ca^{2+} increase in fresh human lymphocytes but no effect was observed on Ca^{2+} pools depletion in these cells. However, our results show that, in K-562 cells, YTX has no effect on cytosolic Ca^{2+} levels in a medium with Ca^{2+} and induces an increase on Ca^{2+} pools depletion followed by a Ca^{2+} influx. As far as Ca^{2+} modulation is concerned these results demonstrate that YTX has a clear opposite effect in tumoural and fresh human lymphocytes. In addition, intracellular Ca^{2+} reservoirs affected by YTX are different than thapsigargin-sensible pools. Furthermore, YTX-dependent Ca^{2+} pools depletion was abolished by cAMP analogue (dibutyryl cAMP), phosphodiesterase-4 (PDE4) inhibitor (rolipram), protein kinase A inhibitor (H89) and oxidative phosphorylation uncoupler carbonyl cyanide p-(trifluoromethoxy) (FCCP) treatments. This evidences the crosstalks between Ca^{2+}, YTX and cAMP pathways. Also, results obtain demonstrate that YTX-dependent Ca^{2+} influx was only abolished by FCCP pre-treatment, which indicates a link between YTX and mitochondria in K-562 cell line. Cytosolic expression of A-kinase anchor proteins (AKAPs), the proteins which integrates phosphodiesterases (PDEs) and PKA to the mitochondria, was determined in both cell models. On the one hand, in human fresh lymphocytes, YTX increases AKAP149 cytosolic expression. This fact is accompanied with a decrease in cAMP levels, and therefore PDEs activation, which finally leads to cell survival. On the other hand, in tumoural lymphocytes, YTX has an opposite effect since decreases AKAP149 cytosolic expression and increase cAMP levels which leads to cell death. This is the first time that YTX and mitochondrial AKAPs proteins relationship is characterised. J. Cell. Biochem. 113: 3752–3761, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: YESSOTOXIN; CYTOSOLIC Ca^{2+}; PDEs; AKAPs

Yessotoxins (YTXs) are phycotoxins produced by microalgae from the group of dinoflagellates *Protoceratium reticulatum* [Satake et al., 1997] and *Gonyaulax polyedra*. When environmental conditions promote the growth of these species, the toxins accumulate in edible tissues of filter-feeding shellfish exposed to these dinoflagelates and may possibly be ingested by humans through seafood consumption [Dell'Ovo et al., 2008]. YTXs was originally isolated from the digestive gland of scallops *Patinopecten yessoensis* contaminated with diarrheic shellfish poisoning (DSP) toxins [Murata et al., 1987]. Since YTXs are non-diarrheagenic, they have been classified and separately regulated from DSP toxins [C. REGULATION, 2011]. Previous studies demonstrate that the mechanism of action of YTXs is not related with phosphatases inhibition, as is the case of OA, from DSP toxins group [Draisci, 2000]. YTXs activates phoshodiesterases (PDEs) and consequently a decrease in adenosine 3′,5′-cyclic monophosphate (cAMP) levels occurs in human lymphocytes [Alfonso et al., 2003]. This study reveals the PDE system as the intracellular target to YTX. In vitro

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studies have shown that YTX further increases the rise of cytosolic Ca^{2+} induced by the marine toxin maitotoxin, throughout a mechanism not sensitive to 1-(β-(3-(4-methoxyphenil)propoxyl)-4-methoxypheyl)-1*H*-imidazole hydrochloride (SKF 96365), a well-known store-operated Ca^{2+} channel (SOC) inhibitor [de la Rosa et al., 2001b]. In addition, YTX produces a Ca^{2+} influx throughout nifedipine and SKF 96365-sensitive channels in human lymphocytes [de la Rosa et al., 2001a].

Apoptosis, or programmed cell death, was described by Kerr et al. in 1972 like a inherently programmed phenomenon with two discrete stages. In the first stage nuclear and cytoplasmic condensation have been occurred. The second stage comprises the apoptotic bodies degradation by lysosomal enzymes derived from the ingesting cells [Kerr et al., 1972]. Mitochondrial apoptosis-inducing factor (AIF) is a molecule associated with programmed cell death and is localised into mitochondria and released in response to death stimuli [Joza et al., 2001]. The permeability transition pore (PTP) is a structure formed by inner membrane proteins, such as the adenine nucleotide translocator, and outer membrane proteins, such as porins. This large conductance channel opens in some apoptosis scenarios, accompanied with mitochondrial inner transmembrane potential collapses [Zamzami and Kroemer, 2001]. Therefore, mitochondria play a crucial role in apoptosis development since this organelle is part of a mayor pathway leading to cell death. The effect of YTX in the mitochondrial membrane potential, mitochondrial morphology and mitochondrial PTP were studied in several articles. First, a decrease in the mitochondrial membrane potential associated with apoptotic changes were observed in YTX-culture cells (BE(2)-M17) [Leira et al., 2002]. In this study YTX and OA were used in BE(2)-M17 cell line and an apoptogenic activity of YTX was described, indicating a lower potency of YTX when compared with OA. In 2003, cytoplasmic protrusions of cardiac muscle cells into pericapillary space and packing of rounded mitochondria in mice treated with YTX, homoYTX a 45-hydroxy-homoYTX have been described. Moreover, any death or signs of toxicity were observed after oral treatment with YTX and its derivatives, while, ultrastructural myocardiocyte alterations like mitochondria assemblage were founded in 45-OH-homoYTX treated cells [Tubaro et al., 2003]. Successively, another study confirmed that YTX opens the PTP of the inner mitochondrial membrane and this effect requires the presence of permissive levels of Ca^{2+} [Bianchi et al., 2004]. Afterwards, a study has shown that apoptotic cell death caspase-dependent was induced by YTX in L6 and BC3H1 myoblast cell lines and considered mitochondria as the major target of YTX-induced apoptosis [Korsnes et al., 2006]. In the present article, the connection between YTX effect on PDEs and mitochondria was studied, with the aim to solve the intriguing biological effect of this elusive group of compounds.

MATERIALS AND METHODS

REAGENTS AND SOLUTIONS

YTX was obtained from CIFGA Laboratories (Lugo, Spain). Hydrochloric acid (HCl) and ethanol absolute were from Panreac Quimica (Barcelona, Spain). Fura-2 acetoxymethyl ester (FURA-2 AM) was purchased from Molecular Probes (Leiden, The Netherlands). Thapsigargin, ionomycin and H-89 were from Alexis Corporation (Laufelfingen, Switzerland). Bovine serum albumin (BSA), $CaCl_2$, dibutyryl AMPc, Carbonyl cyanide p-(trifluoromethoxy) (FCCP) and anti-β-tubulin were from Sigma-Aldrich (Madrid, Spain). Rolipram was from Tocris (Bristol, UK). Anti-AKAP149 was from Santa Cruz Biotechnology (CA). Anti-mouse IgG, cAMP Biotrak Enzymeimmnunoassay (EIA) System and Percoll™ were purchased from GE Healthcare (Barcelona, Spain). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Temecula). Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were purchased from BioRad® (Barcelona, Spain). Protease Inhibitor Complete Tablets and Phosphatase Inhibitor Cocktail Tablets were from Roche (Spain).

Physiological saline solution (Umbreit) composition was (in mM): Na^{+} 142.3; K^{+} 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^{-} 126.2; HCO_3^{-} 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2; glucose 1 g/L was added to the medium giving an osmotic pressure of 290 ± 10 mOsm/kg of H_2O and pH was adjusted to 7.2 with HCl 0.1 N. Ca^{2+}-free solution was made by omitting Ca^{2+} from Umbreit. PBS used for lymphocyte purification consisted of NaCl 137 mM; Na_2HPO_4 8.2 mM; KH_2PO_4 1.5 mM; KCl 3.2 mM and EDTA 2 mM. pH was adjusted to 7.2 with NaOH.

CELL CULTURE

K-562 cell line was purchased from NCI's and maintained in RPMI 1640 medium (Gibco, Invitrogen, Spain) supplemented with 10% foetal bovine serum (FBS) (Gibco) and 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco) at 37°C in a humidified 5% CO_2 atmosphere.

LYMPHOCYTE ISOLATION AND PURIFICATION

Peripheral blood lymphocytes were isolated from freshly human blood from healthy donors. Purification of cells was carried out by means of centrifugation over a 57.5% isotonic Percoll bed. Blood was collected in EDTA-containing tubes and then diluted 1:1 with PBS + EDTA, 4 ml of diluted blood was placed over 3 ml of Percoll, and centrifugation (3,000 r.p.m.) was carried out at room temperature for 25 min. After centrifugation, lymphocytes appearing in the interface were washed twice with PBS + EDTA and finally resuspended in umbreit solution.

cAMP DETERMINATION

Fresh human lymphocytes and K-562 cell line were incubated with YTX 1 and 10 μM at 37°C for 10 min in a final volume of 100 μL. Nine hundred microlitres of 86% ethanol and 1 N HCl (99:1) were added and the tubes were immediately submerged in liquid nitrogen. Samples were stored at −80°C until cAMP determination. cAMP was measured using the protocol described by Amersham for measurement of cAMP by acetylation EIA procedure. Previously samples were thawed and dried by centrifugal evaporation.

CELL VIABILITY

After exposure to 30 nM of YTX during 24 or 48 h in culture medium, cell were centrifuged (1,500 r.p.m., 5 min, 4°C). The pellets were resuspended in saline solution with MTT (250 μg/ml) and then incubated at 37°C during 30 min in darkness. After washing twice with saline solution cells were sonicated for 1 min. The coloured

formazan salt was measured at 595 nm in a spectrophotometer plate reader.

MEASUREMENT OF CYTOSOLIC FREE Ca^{2+}

For Ca^{2+} measurement cells were centrifuged (1,500 r.p.m., 5 min, 4°C) and then washed twice with umbreit containing 0.1% BSA (1,500 r.p.m., 5 min, 4°C). Cells were loaded with FURA-2 AM (0.2 μM) in a bath at 37°C, for 10 min. After this time, loaded cells were washed with saline solution (1,500 r.p.m., 10 min, 4°C). Cells were attached to glass coverslips treated with poly-L-lysine, and these were inserted into a thermostated chamber (Life Sciences Resources, UK). Cells were viewed using a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40× – immersion UV – Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution to the chamber. Cytosolic Ca^{2+} concentrations were obtained from the images collected by fluorescence equipment (Life Sciences Resources). The light source was a 175 W xenon lamp, and the used wavelengths were selected with filters. For FURA-2 AM, the excitation wavelengths were 340 and 380 nm, with emission at 505 nm. The calibration of the fluorescence values versus intracellular Ca^{2+} was made according to the method of Grynkiewicz.

WESTERN BLOTTING ANALYSIS

Cells were incubated with YTX (for 10 min) and then were centrifuged and washed twice with saline solution. Cells were resuspended in a lysis buffer with the following composition: 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM DTT, 2.5 mM PMSF, 40 mg/ml aprotinin, 4 mg/ml leupeptin, 5 mM NaF, 1 mM Na_3VO_4, 1 mg/ml pepstatin A, 1 mg/ml bezamidine, 1X Complete Protease Inhibitor and 1X Phosphatase Inhibitor Cocktail. Bradford assay was the method used to know sample protein concentration and BSA was used as protein standard. Samples were blotted to PVDF membrane by reduced SDS–PAGE. To determine the protein size and also to monitor the progress of electrophoretic runs, Precision Plus Protein™ Standards Kaleidoscope™ molecular weight marker was used. After blockage with 5% non-fat dry milk the membranes were incubated 10 min with anti-AKAP149 (1:1,000), then were washed three times with washing buffer (PBS + 0.1% Tween) and incubated for 10 min with secondary peroxidase-labelled antibody. After three washes chemiluminescence was visualised with SuperSignal® West Pico (Pierce). Relative protein expression was calculated in relation to β-tubulin expression for each experiment. Experiments were carried out three times.

STATISTICAL ANALYSIS

$(Ca^{2+})_i$ values of all cells observed in each experiment were averaged. All the experiments were carried out at least three times by duplicate. A Student's *t*-test was used to examine statistical significance, assumed for $P < 0.05$. Results were expressed as the means ± SEM.

RESULTS

Figure 1A shows that YTX induces a statistical decrease on cAMP levels in fresh human lymphocytes in a medium with Ca^{2+} (cAMP levels decreased around 6 fmol with 1 μM YTX and 14 fmol with 10 μM YTX), whereas in a Ca^{2+}-free medium the toxin effect is opposite and thus, cAMP levels have been increased. cAMP levels were even determined in K-562 cell line, as Figure 1B shows, YTX statistically increases cAMP levels from 19 to 23 fmol or 25 fmol

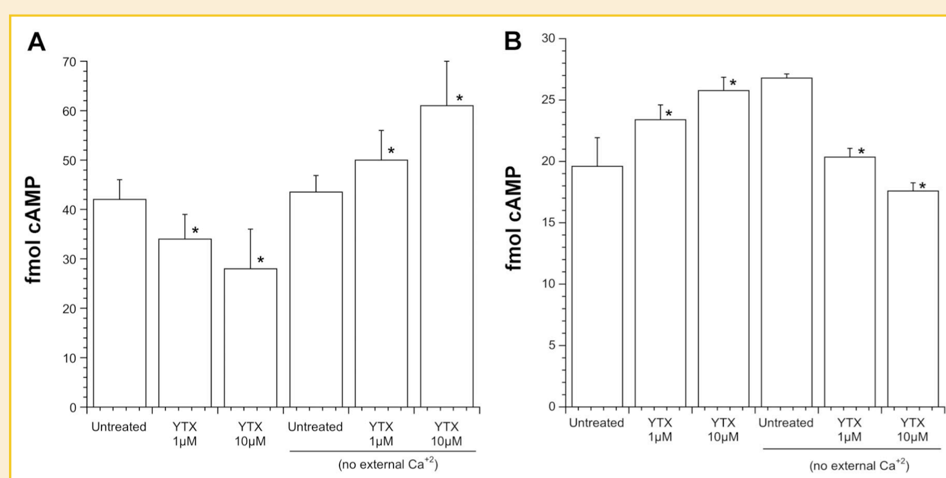

Fig. 1. Effect of YTX on cAMP levels in fresh human lymphocytes and K-562 cells. Femtomoles of cAMP in fresh human lymphocytes (A) and K-562 cell line (B) in the presence of 1 and 10 μM YTX during 10 min (37°C). Mean ± SEM of three experiments. *Significant differences between untreated and YTX-treated cells.

in a medium with Ca^{2+} when 1 μM or 10 μM YTX were used, respectively. Surprisingly, in a Ca^{2+}-free medium, both YTX concentrations have an opposite effect in cAMP levels. Specifically, YTX treatment induces a decrease of cAMP levels from 26 to 20 fmol (with the lower YTX concentration) and 17 fmol (with the highest YTX concentration). Either in fresh human lymphocytes or in K-562 cell line YTX effect is dose-dependent, since cAMP levels modulation is stronger when the higher YTX concentration is used. Since YTX effect on cAMP levels and thus in PDEs activity is different in primary and tumoural lymphocytes, the next step was study if YTX has also different effect on cell viability. To this experiment, a lower toxin concentration (30 nM) was used and cells were incubated during 24 and 48 h. As Figure 2A shows, YTX treatment did not affect fresh human lymphocytes viability after 24 and 48 h treatment. However, in K-562 cell line, cell viability decreases around 20% when the toxin is incubated during 24 h (Fig. 2B). A higher YTX cytotoxic effect was observed when cells were 48 h incubated, in this case the decrease on cell viability due to YTX is around 80%.

To clarify this opposite behaviour and in order to compare with YTX results obtained in fresh human lymphocytes, cytosolic Ca^{2+} levels were studied. Three different toxin concentrations (0.2, 0.5 and 1 μM) were used to analyse a dose–response effect in the cytosolic Ca^{2+} levels. As Figure 3A shows, in a medium with Ca^{2+}, no effects were observed when YTX was added to the cells since intracellular Ca^{2+} levels remained at 100 nM. This effect was observed at all concentrations used. However, in a Ca^{2+}-free medium (Fig. 3B) YTX induces a significant increase on Ca^{2+} pools depletion (Fig. 3C). This Ca^{2+} pools depletion induces an increase on cytosolic Ca^{2+} levels (from 70 nM on untreated cells to 100 nM with YTX 0.2 μM, 120 nM with YTX 0.5 μM and 150 nM with YTX 1 μM). When Ca^{2+} was restored to the medium, the influx from extracellular medium is similar for the three toxin concentrations reached values of 250 nM (Fig. 3B), this Ca^{2+} profile seems to follow the well known capacitative Ca^{2+} influx [Putney, 1986, 1990]. This theory point that Ca^{2+} release from internal stores produces a Ca^{2+} influx from the extracellular medium.

Then, the intracellular pools empty by YTX were studied. First, the effect on the endoplasmatic reticulum was checked by using thapsigargin (a sarcoplasmic/endoplasmic reticulum calcium-dependent ATPase (SERCA) inhibitor). Thapsigargin is a sesquiterpene lactone that effective and irreversibly inhibits the Ca^{2+} SERCA pumps and thus elevates cytosolic Ca^{2+} [Thastrup et al., 1989; Huber et al., 2000]. The effect of co-treatment with thapsigargin and YTX were analysed and results are shown in Figure 4. When thapsigargin was added to the medium an increase on cytosolic Ca^{2+} levels (from 50 to 120 nM) was observed (Fig. 4B). YTX addition induces a summatory effect with thapsigargin and Ca^{2+} levels go from 120 to 300 nM. As Figure 4C shows, YTX addition did not affect Ca^{2+} influx induced by thapsigargin even the decrease observed after 2 min does not appear when YTX is added to the medium.

Next, the relationship between YTX-induced Ca^{2+} pools depletion and cAMP pathway was studied. For this reason, dibutyryl cAMP (cAMP analogue) was incubated in presence of YTX (Fig. 5A). Our results show that calcium pool depletion induced by YTX was abolished when cells were pre-incubated with dibutyryl cAMP (Fig. 5B). As it was previously described YTX increase PDE activity, for this reason, the next compound to be tested was rolipram, a PDE4 inhibitor [Harada et al., 2006; Hirose et al., 2008]. Results obtained with rolipram and rolipram + YTX treated cells are shown in Figure 5C. YTX-induced depletion of calcium pools disappeared when cells were previously treated with rolipram (Fig. 5D). cAMP-dependent protein kinase, protein kinase A (PKA), is intimately related with cAMP and with PDE [McSorley et al., 2006; Rogne et al., 2009]. For this reason, we tested the compound H89, a PKA inhibitor. Figure 5E shows cytosolic Ca^{2+} levels when K-562 cells were treated with H89 and then with YTX. It is also observed that treatment with H89 alone did not affect Ca^{2+} pools depletion

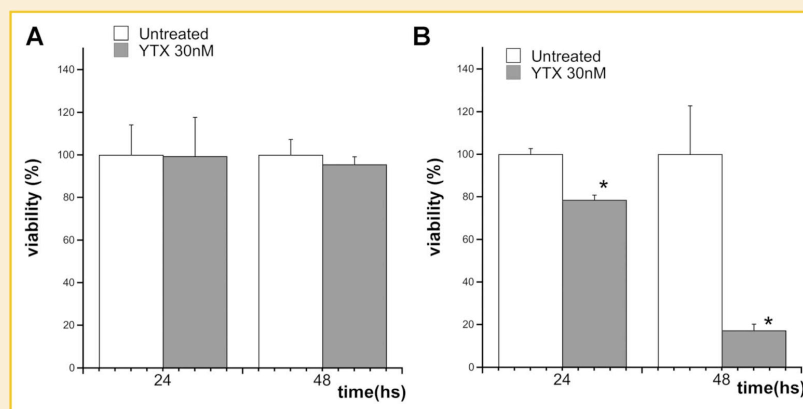

Fig. 2. Effect of YTX on cell viability in human lymphocytes and K-562 cells. Cell viability was tested by MTT test after 24 and 48 h of incubation at 37°C in culture medium with 30 nM of YTX. A: Results obtain in fresh human lymphocytes. B: Results obtain in K-562 cell line.

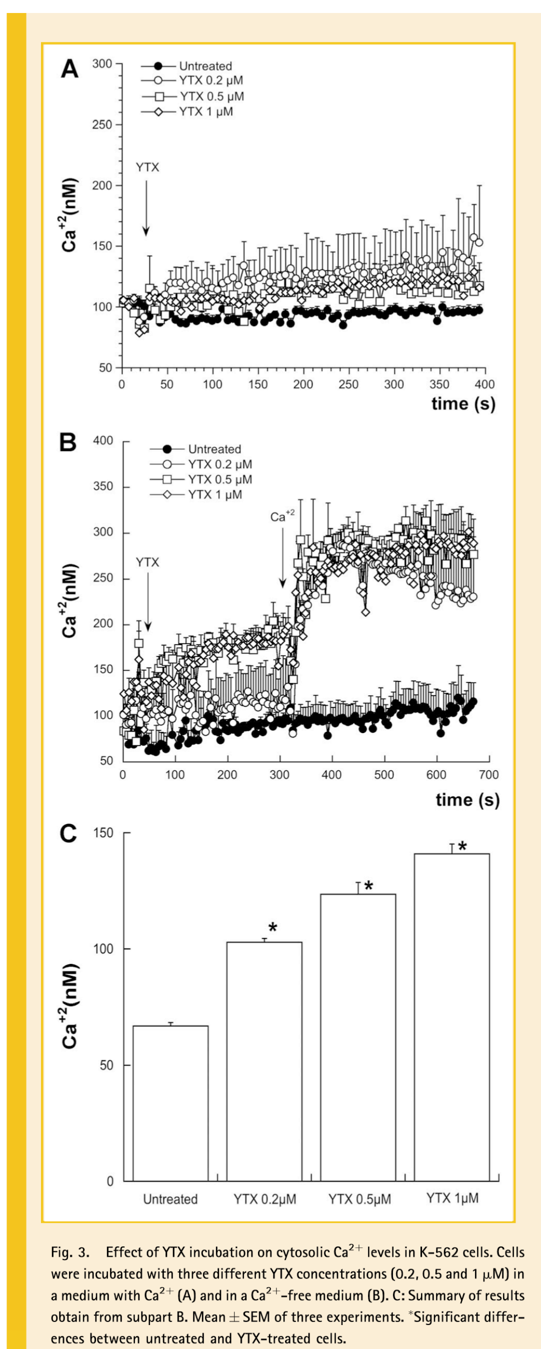

Fig. 3. Effect of YTX incubation on cytosolic Ca^{2+} levels in K-562 cells. Cells were incubated with three different YTX concentrations (0.2, 0.5 and 1 μM) in a medium with Ca^{2+} (A) and in a Ca^{2+}-free medium (B). C: Summary of results obtain from subpart B. Mean $\pm$ SEM of three experiments. *Significant differences between untreated and YTX-treated cells.

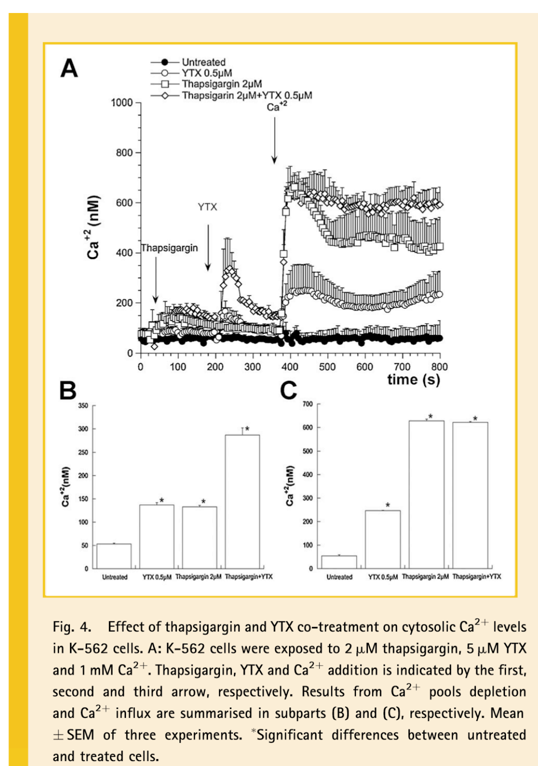

Fig. 4. Effect of thapsigargin and YTX co-treatment on cytosolic Ca^{2+} levels in K-562 cells. A: K-562 cells were exposed to 2 μM thapsigargin, 5 μM YTX and 1 mM Ca^{2+}. Thapsigargin, YTX and Ca^{2+} addition is indicated by the first, second and third arrow, respectively. Results from Ca^{2+} pools depletion and Ca^{2+} influx are summarised in subparts (B) and (C), respectively. Mean $\pm$ SEM of three experiments. *Significant differences between untreated and treated cells.

(Fig. 5F) [Gratschev et al., 2004], whereas co-treatment with PKA inhibitor and YTX induce an inhibition of YTX-induced pools depletion. Therefore, it can be stated that the cAMP pathway seems to be related with YTX effect on cytosolic Ca^{2+}.

The effect of YTX in another important Ca^{2+} reservoir, the mitochondria, was studied. Taking into account the previously described effects of YTX on mitochondria, we studied the oxidative phosphorylation uncoupler effect of carbonyl cyanide p-(trifluoromethoxy) (FCCP) (5 μM) before and after YTX treatment (Fig. 6A and D, respectively). Surprisingly, when oxidative phosphorylation is uncoupled, YTX-induced Ca^{2+} pools depletion did disappear (Fig. 6B), but only if FCCP is added before YTX, if FCCP was added after YTX, toxin effect predominates and FCCP did not change cytosolic Ca^{2+} levels induced by YTX (Fig. 6D,E). Moreover, Ca^{2+} influx induced by Ca^{2+} pools depletion was abolished after FCCP pre-treatment (Fig. 6C). This suppression did not occur when YTX is incubated before oxidative phosphorylation inhibitor (Fig. 6F).

These results indicate that the effect of YTX on Ca^{2+} pools depletion, the cAMP pathway and mitochondrial reservoirs are functionally related. Anchor kinase A proteins (AKAPs) are the proteins that integrate cAMP and mitochondria. PKA and PDE4 are anchored by AKAP149 to the mitochondria. Moreover, AKAP149 and PKA junction is crucial for cell survival. Cytosolic expression of AKAP149 protein was studied in presence of YTX. K-562 cells and fresh human lymphocytes were incubated during 10 min in presence of 5 μM YTX. In tumoural lymphocytes YTX induces a statistical decrease on AKAP149 expression from 1.15 to 0.4 fmol (Fig. 7A). In fresh human lymphocytes YTX has a striking opposite effect

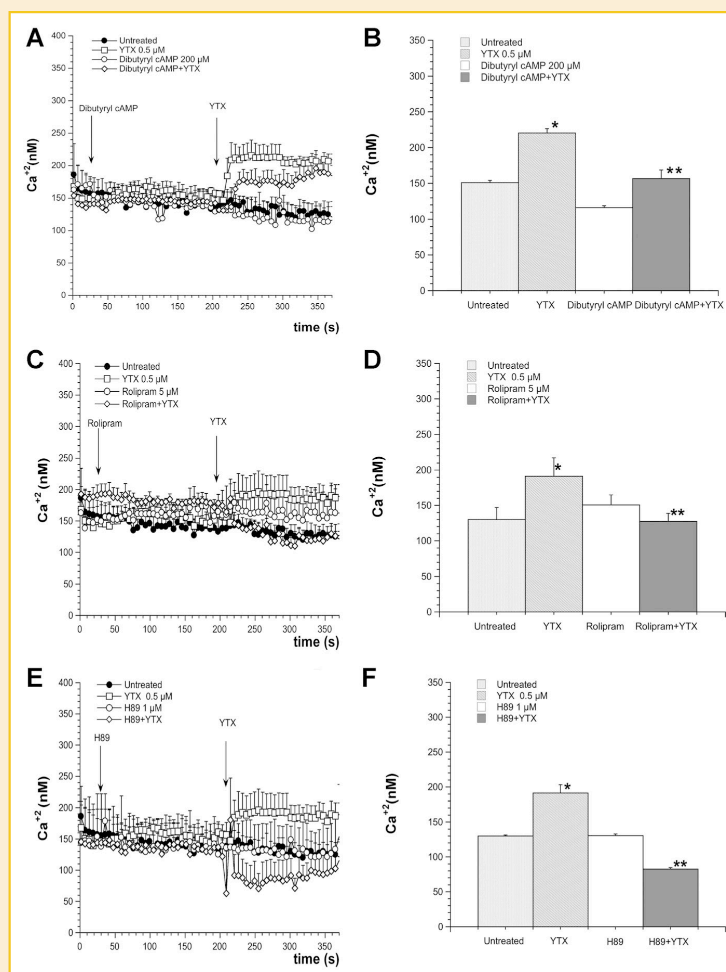

Fig. 5. Effect of dibutyryl cAMP, rolipram and H89 treatments on cytosolic Ca^{2+} levels in K-562 cells treated with YTX. Cells were treated first with 200 μM dibutyryl cAMP (A), 5 μM rolipram (C), 1 μM H89 (E) and afterwards incubated with 5 μM YTX. (B), (E) and (F) summarise results shown in (A), (C) and (E) from Ca^{2+}-pools depletion. Mean ± SEM of three experiments. (*) Significant differences between untreated and treated cells with the different compounds. **Significant differences between co-treated cells and YTX alone-treated cells.

(Fig. 7D), since toxin induces a significant increase on cytosolic AKAP149 levels from 1.1 to 2.4 fmol. To study the relationship between PDE activity and AKAPs levels that have been modulated by YTX, the PDE4 inhibitor, rolipram, was incubated in presence of YTX. Figure 8A shows that PDEs inhibition (treatment with 30 μM rolipram) induces a statistical significant decrease on cytosolic AKAP149 expression, this decrease being lower than that induced by YTX alone. This effect was observed also when cells were treated with rolipram plus YTX. These results support that the effect of YTX is related to AKAP149 protein, and thus it can be concluded that YTX activity pathway is mediated by AKAP149.

DISCUSSION

YTX effect on PDEs pathway has been described in a previous study in which have been determined that the negative effect of YTX on cAMP levels in fresh human lymphocytes in a medium with Ca^{2+} is mediated by an increase on PDE activity [Alfonso et al., 2003]. However, in a Ca^{2+}-free medium YTX effect is opposite and increases cAMP levels. In the present study, K-562 cell line was used to determine YTX effect on cAMP levels in order to known if YTX has the same mechanistic effect in fresh and tumoural lymphocytes. As our results shown, YTX effect in K-562 cell line is completely

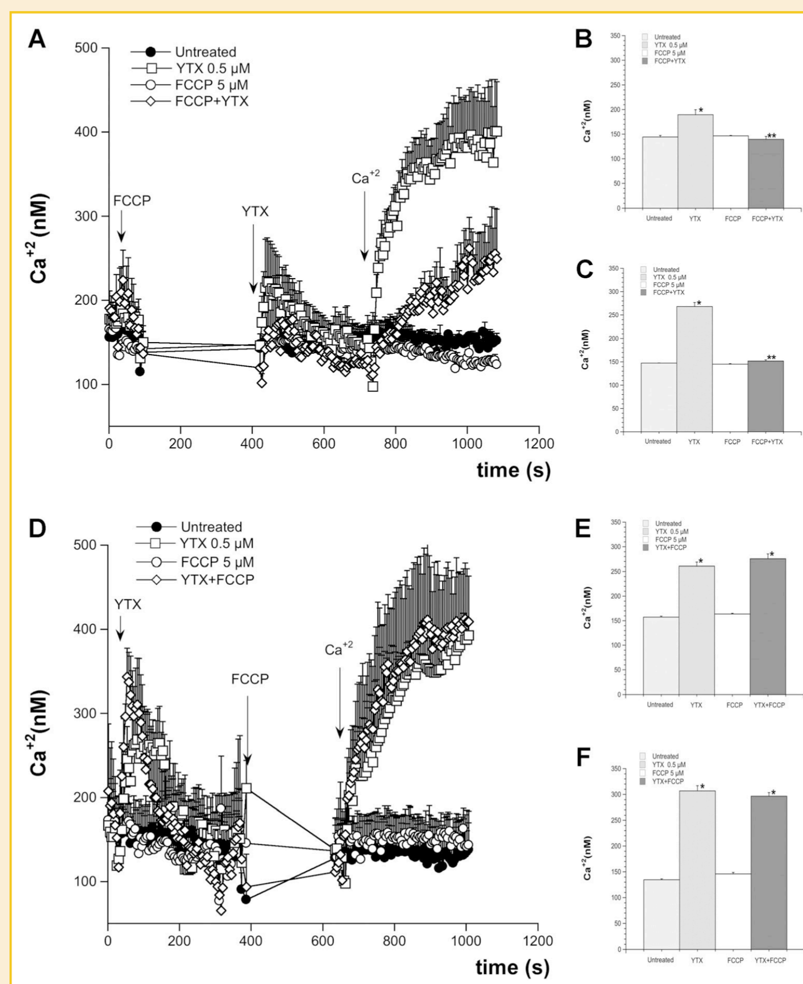

Fig. 6. Effects of FCCP and YTX co-treatments on cytosolic Ca^{2+} levels in K-562 cells. Cytosolic Ca^{2+}profile of cells that were incubated with FCCP 5 μM before (A) and after (D) 5 μM YTX. B,E: Summary of results shown in (A) and (D) from Ca^{2+}-pools depletion. C,F: Summary of results shown in (A) and (D) from extracellular Ca^{2+}-influx. Mean ± SEM of three experiments. *Significant differences between untreated and treated cells with FCCP and YTX. **Significant differences between YTX-treated cells and FCCP + YTX-treated cells.

opposite to that in fresh human lymphocytes, because YTX induces an increase on cAMP levels in a medium with Ca^{2+} whereas in a Ca^{2+}-free medium cAMP levels decrease. It has been previously described that YTX induces an increase of cAMP levels followed by a strong decrease, which results at the end in a significant decrease on cAMP levels related with PDEs activation [Alfonso et al., 2003]. However, in the present study, the increase of cAMP levels due to YTX effect is not accompanied with the subsequently decrease on K-562 cell line. Therefore, it seems that YTX effect is mediated by PDEs activation in fresh human lymphocytes, while tumoural lymphocytes toxin effect is mediated by this enzymes inhibition. Cytotoxic effect of YTX has been studied in several cellular models. Previous studies in BE(2)-M17 neuroblastoma [Leira et al., 2002] and HeLa adenocarcinoma cell lines [Malaguti et al., 2002] demonstrate the apoptotic effect of YTX was induced by two different mechanisms: by changes in mitochondrial membrane potential in the first cell line and by caspase activation in the second. In this study we present data of YTX cytotoxicity in primary human lymphocytes and K-562 cell line. The results shown in this article demonstrate that YTX triggers a mechanism of cytotoxicity to

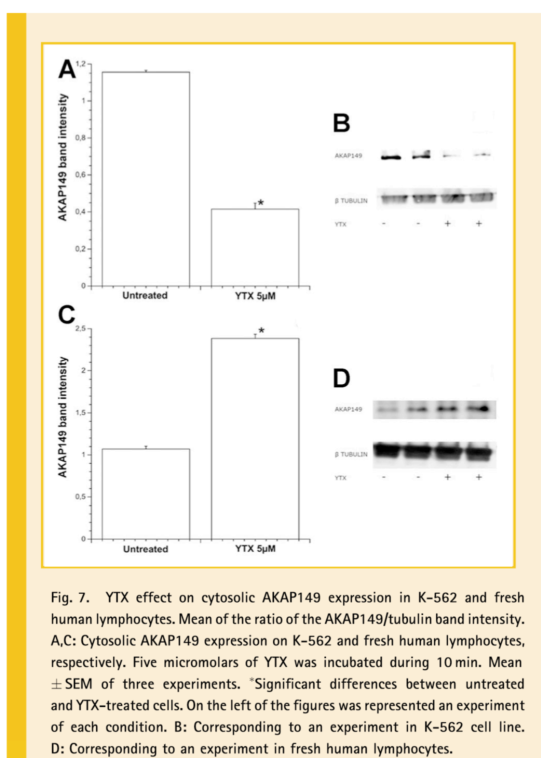

Fig. 7. YTX effect on cytosolic AKAP149 expression in K-562 and fresh human lymphocytes. Mean of the ratio of the AKAP149/tubulin band intensity. A,C: Cytosolic AKAP149 expression on K-562 and fresh human lymphocytes, respectively. Five micromolars of YTX was incubated during 10 min. Mean ± SEM of three experiments. *Significant differences between untreated and YTX-treated cells. On the left of the figures was represented an experiment of each condition. B: Corresponding to an experiment in K-562 cell line. D: Corresponding to an experiment in fresh human lymphocytes.

neoplastic K-562 cells with is totally opposite with regard to primary lymphocytes and the cAMP pathway, since fresh cells are YTX-resistant whereas the tumoural cell line is highly YTX-sensible.

As indicated previously, YTX effect on cAMP pathway is different in a medium with Ca^{2+} and in a Ca^{2+}-free medium. This result indicates that Ca^{2+} presence plays a pivotal role in YTX mechanism of action, and thus the possibility that YTX interacts with intracellular Ca^{2+} compensation systems, as happened in primary lymphocytes [de la Rosa et al., 2001a], has been taken into account. In this previous study, an increase on cytosolic Ca^{2+} levels was observed when YTX was added in a medium with Ca^{2+}. However, our results demonstrate that in K-562 cell line, YTX has no effect in this medium condition. As far as in a Ca^{2+}-free medium is concerned, YTX induces Ca^{2+} pools depletion in K-562 cell line, whereas this effect was not observed in primary lymphocytes [de la Rosa et al., 2001a]. Results in this article demonstrate that YTX effect on cytosolic Ca^{2+} levels in human lymphocytes is completely opposite than in K-562 cell line. Related to this it can be stated that Ca^{2+} reservoirs affected by YTX are different than thapsigargin-sensible pools. Our results clearly demonstrate that YTX-induced and thapsigargin-induced pools depletion have a summation effect when this two compounds were added to the medium. Besides, in this study the relationship between cAMP pathways and intracellular Ca^{2+} oscillations have been described, since the modulation of cAMP pathways (with dibutyryl cAMP, rolipram and H89 treatments) induces an abolition of YTX-dependent pools depletions. Taken into account that YTX is not acting through thapsigargin-sensible reservoirs and the several effects of YTX in the mitochondria [Leira et al., 2002; Tubaro et al., 2003; Bianchi et al., 2004], our hypothesis was that YTX must target its effect in the mitochondrial organelle. Results obtained with treatment with the oxidative phosphorylation uncoupler (FCCP) are in agreement with this hypothesis. In this way, the correct activity of mitochondria is necessary for Ca^{2+} pools depletion induced by YTX. According to this, when cells were firstly incubated with YTX, Ca^{2+} pools depletion was triggered and the subsequent treatment with FCCP did not show an effect on YTX activity.

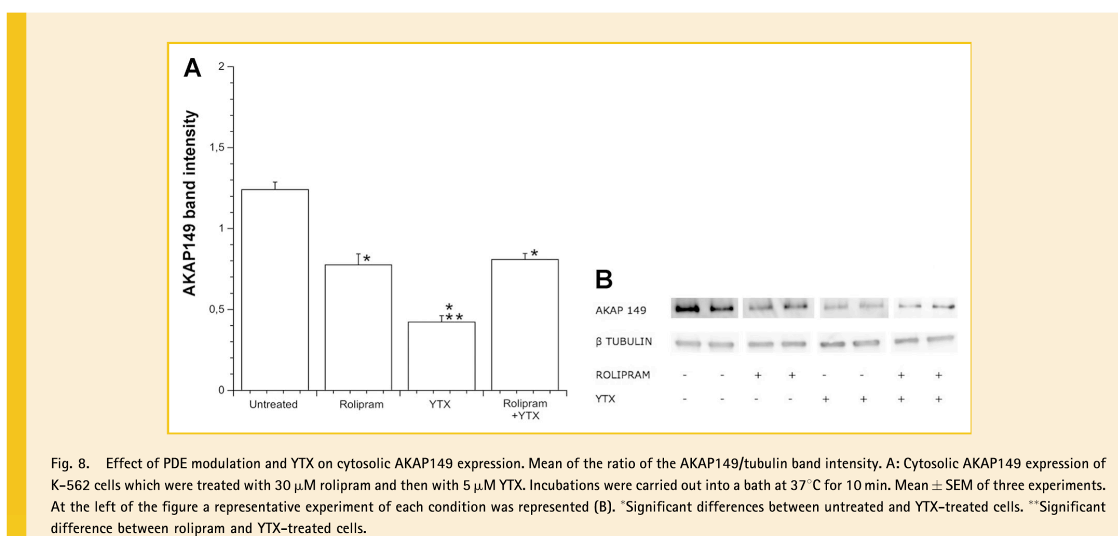

Fig. 8. Effect of PDE modulation and YTX on cytosolic AKAP149 expression. Mean of the ratio of the AKAP149/tubulin band intensity. A: Cytosolic AKAP149 expression of K-562 cells which were treated with 30 µM rolipram and then with 5 µM YTX. Incubations were carried out into a bath at 37°C for 10 min. Mean ± SEM of three experiments. At the left of the figure a representative experiment of each condition was represented (B). *Significant differences between untreated and YTX-treated cells. **Significant difference between rolipram and YTX-treated cells.

A-kinase anchor proteins (AKAPs) are the most important molecules in compartmentalisation of cAMP/PDE4 signalling [Livigni et al., 2006; Omori and Kotera, 2007]. There are several AKAPs families, as AKAP84, AKAP100, AKAP121 and the human homologue AKAP149. These proteins belong to a family that bind to PKA and target the outer membrane of mitochondria, and for this fact they are called mitochondrial AKAPs. AKAPs proteins were studied because they are the proteins linked to the YTX target, PDEs and mitochondria. The present study demonstrates that YTX induces a statistical decrease of AKAP149 (human mitochondrial AKAP) cytosolic expression. Moreover, this effect is Ca^{2+}-independent. The relationship described between YTX and AKAP49 indicates that this protein seems to be the next step after toxin PDEs effect. It has been mentioned that YTX-dependent Ca^{2+} pools depletion was inhibited with PDE4 inhibitor, rolipram, which indicates the relationship between cAMP modulation and YTX-depending Ca^{2+} oscillations. The results obtained on AKAP149 cytosolic levels are in agreement to this. Therefore, co-treatment with rolipram and YTX induce a similar decrease than rolipram alone and lower than the induced by YTX. This finding is essential to understand the effect of YTX in the K-562 cell line. As well as it was previously mentioned, in K-562 cell line, YTX could be acting as PDEs inhibitor. For this, when cell were treated with rolipram, YTX-induced decrease on AKAP149 expression was abolished. Finally, our results demonstrate that PDEs modulation affects AKAP149 expression and thus mitochondrial reservoirs. On the one hand, when PDEs are activated AKAP149 expression is increased and this leads to cell survival in fresh human lymphocytes. On the other hand, in tumour lymphocytes, when PDEs are inhibited AKAP149 expression is decrease which leads to cell death. According to this, it can be mentioned that AKAP149 binding to their substrate is essential for cell survival [Harada et al., 1999; Affaitati et al., 2003]. Moreover, AKAP149 activity is regulated by caspase-dependent manner during apoptotic cell death [Yoo et al., 2008]. Therefore, AKAP149 anchoring is crucial for cellular mitosis [Steen and Collas, 2001] and consequently factors that induce failure on AKAP149 activity are potent agents of cell death, which is the case of YTX in K-562 cell line. All together, these results suggest not only a potential signal pathway for YTX mechanism of action, but also clarifies an important difference in the biochemistry of normal and neoplastic lymphocytes, and this could be used as a future route to define new treatments, in line with the effect of YTX.

ACKNOWLEDGMENTS

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I.2. Interacción entre las mutaciones del c-kit y la PKC: la PKC se trasloca al núcleo únicamente en células HMC-1^{560,816}.

Resumen

En este trabajo se comprueba que la activación de la PKC, durante un periodo de 10 minutos, modula de manera diferente la activación inducida por ionomicina en las dos líneas celulares: HMC-1^{560} y HMC-1^{560,816}. Por un lado, en células HMC-1^{560}, la activación de la PKC potencia la liberación de histamina inducida por el ionóforo de Ca^{2+}, mientras que en células HMC-1^{560,816} el tratamiento con PMA inhibe esta liberación de histamina. Además, se ha comprobado que en presencia de Ca^{2+} en el medio la activación de la PKC estimula la entrada de Ca^{2+} desde el exterior inducida por ionomicina en ambas líneas celulares. Sin embargo, este efecto se observa tan solo cuando empleamos la concentración más baja de ionomicina (0.1 μM) en el caso de las células HMC-1^{560}, mientras que en células HMC-1^{560,816} el efecto se produce tras la incubación con las tres concentraciones de iónoforo empleadas (0.1, 1 y 10 μM). Cuando las células son incubadas en un medio sin Ca^{2+}, la activación de la PKC provoca una disminución del vaciamiento de los reservorios intracelulares de Ca^{2+} en células HMC-1^{560,816}, pero no en HMC-1^{560}. Además, la activación de la PKC en un medio sin Ca^{2+} provoca la traslocación de la proteína al núcleo en HMC-1^{560,816} mientras que en un medio con Ca^{2+} dicha traslocación no tiene lugar. Por otro lado, en células HMC-1^{560}, la activación de la PKC no altera los niveles citosólicos y nucleares de esta proteína.

ARTICLE
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C-Kit Mutations and PKC Crosstalks: PKC Translocates to Nucleous Only in Cells HMC^{560,816}

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ABSTRACT
The human mast cell lines HMC-1^{560} and HMC-1^{560,816} were used to study histamine release, Ca^{2+} signaling and protein kinase C (PKC) localization and expression, with phorbol 12-myristate 13-acetate (PMA). Both sublines carry activating mutations in the proto-oncogene of c-kit that cause autophosphorylation and permanent c-kit tyrosine kinase activation. Both have the Gly-560 → Val mutation but only the second carries the Asp-816 → Val mutation. In this study, it was observed that the stimulation of PKC has different effects in HMC-1^{560} and HMC-1^{560,816} and this would be related to the difference in activating mutations in both mast cell lines. PKC activation increases ionomycin-induced histamine release in HMC-1^{560}. This article demonstrates an opposite histamine response in HMC-1^{560,816} cells, even though classical PKCs are the family of isozymes responsible for this effect in both cellular lines. Furthermore, it can be observed that upon cell stimulation with PMA, primarily cytosolic PKC translocates to the nucleous in HMC-1^{560,816} cells, but not in HMC-1^{560} cell line. J. Cell. Biochem. 112: 2637–2651, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: HMC-1; MAST CELLS; PROTEIN KINASE C; CYTOSOLIC Ca^{2+}; c-kit

Mast cells are tissue-based inflammatory cells produced in the bone marrow and released after their differentiation into peripheral tissues. They are abundant in skin, thymus, and lymphoid tissue as well as around blood vessels and submucosal layer of the digestive tract. Mast cells are part of the immune system and they release vascular active substances in response to danger signals of innate or acquired immunity. One of those substances is histamine. The release of this amine can lead to reactions in the skin, like erythema and edema, in the airways, like mucous secretion and cough as well as nausea, vomiting, diarrhoea, and cramping in the gastrointestinal tract. Ig-E dependent hypersensitivity or diseases of tissue disorders can evoke pathologic increases in mast cell number. The most striking increase occurs in patients with mastocytosis [Prussin and Metcalfe, 2006].

The human mast cell lines HMC-1 express in their membrane the permanent activated receptor tyrosine kinase (TyrK) c-kit [Butterfield et al., 1988]. Two activating mutations in the proto-oncogene of c-kit cause its autophosphorylation and activation of the inner TyrK and induce thereby the ligand-independent proliferation [Furitsu et al., 1993; Kitayama et al., 1995; Ma et al., 1999; Longley et al., 2001]. There are two HMC-1 sublines: HMC-1^{560} and HMC-1^{560,816}. These sublines show different behavior concerning to drug response, phenotype, and growth. Both have the Gly-560 → Val mutation but only the second carries the Asp-816 → Val mutation. The 560 mutation commonly appears in patients with cutaneous mastocytosis, whereas the 816 mutation can be found in 80% of patients with systemic mastocytosis [Valent et al., 2001].

C-kit autophosphorylation in HMC-1^{560} and HMC-1^{560,816} cells lead on to a permanent activation of other transduction pathways including the Ras-MAPK-ERK and the PI3K pathways which are involved in cell survival and proliferation [Furitsu et al., 1993; Kitayama et al., 1995; Valent et al., 2001; Sundstrom et al., 2003]. The natural ligand of c-kit is Stem Cell factor (SCF). In normal mast cells its SCF promotes proliferation and maturation [Tsai et al., 1991a,b]. Further to this, it is able to directly induce murine and

Abbreviations Used: HMC-1, human mast cell line; PKC, protein kinase C; Ca^{2+}, calcium; TyrK, tyrosine kinase.

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human mast cell degranulation and it potentiates Ig-E mediated histamine release at low concentrations. In addition, it is demonstrated that rhSCF-induced histamine release was accompanied by an increase in cytosolic Ca^{2+} in human skin mast cells, which was inhibited by PKC activation [Columbo et al., 1992, 1994; Wershil et al., 1992; Taylor et al., 1995].

PKC is a family of Ser/Thr kinases with different isoforms, subdivided into three groups. The conventional PKCs (α, β, and γ) are Ca^{2+} dependent and activated by diacylglycerol (DAG) or phorbol ester; the novel PKCs (δ, ε, η, and θ), which are Ca^{2+} independent, but require DAG or phorbol ester for their activation and the atypical isoenzymes (ζ, λ, and ι), which seem to be independent of both factors [Lessmann et al., 2006]. The activity of PKC is reversibly regulated by an autoinhibitory pseudosubstrate, which blocks the active site of the enzyme in the absence of activators [Dutil and Newton, 2000]. PKCs are involved in multiple and different biological events representative of their pivotal role in the regulation of cell metabolism which would occur subsequently to their phosphorylation and translocation from cytoskeleton to the plasma membrane [Lanuti et al., 2006]. Moreover, it has been observed that sometimes PKC is translocated from the cytoplasm to the nucleous, where it exhibits catalytic activity [Nishizuka, 1995].

Phorbol 12-myristate 13-acetate (PMA) activates PKC by linking to the DAG binding site. Unlike DAG, PMA is not metabolized and has a longer effect. Its binding activates and translocates the enzyme to the membrane where it phosphorylates subsequent enzymes like MAP-kinases, Raf-kinases, the transcription factor inhibitor, or the epidermal growth factor receptor. PKC affects a variety of important cell processes like morphology, proliferation, differentiation, apoptosis, and production of inflammatory substances in mast cells [Zhao et al., 2004; Sandler et al., 2005; Kuchler et al., 2006; Gobbi et al., 2009]. Activation of phospholipase D and PKC as well as Ca^{2+} mobilization are essential signals for degranulation of mast cells. Diversion of production of phosphatidic acid to phosphatidylbutanol by addition of 1-butanol suppressed both the translocation of diacylglyceride-dependent isoforms of PKC to the membrane and degranulation [Peng and Beaven, 2005]. For signaling to the nucleous, TyrKs associated with growth factor receptors activate MAP-kinases through a series of provocative protein–protein interactions involving Ras and Raf-1 kinases [Nishizuka, 1995].

Depending on the cellular model and the stimuli, PKC activation can increase or inhibit mast cell exocytosis [Botana et al., 1992; Alfonso et al., 2000; Pernas-Sueiras et al., 2005]; therefore the aim of this work was to determine the effect that PKC modulation induces in HMC-1^{560} and HMC-$1^{560,816}$ cells and their relationship with cellular activation.

MATERIALS AND METHODS

CHEMICALS

Ionomycin, Gö6976, GF109203X, rottlerin and chelerythrine, were from Alexis Corporation (Läufelingen, Switzerland). FURA-2 AM and Streptavidin-FITC were from Molecular Probes (Leiden, The Netherlands). Phorbol 12-myristate 13-acetate (PMA), bovine serum albumin (BSA), poly-L-lysine, and Anti β tubulin were from Sigma–Aldrich (Madrid, Spain). Phosphate buffered saline (PBS) was from Invitrogen (Barcelona, Spain). Anti Mouse IgG was purchased from GE Healthcare (Barcelona, Spain). Anti-PKC Clone M110, Goat Anti Mouse IgG, IgM, IgA Biotin Conjugated, Anti Histone H1, and polyvinylidene flouride (PVDF) membrane were from Millipore (Temecula). DNA Prep™ Stain was from Beckman Coulter (Fullerton, CA). Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were from BioRad (Barcelona, Spain). Paraformaldehyde (PFA) was from Aname (Madrid, Spain).

CELL CULTURES

HMC-1^{560} cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and HMC-$1^{560,816}$ cells were kindly provided by Dr. Luis Escribano Mora with permission from Dr. J. Butterfield. They were maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen) and 100 IU/ml penicillin + 100 μg/ml streptomycin (Gibco, Invitrogen) in an atmosphere containing 5% CO_2. The medium was renewed once a week.

CELL PREPARATION

For histamine release assays, cells were centrifuged (1,500 rpm, 5 min, 4°C) and then washed twice with saline solution. The composition of this solution was (mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2, and glucose 1 g/L.

For Ca^{2+} measurement cells were treated in the same conditions, but washed in saline solution plus 0.1%BSA. For cytometry assays cells were washed in PBS (Invitrogen).

In all assays the incubation medium was equilibrated with CO_2 prior to use, to adjust the final pH to 7.20. Experiments were carried out at least three times, by duplicate.

HISTAMINE RELEASE ASSAYS

When the medium reached 37°C, 100 μl of a cell suspension with an approximate density of $1.5–2 \times 10^6$ cells/ml were added to each tube. HMC-1 cells were pre-incubated with the phorbol ester PMA (100 ng/ml) and then stimulated with different concentrations of ionomycin (0.1, 0.5, 1, 5, 10, and 25 μM). In the experiment with PKCs inhibitors, HMC-1 cells were first incubated with Gö6979 100 nM, GF109203X 50 nM, GF109203X 500 nM [Martiny-Baron et al., 1993], rottlerin 10 μM, and chelerythrine 1 μM [Alfonso et al., 2000] for 10 min. Then, cells were incubated with PMA (100 ng/ml) and finally stimulated with ionomycin. Incubations were carried out into a bath at 37°C for 10 min. The incubations were stopped by immersing tubes in a cold bath. After centrifugation at 2,300 rpm for 10 min (4°C), the supernatants were collected and decanted into other tubes for histamine determination. Appropriate controls to determine spontaneous histamine release in the absence of stimuli were executed in each experiment.

Histamine release was tested with a multi-mode plate reader (Synergy™ 4, BioTek Instruments, Vermont) both in pellets and supernatants according to Shore's method [Shore, 1971]. To produce the fluorescent complex, 0.04% orthophthaldialdehyde was used and also trichloroacetic acid (14%) to avoid protein interferences in the histamine determination. To ensure total histamine, pellets were

sonicated for 60 s in 0.2 ml of 0.1 N HCl. Results shown are expressed as the percentage of histamine released from the total histamine content.

MEASUREMENT OF CYTOSOLIC FREE Ca^{2+}

HMC-1 cells were loaded with FURA-2 AM (0.2 μM) in a bath at 37°C for 10 min. After this time, loaded cells were washed with saline solution (1,500 rpm, 10 min, 4°C). Cells were attached to glass coverslips treated with poly-L-lysine, and these were inserted into a thermostated chamber (Life Sciences Resources, UK). Cells were viewed using a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40× − immersion UV − Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution to the chamber. Cytosolic Ca^{2+} concentrations were obtained from the images collected by fluorescence equipment (Life Sciences Resources). The light source was a 175W xenon lamp, and the used wavelengths were selected with filters. For FURA-2 AM, the excitation wavelengths were 340 and 380 nm, with emission at 505 nm. The calibration of the fluorescence values versus intracellular Ca^{2+} was made according to the method of Grynkiewicz et al. [1985].

WESTERN BLOTTING

Cytoplasmic proteins protocol. Cells were incubated first with PMA (100 ng/ml) (10 min) and then with ionomycin (10 μM) (10 min). Afterwards, cells were centrifuged and washed twice with saline solution. After the last centrifugation, pellets were resuspended in lysis buffer specific for cytoplasmic proteins. The composition of this buffer was 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM DTT, 2.5 mM PMSF, 40 mg/ml aprotinin, 4 mg/ml leupeptin, 5 mM NaF, 1 mM Na_3VO_4, 1 mg/ml pepstatin A, and 1 mg/ml bezamidine. The determination of protein concentration was carried out using Bradford assay and BSA as protein standard. For separating proteins according to their molecular weight sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) procedure was used. To determine the protein size and also to monitor the progress of an efectrophoretic run Precision Plus Protein™ Standards Kaleidoscope™ molecular weight marker was used. The cytoplasmic proteins were transferred to a PVDF membrane, which was blocked with 0.25% non-fat dry milk and then it was incubated for 10 min with anti-PKC Clone M110 (1:1,000, Millipore). After two washes with washing buffer (PBS + 0.1%Tween), the membrane was incubated for 10 min with the secondary antibody, Anti-Mouse IgG conjugated with horseradish peroxidase (GE Healthcare). A chemiluminescence detection kit (SuperSignal® West Pico; Pierce) was used to determine the levels of protein expression. Relative protein expression was calculated in relation to β-tubulin expression for each experiment. To chemiluminescence measures Diversity GeneSnap software (Syngene) was used. The experiments were carried out three times by duplicate.

Nuclear fractionation protocol. Control and treated cells were centrifuged and washed twice with saline solution. Pellet was resuspended in 500 μl of buffer containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% NP40, 2.5 mM PMSF, 40 mg/ml aprotinin, 4 mg/ml leupeptin, 5 mM NaF, 1 mM Na_3VO_4, 1 mg/ml pepstatin A, and 1 mg/ml bezamidine at pH 7.9. Cells were centrifuged at 3,000 rpm for 10 min (4°C), the supernatant was kept out, and pellet was resuspended on ice in 374 μl of buffer which contains 5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v) at pH 7.9 and then 26 μl of 4.6 M NaCl was added. Samples were sonicated four times for 10 s. Assay tubes were left on ice for 30 min and centrifuged at 24,000*g* (Beckman J2-21) for 20 min at 4°C. Finally, the supernatant was aliquoted and stored at −20°C. Relative protein expression was calculated in relation to Histone H1 expression.

FLOW CYTOMETRY

About 20×10^6 cells/ml were washed with saline solution and centrifuged at 1,500 rpm, 5 min at 4°C. Samples were incubated with PMA (100 ng/ml) and then with ionomycin (10 μM), as for Western blotting assays. Then the cells were fixed with 4%PFA for 15 min at room temperature and permeabilized using PBS+ 5%BSA+ 0.1%Triton X-100. The cells were incubated for 12 h in the presence of anti PKC Clone M110 (1:1,000, Millipore). After PBS wash, primary antibody was detected with FITC-conjugated secondary antibody (Goat Anti Mouse IgG, IgM, IgA Biotin Conjugated, 1:1,000, Millipore). For propidium iodide staining, the Coulter® DNA Prep™ Stain (Beckman Coulter) was used for 2 min at 37°C. Images files of 10,000 events were collected for each sample using the ImageStream imaging flow cytometer (Amnis Corporation, Seattle, WA) and analyzed using IDEAS software (Amnis Corporation). Negative control of each condition was done and no signal was observed.

STATISTICAL ANALYSIS

Results were analyzed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

RESULTS

To determine the involvement of PKC in HMC-1 activity, several experiments studying histamine release, cytosolic Ca^{2+} levels, and PKC activation were carried out. First, the effect of PMA treatment in the histamine release induced by ionomycin was studied in HMC-1 cells. As Figure 1A shows, in HMC-1^{560} cells 100 ng/ml PMA induces a statistically significant increase in the histamine release at 0.5, 1, and 5 μM ionomycin concentrations. However, in HMC-$1^{560,816}$ cells, the pre-incubation with PMA significantly decreased ionomycin-induced histamine release in concentrations up to 10 μM of the ionophore (Fig. 1B). Several PKCs inhibitors were used with the aim of determining which class of PKCs is involved in this effect. The inhibition of Ca^{2+}-dependent isozymes (or classical PKCs) was induced by Gö6976 100 nM and GF109203X 50 nM; moreover, GF109203X 500 nM was used to inhibit both classes of PKCs: classical PKCs and Ca^{2+}-independent isozymes (or novel PKCs). PKCδ was inhibited by incubation with rottlerin 10 μM. Finally, chelerythrine 1 μM was used as a nonspecific PKC inhibitor. In these experiments 100 ng/ml PMA and two concentrations of

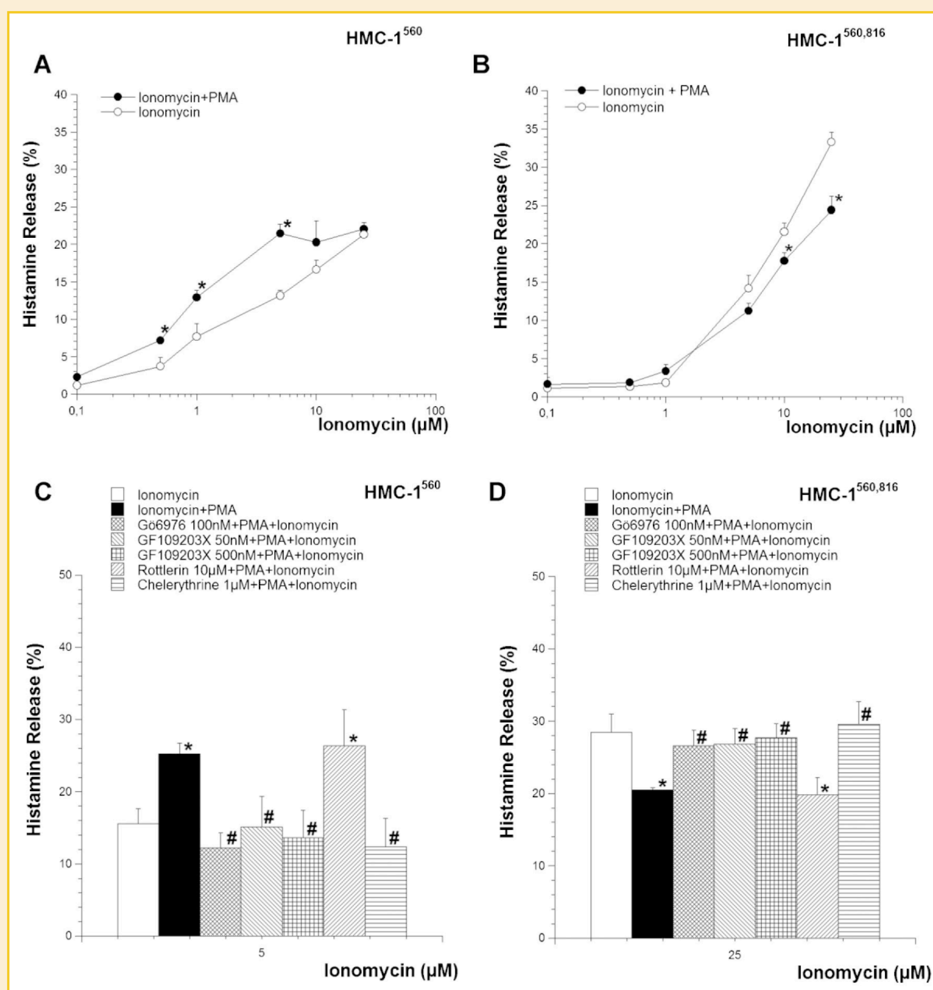

Fig. 1. Effect of PKC stimulation and inhibition on histamine release induced by ionomycin in HMC-1^{560} and HMC-$1^{560,816}$ cells. A: HMC-1^{560} cells and (B) HMC-$1^{560,816}$ cells were pre-incubated with PMA 100 ng/ml and afterwards stimulated with ionomycin. C: HMC-1^{560} cells and (D) HMC-$1^{560,816}$ cells were pre-incubated during 10 min with Gö6976 100 nM, GF109203X 50 nM, GF109203X 500 nM, rottlerin 10 μM, and chelerythrine 1 μM, then cells were stimulated with PMA 100 ng/ml and finally with ionomycin. Incubations were carried out into a bath at 37°C for 10 min. Mean ± SEM of three experiments. *Significant differences between ionomycin and PMA pre-treated cells. #Significant differences between PKC inhibitors pre-treated cells and PMA pre-treated cells.

ionomycin were used to stimulate cells, 5 μM for HMC-1^{560} and 25 μM for HMC-$1^{560,816}$. As Figure 1C shows, the significant increase in ionomycin-induced histamine release due to PMA pre-treatment in HMC-1^{560} cells is avoided by Gö6976, GF109203X, and chelerythrine pre-treatments, while PKCδ inhibition does not block this increase. On the other hand, in HMC-$1^{560,816}$ cells (Fig. 1D) the significant decrease in ionomycin-induced histamine release due to PMA treatment was prevented by Gö6976, GF109203X, and chelerythrine pre-treatments while Rottlerin pre-treatment does not block PMA effect. In this cell line, another concentration of ionomycin was used, 10 μM, and similar results were obtained (data not shown).

In order to clarify the opposite behavior of each cellular model in histamine release, the next group of experiments focuses on studying the effect of PKC activation in cytosolic Ca^{2+} levels. In HMC-1^{560} cells, ionomycin 0.1 μM induces a small increase (around 20 nM) of cytosolic Ca^{2+} levels at the addition point of ionomycin, Figure 2A. The same figure shows that PMA alone did not affect cytosolic Ca^{2+} levels; by contrast, when cells were pre-treated with PMA prior to ionomycin, cytosolic Ca^{2+} levels were significantly

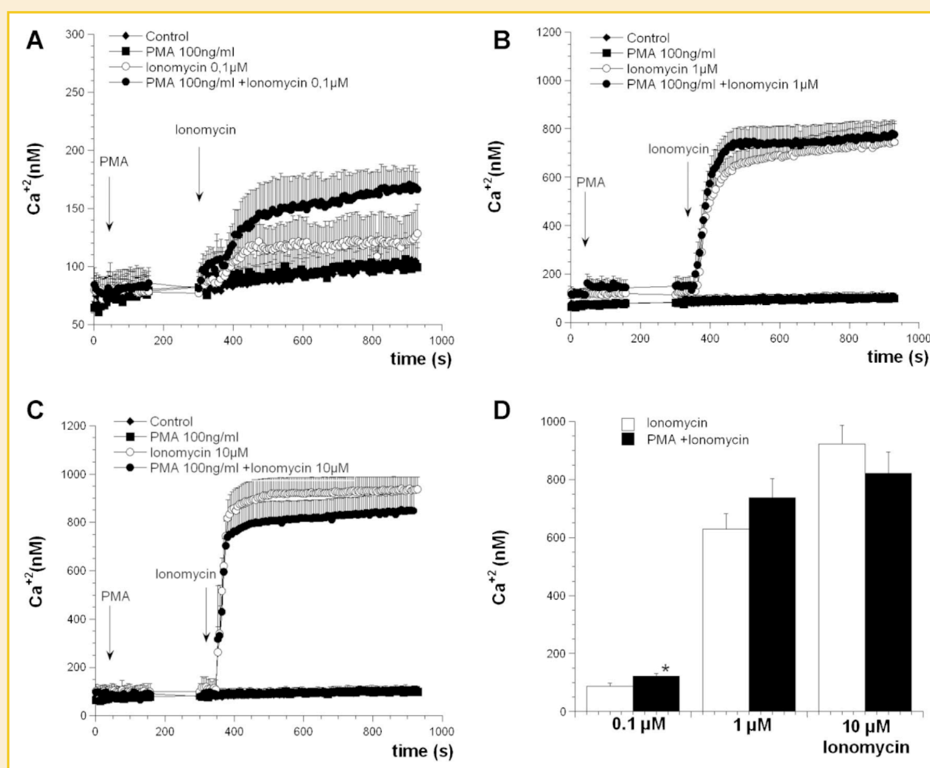

Fig. 2. Effect of PKC activation and ionomycin on cytosolic Ca^{2+} levels of HMC-1[560] cells in a saline solution with calcium. Cytosolic Ca^{2+} profile of cells that were pre-incubated with PMA 100 ng/ml plus (A) ionomycin 0.1 μM or (B) ionomycin 1 μM or (C) ionomycin 10 μM was added. D: Summary of results shown in (A–C) from extracellular Ca^{2+} influx. Mean ± SEM of four experiments. *Significant differences between ionomycin and PMA pre-treated cells.

higher. This increase was kept for 10 min. The effect of PMA was also evident using 1 μM of ionomycin, Figure 2B, since cytosolic Ca^{2+} levels were up from 600 to 700 nM, even though the increase disappears after 5 min. However, when ionomycin 10 μM was used, cytosolic Ca^{2+} levels reached in PMA + ionomycin-treated cells were lower than in ionomycin-treated cells, Figure 2C. These variations in cytosolic Ca^{2+} levels at the addition point of ionomycin are summarized in Figure 2D. In HMC-1[560,816] cells, an increase (not significant) in the cytosolic Ca^{2+} levels was observed at the addition point of ionomycin (0.1 μM) (Fig. 3A). The same figure shows that PMA alone did not increase the levels of this ion. However, when both drugs are present an increase in cytosolic Ca^{2+} levels is observed. This effect was kept and increased after 10 min. The effect was significantly higher when cells were preincubated with PMA and ionomycin 1 μM, Figure 3B, and also in the presence of 10 μM ionomycin, Figure 3C. Figure 3D summarizes all these results in HMC-1[560,816] cells at the addition point of ionomycin. In summary, in HMC-1[560] cells the treatment with PMA + ionomycin induces a significant increase in cytosolic Ca^{2+} levels when ionomycin 0.1 μM was used; when higher concentrations are used no increase or even a lower levels in cytosolic Ca^{2+} are observed (Fig. 2D). On the contrary in HMC-1[560,816] cells, PMA + ionomycin treatment induces a significant increase in Ca^{2+} levels at any concentration checked (Fig. 3D).

Since in the presence of PMA plus ionomycin each cellular line reached different cytosolic Ca^{2+} levels, the effect of PMA on Ca^{2+}-pools depletion and Ca^{2+}-influx from extracellular media was checked. Ionomycin (0.1 μM) induced Ca^{2+} release from intracellular pools (Fig. 4A). This effect was not modified in the presence of PMA. However, when Ca^{2+} is restored to the medium, the presence of PMA increased Ca^{2+} influx (from 300 to 450 nM). Figure 4B shows that the addition of ionomycin (1 μM) induces a cytosolic Ca^{2+} increase due to Ca^{2+}-pools depletion. When Ca^{2+} was restored to the extracellular medium, levels of this ion increased to values about 700 nM. The treatment with PMA prior to ionomycin did not affect neither Ca^{2+}-pools depletion nor extracellular Ca^{2+} influx. When ionomycin 10 μM was added, PMA increased (not significant) Ca^{2+}-pools depletion and inhibited cytosolic Ca^{2+} recovery to basal values. When Ca^{2+} is restored to the medium, no differences between ionomycin and PMA + ionomycin treated cells

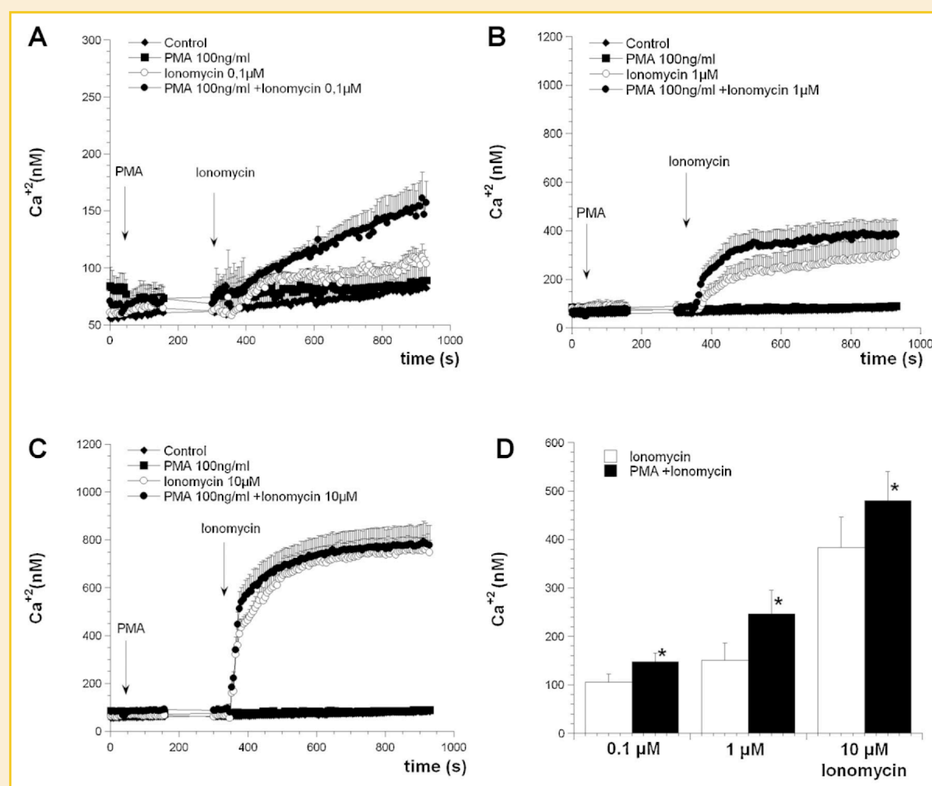

Fig. 3. Effect of PKC activation and ionomycin on cytosolic Ca^{2+} levels of HMC-1^{560,816} cells in a saline solution with calcium. Cytosolic Ca^{2+} profile of cells that were pre-incubated with PMA 100 ng/ml plus (A) ionomycin 0.1 μM or (B) ionomycin 1 μM or (C) ionomycin 10 μM was added. D: Summary of results shown in (A–C) from extracellular Ca^{2+} influx. Mean ± SEM of four experiments. *Significant differences between ionomycin and PMA pre-treated cells.

were observed (Fig. 4C). Figure 5 shows the results obtained in HMC-1^{560,816} cells. Figure 5A shows the cytosolic Ca^{2+} increase at the addition point of ionomycin (0.1 μM), due to the depletion of intracellular Ca^{2+}-pools (125 nM). An increase of 225 nM in cytosolic Ca^{2+} levels was observed when Ca^{2+} was restored to the extracellular medium. In cells treated with PMA alone no effect in cytosolic Ca^{2+} levels was observed. When cells were pre-treated with PMA prior to ionomycin (0.1 μM), Ca^{2+} pools depletion was 20 nM lower (125–105 nM) while Ca^{2+} influx was significantly higher (350–490 nM). The decreasing effect of PMA in intracellular-pools depletion was more evident using 1 μM of ionomycin (47 nM lower than with ionomycin alone), Figure 5B, whereas the enhancing effect on Ca^{2+} influx disappeared. The inhibition of pools depletion by PKC activation was even more notable using 10 μM of ionomycin (86 nM); Figure 5C. In these conditions, when Ca^{2+} was restored to the extracellular medium, the influx of this ion reached the same values than in control cells (ionomycin treated). In summary, in HMC-1^{560} cells PKC activation does not modify Ca^{2+}-pools depletion induced by ionomycin, Figure 4D, while, in HMC-1^{560,816} cells, PMA treatment induces a significant inhibition of ionomycin effect in Ca^{2+}-pools, Figure 5D. After PMA pre-treatment, Ca^{2+}-influx is significantly increased when 0.1 μM ionomycin is used both in HMC-1^{560} and HMC-1^{560,816} cells, while the influx is not affected when the inophore concentration is increased, Figures 4E and 5E.

Different effects either on Ca^{2+} fluxes or on histamine released on HMC-1^{560} and HMC-1^{560,816} cells were observed as consequence of PKC activation; therefore the levels of this protein in the presence of ionomycin, PMA, and Ca^{2+} were checked. A representative experiment for each condition studied is represented in Figure 6A–D. Figure 6E–H shows cytosolic PKC levels (average of band intensity of three experiments). In HMC-1^{560} cells PMA and/or ionomycin treatments did not modify cytosolic PKC levels either in the presence or absence of Ca^{2+} in the extracellular medium (Fig. 6E,G). However, in HMC-1^{560,816} cells, PMA decreased significatively cytosolic PKC levels (Fig. 6F). This decrease was also observed in a Ca^{2+}-free medium and when cells are incubated with ionomycin (Fig. 6H). These results were complemented with the study of nuclear PKC levels. A representative experiment for each condition is shown in Figure 7A–D and Figure 7E–H shows nuclear PKC levels (average of

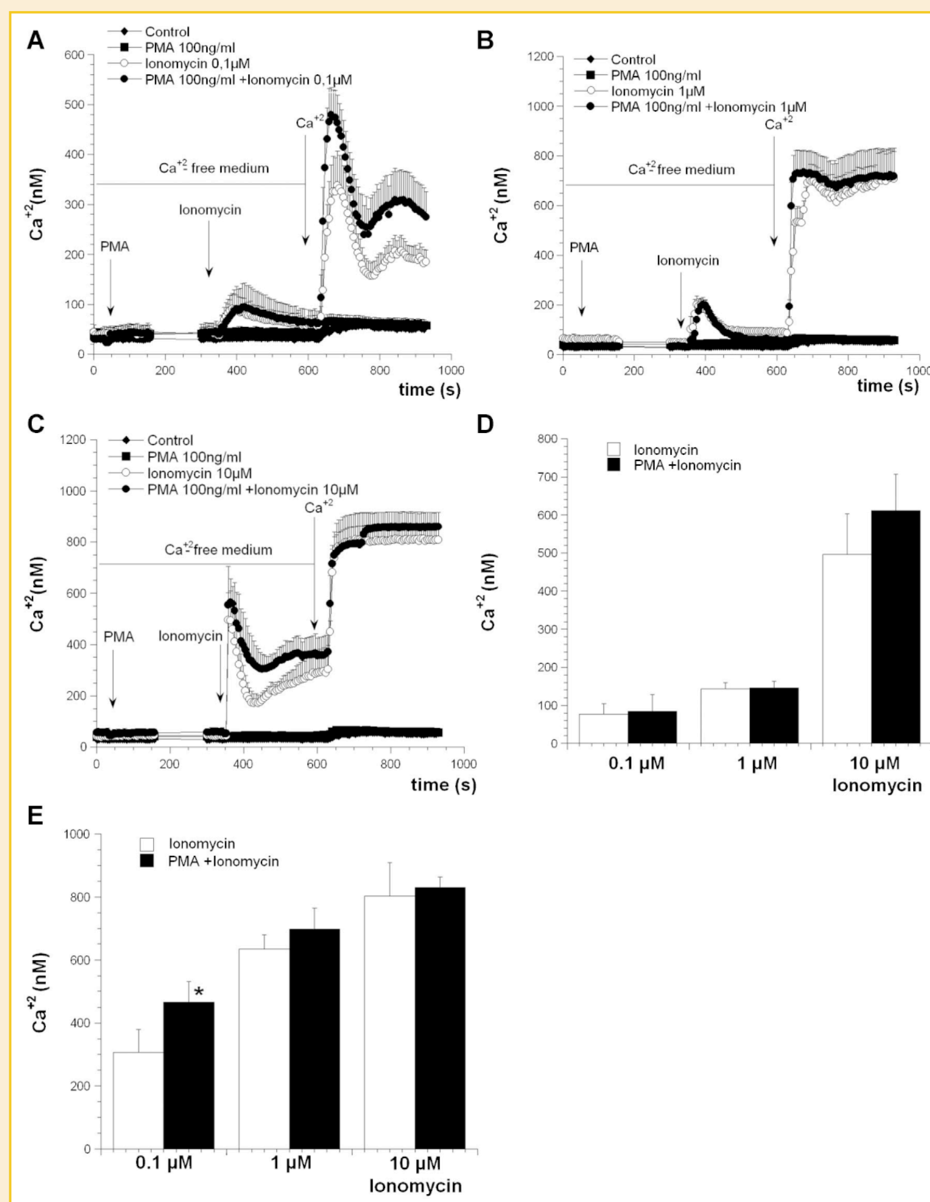

Fig. 4. Effect of PKC activation and ionomycin on cytosolic Ca^{2+} levels of HMC-1^{560} cells in a calcium-free saline solution. Cytosolic Ca^{2+} profile of cells that were pre-incubated with PMA 100 ng/ml plus (A) ionomycin 0.1 μM or (B) ionomycin 1 μM or (C) ionomycin 10 μM was added. D: Summary of results shown in (A–C) from intracellular Ca^{2+} reservoir depletion or extracellular Ca^{2+} influx (E). Mean ± SEM of four experiments. *Significant differences between ionomycin and PMA pre-treated cells.

band intensity of three experiments). In HMC-1^{560} cells PMA and PMA + ionomycin treatments did not modify nuclear PKC levels, both in a saline solution with or without Ca^{2+} (Fig. 7E,G). However, in HMC-1^{560,816} cell line the results were opposite. Surprisingly, when cells were treated with PMA and PMA + ionomycin, nuclear PKC levels (Fig. 7F) were not modified in a saline solution with Ca^{2+}. However, the phorbol ester increased significantly nuclear PKC expression in a Ca^{2+}-free saline solution (Fig. 7H). In these

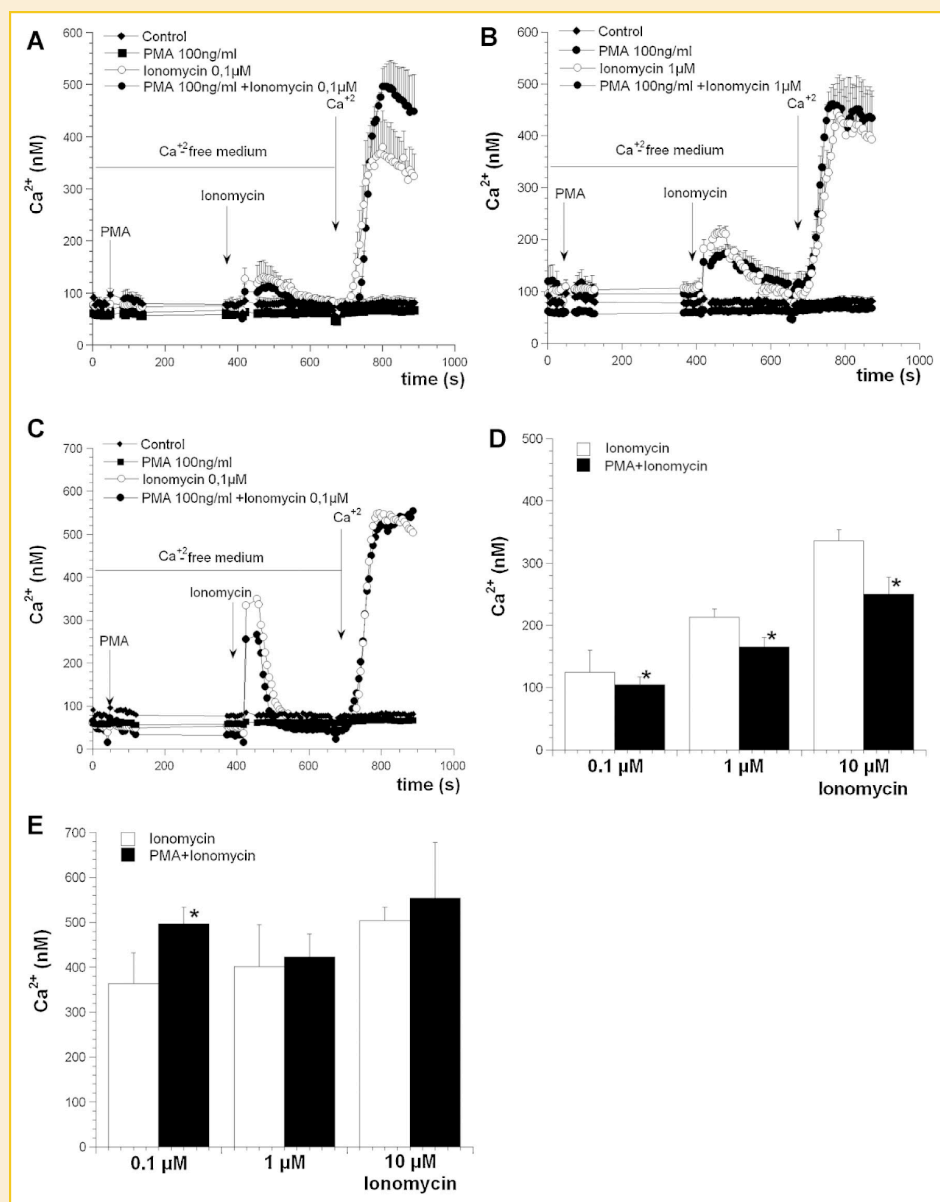

Fig. 5. Effect of PKC activation and ionomycin on cytosolic Ca^{2+} levels of HMC-1[560,816] cells in a calcium-free saline solution. Cytosolic Ca^{2+} profile of cells that were pre-incubated with PMA 100 ng/ml plus (A) ionomycin 0.1 µM or (B) ionomycin 1 µM or (C) ionomycin 10 µM was added. D: Summary of results shown in (A–C) from intracellular Ca^{2+} reservoir depletion or extracellular Ca^{2+} influx (E). Mean ± SEM of four experiments. *Significant differences between ionomycin and PMA pre-treated cells.

conditions, protein expression levels in the presence of PMA are threefold higher than in control or ionomycin-treated cells.

To confirm nuclear PKC localization, several image-flow cytometry experiments were designed in the conditions before described. Double stained methodology was used in order to check the co-localization of nucleous (propidium iodide) and PKC (fluorescein). To quantify the extent of nuclear PKC localization, the degree of pixel intensity correlation between PKC and nuclear

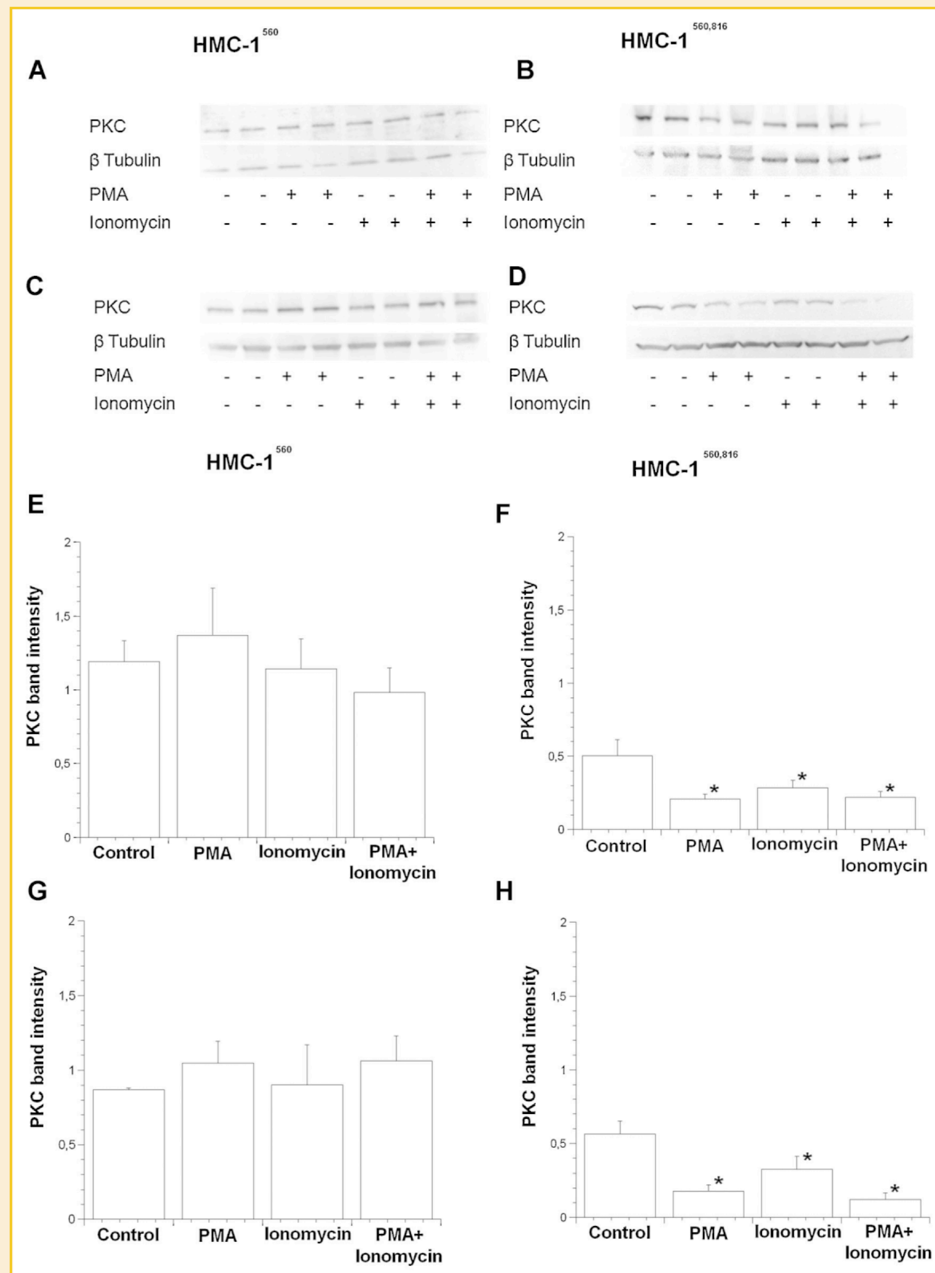

Fig. 6. Effect of PKC activation and Ca^{2+} presence on cytosolic PKC expression in HMC-1 ^{560} and HMC-1 ^{560,816} cells. Mean of the ratio of the PKC/Tubulin band intensity. Cells were incubated during 10 min with PMA 100 ng/ml and afterwards stimulated with ionomycin 10 μM for 10 min. At the top of the figure are represented an experiment of each condition. A: HMC-1 ^{560} and (B) HMC-1 ^{560,816} corresponding to experiments that were carried out in a saline solution with Ca^{2+}, whereas (C) HMC-1 ^{560} and (D) HMC-1 ^{560,816} corresponding to experiments that were carried out in a Ca^{2+}-free saline solution. Cytosolic PKC expression was measured in both cell lines, HMC-1 ^{560} (E) and HMC-1 ^{560,816} (F). The same experiment was carried out in a saline solution without Ca^{2+}. G: From HMC-1 ^{560} cell line and (H) from HMC-1 ^{560,816} cells. Mean ± SEM of three experiments.

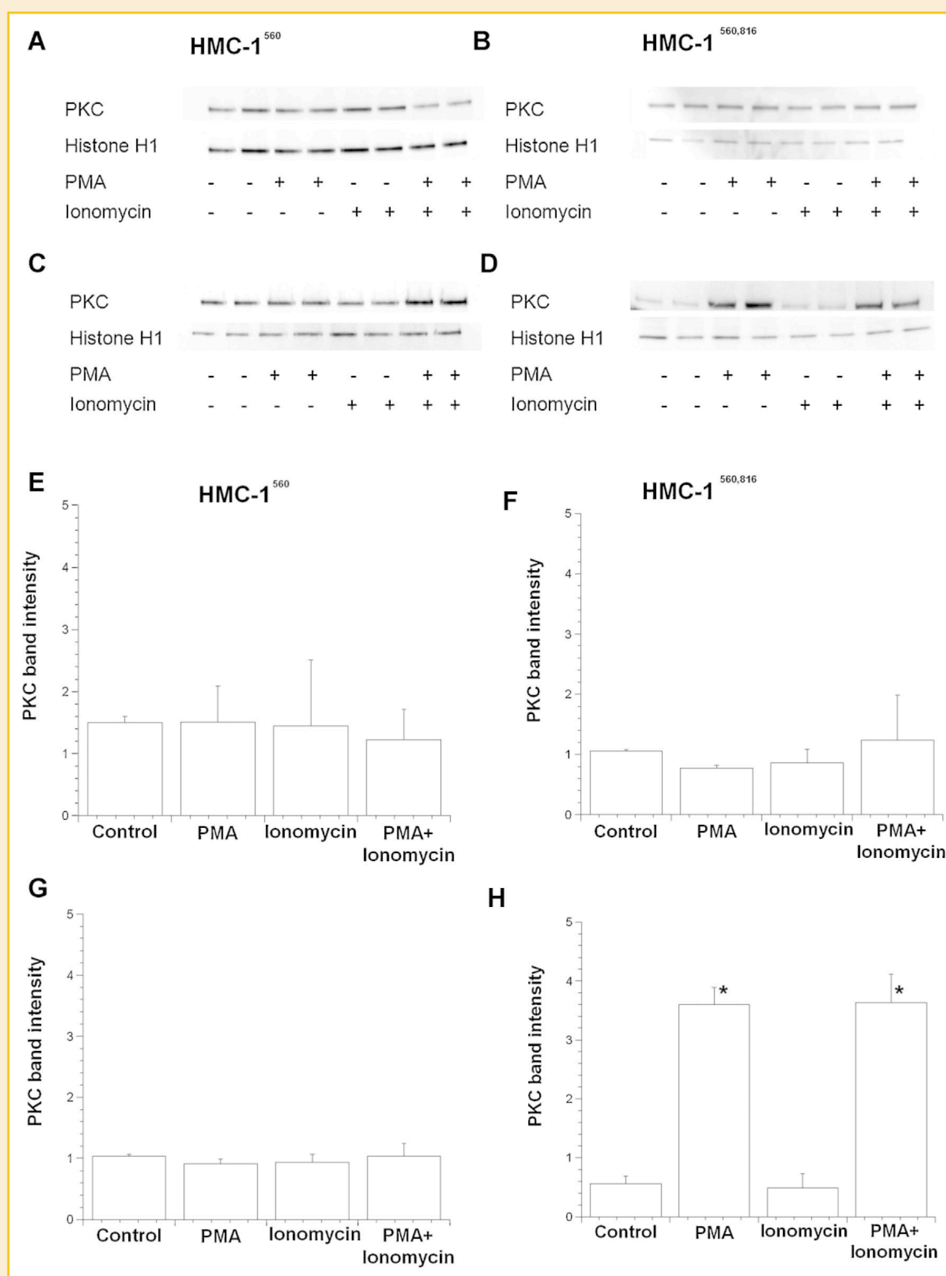

Fig. 7. Effect of PKC activation and Ca^{2+} presence on nuclear PKC expression in HMC-1^{560} and HMC-1^{560,816} cells. Mean of the ratio of the PKC/Histone band intensity. Cells were incubated during 10 min with PMA 100 ng/ml and afterwards stimulated with ionomycin 10 μM for 10 min. At the top of the figure are represented an experiment of each condition. A: HMC-1^{560} and (B) HMC-1^{560,816} corresponding to experiments that were carried out in a saline solution with Ca^{2+}, whereas (C) HMC-1^{560} and (D) HMC-1^{560,816} corresponding to experiments that were carried out in a Ca^{2+}-free saline solution. Cytosolic PKC expression was measured in both cell lines, HMC-1^{560} (E) and HMC-1^{560,816} (F). The same experiment was carried out in a saline solution without Ca^{2+}. G: From HMC-1^{560} cell line and (H) was from HMC-1^{560,816} cells. Mean $\pm$ SEM of three experiments.

images was analyzed. Two features, the correlation coefficient (ρ) and logarithmic transformation of ρ (Similarity), were calculated with the following equations: $\rho = Cov(X,Y)\backslash\sigma_x\sigma_y$, Similarity = $\ln((l+\rho)\backslash(l-\rho))$. Figures 8 and 9 show the images and histograms obtained from a representative experiment. These histograms represent the percentage of translocated cells in the experiment. In this analysis, a large score to positive values indicates a great degree of similarity between FITC and propidium iodide channels and thus PKC translocation; specifically in this study high similarity was visualized at values higher than 3. In addition, the median of the entire "Live" population was shown in the upper left-hand corner of the histograms. The image galleries show four representative cells in brightfield illumination (gray) adjacent to a composite image that represents an overlay of the images from the two fluorescent channels (fluorescein and propidium iodide). At the bottom, the bars plot represents the mean of three experiments. In HMC-1[560] cells, no co-localization was observed at any condition tested (Fig. 8E), in addition the percentage of cells with translocated PKC is lower than 5%. However, as Figure 9E shows, in HMC-1[560,816] cells, PMA treatment induces PKC translocation (nucleous co-localization) in approximately 28% of the cells. These results agree with the increase in nuclear PKC levels observed in Figure 7H.

DISCUSSION

Mast cells express in their membrane the tyrosine kinase receptor c-kit, which is involved in important processes like proliferation, activation, and maturation. The mechanism of down-regulation of the c-kit receptor by the kit ligand was investigated in earlier studies. After binding to the c-kit receptor, kit ligand was internalized and

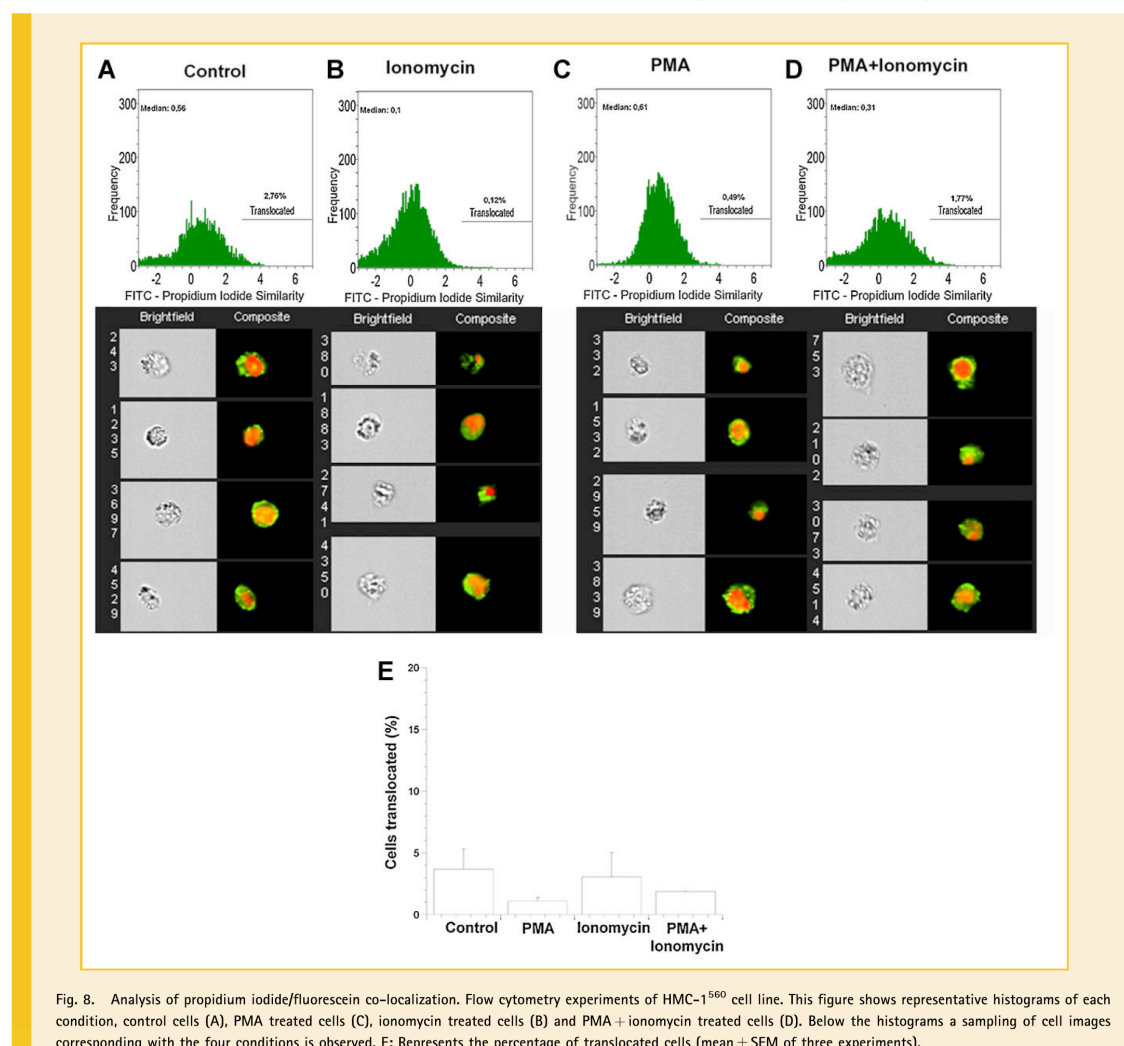

Fig. 8. Analysis of propidium iodide/fluorescein co-localization. Flow cytometry experiments of HMC-1[560] cell line. This figure shows representative histograms of each condition, control cells (A), PMA treated cells (C), ionomycin treated cells (B) and PMA + ionomycin treated cells (D). Below the histograms a sampling of cell images corresponding with the four conditions is observed. E: Represents the percentage of translocated cells (mean ± SEM of three experiments).

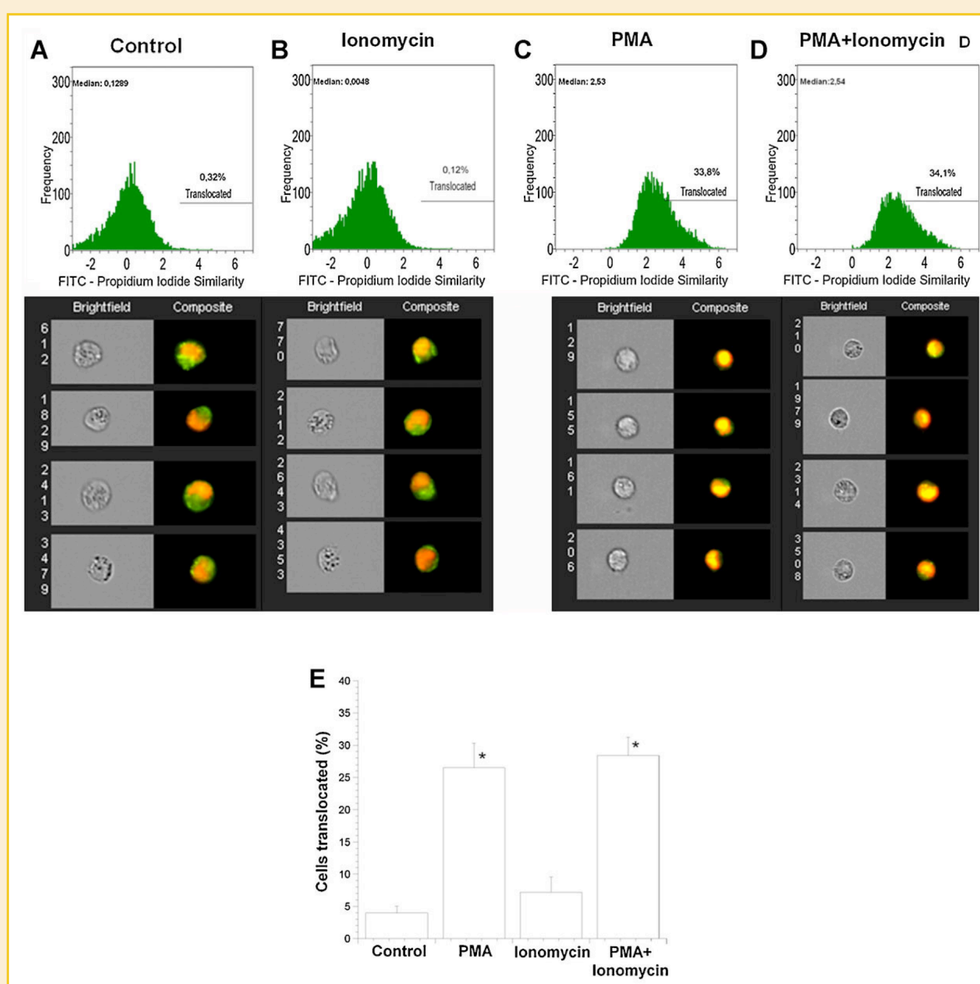

Fig. 9. Analysis of propidium iodide/fluorescein co-localization. Flow cytometry experiments of HMC-$1^{560,816}$ cell line. This figure shows representative histograms of each condition, control cells (A), PMA treated cells (C), ionomycin treated cells (B) and PMA + ionomycin treated cells (D). Below the histograms a sampling of cell images corresponding with the four conditions is observed. E: Represents the percentage of translocated cells (mean ± SEM of three experiments).

accelerated c-kit receptor degradation. Moreover, c-kit receptor transmodulation was produced by the PKC activator PMA and by the Ca^{2+} ionophore ionomycin [Yee et al., 1993]. One pathway to limit c-kit levels proceeds through the internalization and ubiquitin-assisted degradation upon binding of its ligand SCF. This mechanism is rapid and requires an intact kinase activity [Babina et al., 2006]. In addition, it has been described that the activation of c-kit induces or forces mast cell exocytosis and PKC stimulation by PMA down-regulates c-kit expression in mast cell membrane [Columbo et al., 1992, 1994; Wershil et al., 1992; Taylor et al., 1995]. On the other hand, HMC-1^{560} and HMC-$1^{560,816}$ differ in activating mutations in the proto-oncogene c-kit, which cause in both sublines autophosphorylation and permanent activation of the inner TyrK and activate thereby subsequent pathways [Furitsu et al., 1993; Kitayama et al., 1995; Ma et al., 1999; Longley et al., 2001; Sundstrom et al., 2003]. Therefore, PKC has an essential role in c-kit down-regulation. The opposite effect than PKC activation induces in histamine release and calcium fluxes in HMC-1^{560} and HMC-$1^{560,816}$ cells may be related to the activating mutations of c-kit. Ionomycin stimulates histamine release in HMC-1^{560} cells and PKC activation increases exocytosis and Ca^{2+} influx and also cytosolic Ca^{2+} levels. By contrast, the PKC activation negatively affected ionomycin-induced histamine release in HMC-$1^{560,816}$ cells and also intracellular Ca^{2+}-pools depletion while cytosolic Ca^{2+} levels

are increased. These results were surprising and inspired further investigation about the effect of PKC in both HMC-1 sublines. The negative effect of PKC on histamine release in HMC-$1^{560,816}$ cells might be partly regulated by Ca^{2+}-pools depletion, since this was significantly decreased at any ionomycin concentrations after PMA pre-incubation. An earlier study suggested the existence of a feedback mechanism that uses PKC to limit the increase in intracellular Ca^{2+} levels [Barajas et al., 2008]. Results shown in the present article demonstrate that PMA treatment increases cytosolic Ca^{2+} levels in both mast cell lines; however phorbol ester treatment has different effects on ionomycin-induced histamine release and in pools depletion. The increase in histamine release after PKC activation in HMC-1^{560} cell is probably due to the increase in cytosolic Ca^{2+} levels. In HMC-$1^{560,816}$ cells the decrease in histamine release is related to a lower pools depletion. From these results, it can be concluded that PKC acts as a negative regulator of exocytosis in HMC-$1^{560,816}$ cells, but not in HMC-1^{560} cell line. Since calcium levels are increased in both cellular lines, it can be further pointed that either Ca^{2+} has an inhibitory effect in HMC-$1^{560,816}$ line or PKC has a Ca^{2+} independent activity. Data shown in this study confirm that in both cell lines (HMC-1^{560} and HMC-$1^{560,816}$), the effect of PMA on ionomycin-induced histamine release is mediated by classical PKCs. For this reason, when this family of PKCs is inhibited no effect on histamine release is observed. Moreover, it can be affirmed that PKCδ is not involved in the effect of PMA on ionomycin-induced histamine release.

The different effects on histamine release and Ca^{2+} levels in both cell lines can be also related to PKC expression and localization. Some studies have shown that PKC isoforms could be translocated to the membrane or nucleous depending on cellular model. In Mz-ChA-1 cells stimulated with (R)-(alpha)-(−)-methylhistamine dihydrobromide (RAMH), phosphorylation of PKC and mitogen-activated protein kinase isoforms were measured. RAMH induced a shift in the localization of PKCalpha expression from the cytosolic domain into the membrane region of Mz-ChA-1 cells [Francis et al., 2009]. In addition, in NIH 3T3 cells PMA treatment induces the tight association of PKC to the nucleous [Thomas et al., 1988]. Besides, translocation of PKCα to the nucleous in response to PMA was reported in many studies [Eldar et al., 1992; Maissel et al., 2006]. In summary, PKC translocation to the membrane or to the nucleous, when the protein is activated, might be correlated with PKC isoforms and cell lines studied. Furthermore, in this paper it was observed that PKC translocation was different in both mast cell lines, since in HMC-$1^{560,816}$ cells PKC is completely translocated to the nucleous in 28% of the cells but in the other cell line, HMC-1^{560}, PKC activation did not imply their nuclear translocation. For this, PKC activation would down-regulate their cytosolic expression in HMC-$1^{560,816}$ cell line in Ca^{2+}-free medium. In this study it can be stated that PMA and PMA + ionomycin treatments induce the translocation of PKC to the nucleous in a Ca^{2+}-free saline solution in HMC-$1^{560,816}$ cell line. It is necessary to point out that in this cellular line the expression of cytosolic PKC decreases when cells are activated. In Ca^{2+}-free medium, the decrease in cytosolic PKC levels matches the increase in nuclear localization of PKC.

The results obtained show that PKC activation implies a decrease in cytosolic PKC levels that was matched with an increase in nuclear PKC levels. However, this translocation did not occur in a saline solution with calcium. The presence of Ca^{2+} is not needed for the nuclear translocation and the protein would be translocated to another place like plasma membrane [Kimata et al., 1999]. Moreover, ionomycin treatment induces a decrease in cytosolic PKC levels in the absence of Ca^{2+}, but this effect was not accompanied with an increase in nuclear PKC levels. The previous work describes that PKC is translocated into cell membrane along with an increase in intracellular Ca^{+2} concentrations [Izushi and Tasaka, 1992]. In HMC-1^{560} cells PKC activation induces the same effects than in normal mast cells [Friis and Johansen, 1996]. However in HMC-$1^{560,816}$ cells, PKC activation inhibits histamine release and Ca^{2+}-pools depletion. Moreover, in HMC-1^{560} cells PKC activation does not affect cytosolic PKC expression whereas in HMC-$1^{560,816}$ cells PKC activation affects PKC localization and cytosolic expression.

The results shown in this article show for the first time the difference of PKC localization in HMC-1^{560} and HMC-$1^{560,816}$ cell lines when they were activated with PMA. The findings obtained demonstrate that PKC is translocated to the nucleous in 28% of the HMC-$1^{560,816}$ cell line population and that this translocation does not occur in HMC-1^{560}. PKC translocation to the nucleous could be related to the inhibition of pools depletion and histamine release in HMC-$1^{560,816}$ cell line, since all these effects occur in a Ca^{2+}-free saline solution. However, PKC translocation did not happen in HMC-1^{560} cell line and PMA effect on ionomycin-induced histamine release is opposite. Unlike HMC-$1^{560,816}$ cells, in cells with one mutation PMA treatment did not affect calcium pools' depletion. The crosstalks between the two mutations and the final control of PKC remain to be defined. Furthermore, it would be interesting to know the characteristics of the cells that have completely translocated PKC and open a new research line that includes the study of nuclear PKC effect. Finally, the effect of this translocation to the nucleous should be studied in subsequent publications.

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4.3. Sección II: Estudio del efecto de la PKC sobre la inhibición de las enzimas tirosina quinasas en las líneas celulares HMC-1^{560} y HMC-1^{560,816}.

El grupo de las tirosina quinasas posee un papel fundamental en la señalización intracelular ya que incluye tanto receptores como proteínas iniciadoras de la cascada de activación mastocitaria, entre los que destacan el c-kit, el PDGFR, el Bcr-Abl y la Src [130]. La importancia de este grupo no se limita únicamente a su papel como iniciadores de la activación mastocitaria sino que algunos de ellos como el c-kit posee una función esencial en el desarrollo de la mastocitosis sistémica. De esta forma los pacientes con mastocitosis sistémica se caracterizan por presentar una o más mutaciones en el c-kit, provocando una activación permanente del mismo y la proliferación de la célula de manera SCF-independiente. Por esta razón el c-kit es considerado como una importante herramienta en el tratamiento de esta enfermedad [111,273-277]. La CML es una patología muy frecuente en pacientes con mastocitosis sistémica siendo la diana para el tratamiento de la misma el protoncogén Bcr-Abl, ya que el 95% de los pacientes son positivos al mismo [132]. Tanto el c-kit como el protoncogén Bcr-Abl son inhibidos por el grupo de inhibidores de las tirosina quinasas. Este conjunto de fármacos, en el que se incluyen el STI571 (imatinib mesilato) y una segunda generación formada por dasatinib, nilotinib y midostaurina, se ha convertido en el más empleado en el tratamiento de las mastocitosis sistémicas [128,132,148]. Su eficacia ha sido estudiada en la línea celular HMC-1 y se ha comprobado que el compuesto STI571 provoca muerte por apoptosis en la línea celular HMC-1^{560}, mientras que no es efectivo en células HMC-1^{560,816}. Debido al amplio número de pacientes resistentes a este fármaco se procedió al uso de una segunda generación de compuestos efectivos contra las células HMC-1^{560,816}. Sin embargo, se desconoce el papel que juega la PKC, proteína esencial en el proceso de apoptosis celular, en la actividad de este grupo de fármacos en la línea celular HMC-1. Por esta razón se ha procedido al estudio del efecto modulador de la PKC sobre el mecanismo de acción de los inhibidores de tirosina quinasas en ambas líneas celulares: HMC-1^{560} y HMC-1^{560,816}.

A esta sección pertenecen tres publicaciones:

II.1. *PKCδ translocation mediates in HMC-1^{560} apoptosis induced by STI571.*

II.2. *Apoptotic cell death induced by the tyrosine kinase inhibitor dasatinib is PKCδ-dependent in HMC-1 cell lines.*

II.3. *PKC potentiates tyrosine kinase inhibitors STI571 and dasatinib cytotoxic effect.*

II.1. La traslocación de la PKCδ regula la apoptosis inducida por STI571 en la línea celular HMC-1^{560}.

Resumen

La isoforma PKCδ posee un papel fundamental en la muerte de la línea celular HMC-1^{560} mediada por STI571, ya que tras la incubación con este fármaco se produce su traslocación al núcleo a través del citoesqueleto de actina. Además, la PKCδ es imprescindible para que el efecto apoptótico del STI571 sobre las células HMC-1^{560} tenga lugar, ya que la silenciación de esta isoforma bloquea el efecto del fármaco de tal forma que su efecto es tres veces menor. Por otro lado, el proceso apoptótico mediado por el STI571 predomina sobre el efecto de la activación de la PKC en células HMC-1^{560}. En este estudio se observa que el STI571 ejerce una modulación diferente de las PKC clásicas en las líneas celulares HMC-1^{560} y HMC-1^{560,816}, ya que en el caso de las células HMC-1^{560} el STI571 tan solo tiene efecto sobre las PKC clásicas cuando el citoesqueleto de actina está alterado. Por su lado, en la línea celular HMC-1^{560,816}, la inhibición del c-kit aumenta los niveles citosólicos de las isoformas clásicas de la PKC. Además, la activación de la PKC no modifica los niveles citosólicos de PKC clásicas y PKCδ en células HMC-1^{560}, sin embargo en células HMC-1^{560,816} la expresión de ambas está disminuída. Por un lado las PKC clásicas se traslocan a la membrana, migración inhibida por el STI571, mientras que la expresión de la PKCδ se ve disminuída no solo en el citosol sino también en la membrana plasmática y en el núcleo. Además, la inhibición del c-kit evita este descenso de la expresión de PKCδ en citosol y núcleo.

PKCδ translocation mediates in HMC-1^{560} apoptosis induced by STI571.

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Running title: ST571- induced apoptosis is PKCδ-dependent.

Key Words: HMC-1; PKC; c-kit; STI571; PMA.

The authors disclose no conflicts of interest.

Abreviations: Ca^{2+}, Calcium; PKC, Protein Kinase C; cPKC, classical PKCs; HMC-1, Human Mast Cell Line; TyrK, Tyrosine Kinase; PMA, phorbol-12-myristate-13-acetate; MAPKs, Mitogen Activated Protein Kinases; PARP; Poly ADP-ribose polymerase.

ABSTRACT

The effect of c-kit inhibition (STI571 treatment) on Protein Kinase C (PKC) regulation was determined in human mast cell lines HMC-1^{560} and HMC-1^{560,816}. Results obtained demonstrate that c-kit inhibition by STI571 activates PKCδ resulting in mast cell apoptosis in HMC-1^{560}. Apoptotic cell death induced by c-kit inhibition is PKCδ–dependent in these cells, since when this PKC isoform is silenced STI571-apoptotic effect disappears. C-kit inhibition implies nuclear translocation of PKCδ isoform characterized by a clear dependence on actin cytoskeleton integrity. C-kit inhibition does not have the same effect in the other cell line, HMC-1^{560,816}, since nuclear PKCδ translocation was not observed. In HMC-1^{560,816} cell line, c-kit inhibition induces a positive regulation of Ca^{2+}-dependent PKC isoforms. The translocation of Ca^{2+}-dependent PKC isoforms to the plasma membrane was described in HMC-1^{560,816} when PKC is activated. It can be concluded that PKCδ isoform might be considered an important target in the treatment of mastocytosis in which tyrosine kinase inhibition is the pathway selected. Hence, it is important to note that PKCδ modulations can lead to a decrease in the effectiveness of mastocytosis treatment.

INTRODUCTION

Mast cells have a crucial role in the immune response, since their activation is essential for the production of several immune mediators subdivided into three groups: mediators associated to granules (such as histamine), lipid derivatives, cytokines and chemokines (1). Furthermore, mast cell activation might be dependent or independent of the immunoglobulin E (IgE) high-affinity receptor (FcεRI). This receptor has a IgE binding chain, a tetraspanning β chain and a homodimeric disulfide-linked γ subunit (1). The uncontrolled growth of mast cells is called mastocytosis, and these cells can be accumulated in one or more visceral organs (2-4). Mastocytosis is usually associated with somatic gain-of-function point mutations within c-kit receptor (CD117). C-kit is a type III tyrosine kinase (TyrK) receptor expressed in mast cells, hematopoietic progenitors, melanocytes, germ cells, NK cells and interstitial Cajal cells (5, 6). The most common mutation is the c-kit^{D816V} present in HMC-1^{560,816} cell line, resulting from substitution of valine for aspartic acid (7-9). Another important mutation is the substitution of valine for glycine at codon 560 (V560G) present in HMC-1^{560} and HMC-1^{560,816} cell lines. The natural ligand of c-kit is the stem cell factor (SCF, also named mast cell growth factor, steel factor and kit ligand binding domain). Both mutations located on the c-kit receptor imply that these sublines grow *in vitro* without mast cell growth factor and c-kit is phosphorylated in a ligand-independent manner (10-14). The compound STI571 (imatinib mesylate or Gleevec®) is a tyrosine kinase inhibitor that inhibits the kinase activity of c-kit (15-19). Therefore, STI571 induces a cytotoxic effect through an apoptotic pathway activation. Also, the apoptotic cascade is activated by STI571 and several proteins such as caspase-3 or poly ADP-ribose polymerase (PARP) are implicated in STI571 mechanism of action. It has been demonstrated that the combination of STI571 with other cytotoxic compounds, as flubarabine (20) in K-562 cell line or p-glycoprotein (21, 22), results in a synergistic apoptotic effect in malignant glioma cells.

Protein Kinase C (PKC) is a protein involved in multiple and different biological events and thus plays a crucial role in cell metabolism regulation. PKC isoforms have been subdivided into three groups: (1) classical PKCs (α, β and γ), which are calcium (Ca^{2+}) dependent and activated by diacylglycerol (DAG) or phorbol ester; (2) novel PKCs (δ, ε, η and θ), which are Ca^{2+} independent, but require DAG or phorbol ester for their activation and the atypical isoenzymes (ζ, λ and ι), which seem to be independent of both factors (23). The regulation of cell metabolism induced by PKC would occurs subsequently to their phosphorylation and translocation from cytoskeleton to the plasma membrane (24). Therefore, PKC translocation is a response of their activation

and has been described in several cellular models (25), as RBL-2H3 mast cells, in which classical PKCs translocate to the membrane in response to an antigen stimulus (26). However, PKC can translocates also to the nucleus depending on the stimulus (27, 28). Another important pathway related to PKC is apoptotic cell death, since PKC has been associated with apoptosis and subsequently with caspase activation in several cell types, including mast cells (29-31).

In this study, the effect of c-kit inhibition on PKC activated cells and the relationship between STI571-induced cell death and PKC activation has been determined in HMC-1^{560} and HMC-$1^{560,816}$ cell lines.

MATERIALS AND METHODS

Chemicals

STI571 was provided by Dr. Luis Escribano Mora (Centro de Estudios de Mastocitosis de Castilla la Mancha, Hospital Virgen del Carmen, Toledo, Spain). Phorbol 12-myristate 13-acetate (PMA), latrunculin A derived from *Negombata magnifica*, bovine serum albumin (BSA), Tween 20 and anti β-tubulin were from Sigma-Aldrich (Madrid, Spain). Phosphate buffered saline (PBS) was from Invitrogen (Barcelona, Spain). Anti PKCδ was from BD Biosciences (Madrid, Spain). Anti Mouse IgG was purchased from GE Healthcare (Barcelona, Spain). Anti PKC Clone M110, anti β-actin, anti Histone H1 and polyvinylidene flouride (PVDF) membrane were from Millipore (Temecula, USA). Cell Lab ApoScreen™ Annexin V and DNA Prep™ Stain were from Beckman Coulter (Fullerton, CA, USA). Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were from BioRad (Barcelona, Spain). Negative siRNA control (sc-37007) and PKCδ siRNA (sc-36253) were purchased from Santa Cruz Biotechnology (CA, USA). GeneSilencer® was from Genlantis (San Diego, CA, USA).

Cell cultures

HMC-1^{560} cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and HMC-$1^{560,816}$ cells were kindly provided by Dr. Luis Escribano Mora with permission from Dr. J. Butterfield. They were maintained in Iscove´s modified Dulbecco´s medium (IMDM) (Gibco, Invitrogen, Spain) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Spain) and 100 IU/ml penicillin + 100 µg/ml

streptomycin (Gibco, Invitrogen, Spain) in an atmosphere containing 5% CO_2. The medium was renewed once a week.

Cell preparation

HMC-1 cells were incubated with 25 nM STI571, 100 ng/ml PMA and 200 nM latrunculin during 48 hours. Then cells were centrifuged (1,500 rpm, 5 minutes, 4ºC) and washed in saline solution. The composition of this solution was (mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and glucose 1 g/l.

Western blotting

Cytoplasmic proteins protocol: the cells were resuspended in 80 µl of lysis buffer with the follow composition: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1X complete protease inhibitor (Roche, Spain) and 1X phosphatase inhibitor cocktail (Roche, Spain).

Nuclear proteins protocol: the cells were resuspended in 500 µl of buffer containing: 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 5% Triton X-100, 1X complete protease inhibitor (Roche, Spain) and 1X phosphatase inhibitor cocktail (Roche, Spain) at pH 7.9. Cells were centrifuged at 3,000 rpm for 10 minutes at 4ºC, the supernatant was kept out and the pellet was resuspended on ice in 374 µl of buffer which contains 5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v) at pH 7.9 and 26 µl of 4.6 M NaCl were added. Samples were sonicated four times (10 seconds). Assay tubes were refrigerated on ice for 30 minutes and centrifuged at 13,500 rpm for 20 minutes at 4ºC. Finally, the supernatant was collected and stored at -20ºC.

Membrane proteins protocol: the cells were resuspended in lysis buffer with the following composition: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1X complete protease inhibitor (Roche, Spain) and 1X phosphatase inhibitor cocktail (Roche, Spain). Samples were shaken and sonicated for 1 minute and then refrigerated on ice for 20 minutes. Then cells were sonicated 10 seconds/three times and then centrifuged (12,000 rpm, 20 minutes, 4ºC). Finally, the supernatant was stored at -20ºC.

Once the proteins have been lysated following the three protocols previously described the determination of protein concentration was carried out by using Bradford assay and BSA as protein standard. To separate proteins according to their molecular

weight sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure was used. Proteins were transferred to a PVDF membrane which was blocked with 0.25% non-fat dry milk and then it was incubated for 10 minutes with anti-PKC clone M110 (1:1,000, Millipore), or anti PKCδ (1:1,000, BD Bioscience). After three washes with washing buffer (PBS+0.1% Tween 20), the membrane was incubated for 10 minutes with secondary antibody, anti-Mouse IgG conjugated with horseradish peroxidase (GE Healthcare). A chemiluminescence detection kit (SuperSignal West Femto; Pierce) was used to determine the levels of protein expression. Relative protein expression was calculated in relation to β-actin (0.3:1,000, Millipore) for cytoplasmic and membrane proteins. For experiments with latrunculin, relative protein expression was calculated in relation to β-tubulin (0.3:1,000, Sigma Aldrich). Histone H1 expression (1:1,000, Millipore) was used to calculate relative protein expression for nuclear proteins.

Apoptotic and necrotic cell death determination by flow cytometry

Apoptosis was detected by Annexin-V-FITC/PI staining using the Cell Lab ApoScreen™ Annexin V kit. The cells were incubated with 25 nM STI571 for 48 hours. Then cells were prepared exactly following manufacturer's instructions assay.

Transfection by lipid-based method (Genlantis)

GeneSilencer® (Genlantis) was composed by the transfection reagent and the diluent. Manufacturer's instructions were followed carefully. On the day of transfection two solutions were prepared. On the one hand, solution A, composed by: diluent, FBS/Antibiotic –free IMDM medium and PKCδ siRNA. Control siRNA (sc-37007) was used as negative control for evaluating RNAi off-target effects. On the other hand, solution B was composed by transfection reagent diluted in FBS/Antibiotic-free IMDM medium. Solutions A and B were mixed and incubated for 5 minutes at room temperature. HMC-1^{560} cells were incubated in a total volume of 500 μl in a FBS/Antibiotic-free IMDM medium. The concentration of the cells was $2x10^6$/ml. After 5 hours of transfection 500 μl of IMDM (supplemented with 20% FBS and Penicillin/Streptomicyn 2x) was added to the HMC-1^{560} cells. 19 hours after the cells were incubated with 25 nM STI571 for 48 hours.

Statistical Analysis

Results were analyzed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

RESULTS

As it was well described elsewhere, STI571 induces a cytotoxic effect in HMC-1^{560} cell line and inhibits their proliferation (15-19, 32, 33). In order to determine STI571 involvement on PKC activity in HMC-1 cell lines, several experiments were carried out in this study. First, the effect of 25 nM STI571 on Ca^{2+}-dependent PKCs (cPKC) levels was studied in HMC-1^{560} cells. As Fig. 1B shows tyrosine kinase inhibition does not modify cytosolic cPKC levels, as well as PKC activation. Also, after STI571+PMA co-treatment no modification has been observed. The novel PKCδ isoform has been related with cell death including wild-type kit and oncogenic kit mutant (34) and produces DNA damage through caspase-3 apoptotic pathway in HMC-1 cell line (35-37). Therefore, the effect of STI571 treatment on cytosolic PKCδ levels has been also determined. As it can be seen in Fig. 1C, STI571 treatment induces a decrease of cytosolic PKCδ levels (from 1 to 0.2 ratio intensity values). This negative STI571 effect prevails even when PKC is activated, whereas PMA does not modify cytosolic PKCδ levels. cPKC levels were then determined in HMC-$1^{560,816}$ cell line. In this case, STI571 induces an increase of cPKC levels (protein levels are two-fold higher than in untreated cells), whereas PKC activation has the opposite effect (Fig. 1E). However, after STI571+PMA treatment no modification was observed. As well as for HMC-1^{560} cell line, PKCδ levels were studied in cells with two c-kit mutations. Contrary with the results obtained in the HMC-1^{560} cell line, STI571 does not modify cytosolic PKCδ levels (Fig. 1F), whereas PKC activation induces a significant decrease of cytosolic PKCδ expression (ratio intensity values fall down from 1 to 0.3) and also, the incubation with STI571 prevents this negative PMA effect.

Since cytosolic PKCδ levels decrease in both mast cell lines, nuclear PKCδ levels have been determined with the objective to determine nuclear translocation of this isoform. As Fig. 2C shows, PKCδ translocates to the nucleus after STI571 treatment in HMC-1^{560} cell line, since nuclear levels of this isoform significantly increase from 1 to 3 (ratio intensity values) in the presence of this drug. In the same figure, it can be observed that PMA does not induce any effect. Although PKC activation does not prevent this translocation, PKCδ levels are lower than in the case of STI571 treatment alone. Contrary with the observed in HMC-1^{560} cell line, PKCδ does not translocate to the nucleus in cells with two mutations after STI571 treatment (Fig. 2D). Also, PKC activation induces a significant decrease on nuclear PKCδ levels, while c-kit inhibitor STI571 blocks the effect of PMA. Furthermore, it has been described that

several PKC isoforms translocate to the plasma membrane (38). In this sense, the determination of PKCδ plasma membrane levels was done in order to know if the decrease observed in PKCδ cytosolic expression correlates exclusively with the increase of nuclear expression in HMC-1^{560} cell line. As Fig. 3C shows, STI571 does not cause PKCδ membrane translocation, since the levels of this isoform in the membrane significantly decrease and no increase is observed. Besides, STI571+PMA co-treatment also induces a significant decrease of PKCδ membrane levels. The treatment with PMA alone does not modify plasma membrane levels of this isoform. cPKC and PKCδ plasma membrane levels were determined in order to know a possible translocation to this subcellular place in HMC-1^{560,816} cell line. As it can be seen in Fig. 3D, STI571 does not induce any alteration on cPKC levels, however, a significant increase was observed after PMA treatment. Also, STI571+PMA co-treatment induces a no-significant increase of cPKC levels. Fig. 3E shows that STI571 does not modify PKCδ levels, however, an important decrease on plasma membrane PKCδ band intensity was observed when PKC is activated and after STI571+PMA co-treatment.

Both proteins, tyrosine kinase and PKC, are related with actin cytoskeleton. On the one hand, apoptotic effect of STI571 in HMC-1^{560} cell line has been associated with actin cytoskeleton alteration, accompanied by actin filamentous aggregation and decrease in actin polymerization state (33). On the other hand, it has been described that PKC isoforms binding to F-actin enhances the level of isozyme activity (39). In addition to F-actin, PKC isoforms can be associated with other cytoskeletal proteins (vimentin and cytokeratins). Hence, in order to determine if actin cytoskeleton participates in nuclear PKCδ translocation STI571-induced, the effect of latrunculin, which affects actin cytoskeleton by sequestering G-actin (monomeric) (40), was studied on STI571 treated cells. Although c-kit inhibition does not affect cytosolic cPKC levels in HMC-1^{560} cell line, the levels of these isoforms were determined in the presence of latrunculin. While STI571 and latrunculin do not induce any modification of cPKC levels separately, when both compounds are simultaneously incubated a significant decrease on cytosolic cPKC levels was observed (Fig. 4B). PKCδ cytosolic levels were also determined in HMC-1^{560} cell line in the presence of latrunculin. As Fig. 4C shows, latrunculin partially prevents STI571-negative effect over PKCδ cytosolic expression, since PKCδ levels up from 0.2 to 0.5 ratio intensity values. Moreover, latrunculin does not have any effect by itself. The same study with latrunculin has been carried out in HMC-1^{560,816} cell line. As Fig. 4E shows, latrunculin does not modify cytosolic cPKC levels by itself. Also, latrunculin avoids the enhanced effect induced by STI571

previously described. In the case of PKCδ, no modifications were observed in cytosolic PKCδ levels with any treatment tested (Fig. 4F). Latrunculin has a negative effect in both cPKC or PKCδ cytosolic levels, and consequently, the next step was to determine if latrunculin had any effect on cPKC and PKCδ nuclear levels when c-kit was inhibited in the HMC-1^{560} cell line. As Fig. 5B shows nuclear cPKC levels are not modified at any concentration tested. However, latrunculin partially avoids the nuclear tranlocation of PKCδ induced by STI571, as shown in Fig. 5C (ratio intensity values of nuclear PKCδ falls from 3 to 1.3).

In order to clarify the cytotoxic effect of STI571 an Annexin V-FITC technique was used in HMC-1^{560} cell line. This method allows us to determine the distribution of the three different cellular populations; live, apoptotic and necrotic. As Fig. 6 shows, the percentage of viable cells in untreated sample is 70% whereas necrotic cells represents the other 30%. When HMC-1^{560} cells were incubated with 25 nM STI571, live cells represent around 40% of the total, apoptotic cells population is 39%, while necrotic population is around 15%.

Apoptotic cell death has been related to the PKCδ isoform, specifically with its activation and translocation in several cell lines (29). As it was previously described PKCδ isoform translocates to the nucleus in HMC-1^{560} cells with c-kit receptor inhibited. Also, the inhibition of c-kit receptor causes apoptotic cell death in the 39% of HMC-1^{560} population. In this sense, the possible relationship between apoptotic cell death STI571-induced and PKCδ isoform has been analyzed in HMC-1^{560} cells. Therefore, the next step was to silenciate PKCδ and evaluate the effect of tyrosine kinase inhibitor in the absence of this protein. The first step was focused on demonstrating the effectiveness of PKCδ silencing in HMC-1^{560} cells, using a tested siRNA PKCδ (41-44). For this, a determination of cytosolic PKCδ levels was carry out during STI571 treatment (48 hours). As it can be observed in Fig. 7B, cytosolic expression of PKCδ isoform is significantly decreased (from 0.46 to 0.16) in HMC-1^{560} transfected cells. Therefore, it can be concluded that an inhibition around 65% of cytosolic PKCδ expression has been obtained. Cytotoxic effect of STI571 when PKCδ isoform is silenced has been determined by Annexin V/FITC technique. As it can be seen in Fig. 8, the distribution of three populations in untreated cells is similar either in PKCδ-silenced or no transfected cells. However, when HMC-1^{560} cells were treated with STI571, the apoptotic cell death induced by this tyrosine kinase inhibitor disappears in PKCδ-silenced cells, since apoptotic population falls down from 40 to 15%.

Hence, results obtained in this paper clearly demonstrate that the apoptotic cell

death induced by c-kit inhibitor STI571 is PKCδ-dependent in HMC-1[560] cell line. Also, PKCδ isoform translocates to the nucleus in response to c-kit inhibition and to achieves this translocation actin cytoskeleton integrity is essential in this cell line.

DISCUSSION

The results shown in this paper demonstrate that c-kit inhibition is clearly linked with PKC activity. In HMC-1[560] cell line, cPKCs seem to be less sensible to STI571-regulation, since tyrosine kinase inhibitor has only effect when actin cytoskeleton is altered (through latrunculin presence). This fact seems to suggest that actin disruption favors down-regulation effect of c-kit inhibition over cPKCs. Also, in HMC-1[560] cell line, PKC activation does not induce a decrease either on cPKCs or PKCδ cytosolic levels. This observation is in accordance with a previous study in which the PKC was only activated for 10 minutes and no modifications on cPKC levels were observed (28). Also, it was observed that c-kit inhibition induces nuclear translocation of PKCδ isoform in HMC-1[560] cell line, which is accompanied with a decrease on cytosolic and membrane levels. Moreover, it has been demonstrated that PKCδ translocation to the nucleus takes place through actin cytoskeleton, since when this is disrupted the PKCδ translocation is lower. This fact indicates that apoptotic effect due to c-kit inhibition is actin cytoskeleton-dependent, specifically, it has been described in another study that apoptotic effect induced by c-kit inhibition is accompanied with actin filamentous instability in colon adenocarcinoma cells (33). Also, it was observed that nuclear PKCδ translocation induced by c-kit inhibition predominates over PKC activation. Besides, the inhibitory effect due to c-kit inhibition has been described over other different kinases including PKCε and protein tyrosine kinase 2 (PyK2) accompanied with a negative STI571 effect on cytosolic expression of these proteins in T98G malignant glioma cells, which indicates that these kinases might be implicated as targets for the drug (22).

In HMC-1[560,816] cell line, the c-kit inhibition has an opposite effect in cPKC levels, since in this case tyrosine kinase inhibition induces an increase of cPKC levels, whereas does not have any effect on PKCδ expression. The positive effect of c-kit inhibition over cPKC expression was also described in T98G cells (malignant glioma cells) in which 5 µM STI571 increases PKCα and βI cytosolic expression (22), both belong to cPKC family, the same group as it was observed in the present study. As in

T98G cells, in this paper a positive relationship between c-kit inhibition and cPKC was described in HMC-1^{560,816} cell line. Moreover, the effect of c-kit inhibition on cell degranulation was previously study in HMC-1^{560,816} cell line, since the inhibition of c-kit negatively affects spontaneous histamine release (45). Therefore, the enhance effect that c-kit inhibition has over cPKC cytosolic expression might be related with HMC-1^{560,816} cells activation. As it was described, PKC activation induces membrane translocation of cPKC in these cells, which is in agreement with a decrease on cytosolic cPKC levels. However, results shown in this paper demonstrate that when PKC activation and c-kit inhibition take place simultaneously, cytosolic cPKC levels are not modified, therefore, this fact might indicate a possible competitive action between both compounds (PMA and STI571). This conclusion is in accordance with the results obtained when membrane cPKC levels were determined, since the competition of both drugs avoids membrane cPKC translocation. This relationship does not exist in HMC-1^{560} cell line, since tyrosine kinase inhibition does not have any effect on cPKC isoforms, as it only modifies PKCδ isoform. Moreover, in HMC-1^{560,816} cell line, PMA induces a decrease on cytosolic cPKC and PKCδ levels. On the one hand, cPKC isoforms translocate to the membrane when they are activated. It is important to note that the plasma membrane is a subcellular localization very common when this kinase is activated (38, 46). On the other hand, PKCδ isoform does not translocate to the plasma membrane or to the nucleus, in fact, membrane PKCδ levels decrease when this kinase is activated. Hence, this suggests an intense down-regulation of this isoform in the HMC-1^{560,816} cell line. Besides, this regulation on cytosolic PKCδ expression when this kinase is activated was also described in liver tumor, suggesting an inhibitory role that this isoform may have in tumor cell proliferation (47). In HMC-1^{560,816} cell line, this down-regulation of PKCδ expression is not only observed in the cytosol but also in plasma membrane and nuclear fractions. It has been described in a previous study in RBL-2H3 cell line that the treatment for 30 minutes (short-term) with PMA implies the translocation of the protein from the cytosol to the membrane and also an increase on cell immunological response. However, when PKC is activated for a long period of time (18 hours), the result is the complete absence of degranulation and the absence of cytosolic PKC. Moreover, in this study it was not explained which family or isoform was lost (48). With the results obtained in our study, it can be confirmed that in HMC-1^{560,816} cell line, a long period of exposure to PMA implies the degradation of a specific isoform, PKCδ. Taking into account that PKC has a central role in mast cell activation and degranulation (45, 49-52), these results might indicate a specific role of PKCδ in HMC-1^{560,816} cell line activation that is not present in the other cell line HMC-1^{560}. Besides, c-kit inhibition blocks the degradation of PKCδ isoforms when they are

activated in the HMC-1^{560,816} cell line, both in cytosol and nucleus, but not in the membrane, which suggests the important relationship between c-kit and PKC activation pathways in the intracellular space.

The PKCδ translocation to the nucleus is closely related with cell death in HMC-1^{560} cells, since as it was described in other studies, PKCδ translocates from the cytoplasm to the nucleus in response to apoptotic stimuli (17), which in our case is STI571 treatment. Therefore, our results confirm that apoptosis induced by c-kit inhibition takes place through PKCδ activation. Also, further experiments need to be carry out in order to determine the mechanism used by STI571 to induces PKCδ activation. In this sense, several intracellular changes might be occur in STI571-induced cell death as well as nuclear PKCδ translocation, since caspase-3, mitogen activated protein kinases (MAPKs) and PARP are proteins also involved in STI571-apoptotic cell death (53, 54). It is important to note that actin cytoskeleton plays an important role in this translocation, but also in the regulation of cPKC levels either in HMC-1^{560} or HMC-1^{560,816} cell line when c-kit is inhibited. Our results show that in HMC-1^{560} cell line, c-kit inhibition does not have any effect on cPKC cytosolic levels by itself, however, actin cytoskeleton disruption favours a decrease on cytosolic levels of these isoforms. Also, this decrease on cPKC levels is not accompanied with an increase in the nucleus. Therefore, cPKC isoforms might be degraded or translocated to another subcellular place such as the plasma membrane. In cells with two mutations actin cytoskeleton has also a crucial assignment on cPKC levels when c-kit is inhibited. As it was previously described c-kit inhibition induces an increase on cytosolic cPKC levels abolished by actin cytoskeleton disruption, as well as PKC activation. This fact clearly indicates that c-kit regulation over cPKC depends on PKC activation and actin cytoskeleton integrity in HMC-1^{560,816} mast cell line. The regulation due to STI571 over other several kinases (CDK4, CDK1 or ERK) was also described in malignant glioma cell lines (U87MG, T98G, LNZ308, RG, G44, G112, G130, and G168) (22).

Results obtained in the present paper demonstrate that PKCδ isoform is related with apoptotic cell death in HMC-1^{560} mast cell line. Also, in the present study PKCδ was silenced in HMC-1^{560} mast cell line for first time. Transfection success was evident, since a significant decrease (around 65%) on cytosolic PKCδ levels was observed. Moreover, this study demonstrates that apoptotic cell death induced by c-kit inhibition is clearly PKCδ-dependent in HMC-1^{560} cell line. In this way, when PKCδ expression is abolished, apoptotic effect induced by c-kit inhibition is three-fold lower. For this, it can be stated that PKCδ plays an essential role in apoptotic cell death induced by STI571. This conclusion is of potential relevance, and should be beared in mind in patients with

STI571 treatment, since any factor or secondary effect that could down-regulate PKCδ might diminish three fold the apoptotic cell death induced by the tyrosine kinase inhibitor STI571.

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FIGURE LEGENDS

Figure 1. Effect of STI571 treatment and PKC activation on cytosolic cPKC and PKCδ levels in HMC-1^{560} and HMC-1^{560,816} cell lines. (A) and (D) are a representative experiment of each condition in HMC-1^{560} and HMC-1^{560,816} respectively. (B) and (C) represent cytosolic cPKC and PKCδ levels for HMC-1^{560} cell line, whereas (E) and (F) represent cytosolic cPKC and PKCδ levels for HMC-1^{560,816} cell line. Protein levels are represented in relation with β-actin cytosolic levels. Mean ± SEM of three experiments. (*) significant differences between untreated and treated cells.

Figure 2. Effect of STI571 treatment and PKC activation on nuclear PKCδ levels in HMC-1^{560} and HMC-1^{560,816} cell line. (A) and (B) show a representative experiment of each condition in HMC-1^{560} and HMC-1^{560,816} respectively. (C) HMC-1^{560} and (D) HMC-1^{560,816} nuclear PKCδ expression was represented in relation with Histone H1 protein band intensity. Mean ± SEM of three experiments. (*) significant differences between untreated and treated cells.

Figure 3. Effect of STI571 treatment and PKC activation on plasma membrane cPKC and PKCδ levels in HMC-1^{560} and HMC-1^{560,816} cell line. (A) and (B) show a representative experiment of each condition in HMC-1^{560} and HMC-1^{560,816} respectively. HMC-1^{560} (C) and HMC-1^{560,816} (E) plasma membrane PKCδ expression was represented in relation with β-actin plasma membrane expression. (D) plasma membrane cPKC expression in relation with β-actin in HMC-1^{560,816} cell line. Mean ± SEM of three experiments. (*) significant differences between untreated and treated cells.

Figure 4. Effect of STI571 and PKC activation on cytosolic cPKC and PKCδ levels in HMC-1^{560} and HMC-1^{560,816} cell lines-latrunculin incubated. (A) and (D) are a representative experiment of each condition in HMC-1^{560} and HMC-1^{560,816} respectively. (B) and (C) represent cytosolic cPKC and PKCδ levels for HMC-1^{560} cell line, whereas (E) and (F) represent cytosolic cPKC and PKCδ levels for HMC-1^{560,816} cell line. Mean ± SEM of three experiments. (*) significant differences between untreated and treated cells.

Figure 5. Effect of STI571 and PKC activation on nuclear cPKC and PKCδ levels in HMC-1^{560} cell line-latrunculin incubated. (A) representative experiment of each condition. cPKC (B) and PKCδ (C) nuclear expression was represented in relation with Histone H-1 band intensity. Mean ± SEM of three experiments. (*) significant differences between untreated and treated cells. (#) significant differences between

STI571 and STI571+Latrunculin treated cells.

Figure 6. Determination of live, apoptotic and necrotic HMC-1^{560} cells treated with 25 nM STI571. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic (upper panel) cells were detected by Annexin-V-FITC/PI staining. Graphs represent one of three repeated experiments: (A) untreated cells and (B) STI571-treated cells. The mean ± SEM of three experiments is represented in Fig. 6C. (*) represents significant differences between untreated and treated cells.

Figure 7. Determination of PKCδ silencing efficiency by western blotting analysis in HMC-1^{560} cell line. (A) shows a representative image of each contition. (B) mean ± SEM of three experiments. Cytosolic PKCδ levels were represented respect to cytosolic β-actin expression. (*) significant differences between no transfected and PKCδ-silenced cells.

Figure 8. Determination of live, apoptotic and necrotic population in PKCδ-silenced HMC-1^{560} cells treated with 25 nM STI571. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic (upper panel) cells were detected by Annexin-V-FITC/PI staining. Graphs represent one of three repeated experiments: (A) untreated cells and (B) STI571-treated cells. The mean ± SEM of three experiments is represented in Fig. 8C. (*) statistical significance difference between no transfected and PKCδ transfected HMC-1^{560} cells.

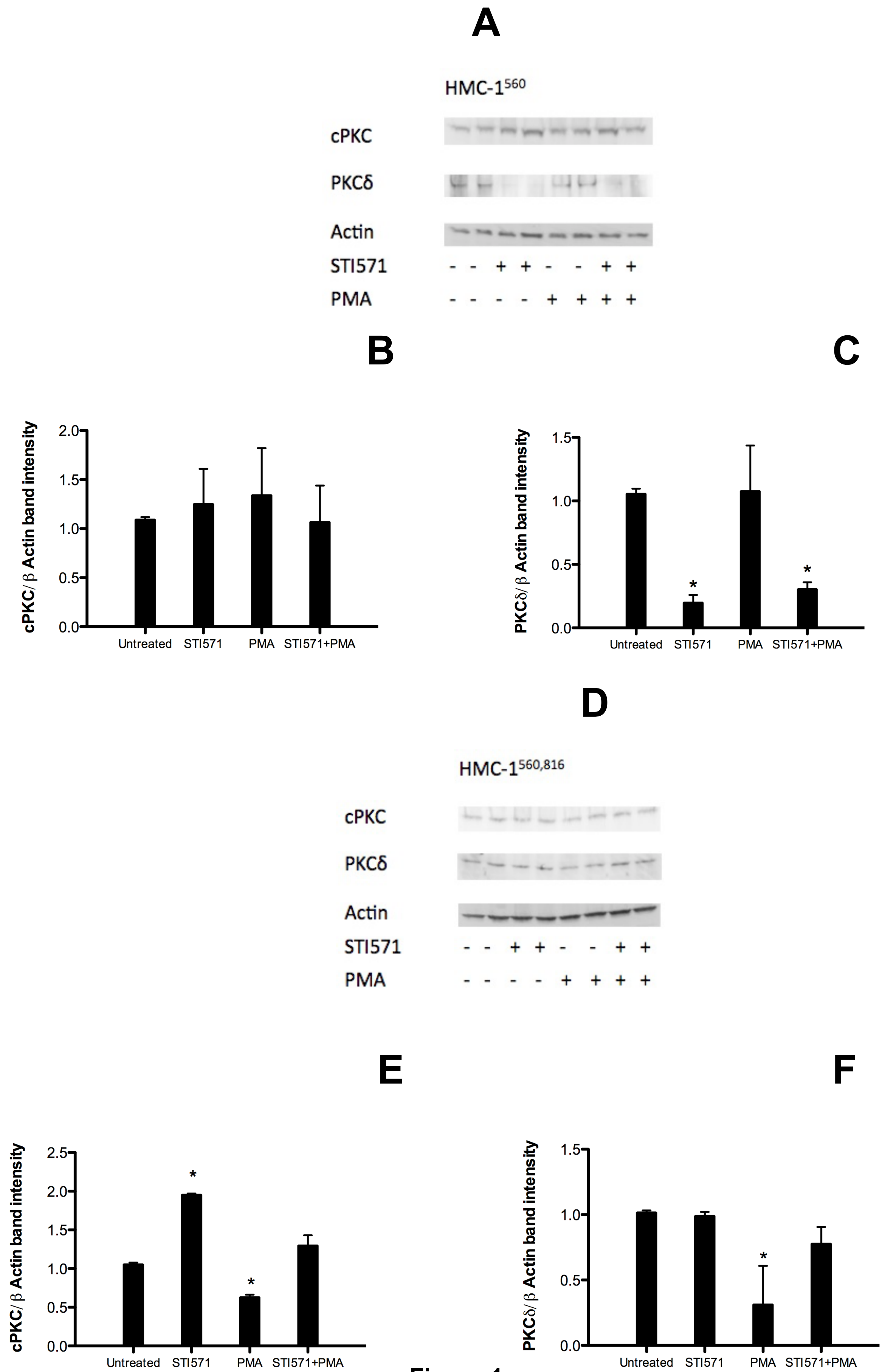


Figure 1

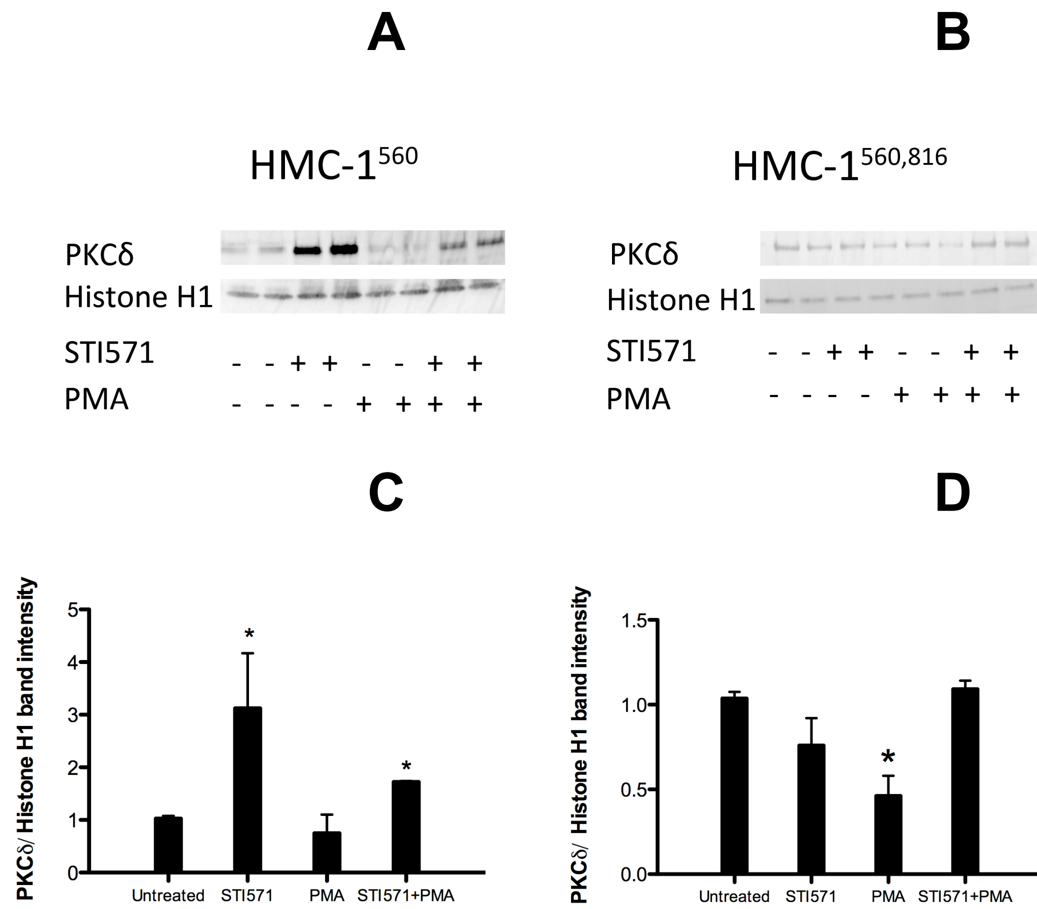


Figure 2

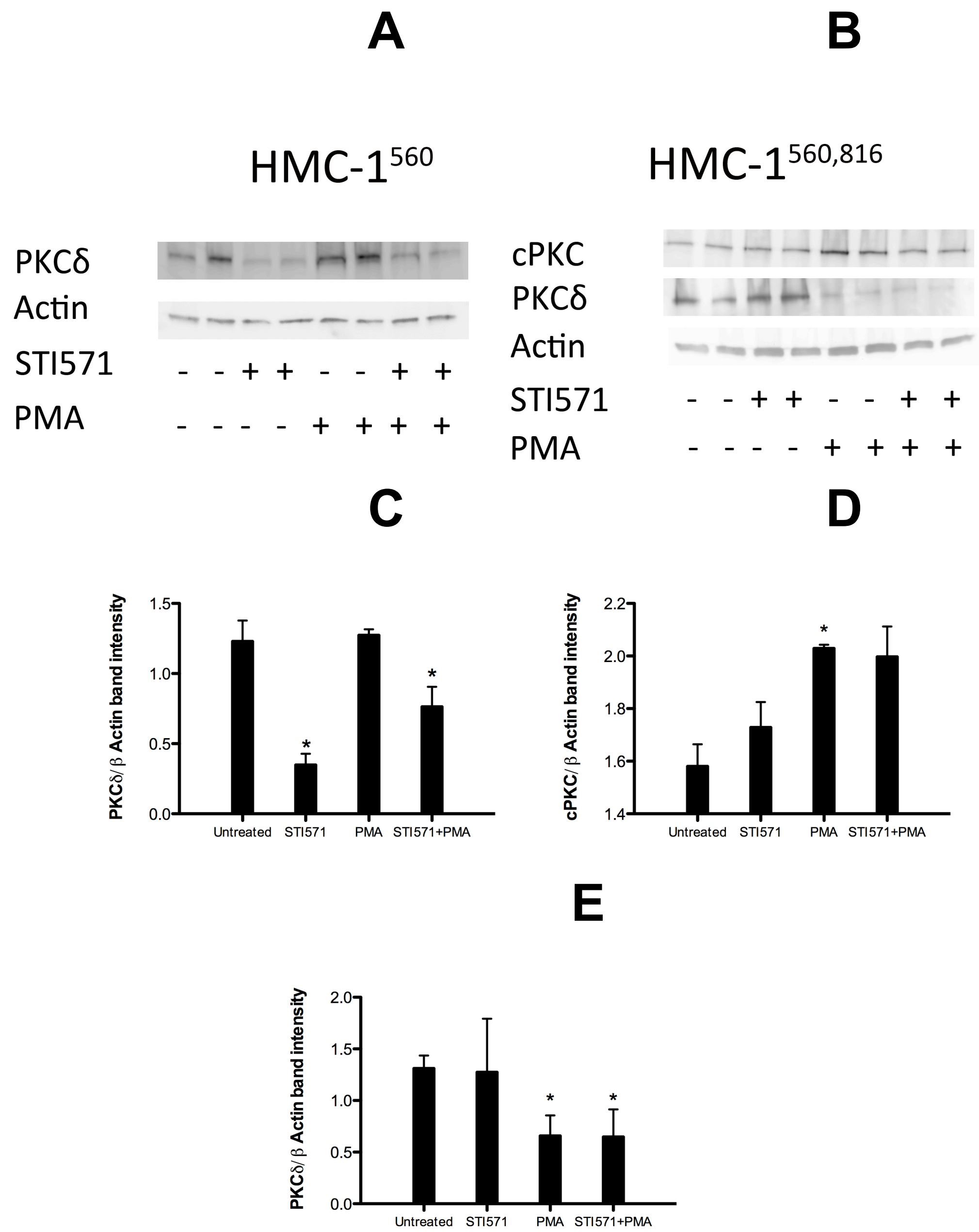


Figure 3

A

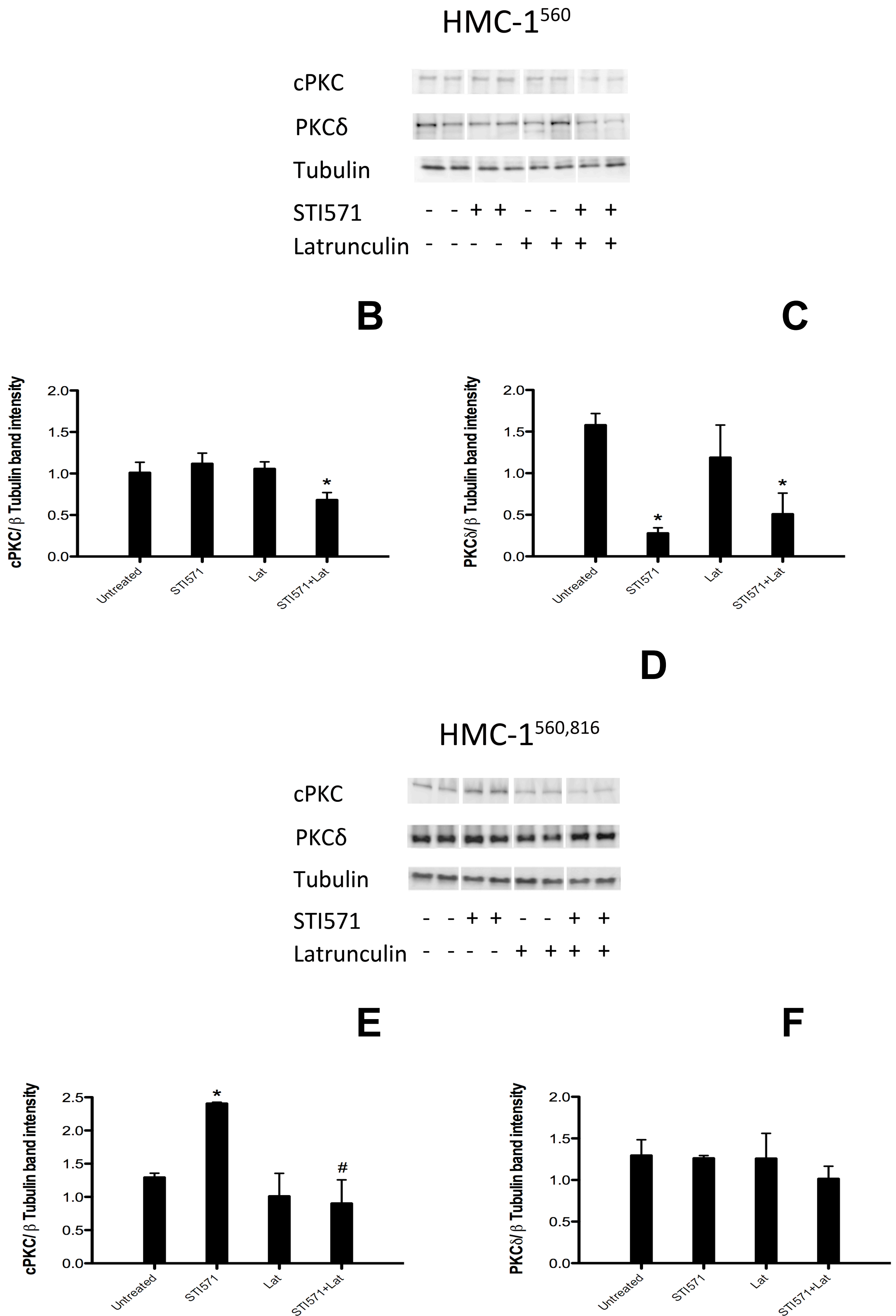


Figure 4

A

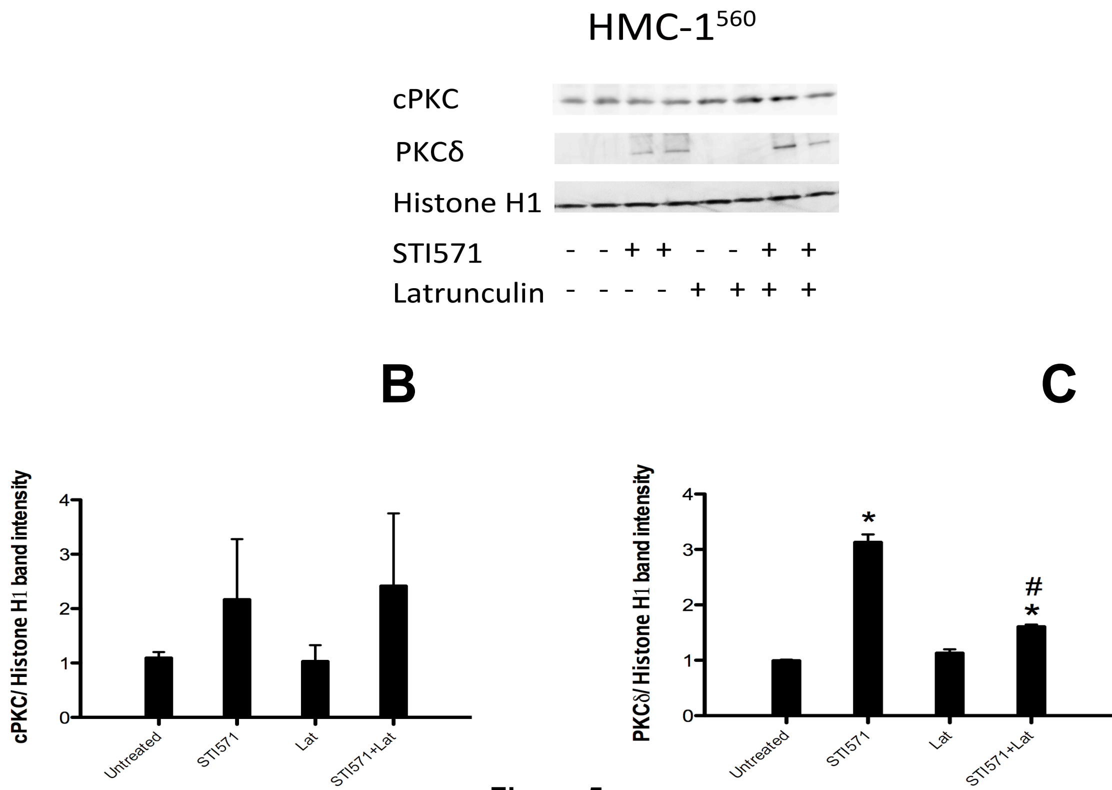
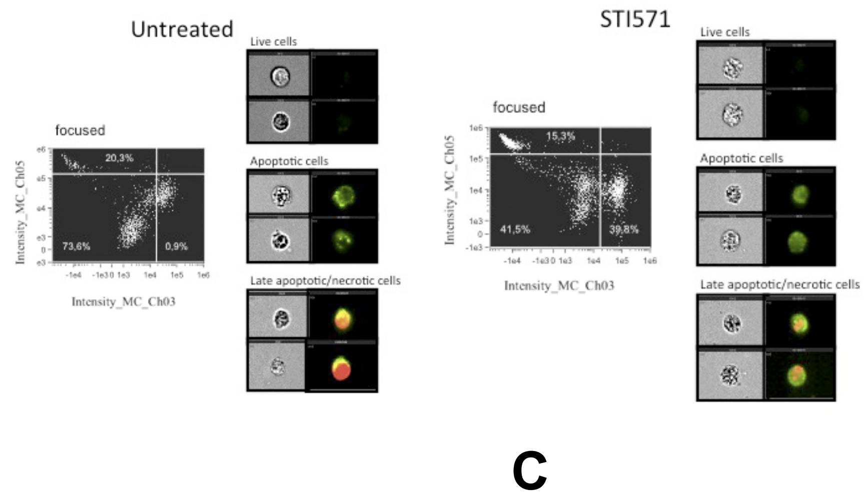


Figure 5



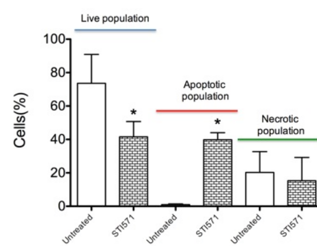


Figure 6

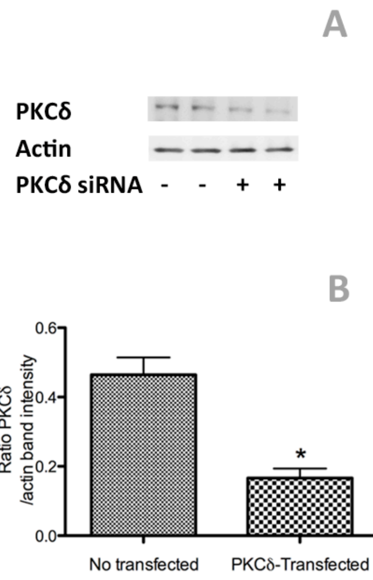


figure 7

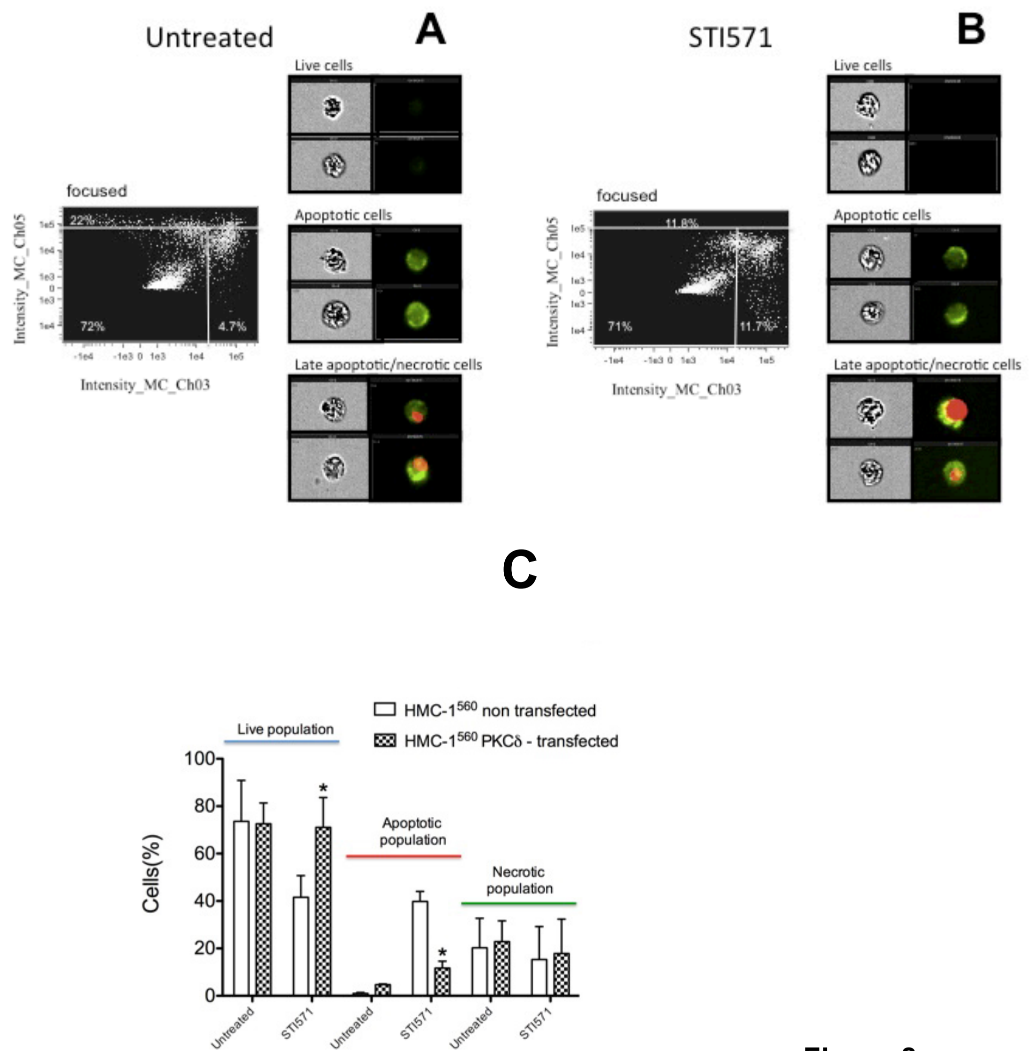


Figure 8

II.2. La muerte apoptótica inducida por el inhibidor de tirosina quinasas dasatinib es dependiente de PKCδ en las líneas celulares HMC-1.

Resumen

En este trabajo se estudia la citotoxicidad de varios inhibidores de tirosina quinasas como el dasatinib, nilotinib, midostaurina y el análogo de nucleósido cladribina. Los resultados nos indican que el fármaco con mayor poder citotóxico frente a células HMC-1^{560} es el nilotinib, seguido del dasatinib y la midostaurina. En el caso de la línea celular HMC-$1^{560,816}$ el compuesto que induce una mayor citotoxicidad es la midostaurina, mientras que el dasatinib y el nilotinib ocupan el segundo y tercer lugar. Es importante destacar que la cladribina tan solo tiene efecto citotóxico en células HMC-$1^{560,816}$, con ausencia de efecto sobre la línea celular HMC-1^{560}. Teniendo en cuenta las dos líneas celulares el dasatinib ha resultado ser el fármaco más potente, por lo que es seleccionado para realizar un estudio comparativo entre las dos líneas celulares del posible efecto modulador de la PKC sobre el mecanismo de acción de los inhibidores de tirosina quinasas. Los resultados obtenidos en este trabajo nos muestran que el dasatinib estimula la liberación espontánea de histamina en ambas líneas celulares, al igual que la activación de la PKC. Este efecto se mantiene tras la modulación de ambas rutas: PKC y tirosina quinasas. Además el dasatinib inhibe claramente la expresión de las isofomas clásicas de la PKC y de la PKCδ en células HMC-1^{560}. Sin embargo en células HMC-$1^{560,816}$, el dasatinib provoca la traslocación de las isoformas clásicas a la membrana y la de la PKCδ al núcleo. Finalmente se ha comprobado que la isoforma PKCδ es imprescindible para que el dasatinib posea efecto apotótico en ambas líneas celulares, ya que la silenciación de esta proteína implica la pérdida de actividad citotóxica del fármaco.

Apoptotic cell death induced by the tyrosine kinase inhibitor dasatinib is PKCδ-dependent in HMC-1 cell lines.

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Abreviations: PKC, Protein Kinase C; HMC-1, Human Mast Cell Line; TyrK, Tyrosine Kinase; PMA, Phorbol-12-Myristate-13-Acetate; MAPK, Mitogen Activated Protein Kinase; PDGFR, Platelet-Derived Growth Factor Receptor; FLT-3, Fms-like Tyrosine Kinase 3.

Key Words: HMC-1; PKC; c-kit; Dasatinib; PMA.

ABSTRACT

Human mast cell (MC) lines, HMC-1^{560} and HMC-1^{560,816}, are frequently found in patients with mastocytosis. Dasatinib, nilotinib and midostaurin belong to the second generation of tyrosine kinase inhibitors and have been described as a useful tool in systemic mastocytosis treatment. Results obtained demonstrate that nilotinib is the compound that has the highest cytotoxicity against HMC-1^{560} cells, while midostaurin is the most potent in HMC-1^{560,816} cells. A positive effect of dasatinib over spontaneous histamine release has been described in both MC lines. In this sense, this enhancer effect might be related with the secondary reactions described in dasatinib-treated patients. PKC activation is an important signal to MC degranulation. This PKC role is clearly demonstrated in this study, since an increase on spontaneous histamine release is observed after PKC activation either in HMC-1^{560} or HMC-1^{560,816} cells. In HMC-1^{560,816} cells, tyrosine kinase inhibitor dasatinib induces Ca^{2+}-dependent PKCs translocation from the cytosol to the membrane, whereas PKCδ is translocated from the cytosol to the nucleous. PKCδ isoform is essential on the apoptotic cell death dasatinib-induced in both MC lines. In this sense, apoptosis induced by dasatinib is abolished when PKCδ expression is inhibited, whereas when this isoform is expressed in normal conditions, tyrosine kinase inhibitor has their normal activity. Therefore, the apoptotic downstream dasatinib-induced is accompanied with nuclear PKCδ translocation in HMC-1^{560,816} cells, whereas in cells with one mutation PKCδ isoform does not translocate to the nucleous. It can be concluded that in HMC-1 cell line, apoptotic cell death induced by dasatinib is PKCδ-dependent.

INTRODUCTION

Mast cells (MCs) are derived from $CD13^{+}$, $CD34^{+}$ and c-kit^{+} (also called CD117) hematopoietic lineage cells (1). These cells are responsible of the defense against external pathogens and are also involved with antigen reactions during atopy and anaphylaxis (2-4). The inflammatory reactions are distinguished by the release of potent mediators subdivided into three groups: (1) granule-associated mediators (histamine, proteoglycans and neutral proteases), (2) lipid-derived mediators (leukotriene B_4 or prostaglandin D_2) and (3) citokines and chemokines (interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, tumor necrosis factor α, chemokines CCL-2, CCL-3, and CCL-5) (5-9). Mastocytosis is a rare disorder characterized by uncontrolled expansion and accumulation of tissue MCs in one or various organs (10-12). Therefore, depending on the localization of the lesions, mastocytosis has been classified as cutaneous mastocytosis (CM), defined by typical skin lesions, systemic mastocytosis (SM) and localized MCs tumors. World Health Organization describes several types of CM, such as urticaria pigmentosa (UP) and diffuse CM (DCM) and various subvariants of SM: indolent systemic mastocytosis (ISM) or smoldering systemic mastocytosis (SSM) (11-14). The presence of activated c-kit mutations explains the autonomous development of neoplastic MCs. In this sense, it has been described two different Human Mast Cell Lines (HMC-1), HMC-1^{560} and HMC-$1^{560,816}$. Both carries Gly-560 → Val ($V560G^{+}$) mutation in the 560 position of c-kit receptor. However, only the second one, HMC-$1^{560,816}$ has the other mutation: Asp-816 → Val. The first mutation in the position 560 occurs in the juxtamembrane region of c-kit and leads to a permanent ligand (Stem Cell Factor) independent activation of c-kit in both MCs lines. The Asp-816 → Val mutation is detectable in the most part of the patients with SM, including aggressive SM, indolent SM or mast cell leukemia, whereas in CM codon 816 c-kit mutation is rare and only founded in atypical pediatric cases and a group of adults. In fact, c-kit mutations are generally not detectable in CM (11). The role of Asp-816 → Val mutation in SM is very important since contributes to growth of MCs.

Chronic myeloid leukemia (CML) is a malignant myeloproliferative disease characterized by the translocation of chromosomes 9 and 22, resulting the short Philadelphia chromosome carrying the Breakpoint Cluster Region-Abelson Leukaemia (Bcr-Abl) fusion gene (15). The majority of CML patients (95%) express the splice variant (p210 with 210-kDa) of this protein, this fact converts this protein into the main target for CML treatment. Two different phases have been described in CML. First, a chronic phase due to an activated Abl tyrosine kinase (TyrK) domain takes place. The

expansion of granulocyte and macrophage progenitor cells appears in this first stage. Then, the presence of clones that express mutant forms of Bcr-Abl emerge inducing the advanced phases of CML (15). Imatinib (Signal Transduction Inhibitor (STI) 571 or Gleevec®) is the first generation of TyrK inhibitors that acts by inhibiting the c-kit receptor, Brc-Abl and platelet derived growth factor receptor (PDGFR) (16). This compound has been widely used in CML treatment as the first therapy option. However, imatinib treatment is not efficient in patients with CML in advanced phases. This is probably because the leukaemic population of quiescent stem cells (non-cycling G_0), created by mutated forms of Brc-Abl, are imatinib-resistant (15). Besides, imatinib is not able to induce cell death in HMC-$1^{560,816}$ MC line and therefore several imatinib-resistant patients were identified (16, 17). In this sense, more studies have been performed in order to identify compounds that interfere with c-kit activity, specifically against Asp-816 → Val mutation. These compounds are the second generation TyrK inhibitors. In this way, dasatinib (formerly BMS-354825), midostaurin (PKC412) and nilotinib (AMN107) are included in the second-generation of TyrK inhibitors that induce HMC-$1^{560,816}$ cell death. Dasatinib is a novel, oral and multitargeted TyrK inhibitor. As well as c-kit this drug inhibits other TyrKs such as Bcr-Abl, Src, ephrin receptor kinase, p38 MAPK and PDGFR (15, 18, 19). Scr family kinases (SFKs) are a group of non-receptor TyrKs composed by eleven members (including Lyn) that have been described as mediators in cell migration, adhesion and cellular survival (20). Dasatinib is the dual inhibitor, Bcr-Abl and Src kinases, most commonly used in CML patients. In 2010, dasatinib was approved as a first-line treatment of patients with CML (100 mg once a day) and it has been described that is 300-fold more potent than imatinib against wild-type Bcr-Abl (19, 21, 22). In spite of having multiple targets, Src kinase Lyn was defined as the most important dasatinib target in CML and breast cancer lines (23, 24). Moreover, Bruton tyrosine kinase (Btk) as well as Lyn were identified as the two protein targets for dasatinib (25). Both, Lyn and Btk, are c-kit-independent signaling molecules in both MC lines, HMC-1^{560} and HMC-$1^{560,816}$. These proteins were described as an activated stage in both mast cell lines and collaborate with the growth of neoplastic MCs (26). Specifically, dasatinib binds to Btk and Lyn and blocks their activity either in HMC-1^{560} or HMC-$1^{560,816}$ MC lines. The alkaloid derivative of staurosporin, midostaurin, inhibits various TyrKs: c-kit, FLT-3 and PDGFR, as well as a PKC (27). Besides, midostaurin colaborates with dasatinib and nilotinib inducing apoptotic cell death in neoplastic MCs (27-29). With regard to nilotinib, this TyrK inhibitor has several receptor targets in common with imatinib, such as Brc-Abl, c-kit and PDGFR tyrosine kinases (30). Although nilotinib is effective against HMC-1^{560} MC line, its cytotoxic effect over HMC-$1^{560,816}$ cells is very low (27). As with TyrK inhibitors,

other compounds such as the nucleoside analog cladribine has been used as a useful tool for treatment of SM (31). However, cladribine is not effective in diseases characterized by fast progress, like aggresive SM (ASM) or mast cell leukemia (MCL), therefore, alternative drugs are necessary for the treatment of these diseases (31).

Protein Kinase C (PKC) is a family of serine/threonine kinases that belong to the ACG superfamily, that includes cAMP-dependent, cGMP-dependent protein kinases and PKC (32). PKC family is composed by different isoforms divided into three classes depending on their sensitivity to calcium (Ca^{2+}) and phorbol esters: (1) Ca^{2+}-dependent isozymes (or cPKCs) (α, β1, β2 and γ) that can be activated by diacylglycerol (DAG) or 12-O-tetradecanoylphorbol-13-acetate (PMA); (2) Ca^{2+}-independent isozymes (or novel PKCs) (δI-III, ε, η, and θI-II) activated by DAG and finally; (3) atypical isozymes (ζ, PKMζ and ι/λ), which are DAG and Ca^{2+}-independent (33). PKC plays a primordial role on MCs activation and degranulation, therefore, the effect of this kinase in mastocytosis has been reported in several studies (34-38). PKC activation has been described as a requirement for MC exocytosis and cell migration and adhesion (39, 40). Therefore, since PKC plays an essential role in MC activity, the relationship between TyrK inhibition and PKC activation was studied in detail in this work.

MATERIALS AND METHODS

Chemicals

Dasatinib (sc-358114), nilotinib (sc-202245), negative siRNA control (sc-37007) and PKCδ siRNA (sc-36253) were purchased from Santa Cruz Biotechnology (CA, USA). Midostaurin (M1323), cladribine (C4438), PMA, Tween 20, bovine serum albumin (BSA) were from Sigma-Aldrich (Madrid, Spain). Orthophthaldialdehyde was from Sigma-Aldrich (MO, USA). Phosphate buffered saline (PBS) was from Invitrogen (Barcelona, Spain). Anti Mouse IgG was purchased from GE Healthcare (Barcelona, Spain). Trichloroacetic acid and hydrochloric acid were from Panreac (Barcelona, Spain). Anti PKC Clone M110, anti β-actin, anti Histone H1 and polyvinylidene flouride (PVDF) membrane were from Millipore (Temecula, USA). Anti PKCδ was from BD Biosciences (Madrid, Spain). Cell Lab ApoScreen™ Annexin V and DNA Prep™ Stain were from Beckman Coulter (Fullerton, CA, USA). Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were from BioRad (Barcelona, Spain). GeneSilencer® was from Genlantis (San Diego, CA, USA).

Cell cultures

HMC-1^{560} cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and HMC-$1^{560,816}$ cells were kindly provided by Dr. Luis Escribano Mora with permission from Dr. J. Butterfield. They were maintained at 37ºC in Iscove´s modified Dulbecco´s medium (IMDM) (Gibco, Invitrogen, Spain) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Spain) and 100 IU/ml penicillin + 100 µg/ml streptomycin (Gibco, Invitrogen, Spain) in an atmosphere containing 5% CO_2. The medium was renewed once a week.

MTT assay

HMC-1^{560} and HMC-$1^{560,816}$ cell lines were incubated with different drugs for 48 hours at 37ºC. Then cells were washed with saline solution and then incubated with MTT (250 µg/ml) for 30 minutes. After washed with saline solution the cells were resuspended in 200 µl of water and sonicated for 1 minute. Absorbance was determined in a Bio-Tek Synergy 4 plate reader at 595 nm.

Histamine Release Assays

Cells were washed with saline solution with the following composition (mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and glucose 1 g/l. Then cells were centrifuged (1,500 rpm, 5 minutes, 4ºC) and resuspended in saline solution. HMC-1 cells were incubated with 100 ng/ml PMA and with 0.012 µM (HMC-1^{560}) and 0.6 µM (HMC-$1^{560,816}$) dasatinib for 48 hours. The incubations were stopped by immersing tubes in a cold bath. After centrifugation at 2,300 rpm for 10 minutes (4ºC), the supernatants were decanted into other tubes for histamine determination and pellets were diluted in 200 µl hydrochloric acid 0.1 N. To ensure total histamine, pellets were sonicated for 60 seconds. Then, 200 µl trichloroacetic acid (5%) was added to each tube to avoid protein interferences in the histamine determination. Next, the samples were mixed (20 seconds/3 times). Histamine release was tested with a multi-mode plate reader (Synergy^{TM} 4, BioTek Instruments, Vermont, USA) both in pellets and supernatants according to Shore's method (41). To produce the fluorescent complex, 0.04% orthophthaldialdehyde was used.

Western blotting

Cells were incubated with 0.012 μM and 0.6 μM dasatinib for HMC-1^{560} and HMC-1^{560,816} respectively. Then, three different protocols were carried out:

Cytoplasmic proteins protocol: the cells were resuspended in 80 μl lysis buffer with the follow composition: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1X complete protease inhibitor (Roche, Spain) and 1X phosphatase inhibitor cocktail (Roche, Spain).

Nuclear proteins protocol: the cells were resuspended in 500 μl of buffer containing: 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 5% Triton X-100, 1X complete protease inhibitor (Roche, Spain) and 1X phosphatase inhibitor cocktail (Roche, Spain) at pH 7.9. Cells were centrifuged at 3,000 rpm for 10 minutes at 4ºC, the supernatant was kept out and the pellet was resuspended in 374 μl of buffer which contains 5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v) at pH 7.9 and 26 μl of 4.6 M NaCl were added. Samples were sonicated four times (10 seconds). Assay tubes were refrigerated on ice for 30 minutes and centrifuged at 13,500 rpm for 20 minutes at 4ºC. Finally, the supernatant was collected and stored at -20ºC.

Membrane proteins protocol: the cells were resuspended in lysis buffer with the following composition: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1X complete protease inhibitor (Roche, Spain) and 1X phosphatase inhibitor cocktail (Roche, Spain). Samples were shaken and sonicated for 1 minute and then refrigerated on ice for 20 minutes. Then cells were sonicated 10 seconds/three times and then centrifuged (12,000 rpm, 20 minutes, 4ºC). Finally, the supernatant was stored at -20ºC.

Once the proteins have been lysated following the three protocols previously described the determination of protein concentration was carried out by using Bradford assay and BSA as protein standard. To separate proteins according to their molecular weight sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure was used. Proteins were transferred to a PVDF membrane which was blocked with 0.5% BSA and then it was incubated for 10 minutes with anti-PKC clone M110 (1:1,000) or anti PKCδ (1:1,000). After three washes with washing buffer (PBS+0.1% Tween 20), the membrane was incubated for 10 minutes with secondary antibody, anti-Mouse IgG conjugated with horseradish peroxidase. A

chemiluminescence detection kit (SuperSignal West Pico®; Pierce) was used to determine the levels of protein expression. Relative protein expression was calculated in relation to β-actin (0.3:1,000) for cytoplasmic and membrane proteins. Histone H1 expression (1:1,000) was used to calculate relative protein expression for nuclear proteins.

Apoptotic and necrotic cell death determination by flow cytometry

Apoptosis was detected by Annexin-V-FITC/PI staining using the Cell Lab ApoScreen™ Annexin V kit. The cells were incubated with 0.012 µM or 0.6 µM dasatinib for HMC-1^{560} and HMC-$1^{560,816}$ cells respectively. Incubation time was 48 hours (37ºC). Then cells were prepared exactly following manufacturer's instructions assay.

Transfection by lipid-based method (Genlantis)

GeneSilencer® (Genlantis) was composed by the transfection reagent and the diluent. Manufacturer's instructions were followed carefully. On the day of transfection two solutions were prepared. On the one hand, solution A, composed by: diluent, FBS/Antibiotic –free IMDM medium and PKCδ siRNA. Control siRNA (sc-37007) was used as negative control for evaluating RNAi off-target effects. On the other hand, solution B was composed by transfection reagent diluted in FBS/Antibiotic-free IMDM medium. Solutions A and B were mixed and incubated for 5 minutes at room temperature. Cells were incubated in a total volume of 500 µl in a FBS/Antibiotic-free IMDM medium. The concentration of the cells was $2x10^6$/ml. After 5 hours of transfection 500 µl of IMDM (supplemented with 20% FBS and Penicillin/Streptomicyn 2x) was added to the cells. 19 hours after HMC-1 cells were incubated with 100 ng/ml PMA and 0.012 µM or 0.6 µM dasatinib for HMC-1^{560} and HMC-$1^{560,816}$ cells respectively. Incubation time was 48 hours at 37ºC.

Statistical Analysis

Results were analyzed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

RESULTS

Tyrosine kinases are a good target for cancer treatment, specifically for mastocytosis and other hematological diseases (19). The first step in this study was to determine the cytotoxicity of several TyrK inhibitors, dasatinib, midostaurin, nilotinib and the nucleoside analog cladribine in HMC-1^{560} and HMC-$1^{560,816}$ lines. HMC-1 cells were incubated for 48 hours in presence of these drugs. Figures 1A and 1B show the cytotoxic effect of various concentrations of dasatinib (0.0005 µM to 10 µM) over HMC-1^{560} and HMC-$1^{560,816}$ cells. As figure 1A and 1B show, dasatinib induces a decrease on cell viability either in HMC-1^{560} or HMC-$1^{560,816}$ cells. Specifically, HMC-1^{560} is more sensitive to this TyrK inhibitor (IC_{50}: 0.012 µM) than HMC-$1^{560,816}$ cell line (IC_{50}: 0.6022 µM). Dasatinib treatment leads to a decrease on cell viability around 77% (in HMC-1^{560}) and 90% (in HMC-$1^{560,816}$) with the higher concentration used (10 µM). Moreover, cytotoxic effect of TyrK inhibitor midostaurin (0.001 µM to 5 µM) is studied in HMC-1^{560} (figure 1C) and HMC-$1^{560,816}$ (figure 1D) cell lines. In this case, a similar response to midostaurin treatment is observed in both MC lines, since IC_{50} is 0.2607 µM and 0.4456 µM for HMC-1^{560} and HMC-$1^{560,816}$ respectively. Furthermore, 5 µM midostaurin induces a decrease of cell viability of 80% approximately in both cell lines. The third TyrK inhibitor used in this study is the nilotinib (0.001 µM to 5 µM). Figure 1E shows nilotinib effect on cell viability in HMC-1^{560} cell line, whereas figure 1F represents the effect in HMC-$1^{560,816}$ cell line. Different effect is observed in both cell lines, since HMC-$1^{560,816}$ cells seem to be less sensitive to the drug (IC_{50}: 1.35 µM) than HMC-1^{560} cells (IC_{50}: 0.0025 µM). In addition, nilotinib causes an important decrease on HMC-1^{560} viability (around 80%), while in cells with two mutations this decrease is only around 50%. Finally, the effect of nucleoside analog cladribine (0.035 µM to 35 µM) on cell viability is tested in both MC lines. Figure 1G shows that cladribine does not have any dramatic effect on cell viability in cells with one mutation, since even with the highest cladribine concentration (35 µM) only a decrease on cell viability around 15% is observed. Nevertheless, the effect of this compound in HMC-$1^{560,816}$ is different (figure 1H). In this case, a decrease of 50% on cell viability is observed when HMC-$1^{560,816}$ cells are incubated with 35 µM cladribine and the IC_{50} is 4.25 µM. Considering both MC lines, dasatinib is the most potent compound tested, for this, it has been selected for the study of TyrK inhibition effects on histamine release and PKC activation.

As it was described in previous studies, TyrK inhibition affects histamine release after cellular stimulation in several cell types (42-45). As well as TyrK inhibition, PKC activation also affects MC exocytosis (39). Therefore, the effect of the TyrK inhibitor dasatinib treatment and the PKC activation on MC degranulation is next studied in both

cell lines. As figure 2A shows, dasatinib induces a significant increase of spontaneous histamine release (7%) in HMC-1^{560} cell line. PKC activation also stimulates spontaneous MC degranulation (around 8%), as well as dasatinib+PMA co-treatment (10%). In HMC-1^{560,816} cells, dasatinib significantly increases spontaneous histamine release (4% of increase). A enhancer effect is also observed after PMA or dasatinib+PMA co-treatment, 8 and 10% of increase of histamine release respectively is observed (figure 2B).

Since dasatinib affects spontaneous histamine release in both MC lines, as well as PKC activation, then, the effect of dasatinib treatment over cytosolic levels of this protein were determined. It has been reported that the TyrK inhibitor dasatinib induces apoptotic cell death in HMC-1 cell line, therefore, in addition to cPKCs, cytosolic levels of an isoform intimately related with apoptotic cell death (26), namely PKCδ, were also determined in this study (figure 3) (28, 46-48). As figure 3B shows, in HMC-1^{560} cell line, dasatinib treatment induces a significant decrease of cytosolic cPKCs levels (from 1 to 0.34 ratio intensity values). In HMC-1^{560,816} cells, dasatinib has also a negative effect over cytosolic cPKCs levels, inducing a significant decrease (from 1 to 0.5). In addition, this drug decreases cytosolic PKCδ levels (figure 3C), since ratio intensity values decrease from 1.2 to 0.2 in HMC-1^{560}. A similar effect is observed in HMC-1^{560,816}, since the signal falls down from 0.92 to 0.47. These results indicate that dasatinib has a negative role over cPKC and PKCδ cytotolic levels. As it has been widely described, PKCs isoforms translocate after their activation to different subcellular localizations, such as the membrane, the nucleous or the mitochondria (33, 49). Therefore, in order to know if a translocation of these isoforms is taking place, nuclear levels of these proteins were determined (figure 4). TyrK inhibitor dasatinib induces a significant decrease on nuclear cPKCs levels, from 1.1 to 0.21 (figure 4B) in cells with one mutation. However, in HMC-1^{560,816} cell line, dasatinib did not modify nuclear cPKCs levels. In relation to nuclear PKCδ levels, in HMC-1^{560} cells, dasatinib causes a decrease of this isoform levels from 1.34 to 0.28 (figure 4C). Surprisingly, a sharp increase of PKCδ in the nucleous is observed after TyrK inhibitor treatment in HMC-1^{560,816} cells (levels up from 0.94 to 1.97). The next step was to determine the PKC levels in the plasma membrane (figure 5). Figures 5A and 5B show that TyrK inhibitor dasatinib provokes a significant decrease of plasma membrane cPKCs levels in HMC-1^{560} cells. Nevertheless, dasatinib induces an increase of plasma membrane cPKCs levels in HMC-1^{560,816} cell line (relative values rise from 1.25 to 1.93). Figure 5C shows that TyrK inhibitor does not modify plasma membrane PKCδ levels in HMC-1^{560} cells, whereas in HMC-1^{560,816} a significant decrease is observed after dasatinib

treatment (from 1.2 to 0.46).

As figures 1A and 1B show, dasatinib induces a cytotoxic effect. In order to determine the distribution of apoptotic versus necrotic population the Annexin V/FITC-PI staining and flow cytometry technique was employed. This method allow us to differentiate the distribution of the three different populations: live (negative with both fluorochromes), apoptotic (FITC positive) and necrotic (FITC and PI positive) after dasatinib incubation. Figures 6A and 6B show the distribution of the untreated and dasatinib treated cells respectively, regarding to the intensity of both fluorochromes. Moreover, an example of two single cells of each population is shown. Figure 6C represents the distribution of the three populations: live, apoptotic and necrotic. As figure 6C shows, dasatinib treatment induces a significant decrease on live population (around 30%) in HMC-1^{560} cell line, while apoptotic population increases from 4 to 36%. Also, the necrotic population show a non significant increase from 8 to 17% after dasatinib treatment. For HMC-1^{560,816} cells, the distribution of untreated and dasatinib treated cells is shown in figures 7A and 7B respectively, accompained with two representative cells of each population. In HMC-1^{560,816} cell line, a 35% decrease of live population is observed after dasatinib treatment (figure 7C). Besides, dasatinib affects apoptotic population, since it is increased from 3 to 28%. Also, the TyrK inhibitor does not cause any significant effect on the necrotic population, since the percentage is still 6% after dasatinib treatment. As it was described in other studies, PKCδ isoform has been closely related with apoptotic cell death (46, 50), unlike other isoforms that are associated with different intracelular pathways, such as the case of PKCβ in mast cell exocytosis or insulin release (39, 51). Results previously described show that PKCδ has an important role in HMC-1 cell line. Therefore, another experiment was carried out in order to clarify if apoptotic cell death induced by dasatinb is related with PKCδ isoform. In this sense, the effect of PKCδ silencing over apoptotic cell death due to dasatinib was evaluated in both MC lines. First, the efficience of the lipid-based method transfection was tested by western blot analysis. As figure 8A and 8C shows, this transfection technique is clearly capable of inducing a decrease over cytosolic PKCδ levels in HMC-1^{560} cell line, since cytosolic levels of this isoform are significantly diminished after 36 and 48 hours of silencing (the decrease is about 46 and 65% for 36 and 48 hours respectively). On the other hand, a similar result is obtained in HMC-1^{560,816} cells, since cytosolic PKCδ levels are significantly decreased with 36 and 48 hours after silencing (figure 8B and 8D). After determining that our transfection method is effective, secondly, we determined the effect of dasatinib on live, apoptotic and necrotic populations in PKCδ-silenced cells. For HMC-1^{560} cells, figures 9A and 9B

show the distribution of untreated and dasatinib-treated cells regarding the intensity of both fluorochromes and two representative cells of each population. In the case of HMC-1^{560} cells, the TyrK inhibitor dasatinib does not induce any modification on the live population when PKCδ is silenced (figure 9C). Moreover, no modifications were observed in apoptotic population after dasatinib treatment. Also, necrotic population has also not undergone any change. Figure 10 shows the results obtained in cells with two mutations. A representative plot of untreated and dasatinib treated cells acompanied with cells belonging to the three different populations is shown in figures 10A and 10B, respectively. When PKCδ is silenced in cells with two mutations, the effect of dasatinib disappears (figure 10C), as for the case of HMC-1^{560} cell line. The distribution of the three different populations prevails after TyrK inhibitor incubation. As it can be observed in figure 10C, live population is maintained at 70% after dasatinib treatment. Moreover, dasatinib does not modify the distribution of apoptotic or necrotic populations.

DISCUSSION

In recent years, various TryK inhibitors have been studied and approved as treatment for human cancer (19, 27). Their capacity to inhibit the ATP-binding site of the protein and consequently block the phosphorylation of several proto-oncogenes implies that this group is considered a useful tool in neoplastic diseases. Therefore, TyrK inhibitors prevent the downstream of intracellular transduction pathways in tumour cells (52). Our results demonstrate that nilotinib is the compound that induces the highest cytotoxicity in HMC-1^{560} cell line, whereas dasatinib and midostaurin are the second and the third most efficient drugs. In fact, all possible interactions between these three different TyrK inhibitors induces a synergistic effect in cells with one mutation (28). In the case of HMC-1^{560,816} cells, midostaurin is the most potent TyrK inhibitor of the three compounds tested, followed by dasatinib and nilotinib. It is important to note that nilotinib has an extremely higher effect in HMC-1^{560} than in HMC-1^{560,816} cells. On the one hand the highest concentration of nilotinib (5µM) induces a decrease around 80% of cell viability in cells with one mutation, whereas in HMC-1^{560,816} cells, this decrease is only of 50%. These results are in agreement with those described in a previous study (53). Besides, and contrary with the observed in HMC-1^{560} cells, in cells with two mutations, only the co-treatment of dasatinib with midostaurin or cladribine induces a synergistic effect (28). The enhancer effect

obtained with dasatinib+midostaurin combination seems to be a consequence of a dual effect: the inhibition of Lyn and Btk due to dasatinib and on the other hand the blockage of c-kit by midostaurin (26). Our results are in accordance with this synergistic relationship since the TyrK inhibitor midostaurin inhibits cPKCs isoforms, but does not have any effect on PKCδ isoform (54). In this regard, PKCδ isoform is free to participate on apoptotic cell death induced by dasatinib (54, 55), as our data indicate. Interestingly, results obtained in this paper show that cladribine induces cytotoxicity in HMC-1^{560,816} cell line, but not in HMC-1^{560}, which might explain that the summatory effect observed with dasatinib occurs only in HMC-1^{560,816} cells. Besides, our results corroborate the effect described in a 24 hours-incubation of cladribine in both MC lines (31). Therefore, it can be concluded that cladribine might be considered as a usable treatment of neoplastic MCs with Asp-816 → Val mutation.

As it was previously described PKC has an essential role in MC exocytosis, in fact, PKC activation has been described as a prerequisite for MC degranulation (39). Specifically, PKC translocation to the membrane has been described after IgE stimulation in human cultured mast cells (HCMC) (56). Our results clearly demonstrate the important role that PKC has on MC exocytosis, since their activation stimulates spontaneous histamine release in both MC lines. This is in accordance with the enhancer effect that have the phorbol ester PMA over histamine release also observed in previous studies (57). Besides, TyrK inhibitor dasatinib potentiates spontaneous histamine release in both MC lines. The effect of this compound is opposite to the observed when HMC-1^{560} and HMC-1^{560,816} cells were incubated with other TyrK inhibitor, imatinib (38, 44). It has been described that the TyrK inhibitors imatinib, nilotinib and midostaurin do not affect spontaneous histamine release in both HMC-1 cell lines (29). However, an opposite effect has been described on IgE-induced histamine release, since midostaurin blocks IgE-dependent histamine secretion in cultured human cord blood cell-derived mast cells and basophils (29). It is very important to note that, Btk and Lyn, the two primary targets described for dasatinib in HMC-1 cell lines are related with IgE-dependent exocytosis. Therefore, although dasatinib inhibits IgE-induced histamine release in blood basophils at high drug concentrations (1 μM), it has the contrary effect at low concentrations (45). This dasatinib enhancer effect was described specially in allergic patients characterized by the presence of edema and effusions (45). Also, the blockage of dasatinib-enhanced histamine release with glucocorticosteroids treatment was described in a recent study in basophils (58). Moreover, our data indicate that the enhancer effect that have TyrK inhibitor dasatinib over histamine release might be related with the appearance of

secondary reactions, including cutaneous adverse effects like skin rash and peripheral oedema in the patients (45, 52). In conclusion, our results allow us to explain the origin of secondary effects observed in patients treated with dasatinib, since they might be related with spontaneous histamine release TyrK inhibitor-induced.

The present results confirm that dasatinib is a potent apoptosis inductor in HMC-1^{560} and HMC-$1^{560,816}$ cells, inducing about 40% of apoptotic cells in both MC lines after 48 hours treatment. The apoptotitc effect of TyrK inhibitor described in our paper is in agreement with the conclusions observed in a previous study (28). Even though apoptotic populations were smaller than those obtained in the present paper in both MC lines, it is necessary to bear in mind that the concentrations used in this previous study were lower (0.003 and 0.3 μM) and 24 hours, instead of 48 hours, was the incubation time established (28). Another relevant fact is that dasatinib induces a clear inhibition of cPKCs and PKCδ isoforms, since their levels are significantly decreased after TyrK treatment in HMC-1^{560} cell line. This inhibitory effect is very potent since cPKCs levels are diminished in the cytosol, the nucleous and the plasma membrane subplaces after dasatinib treatment. The effect over PKCδ is slightly different since this decrease is observed in the cytosol and the nucleous (but not in the plasma membrane). This fact could be related with a possible down-regulatory effect of dasatinib over PKCδ isoform, since it has been demonstrated that the association of PKC to the plasma membrane is an important requisite for down-regulation (59, 60). Therefore, this fact indicates that dasatinib apoptotic effect on HMC-1^{560} is intimately linked to PKCδ, a fact confimed after the disappearance of apoptotic TyrK inhibitor effect in PKCδ-silenced cells. In the case of HMC-$1^{560,816}$ cells, dasatinib induces the translocation of cPKCs from the cytosol to the plasma membrane. PKC translocation to the plasma membrane is considerer as a step that follows protein activation, therefore it means that dasatinib might acts as a cPKC activator in this cell line (33, 56, 61, 62). Interestingly, apoptotic cell death induced by dasatinib is accompained with nuclear PKCδ translocation in this cell line, but not in HMC-1^{560}. It should be noted that the apoptotic cell death (induced by genotoxins, phorbol ester, oxidative stress or death receptors) is accompained with nuclear translocation of this isoform (63, 64). Once in the nucleous PKCδ interacts with several mediators (like DNA-dependent protein kinase, lamin B and topoisomerase IIα) which lead to cell death (50, 63, 64). This relationship between apoptosis and PKCδ, far from weak and light is very strong, since dasatinib apoptotic effect is blocked after partial PKCδ silencing. Moreover, apart from being related with TyrK inhibition induced by dasatinib, PKCδ has a wide range of funtions. In this sense, PKCδ has been described as a proliferative inductor (in Rat1

fibroblasts) or antiproliferative isoform (in NIH 3T3 fibroblast or U-251 human glioma cell line) (65-67). Another important controversial function has been found in human neutrophils, since PKCδ is considerer an anti-apoptotic inductor in TNF-α-dependent apoptosis (67).

With these results it can be concluded that PKCδ isoform is an essential target for dasatinib, as well as the previously described kinase Lyn (26). Interestingly, the interaction between dasatinib and PKCδ described in the present study might be related with the relationship Lyn-PKCδ previously defined in RBH-2H3 cells, resulting in Lyn inhibition (68). Furthermore, it is important to bear in mind that although the effectiveness of the multitarget TyrK inhibitor dasatinib is extremely dependent on PKCδ in both MC lines, the intracellular pathways that PKCδ follows in both cases are different. The nuclear PKCδ translocation only takes place in HMC-$1^{560,816}$ cells after dasatinib treatment, which means that in cells with one mutation, this translocation is not necessary to induce apoptotic cell death and consequently PKCδ is acting through another pathway. The route followed in HMC-$1^{560,816}$ by PKCδ has been widely described in other studies, in which PKCδ translocates from the cytoplasm to the nucleous in response to apoptotic stimuli (69), as happens with the dasatinib in our case. For this, it is essential to note that PKCδ silencing blocks apoptotic cell death induced by dasatinib. Therefore, any PKCδ alteration that leads to a decrease of its expression might induces the abolishment of dasatinib apoptotic effect, since this compound acts through PKCδ isoform in both MC lines, HMC-1^{560} and HMC-$1^{560,816}$.

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AUTHOR CONTRIBUTIONS

The design of the experiments has been done by Araceli Tobío, Amparo Alfonso and Luis M. Botana. The experiments, data collection, analysis and interpretation as well as statistical analysis and the writing of the manuscript have been done by Araceli Tobío. The manuscript has been supervised by Amparo Alfonso and Luis M. Botana.

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FIGURE LEGENDS

Figure 1. Effect of tyrosine kinase inhibitors and nucleoside analog cladribine on cell viability in HMC-1 cell lines. Cells were incubated for 48 hours with each compound. Figures (A), (C), (E) and (G) represent the effect of dasatinib, midostaurin, nilotinib and cladribine respectively in HMC-1^{560} cell line. Figures (B), (D), (F) and (H) represent the effect of dasatinib, midostaurin, nilotinib and cladribine respectively in HMC-$1^{560,816}$ cell line. Mean ± SEM of three experiments.

Figure 2. Effect of dasatinib and PKC activation on spontaneous histamine release in HMC-1 cell lines. (A) HMC-1^{560} and (B) HMC-$1^{560,816}$ cells were incubated for 48 hours with 0.012 µM and 0.6 µM dasatinib respectively. The concentration of PMA was 100 ng/ml for both cell lines. Then, spontaneous histamine release was determined. Mean ± SEM of three experiments. (*) significant differences between untreated and drug pre-incubated cells.

Figure 3. Effect of dasatinib and PKC activation on cytosolic cPKC and PKCδ levels in HMC-1 mast cell lines. HMC-1^{560} and HMC-$1^{560,816}$ cells were incubated for 48 hours in the presence of 0.012 µM and 0.6 µM dasatinib respectively. (A) representative experiment of each condition. cPKC (B) and PKCδ (C) cytosolic levels were calculated in relation with actin cytosolic expression. Mean ± SEM of three experiments. (*) and (**) significant differences between untreated and drug pre-incubated HMC-1^{560} and HMC-$1^{560,816}$ cells respectively.

Figure 4. Effect of dasatinib and PKC activation on nuclear cPKC and PKCδ levels in HMC-1 mast cell lines. HMC-1^{560} and HMC-$1^{560,816}$ cells were incubated for 48 hours in the presence of 0.012 µM and 0.6 µM dasatinib respectively. (A) representative experiment of each condition. cPKC (B) and PKCδ (C) nuclear levels were calculated in relation with histone-H1 nuclear expression. Mean ± SEM of three experiments. (*) and (**) significant differences between untreated and drug pre-incubated HMC-1^{560} and HMC-$1^{560,816}$ cells respectively.

Figure 5. Effect of dasatinib and PKC activation on plasma membrane cPKC and PKCδ levels in HMC-1 mast cell lines. HMC-1^{560} and HMC-$1^{560,816}$ cells were incubated for 48 hours in the presence of 0.012 µM and 0.6 µM dasatinib respectively. (A) representative experiment of each condition. cPKC (B) and PKCδ (C) plasma membrane levels were calculated in relation with actin plasma membrane expression. Mean ± SEM of three experiments. (*) and (**) significant differences between untreated and drug pre-incubated HMC-1^{560} and HMC-$1^{560,816}$ cells respectively.

Figure 6. Live, apoptotic and necrotic population in HMC-1^{560} cell line after dasatinib treatment. HMC-1^{560} cells were incubated for 48 hours in the presence of 0.012 μM dasatinib. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic (upper panel) cells were detected by Annexin V-FITC/PI staining. (A) and (B) represent one representative experiment of untreated and dasatinib-treated cells respectively. (C) mean ± SEM of the three experiments. (*) significant differences between untreated and dasatinib-incubated HMC-1^{560} cells.

Figure 7. Live, apoptotic and necrotic population in HMC-1^{560,816} cell line after dasatinib treatment. HMC-1^{560,816} cells were incubated for 48 hours in the presence of 0.6 μM dasatinib. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic (upper panel) cells were detected by Annexin V-FITC/PI staining. (A) and (B) represent one representative experiment of untreated and dasatinib-treated cells respectively. (C) mean ± SEM of the three experiments. (*) significant differences between untreated and dasatinib-incubated HMC-1^{560,816} cells.

Figure 8. PKCδ silencing efficiency in HMC-1 mast cell lines. PKCδ siRNA was incubated for 24, 36 and 48 hours in HMC-1^{560} and HMC-1^{560,816} cell lines. (A) and (B) show a representative image of each condition in HMC-1^{560} and HMC-1^{560,816} cell line respectively. (C) and (D) is the mean ± SEM of three experiments in HMC-1^{560} and HMC-1^{560,816} cell line respectively. Cytosolic PKCδ values were calculated respect to actin band intensity. (*) significant differences between no-transfected and PKCδ-transfected cells.

Figure 9. Live, apoptotic and necrotic population in PKCδ–silenced HMC-1^{560} cells after 0.012 μM dasatinib. HMC-1^{560} cells were incubated for 48 hours. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic (upper panel) cells were detected by Annexin V-FITC/PI staining. (A) and (B) represent one representative experiment of untreated and dasatinib-treated cells respectively. (C) mean ± SEM of the three experiments.

Figure 10. Live, apoptotic and necrotic population in PKCδ–silenced HMC-1^{560,816} cells after 0.6 μM dasatinib. HMC-1^{560,816} cells were incubated for 48 hours. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic (upper panel) cells were detected by Annexin V-FITC/PI staining. (A) and (B) represent one representative experiment of untreated and dasatinib-treated cells respectively. (C) mean ± SEM of the three experiments.

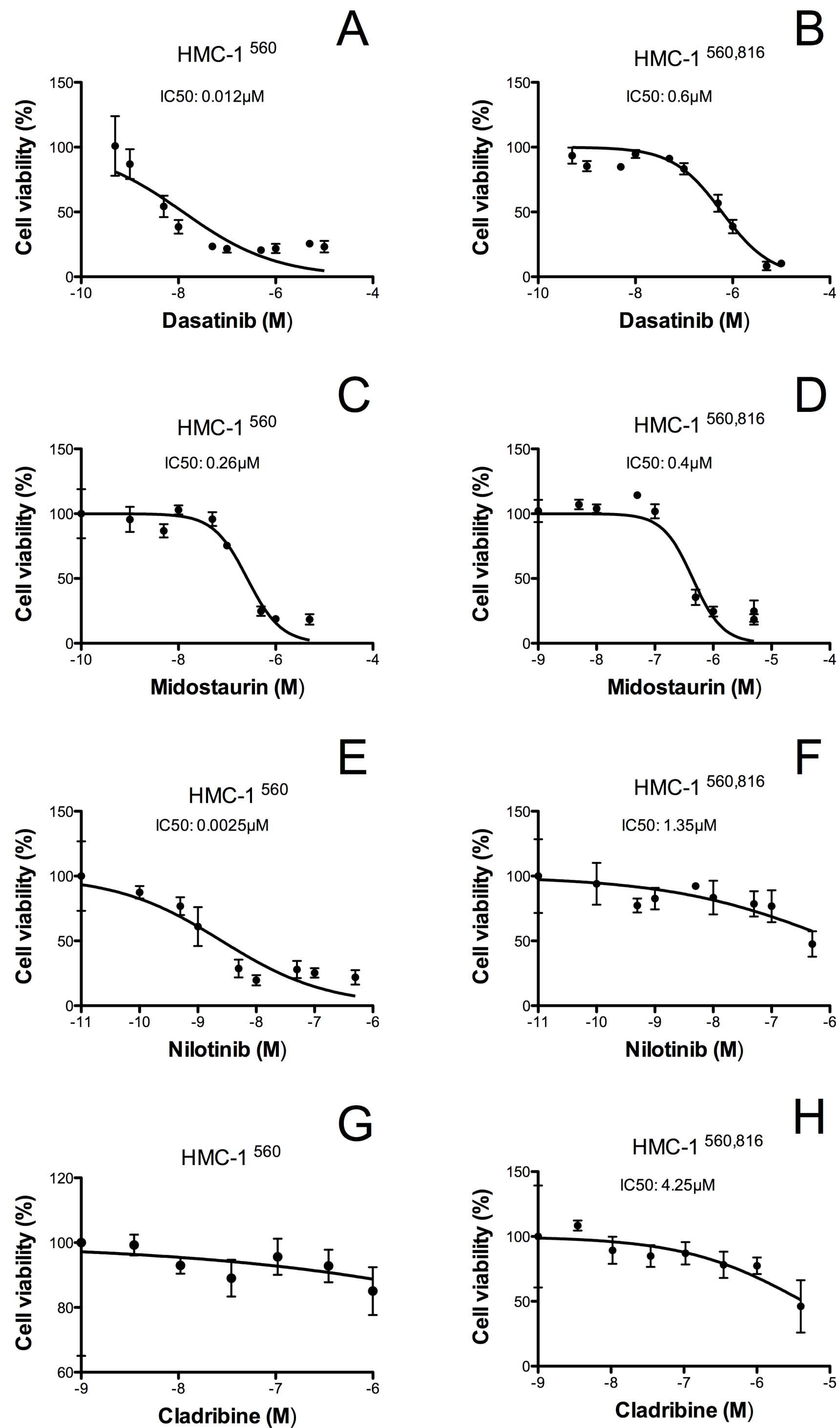
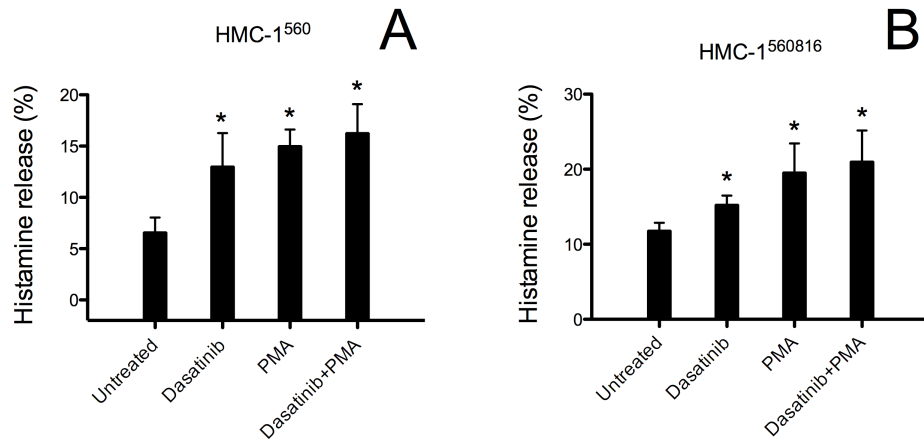


figure 1



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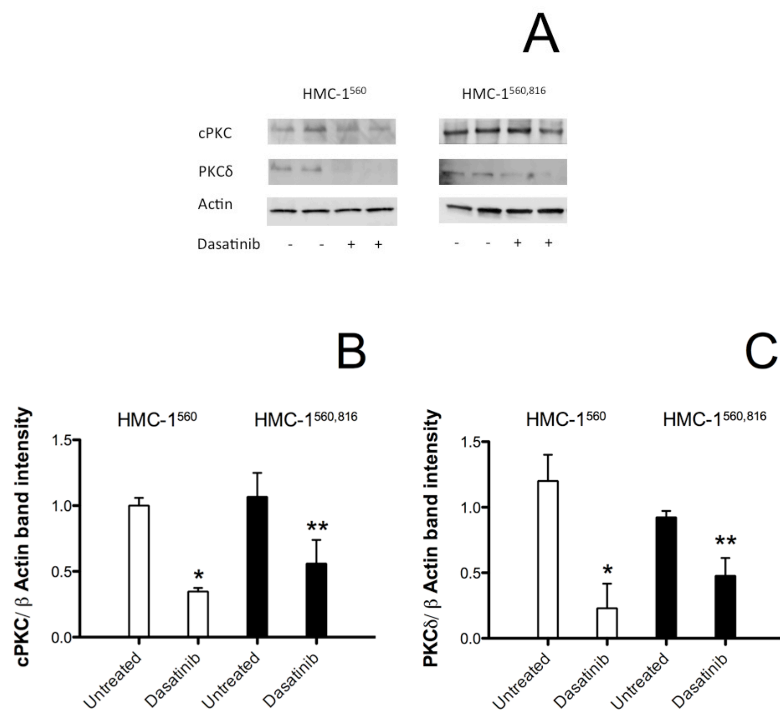

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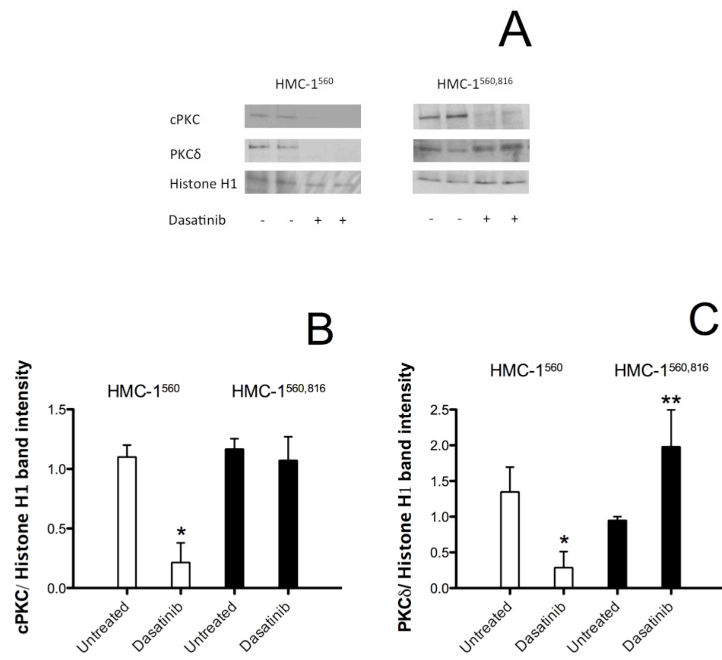

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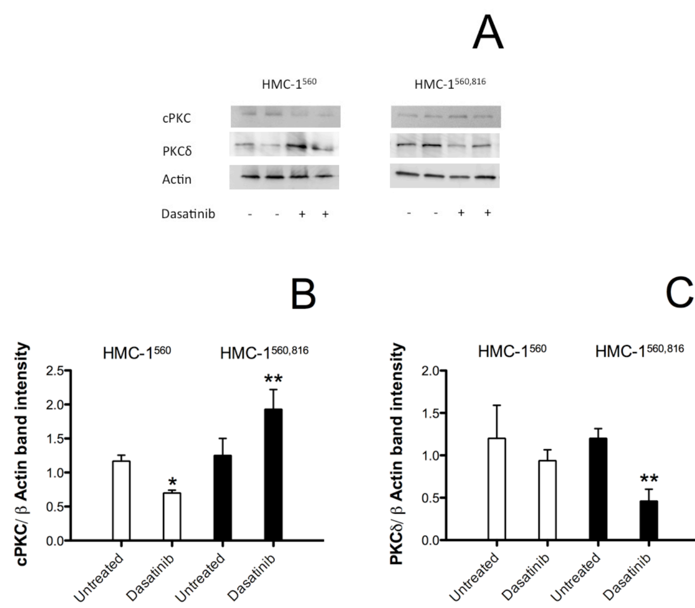

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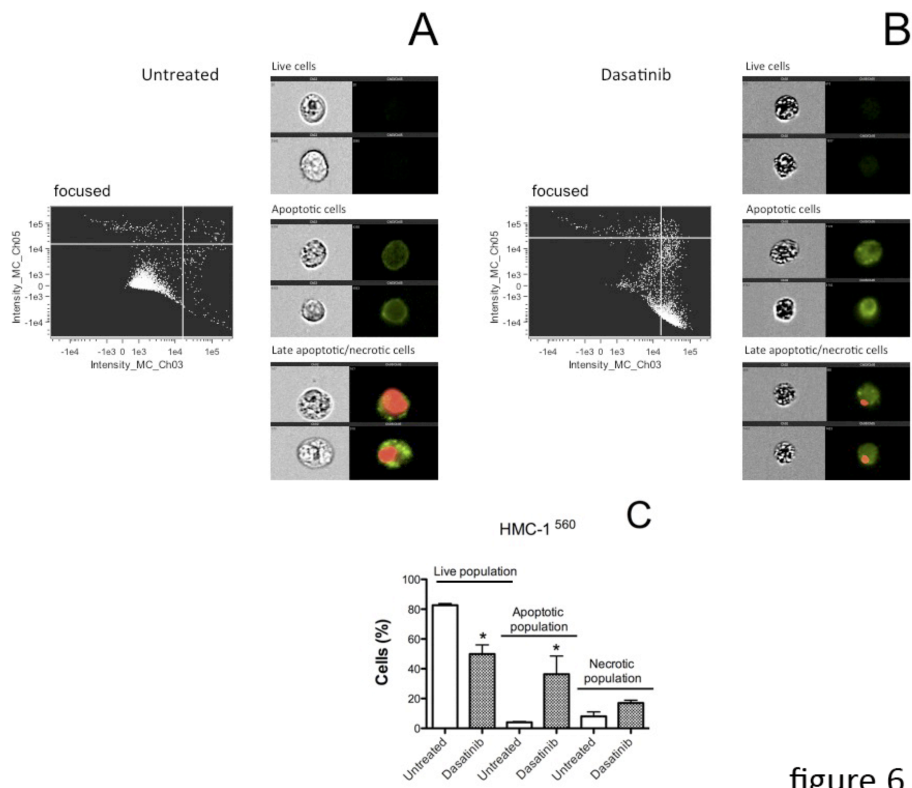

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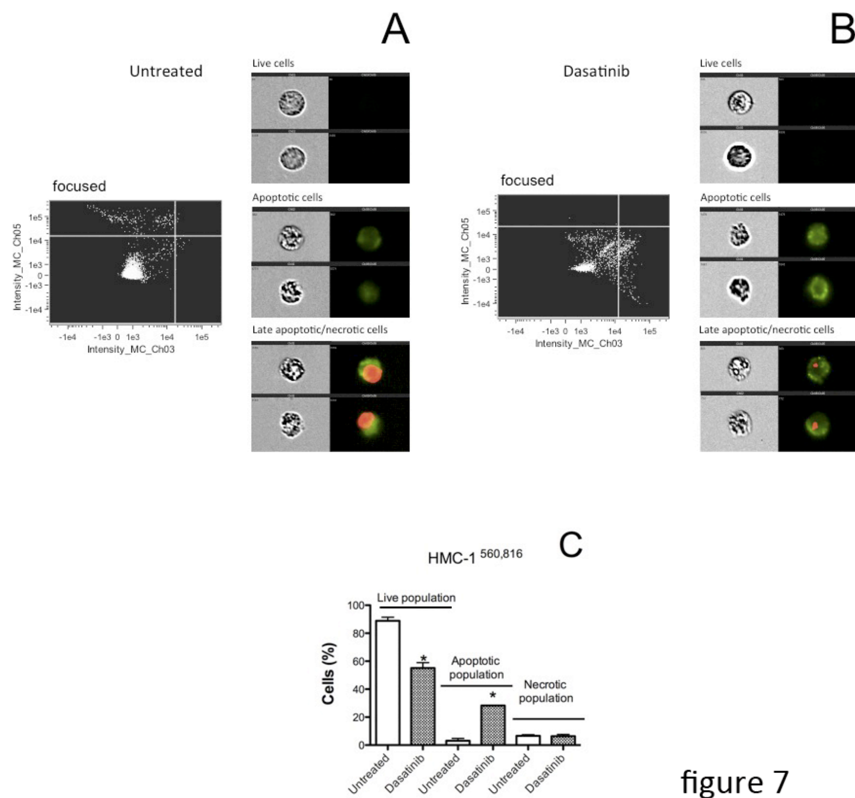

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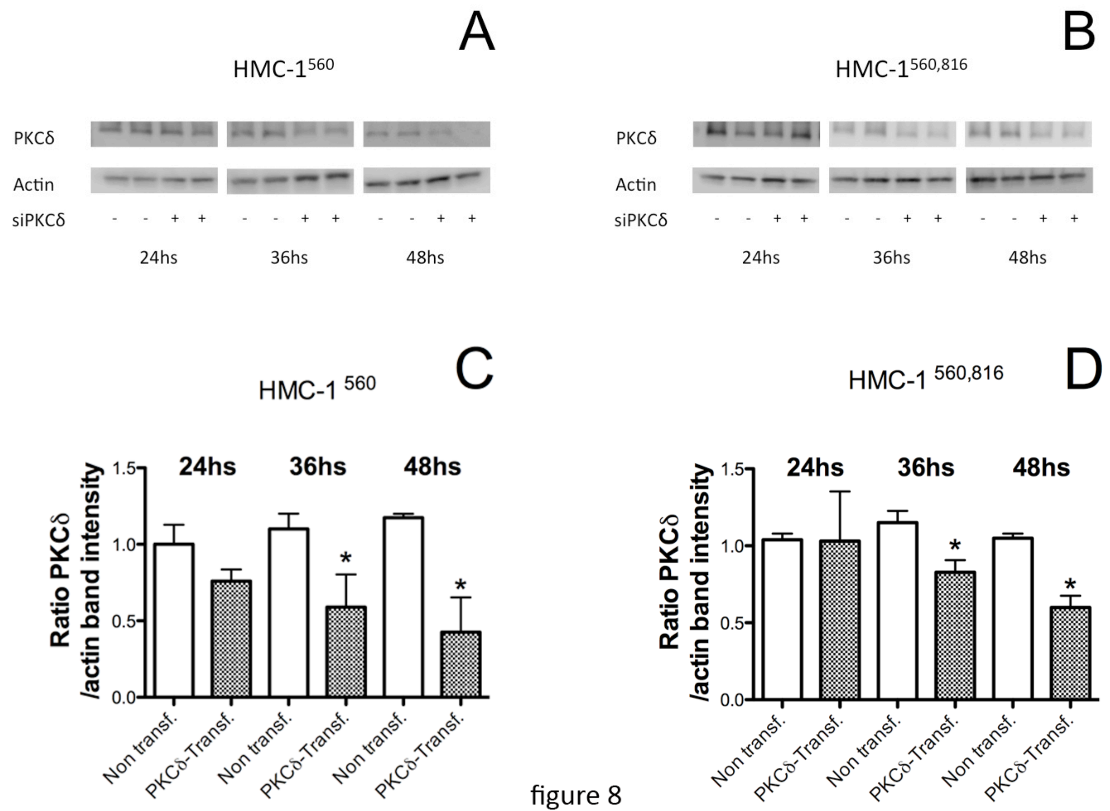

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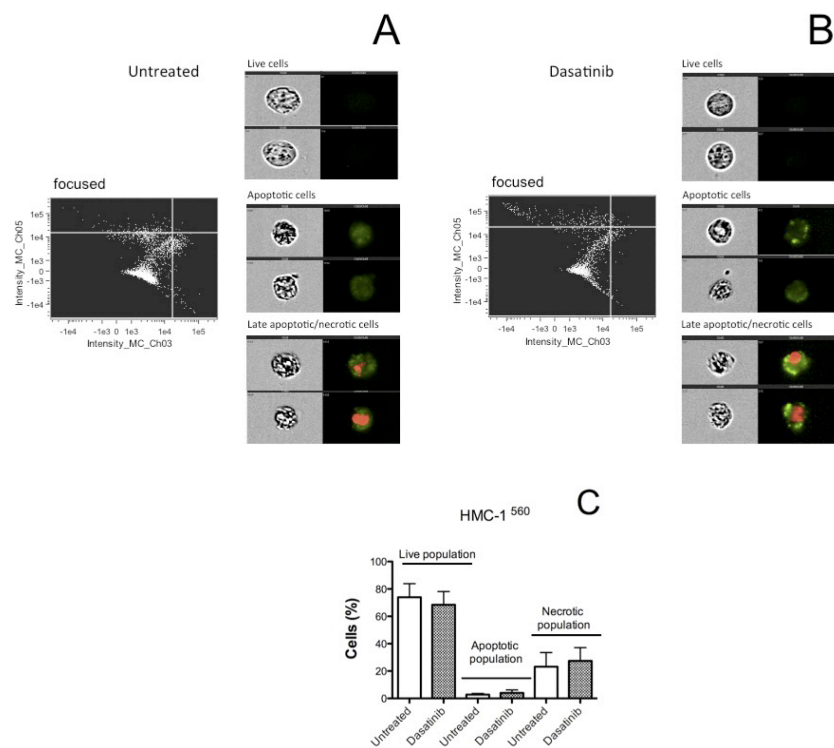

figure 9

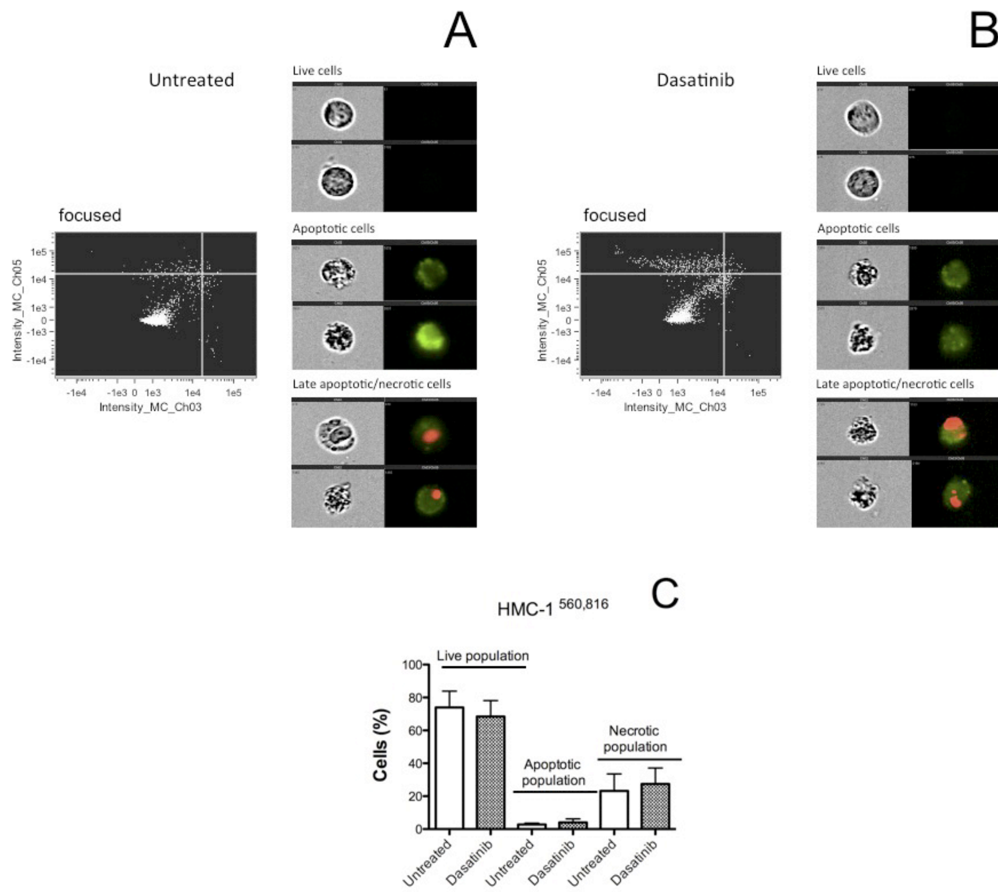

figure 10

II.3. La PKC potencia el efecto citotóxico de los inhibidores de tirosina quinasas STI571 y dasatinib.

Resumen

En el tratamiento de la mastocitosis se emplean varios grupos de fármacos entre los que se encuentran los inhibidores de tirosina quinasas STI571 y dasatinib. Además de las tirosina quinasas se han considerado otras proteínas diana, como es el caso de la PKC, en el tratamiento de esta enfermedad. En este estudio se describe el efecto ejercido por la activación prolongada de la PKC sobre la apoptosis inducida por STI571 en la línea de mastocitos tumorales HMC-1^{560} y por el dasatinib en ambas líneas celulares HMC-1: HMC-1^{560} y HMC-1^{560,816}. En primer lugar se observa que la activación de la PKC causa muerte celular por apoptosis dependiente de la isoforma PKCδ en ambas líneas celulares HMC-1. Además, el tratamiento combinado de STI571 y PMA incrementa el efecto citotóxico del inhibidor de tirosina quinasas sobre las células HMC-1^{560}, resultando en un aumento de la población necrótica. Este efecto también es observado después del tratamiento con dasatinib y PMA en células HMC-1^{560,816}, pero no en células HMC-1^{560}. Con el objetivo de aclarar este efecto se estudió la posible implicación de la isoforma PKCδ, ampliamente relacionada con el proceso de apoptosis celular. Los resultados obtenidos indican que este efecto potenciador del PMA sobre las citotoxicidades de STI571 y dasatinib desaparece tras la silenciación de esta isoforma. Por lo tanto puede concluirse que la PKCδ es esencial para el tratamiento de pacientes con mastocitosis ya que el efecto citotóxico observado tras el tratamiento de los inhibidores de tirosina quinasa y PMA es dependiente de esta isoforma.

PKC potentiates tyrosine kinase inhibitors STI571 and dasatinib cytotoxic effect.

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Abreviations: PKC, Protein Kinase C; HMC-1, Human Mast Cell Line; TyrK, Tyrosine Kinase.

Key Words: HMC-1; PKC; c-kit; STI571; Dasatinib; PMA.

ABSTRACT

HMC-1^{560} and HMC-1^{560,816} cells are growth-factor independent cells present in patients with mastocytosis. For the treatment of this disease several groups of compounds have been used, such as the tyrosine kinase inhibitors STI571 and dasatinib. As well as tyrosine kinase, other proteins such as PKC, have been defined as an useful target for the treatment of HMC-1 uncontrolled proliferation. Results obtained in this paper describe that long-time incubation with PKC activation induces apoptosis in both mast cell lines. Moreover, PKC activation potentiates cytotoxic effect due to STI571 in HMC-1^{560}. Specifically, PKC activation enhances STI571-cytotoxicity increasing necrotic population. However, this effect was not observed when PMA was combined with dasatinib in cells with one mutation. In HMC-1^{560,816} cell line, PKC activation stimulates cytotoxicity due to dasatinib. Besides, this enhancer effect is accompanied with a significant increase of necrosis. In order to clarify this PKC effect, the isoform related with cell death PKCδ, has been silenced in both lines. Results obtained evidence that either STI571 or dasatinib-apoptotic cell death, are PKCδ-dependent even though STI571 shows less PKCδ-dependence than dasatinib. Moreover, PKC enhancer effect over STI571 and dasatinib cytotoxicities disappears after PKCδ-silencing in both cell lines. Therefore, PKC activation increases cytotoxicity by the stimulation of necrotic cell death in HMC-1^{560} STI571-treated cells and in HMC-1^{560,816} dasatinib-treated. In conclusion, our results demonstrate that the modulation of PKCδ isoform is essential and determines the effectiveness of the treatment in patients with mastocytosis, since the effect of TyrK inhibition is PKCδ-dependent.

INTRODUCTION

Allergic responses are characterized by a cascade of events that leads to a immediate hypersensitivity reaction in which mast cells (MCs) have a crucial role. Mast cells are derived from $CD34^+$, $CD117^+$ ($c\text{-}kit^+$) and $CD13^+$ bone marrow progenitors and after their activation they release several inflammatory mediators to the bloodstream (1, 2). MCs produce several mediators and express the high-affinity IgE receptor (FCεRI). Two different subtypes, MC_T and MC_{TC}, were described after immunohistochemical studies in human tissues, depending on the presence of tryptase (MC_T) or tryptase+chymase (MC_{TC}) simultaneously. However, this classification is controversial since recent studies have demonstrated that all MCs are able to produce chymase. Moreover, it has been determined that all MCs have the same common MC progenitor (3). Of all the inflammatory mediators produced by MCs, histamine, prostaglandin D_2 and platelet-activating factor (PAF) (4), contribute to induce the symptoms after MC activation (MCA). MCA syndrome (MCAS) is a term applied when one or various of these three criteria are present: 1) chronic or recurrent clinical signs, 2) MCs are present and 3) the symptoms respond to mast cell-stabilizing agents therapy, or drugs against MC mediators are used (5-7). In this context, MCAS is subdivided into three variants: 1) primary MCAS, with the presence of c-kit $D816V^+$-mutated clonal MCs, 2) secondary MCAS, in patients with allergy or atopic disorder without clonal MCs, 3) idiopathic MCAS, specific of patients that fulfill the three criteria but do not have allergen-specific IgE and clonal MCs (8). Along with MCAS, two other MC disorders have been described, the MC hyperplasia and mastocytosis (with eight subtypes). First, MC hyperplasia is defined as an increase of tissue MCs as consequence of different disorders like chronic infections or cancer. Second, the increase of (mono)clonal MCs is named mastocytosis and can be subdivided into cutaneous mastocytosis (CM), systemic mastocytosis (SM), mastocytoma (benign and localized) and MC sarcoma (aggressive and localized) (4).

The c-kit ligand stem cell factor (SCF), also named mast cell growth factor, steel factor or kit ligand binding domain, activates MCs. Nevertheless, human mast cell line (HMC)-1 is SCF-independent. Two different HMC-1 sublines have been described: $HMC\text{-}1^{560}$ and $HMC\text{-}1^{560,816}$ (9). Both MC lines differ from human tissue MCs in the absence of FCεRI receptor in the cellular surface (10-12). Either the $HMC\text{-}1^{560}$ or $HMC\text{-}1^{560,816}$ sublines have the Gly-560-> Val mutation at codon 560 in the juxtamembrane position of the c-kit. This mutation implies that c-kit receptor is constitutively phosphorylated and therefore does not need the SCF for their activation (13, 14). On the other hand, the second subline has another c-kit mutation; Asp-816-> Val at codon

816 within c-kit exon 17 (15). Mastocytosis is characterized by the abnormal growth and expansion of c-kit mutant MCs (for example: Asp-816-> Tyr or Asp-816-> Phe). Specifically, Asp-816-> Val^+ cells are present in 80% of the patients with SM (16). This Asp-816-> Val mutation was firstly described in adult patients with SM and was defined as rare in pediatric cases (17). However, more recent data describe the presence of Asp-816-> Val^+ cells in the 42% of pediatric patients (18). C-kit mutations are not only present in matocytosis, also in gastrointestinal stromal and Ewing's tumors, thymic and ovarian cancer, neuroblastoma or adenoid cystic carcinoma (19-21). For the treatment of SM, several drugs have been used, such as interferon-α (IFN-α), the nucleoside analog 2-chlorodeoxyadenosine cladribine and the group of tyrosine kinase (TyrK) inhibitors. Chronic myeloid leukemia (CML) is a disorder that occurs in patients with SM, in these cases, the variant of SM is named as SM with associated clonal haematological non-mast cell lineage disease (SM-AHNMD) (22, 23). In fact, CML was the first cancer associated with an oncogene marker, the Philadelphia chromosome (24), and it is characterized by a chromosome translocation which leads to the formation of Breakpoint Cluster Region-Abelson Leukaemia (Bcr-Abl) oncogene. TyrK inhibitors have been widely used in SM-AHNMD treatment, since most of them inhibit other src family kinases as well as c-kit repector (25-28). The first TyrK inhibitor used was imatinib (Signal Tranduction Inhibitor (STI) 571 or Gleevec®), with activity against several oncogenic TyrKs: Bcr-Abl, c-kit, platelet-derived growth factor receptor, discoidin domain receptor and colony-stimulating factor receptor-1 (29). The emergence of imatinib resistances and the fact that it is not effective against HMC-$1^{560,816}$ cell line gave birth to the second generation of TyrK inhibitors, including compounds such as dasatinib (BMS354825), nilotinib (AMNI07), midostaurin (PKC412) and bosutinib (SKI-606) (26, 28). The multikinase inhibitor dasatinib is able to inhibit Bcr-Abl, c-kit, PDGRF and ephrin receptor kinase (26). Interestingly, it is a good choice to induce cell death in HMC-$1^{560,816}$ cells, unlike imatinib (30). Also, the combination of dasatinib+midostaurin show a synergistic effect indicating that the combination of different TyrK inhibitors is a usable tool for the treatment of SM and CML (31).

In mast cells, PKC plays a primordial role on activation and degranulation, therefore, the effect of this kinase in mastocytosis has been described in several studies (32-37). PKC is a family of serine/threonine kinases with different isoforms divided into three classes depending on their sensitivity to Ca^{2+} and phorbol esters: (1) Ca^{2+}-dependent isozymes (or cPKCs); α, β_1, β_2 and γ that are activated by diacylglicerol (DAG) or 12-O-tetradecanoylphorbol-13-acetate (PMA); (2) Ca^{2+}-independent isozymes (or novel PKCs); δ, ε, η, μ and θ, activated by PMA; and (3)

atypical isozymes (ζ and ι/λ), which are PMA and Ca^{2+}-independent. PKC activation has been described as an essential signal for mast cell exocytosis (38). Src TyrKs family is considered as a proximal event in mast cell activation and their relationship with PKC isoforms has been widely studied. Scr belongs to a family of eleven members including Lyn (39). Syk, Btk, Lyn or Fyn are proteins related with exocytosis granules (40). In order to determine the role of the receptor-proximal TyrKs on secretory granules in mast cells, gene-inactivated mice were used. Out of all, Lyn is the only protein that is not essential for mast cell exocytosis, whereas exocytosis is abrogated in Fyn, Syk and Btk-deficient mast cells (41-44). Interestingly, PKCα and PKCβ II activation is increased upon FcεRI stimulation in $lyn^{-/-}$ mast cells (43, 45, 46). Also, cell degranulation is restored by PKCβ and Ca^{2+} in RBL-2H3 cells, together with membrane translocation of this PKC isoform. In addition, in PKCβ-deficient mast cells, the production of IL-6 and the degranulation are inhibited (47). Another important isoform related with mast cell exocytosis regulation is PKCδ (48). Regulation of mast cell exocytosis is dependent on several cellular signaling proteins and SHIP/Shc/PKCδ complex is essential to regulate mast cell exocytosis. Therefore, the molecules that interact with SHIP might modulate mast cell degranulation (49).

As it has been shown, either PKC or TyrK inhibitors take part on intracellular pathways in which src proteins are involved. The relationship between PKC and TyrK proteins has been described in several studies and the combination of PKC and TyrK inhibition is a useful tool in the treatment of patients with aberrant mast cells (50). In the present paper, we study the effect of PKC activation over two TyrK inhibitors, STI571 and dasatinib, and the effectiveness against $HMC\text{-}1^{560}$ and $HMC\text{-}1^{560,816}$ proliferation.

The protein kinases PKC and TyrK are considered a usable target in the treatment of systemic mastocytosis (28, 51). In this sense, the combination of TyrK and PKC inhibitors is a highly effective therapy in patients with this disease. Among all TyrK inhibitors that have cytotoxic effect against HMC-1 cell line, STI571 and dasatinib are two of the most potent studied (30, 52). Moreover, long-term treatment with PKC activator PMA has been described as a potent cell death inductor in several cell lines (53-55). Also, PKC potentiates the effect of several TyrK inhibitors such as dasatinib and nilotinib in HMC-1 cell line (50). Therefore, since the effect of PKC inhibition over Tyrk inhibitors effect has been already described, the purpose of this study is to clarify the consequences of PKC modulation, this time through its activation, in HMC-1 cells treated with two different TyrK inhibitors, STI571 and dasatinib.

MATERIALS AND METHODS

Chemicals

STI571 was provided by Dr. Luis Escribano Mora (Centro de Estudios de Mastocitosis de Castilla la Mancha, Hospital Virgen del Carmen, Toledo, Spain). Dasatinib (sc-358114), negative siRNA control (sc-37007) and PKCδ siRNA (sc-36253) were purchased from Santa Cruz Biotechnology (CA, USA). PMA, bovine serum albumin (BSA) were from Sigma-Aldrich (Madrid, Spain). Phosphate buffered saline (PBS) was from Invitrogen (Barcelona, Spain). Anti Mouse IgG was purchased from GE Healthcare (Barcelona, Spain). Anti β-actin and polyvinylidene flouride (PVDF) membrane were from Millipore (Temecula, USA). Anti PKCδ was from BD Biosciences (Madrid, Spain). Cell Lab ApoScreen™ Annexin V and DNA Prep™ Stain were from Beckman Coulter (Fullerton, CA, USA). Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were from BioRad (Barcelona, Spain). GeneSilencer® was from Genlantis (San Diego, CA, USA).

Cell cultures

HMC-1^{560} cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and HMC-$1^{560,816}$ cells were kindly provided by Dr. Luis Escribano Mora with permission from Dr. J. Butterfield. They were maintained at 37ºC in Iscove´s modified Dulbecco´s medium (IMDM) (Gibco, Invitrogen, Spain) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Spain) and 100 IU/ml penicillin + 100 µg/ml streptomycin (Gibco, Invitrogen, Spain) in an atmosphere containing 5% CO_2. The medium was renewed once a week.

MTT assay

HMC-1^{560} and HMC-$1^{560,816}$ cell lines were incubated with STI571, dasatinib and PMA for 48 hours at 37ºC. Then cells were washed with saline solution and then incubated with MTT (250 µg/ml) for 30 minutes. After washed with saline solution the cells were resuspended in 200 µl of water and sonicated for 1 minute. Absorbance was determined in a Bio-Tek Synergy 4 plate reader at wave length of 595 nm.

Apoptotic and necrotic cell death determination by flow cytometry

Apoptosis was detected by Annexin-V-FITC/PI staining using the Cell Lab ApoScreen™ Annexin V kit. HMC-1^{560} and HMC-$1^{560,816}$ cells were incubated with STI571, dasatinib and PMA for 48 hours (37ºC). Then cells were prepared exactly following manufacturer's instructions assay.

Transfection by lipid-based method (Genlantis)

GeneSilencer® (Genlantis) was composed by the transfection reagent and the diluent. Manufacturer's instructions were followed carefully. On the day of transfection two solutions were prepared. On the one hand, solution A, composed by: diluent, FBS/Antibiotic –free IMDM medium and PKCδ siRNA. Control siRNA (sc-37007) was used as negative control for evaluating RNAi off-target effects. On the other hand, solution B was composed by transfection reagent diluted in FBS/Antibiotic-free IMDM medium. Solutions A and B were mixed and incubated for 5 minutes at room temperature. HMC-1^{560} cells were incubated in a total volume of 500 µl in a FBS/Antibiotic-free IMDM medium. The concentration of the cells was $2x10^6$/ml. After 5 hours of transfection 500 µl of IMDM (supplemented with 20% FBS and Penicillin/Streptomicyn 2x) was added to the HMC-1^{560} cells. 19 hours later the cells were incubated with STI571, dasatinib and PMA. The cells were incubated with the different compounds for 48 hours at 37ºC.

Western blotting

Cells were re-suspended in 80 µl lysis buffer with the follow composition: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1%Triton X-100, 2 mM DTT, 2.5 mM PMSF, 40 mg/ml aprotinin, 4 mg/ml leupeptin, 5 mM NaF, 1 mM Na_3VO_4, 1 mg/ml pepstatin A and 1 mg/ml bezamidine, 1X complete protease inhibitor (Roche, Spain) and 1X phosphatase inhibitor cocktail (Roche, Spain). The determination of protein concentration was carried out by using Bradford assay and BSA as protein standard. For separating proteins according to their molecular weight sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure was used. Proteins were transferred to a PVDF membrane which was blocked with 0.5% BSA and then it was incubated for 10 min with anti PKCδ (1:1,000). After two washes with washing buffer (PBS+0.1%Tween), the membrane was incubated for 10 minutes with secondary antibody, anti-Mouse IgG conjugated with horseradish peroxidase. A chemiluminescence detection kit (SuperSignal West Femto; Pierce) was used to determine the levels of protein expression. Relative protein expression was calculated in relation to β-actin (0.3:1,000).

Statistical Analysis

Results were analyzed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

RESULTS

A previous study demonstrates that 25 nM STI571 induces a 50% of cell death in HMC-1^{560} cell line, whereas the IC_{50} for HMC-1^{560} and HMC-$1^{560,816}$ are 0.012 µM and 0.6 µM dasatinib, respectively. In order to determine the effect on cell viability of long-time PMA incubation and co-incubation with the TyrK inhibitors STI571 and dasatinib, several experiments were done. First, MTT assays were carried out in order to determine cytotoxic effect of TyrK inhibitors and PMA treatment for 48 hours in HMC-1^{560} and HMC-$1^{560,816}$ cells (figures 1A and 1B, respectively). As figure 1A shows, 25 nM STI571 elicits a significant 50% decrease of cell viability whereas dasatinib induces a decrease of 40%. Also, PKC activation significantly decreases cell viability by 36% and its co-incubation with STI571 causes a higher decrease (73%) than that observed with the TyrK inhibitor alone. However, the co-incubation PMA+dasatinib does not modify the effect of dasatinib alone and the decrease observed on cell viability is still 40% approximately. To know the effect of PKC activation on the cytotoxic activity of dasatinib on HMC-$1^{560,816}$ cells, a MTT assay was carried out. Figure 1B shows that 0.6 µM dasatinib causes a significant 40% decrease on cell viability, and PKC activation induces a significant 43% decrease. Moreover, a higher 54% decrease than that induced by dasatinib alone is observed with the simultaneous use of both compounds, dasatinib+PMA.

Results obtained in figures 1A and 1B demonstrate that PMA has a potent cytotoxic effect against both MC lines. Also, PMA co-incubation with STI571 and dasatinib in HMC-1^{560} and HMC-$1^{560,816}$ respectively has a summatory cytotoxic effect. As it was previously described in other studies, TyrK inhibitors STI571 and dasatinib cause apoptotic cell death in HMC-1^{560} and HMC-$1^{560,816}$ sublines respectively (31, 56, 57), however, the type of cell death induced by PKC activation alone or in combination with the different TyrK inhibitors on HMC-1^{560} and HMC-$1^{560,816}$ cells remains to be defined. In this sense, the Annexin V-FITC /PI technique was employed next to determine the distribution of the three cellular populations: live, apoptotic and necrotic in HMC-1^{560} cell line after 48 hours treatment (figure 2). Figures 2A to 2F represent the intensity of Annexin V-FITC (X axis) and PI (Y axis) in the different conditions and figure 2G shows the results of three experiments. The percentage of live cells is 81% when no treatment was added to HMC-1^{560} cells, whereas apoptotic and necrotic populations represent 5 and 14%, respectively. Moreover, STI571 induces a significant decrease of viability from 81 to 41%, whereas the apoptotic population increases by 36% and the necrotic population is not modified in the HMC-1^{560} cell line. On the other hand, dasatinib elicits a significant decrease on the live population, with a reduction

from 81 to 43%. Besides, the apoptotic population increases from 3 to 48% after dasatinib treatment and necrotic population slightly increases from 5 to 15%. In the case of the PMA treatment, a long-time incubation with this phorbol ester has a similar effect than the one induced by TyrK inhibitors tested, STI571 and dasatinib. PKC activation causes a decrease of live cells from 81 to 46%, while apoptotic cells are significantly increased from 3 to 41%. As it happens after treatment with TyrK inhibitors, PMA does not induce any significant modification on the necrotic population. The combination of TyrK inhibitor STI571 with PMA induces a decrease of cell viability even higher than that observed with STI571 alone, since the apoptotic population increases from 3 to 53%, whereas the necrotic population is also significantly increased (the percentage of necrotic cells rise from 5 to 30%). Besides, dasatinib+PMA co-treatment causes a significant decrease on viable cells (from 81 to 53%) with an increase of apoptotic population of 32%, while dasatinib+PMA co-incubation does not change the distribution of the necrotic population.

The same experiment was carried out in HMC-$1^{560,816}$ cells (figure 3). The distribution of live, apoptotic and necrotic populations was determined after treatment with dasatinib, long-term PMA exposure and the combination of both drugs. The distribution of cellular population in untreated, dasatinib-treated, PMA and dasatinib+PMA treated cells is shown in figures 3A, 3B, 3C and 3D, respectively, and figure 3E shows the average results of the three experiments. As shown in figure 3E, the population distribution of untreated cells is 85% live, 1% apoptotic and 14% necrotic. Dasatinib causes a 33% decrease of viable cells, while the apoptotic cell population significantly rises from 3 to 28% and the percentage of necrotic cells remains unchanged. When HMC-$1^{560,816}$ cells are incubated with PMA, a significant decrease, from 88 to 52% on live population, is observed. In apoptotic cells, a significant increase appears after PMA treatment (from 3 to 37%) and as it happens with dasatinib treatment, no effect is observed on the necrotic population. An acute decrease on live cells can be observed after the combination of both drugs, since the percentage of live cells decreases from 88 to 40%, whereas apoptotic population is significantly increased. Surprisingly, when both compounds are incubated simultaneously, the necrotic population rises from 16 to 29 % and achieves a similar percentage than apoptotic cells. This result differs from that observed when cells were treated with dasatinib or PMA alone, since in this case the necrotic population was not modified.

Therefore, results obtained in figures 2 and 3 demonstrate that PKC activation potentiates TyrK inhibitors effect on cell viability either in HMC-1^{560} or HMC-$1^{560,816}$. It

was reported that PKC is related with apoptotic cell death in several cell lines. Specifically, the Ca^{2+}-independent isoform PKCδ is described as the most important PKC isoform associated with the apoptotic pathway. Therefore, in order to determine if apoptotic cell death induced by PKC activation is also related with the PKCδ isoform, the determination of live, apoptotic and necrotic cells was carried out in PKCδ-silenced cells. The effectiveness of lipid-based transfection method is determined in both MC lines (figure 4). Cytosolic PKCδ levels were determined in control and PKCδ-silenced HMC-1^{560} and HMC-$1^{560,816}$ cells after 24, 36 and 48 hours of silencing. As figure 4A and 4C show, cytosolic PKCδ levels significantly diminished after 24, 36 and 48 hours. After 24 hours, the levels of the PKCδ isoform decrease around 25%, whereas a higher diminution is observed after 36 hours (37%). Moreover, a high decrease (50%) is induced after 48 hours incubation. Therefore, an inhibition of PKCδ expression around 50% is obtained with this transfection method in cells with one c-kit mutation. In HMC-$1^{560,816}$ cell line, a significant decrease on cytosolic PKCδ levels can be observed at all times tested (figures 4B and 4D). In this case, the PKCδ ratio intensity values diminish 29%, 55% and 62% after 24, 36 and 48 hours respectively (figure 4D). Then cell viability determination of HMC-1^{560} cells after 25 nM STI571, 0.012 μM dasatinib and 100 ng/ml PMA treatments was carried out with MTT assay in PKCδ-silenced cells. As figure 5A shows, PKCδ silencing does not affect the percentage of viability of untreated cells. However, the cytotoxicity on STI571-treated cells is lower when PKCδ is silenced, since the percentage of viability rise from 52 to 70% even though the decrease of cell viability caused by STI571 is still significant. The effect of PKCδ silencing is even higher in the case of dasatinib-treated cells because the cytotoxicity of this compound is significantly lower. Besides, when PKC is activated the silencing of PKCδ causes the absence of cell death and therefore an increase of cell viability is observed (from 58 to 68%). Also, when the TyrK inhibitor STI571 is combined with PMA the silencing of PKCδ isoform provokes an increment on cell viability from 27 to 76%. On the other hand, the cytotoxicity induced by dasatinib+PMA co-treatment seems to be also PKCδ-dependent, since the silencing of this isoform significantly prevents cell death in the presence of both compounds. In the case of cells with two mutations (figure 5B), dasatinib cytotoxicity is also PKCδ dependent, as for HMC-1^{560} cell line, since cell viability increases from 65 to 89% when PKCδ isoform is silenced. Furthermore, the PMA cytotoxic effect partially disappears after PKCδ silencing (cell viability rises from 57 to 82%). The potent cytotoxicity induced by dasatinib and PMA co-incubation is also affected by the decrease of PKCδ expression, causing an increase from 46 to 79% of cell viability.

The determination of live, apoptotic and necrotic populations in HMC-1^{560}-PKCδ silenced cells after STI571, dasatinib and PMA treatments was next studied. Figures 6A, 6B, 6C, 6D, 6E and 6F represent the cellular distribution at the different conditions, whereas the average distribution obtained with all the treatments is represented in figure 6G. As figure 6G shows, the distribution of the three populations in untreated-PKCδ silenced cells is 72% live population, 4% apoptotic and 22% necrotic cells. However, the apoptotic population is increased after STI571 treatment (the percentage changes from 4 to 11%), whereas live and necrotic populations do not suffer any modification. The distribution of the three populations in dasatinib-treated cells does not differ from untreated cells, as for the case of PKC activated cells. When TyrK inhibitors are co-incubated with PKC activator PMA no differences are observed respect to untreated cells. The distribution of the three populations is 63%, 5% and 31% for live, apoptotic and necrotic population respectively for the case of STI571+PMA co-incubated cells. Therefore, it is important to note that the increase of necrotic population previously observed in STI571+PMA treated cells is inhibited after PKCδ silencing. When PKCδ-silenced cells are co-treated with dasatinib and PMA, the distribution of the three populations is similar to the untreated cells with 78% live and 2% apoptotic cells, while the necrotic fraction represents a 18% of the total. The same experiment was carried out in HMC-1^{560,816} cell line. As figure 7G shows, the distribution of untreated cells is 73% live cells, 4% apoptotic and 23% of necrotic cells. When PKCδ-silenced cells are treated with 0.6 μM dasatinib for 48 hours, no changes are observed and the percentage of the different subpopulations is similar to untreated cells, with 75% live, 2% apoptotic and 22% necrotic cells. Moreover, no effect was observed after PKC activation and dasatinib+PMA treatments in HMC-1^{560,816} PKCδ-silenced, since the distribution in both cases of live, apoptotic and necrotic cells still around 75%, 2% and 20% respectively. Hence, the increase of necrotic cells previously observed in dasatinb+PMA-treated cells (figure 3E) is abolished with PKCδ silencing.

Therefore, in addition to the apoptotic effect observed after STI571 and dasatinib in HMC-1^{560} and HMC-1^{560,816} cells, respectively, it can be concluded that PKCδ activation stimulates necrosis when TyrK are inhibited, increasing the high cytotoxicity of TyrK inhibitors.

DISCUSSION

One of the most important group of compounds used in the treatment of SM is TyrK inhibitors. Their effectiveness against HMC-1 cell line and bone marrow cells has been widely described (28). Either STI571 or dasatinib are characterized by their cytotoxic effect against cells that have bcr-abl oncoprotein in a constitutively activated state, but other TyrK receptors, such as c-kit are also an important target for both compounds. The presence of Asp-816-> Val mutation avoids STI571 cytotoxic effect by interfering with the binding of the drug on HMC-1^{560,816} cells, but not with the Gly-560-> Val activating mutation (58). On the other hand, the Asp-816-> Val mutation does not affect the dasatinib mechanism of action and consequently this compound provokes HMC-1^{560,816} cell death. Results shown in this paper indicate that TyrK inhibitor STI571 activates apoptosis in HMC-1^{560} cells. These results are in accordance with those obtained in other cell lines (human colon adenocarcinoma cells and CML cell lines bcr/abl positive), in which STI571 increases caspase-3 activity, an indicator of apoptosis activation (59, 60). These results demonstrate that, as for STI571, dasatinib also induces apoptotic cell death in HMC-1^{560} and HMC-1^{560,816} cell lines, in agreement with the results obtained for HMC-1^{560,816} cells in a previous study (61). Apoptotic activities of both TyrK inhibitors (dasatinib and SIT571) are PKCδ-dependent, since a decrease on cytosolic levels of this protein isoform induces a decrease on TyrK inhibitors effectiveness. It is important to note that dasatinib presents a higher PKCδ dependence than STI571. In this sense, in spite of having silenced the PKCδ, an increase of the apoptotic population is observed after STI571 treatment in HMC-1^{560} cells, unlike that observed after dasatinib treatment. This fact might be related with the targets (Btk and Lyn proteins) described for dasatinib (61). Lyn is a protein related to the PKCδ isoform, since both take part in the degranulation pathway regulated by the Src homology 2 domain-containing inositol-5'-phophatase (SHIP). In this regard, the relationship between PKCδ isoform and Lyn kinase has been widely described in antigen-induced MC degranulation (62). Therefore, considering the strong dependence of the dasatinib mechanism of action by PKCδ, this isoform PKCδ may be considered an important target for the TyrK inhibitor dasatinib.

The above results demonstrate that PKC activation induces apoptotic cell death both in HMC-1^{560} and HMC-1^{560,816} cells that is completely dependent of PKCδ. The role of phorbol esters over cell death is controversial, since the effect of PMA after a short period of incubation is usually described as cytoprotective, and the treatment with PMA for 5 minutes inhibits thapsigargin-induced cell death in smooth muscle cells (63). Moreover, anti-apoptotic role of PMA has been observed in thymocytes, T lymphocytes

and chronic lymphocytic leukemia B-cells after short incubations (64-66). Also, it has been described that the DAG analog phorbol 12,13-dibutyrate (PDBu) inhibits apoptotic Fas-mediated cell death in Jurkat Leukemic T cells after incubation for 2 hours (67). A similar effect of PKC activation was observed in nerve cells, since it was confirmed that tetradecanoylphorbol acetate (TPA) inhibits cell death. In this case, TPA neuroprotection occurs through an extensive phosphorylation pathway which are involves extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and finally PKCδ (68). On the other hand, it has been described an opposite effect due to PKC activation in several studies. For example, in human bronchial epithelial cells, PKCδ is related with cell death induced by the carcinogen asbestos. Also, asbestos-induced cell death is described as PKCδ–dependent and the translocation to the nucleous of this isoform takes place after their activation (54). Therefore, although several studies described an inhibitory proliferation effect for PKCδ (69, 70), others described this isoform as a cell growth inductor (71, 72). Hence, this means that PKC activation can protect the cells against cytotoxic agents or conversely to induce or potentiate cell death. In our case, PKC activation has an evident cytotoxic effect on both HMC-1 cell lines. It is important to note that PKCδ activity is not exclusively related with apoptotic cell death, as it was widely described in the present study, but also with necrotic pathway (73-77). In fact, the PKCδ dual role has been previously described as dependent on the localization of the protein and the presence of pro- or antiapoptotic mediators (78).

PKC is a protein intimately related with MC activation, adhesion and migration (38, 79-83). Specifically, MC activation is a process in which an extensive number of proteins crosstalk. This activation starts after antigen aggregation to FCεRI and is enhanced by the binging of SCF to the c-kit. Intracellular pathways that follow c-kit activation are dependent of TyrK activity, however, src family of TyrKs (including Lyn kinase) are necessary for IgE-dependent degranulation. Also, Btk is the responsible of upregulating MC activation through the FCεRI pathway. Specifically, Btk and Lyn have been described as the most important targets for TyrK inhibitor dasatinib in neoplastic MC (61) and TyrK inhibitors were defined as potent modulators of MC degranulation (84, 85). Dasatinib inhibits IgE-dependent histamine release in human basophils (85) while STI571 induces a decrease on spontaneous histamine release in both HMC-1^{560} and HMC-1^{560,816} (36, 37). Therefore, both TyrK inhibition or PKC activation regulates MC activation. Surprisingly, the cytotoxic effect is completely different when PKC activation is combined with STI571 or dasatinib treatment in cells with one mutation. Whereas the cytotoxic effect is increased after TyrK inhibition with STI571 and PKC

activation, in the presence of dasatinib the activation of PKC does not modify the inhibitor effect of TyrK. Therefore, the combination of the TyrK inhibitor STI571 and PKC activation implies a higher necrotic effect on HMC-1^{560} cells. On the other hand, in HMC-1^{560} cells, the cytotoxicity induced by dasatinib and PKC activation might be sharing a similar pathway, probably through PKCδ, and therefore the final cytotoxic effect is similar when both compounds (dasatinib and PMA) are incubated alone or combined. In HMC-$1^{560,816}$ cells, the cytotoxicity induced by PKC activation and dasatinib are additive, and as for STI571 in HMC-1^{560} cells, this is due to the increase of the necrotic population. Interestingly, previous results obtained in our laboratory demonstrate that the inhibition of TyrKs due to STI571 (in HMC-1^{560}) or dasatinib (in HMC-$1^{560,816}$) implies the translocation of PKCδ from the cytosol to the nucleus. Therefore, the combination of PKCδ translocation with long-term PKC activation indicate that the cell might be suffering an extreme final damage that leads to necrotic cell death in 25% of the population. Hence, it can be stated that PKC activation affects apoptosis induced by TyrK inhibitors STI571 and dasatinib, both in HMC-1^{560} or HMC-$1^{560,816}$ cells. In addition to PKC activation, the inhibition of this protein has been also described as a potential target to induce cell death in multiple myeloma cells (86). In this regard, it is necessary to clarify that PKC inhibitors were previously described as an anti-cancer drugs (86). Specifically, midostaurin, a compound that inhibits Ca^{2+}-dependent PKCs as well as acts as a TyrK inhibitor, has a high effectiveness against HMC-1 cells (50). Interestingly, a synergistic effect of midostaurin and dasatinib has been found in HMC-1 cell line (31). This fact implies a crosstalk between PKC regulation and TyrK inhibition. In summary, the results obtained in this paper demonstrate that PKC activation can be used as a potential tool, in combination with TyrK inhibition, in the treatment of SM, inducing necrotic effect in STI571 (HMC-1^{560}) and dasatinib (HMC-$1^{560,816}$) treatments.

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FIGURE LEGENDS

Figure 1. Effect of tyrosine kinase inhibitors, STI571 and dasatinib, and PKC activation on cell viability in HMC-1 cell line. (A) HMC-1^{560} cells and (B) HMC-$1^{560,816}$ cells. Cells were incubated for 48 hours with 25 nM STI571, 0.012 µM (HMC-1^{560}) and 0.6 µM (HMC-$1^{560,816}$) dasatinib and 100 ng/ml PMA at 37ºC. Mean ± SEM of three experiments. (*) significant differences between untreated and treated cells. (#) significant differences between STI571 and STI571+PMA treated cells.

Figure 2. Effect of STI571, dasatinib and PMA treatments on population distribution (live, apoptotic and necrotic) in HMC-1^{560} cell line. HMC-1^{560} cells were incubated for 48 hours in the presence of 25 nM STI571, 0.012 µM dasatinib and 100 ng/ml PMA at 37ºC. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic cells (upper panel) were detected by Annexin V-FITC/PI staining. (A), (B), (C), (D), (E) and (F) represent one representative experiment of untreated, STI571, dasatinib, PMA, STI571+PMA and dasatinib+PMA treatments respectively. (G) mean ± SEM of the three experiments. (*) significant differences in live populations between the treatments. (#) significant differences in apoptotic populations between the treatments. (**) significant differences in necrotic populations between the treatments. (Δ) significant differences in live population between STI571 and STI571+PMA treated cells.

Figure 3. Effect of dasatinib and PMA treatments on population distribution (live, apoptotic and necrotic) in HMC-$1^{560,816}$ cell line. HMC-$1^{560,816}$ cells were incubated for 48 hours in the presence 0.6 µM dasatinib and 100 ng/ml PMA at 37ºC. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic cells (upper panel) were detected by Annexin V-FITC/PI staining. (A), (B), (C) and (D) represent one representative experiment of untreated, dasatinib, PMA and dasatinib+PMA treatments respectively. (E) mean ± SEM of the three experiments. (*) significant differences in live populations between the treatments. (#) significant differences in apoptotic populations between the treatments. (**) significant differences in necrotic populations between the treatments. (Δ) significant differences in live population between dasatinib and dasatinib+PMA treated cells.

Figure 4. Determination of PKCδ silencing efficiency by western blotting analysis in HMC-1 mast cell lines. PKCδ siRNA was incubated for 24, 36 and 48 hours in HMC-1^{560} and HMC-$1^{560,816}$ cell lines. (A) and (B) show a representative image of each condition in HMC-1^{560} and HMC-$1^{560,816}$ cell line respectively. (C) and (D) is the mean ± SEM of three experiments in HMC-1^{560} and HMC-$1^{560,816}$ cell line respectively. Cytosolic

PKCδ values were calculated respect to actin band intensity. (*) significant differences between no-transfected and PKCδ-transfected cells.

Figure 5. Effect of tyrosine kinase inhibitors, STI571 and dasatinib, and PKC activation on cell viability in PKCδ-silenced HMC-1^{560} and HMC-1^{560,816} cell lines. Cells were incubated for 48 hours with STI571, dasatinib and PMA at 37ºC. (A) and (B) results obtained in HMC-1^{560} and HMC-1^{560,816} cell lines respectively. Mean ± SEM of three experiments. (*) significant differences between transfected and no-transfected cells. (#) significant differences between untreated and STI571 PKCδ-silenced cells.

Figure 6. Effect of 25 nM STI571, 0.012 µM dasatinib and 100 ng/ml PMA treatments in PKCδ–silenced HMC-1^{560} cells. HMC-1^{560} cells were incubated for 48 hours at 37ºC with the different compounds. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic cells (upper panel) were detected by Annexin V-FITC/PI staining. (A), (B), (C), (D), (E) and (F) represent one representative experiment of untreated, STI571, dasatinib, PMA, STI571+PMA and dasatinib+PMA treatments respectively. (G) mean ± SEM of the three experiments. (**) significant difference between untreated and STI571-treated cells on apoptotic population. (#) significant difference between live and apototic population in STI571-treated cells.

Figure 7. Effect of 0.012 µM dasatinib and 100 ng/ml PMA treatments in PKCδ–silenced HMC-1^{560,816} cells HMC-1^{560,816} cells were incubated for 48 hours at 37ºC with the different compounds. Live (botton left panel), apoptotic (botton right panel) and late apoptotic/necrotic cells (upper panel) were detected by Annexin V-FITC/PI staining. (A), (B), (C) and (D) represent one representative experiment of untreated, dasatinib, PMA and dasatinib+PMA treatments respectively. (E) mean ± SEM of the three experiments.

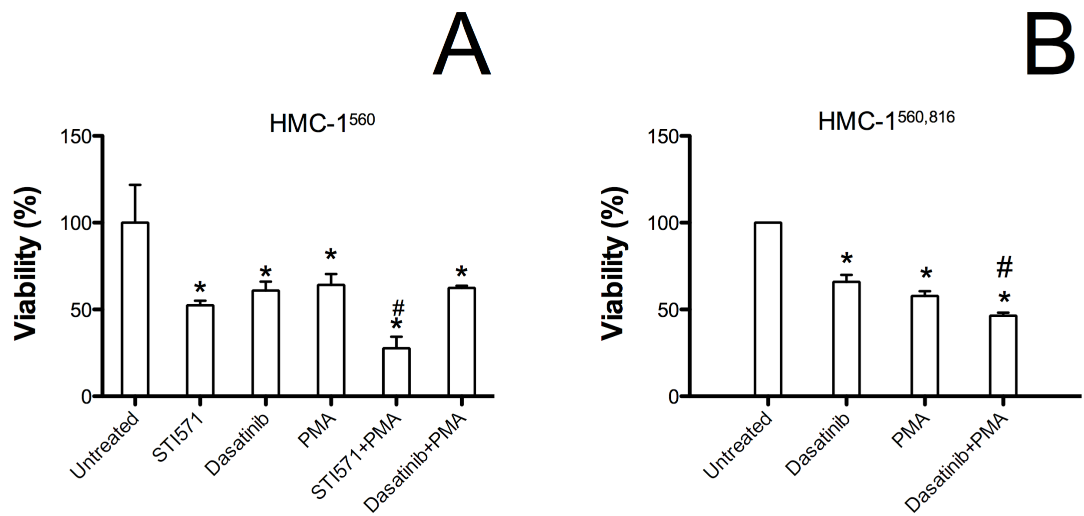
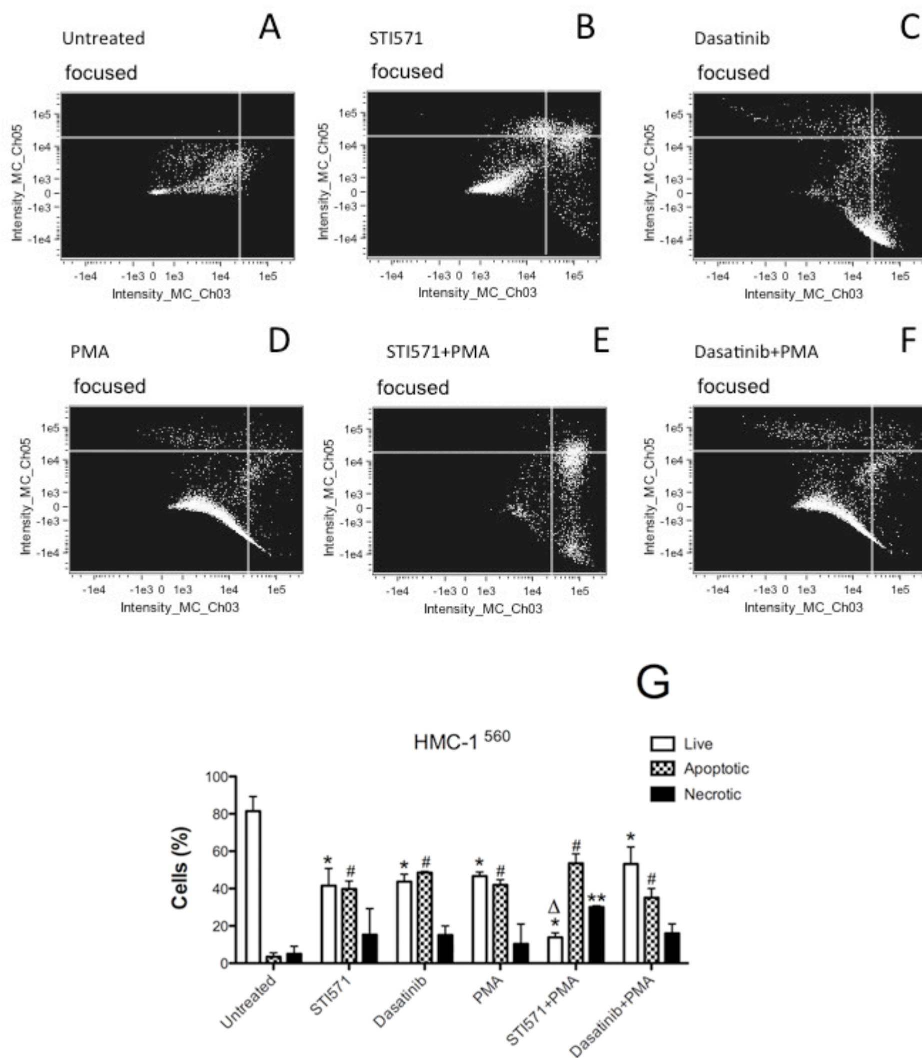


figure 1



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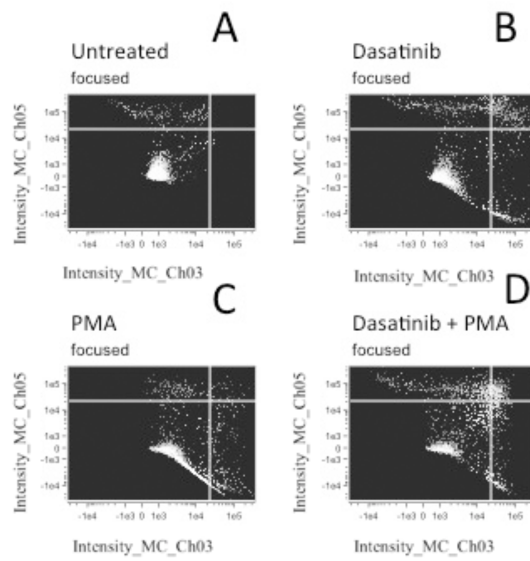

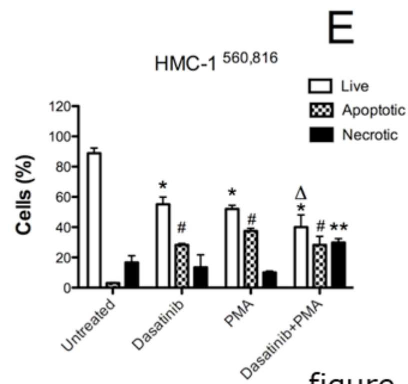

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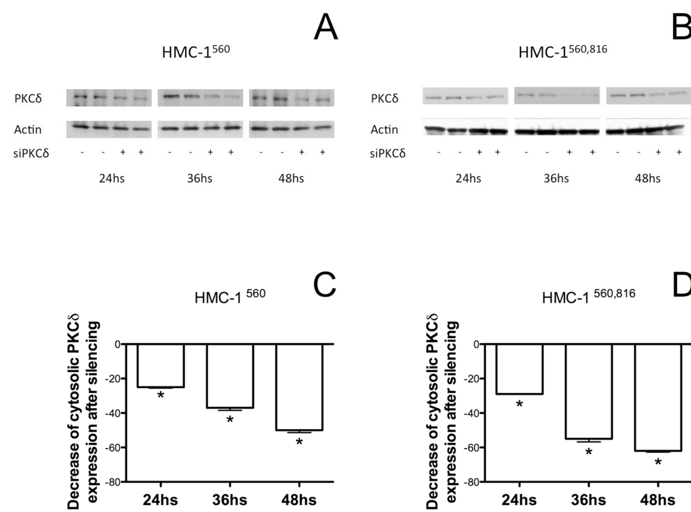

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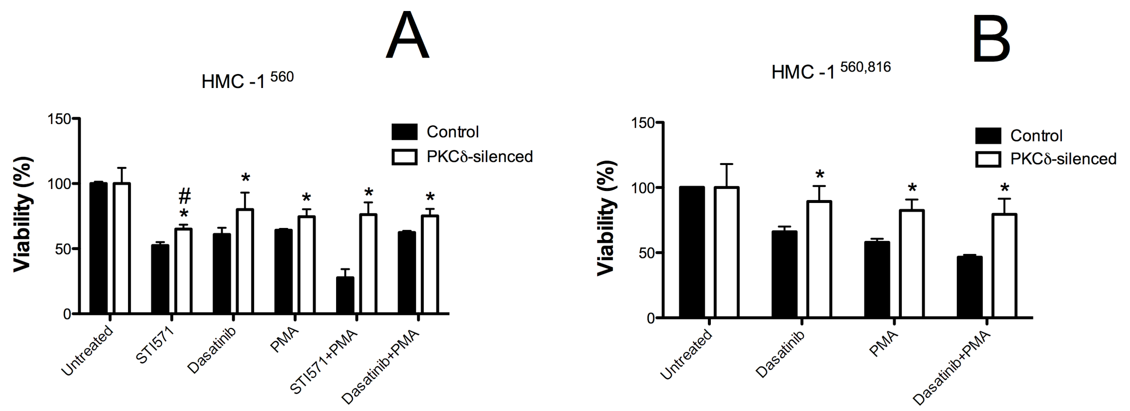
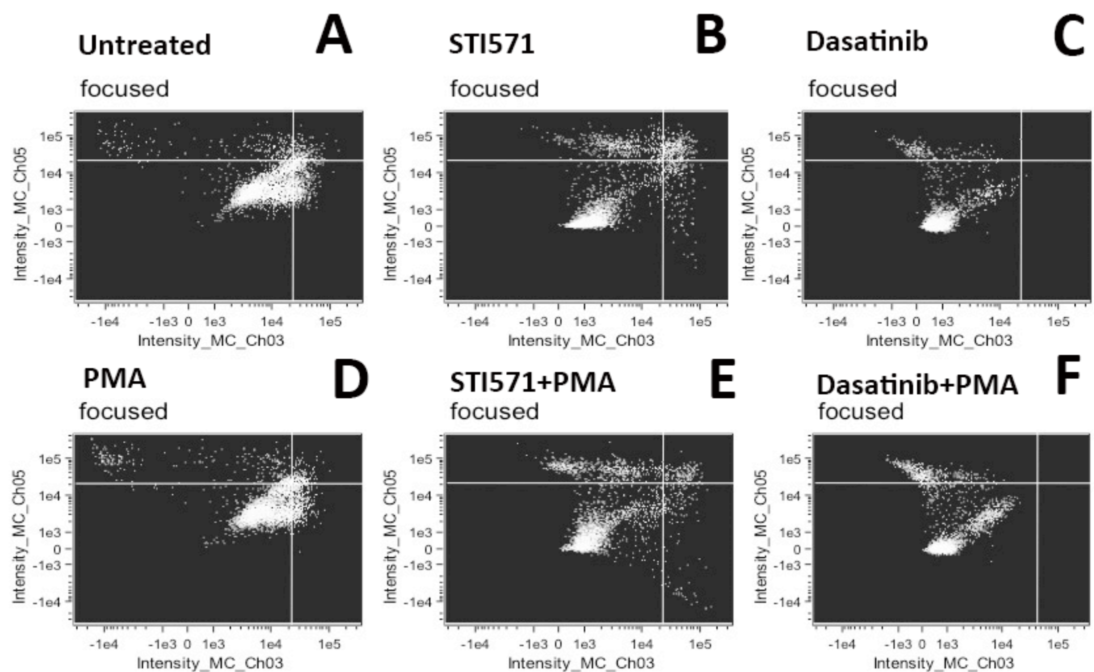


figure 5



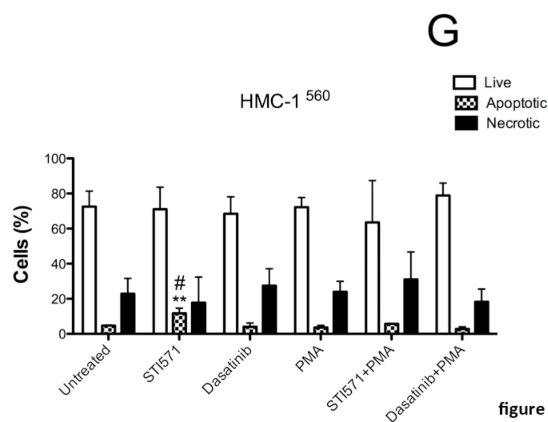
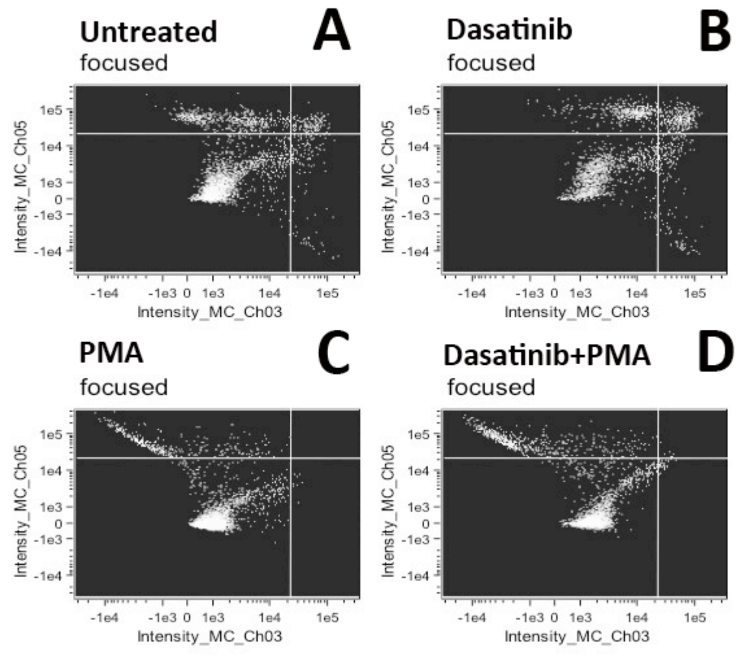


figure 6



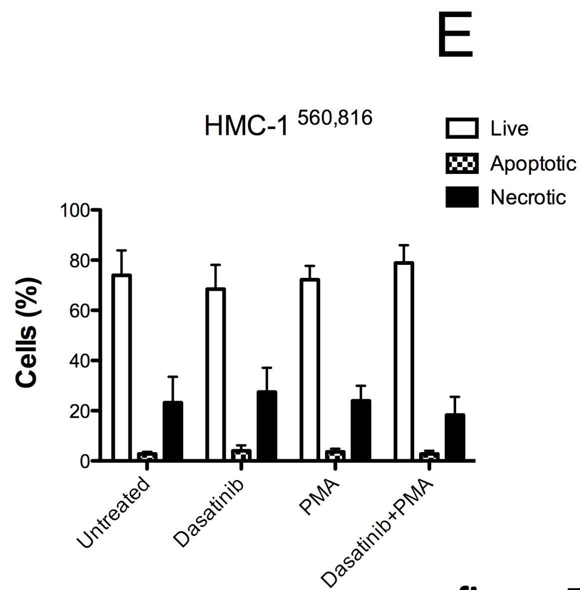


figure 7

4.4. Sección III: Estudio de la inhibición de la aurora quinasa por el CCT129202 en la línea celular HMC-1^{560,816}.

La aurora quinasa es una proteína que posee tres isoformas: A, B y C, relacionadas con una serie de eventos que tienen lugar durante la mitosis celular como son la alineación y segregación cromosómica, la maduración del centrosoma, la formación de los husos mitóticos y la citocinesis. Se encuentra sobreexpresada en numerosos tipos de cáncer y se ha demostrado que su inhibición provoca la muerte celular en varias líneas tumorales [278,279]. De esta forma se considera una proteína diana en el tratamiento antitumoral convirtiéndose en objeto de numerosos estudios relacionados con la obtención de fármacos anticancerígenos [280]. El inhibidor de la aurora quinasa CCT129202 pertenece al grupo más importante de inhibidores de esta proteína: los derivados de imidazol [264]. Este compuesto presenta una alta especificidad frente a la aurora quinasa y se ha comprobado que provoca apoptosis en varios tipos celulares tumorales, entre ellos en la línea HCT116 de cáncer de colon humano y en varios tipos celulares de carcinoma incluyendo el mamario y el oral [264,281]. Sin embargo, no se ha determinado el efecto del CCT129202 en la línea celular HMC-1^{560,816}, presente en el 80% de los pacientes con mastocitosis sistémica [122]. Por esta razón se ha procedido al estudio del efecto de la inhibición de la aurora quinasa, a través del CCT129202, en la línea celular HMC-1^{560,816}.

A esta sección corresponde la siguiente publicación:

III.1. *Protein kinase C modulates aurora-kinase inhibition induced by CCT129202 in HMC-1^{560,816} cell line.*

III.1. La proteína quinasa C modula la inhibición de la aurora quinasa inducida por el CCT129202 en la línea celular HMC-1^{560,816}.

<u>Resumen</u>

En este estudio se comprueba que el compuesto CCT129202 estimula la apoptosis en la línea celular HMC-1^{560,816} tras un periodo de incubación de 48 horas.

Los resultados obtenidos nos indican que el mecanismo de acción de este inhibidor tiene lugar a través de las caspasas, enzimas esenciales en la señalización del proceso apoptótico. De este modo, se observa que el CCT129202 aumenta la actividad de las caspasas 8 y 3. Además, la PKC regula la actividad de este fármaco, de tal forma que la inhibición de esta proteína implica la anulación del efecto apoptótico mediado por CCT129202. En primer lugar se observa que la inhibición de las isoformas clásicas de la PKC anula el efecto del CCT129202 sobre la actividad de la caspasa-8, sin embargo no altera el efecto del mismo sobre la actividad de la caspasa-3. Por otro lado, al inhibir las isoformas nuevas de la PKC se anula el efecto estimulador del CCT129202 sobre la caspasa-3 pero no sobre la 8. Además, la dependencia del inhibidor CCT129202 por la PKC se corrobora tras silenciar esta proteína. De tal forma que la ausencia de expresión de la PKC anula el efecto estimulador del CCT129202 sobre la actividad de la caspasa-3. Finalmente, se describe que el CCT129202 no modifica la expresión citosólica de las isoformas clásicas ni tampoco de la PKCδ, ampliamente relacionada con el proceso de apoptosis celular, pero sí provoca la retención en la fase mitótica de una parte de la población celular.

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Protein Kinase C Modulates Aurora-kinase Inhibition Induced by CCT129202 in HMC-1^{560,816} Cell Line

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Abstract: The human mast cell line HMC-1^{560,816} carries activating mutations in the proto-oncogene of c-kit that cause autophosphorylation and permanent c-kit receptor activation. The compound CCT129202 is a new and selective inhibitor of Aurora kinase A and B that decreases the viability of a variety of human tumor cell lines. The effect of Aurora kinase inhibition was assessed in the HMC-1^{560,816} line in order to find a suitable tool for mastocytosis treatment. CCT129202 treatment induces a significant decrease in cell viability in HMC-1^{560,816} cells after 48 hours of treatment. Moreover, caspase-3 and caspase-8 activation was induced after incubation of HMC-1^{560,816} cells in the presence of CCT129202. It has been demonstrated that Protein Kinase C (PKC) plays a crucial role in mast cell activation as well as cell migration, adhesion and apoptotic cell death. Co-treatment of Ca^{2+}-independent PKCs (δ, ε and θ) inhibitor GF109203X with CCT129202, reduces caspase-3 activation which controls cell levels. In contrast, Gö6976, an inhibitor of Ca^{2+}-dependent PKCs, increases caspase-3 activation. Oppositely, GF109203X does not modify CCT129202-induced apoptosis through the caspase-8 pathway whereas Gö6976 treatment abolishes the increase on caspase-8 activity due to CCT129202. This implies that Ca^{2+}-independent PKC isoforms seems to be related with CCT129202-induced apoptosis through the caspase-3 pathway, whereas Ca^{2+}-dependent PKC isoforms are related with the CCT129202 effect on the caspase-8 pathway. Interestingly, CCT129202 cytotoxic effect remains even though Ca^{2+}-dependent PKCs are inhibited, which shows that the Aurora kinase inhibitor effect is acting through the caspase-3 pathway. On the other hand, Ca^{2+}-independent PKCs inhibition does not affect the final apoptotic CCT129202 effect because this seems to be mediated by the caspase-8 pathway. Moreover, CCT129202 does not affect PKCδ and Ca^{2+}-dependent PKC translocation, which indicates that PKC translocation pivots on its activation. This demonstrates that Aurora kinase inhibition is not related to this process. Finally, when PKC is silenced in HMC-1^{560,816} cells, the effect of CCT129202 on the caspase-3 pathway disappears, which indicates that the CCT129202 effect is clearly PKC-dependent.

Keywords: HMC-1, Aurora kinase, CCT129202, PKC, caspases.

INTRODUCTION

Mastocytosis is a mast cell disease characterised by an abnormal growth and accumulation of mast cells in one or more tissues [1]. HMC-1 (Human Mast Cell) is a human mast cell line isolated from a patient with mast cell leukemia, an aggressive form of systemic mastocytosis (SM). The maturation of mast cell precursors is influenced by cytokines like interleukin 4 (IL-4), IL-9, nerve growth factor and stem-cell factor (SCF). Moreover, SCF binding to its c-kit receptor seems to be the main drive for cell differentiation and survival. There are two sublines, HMC-1^{560} and HMC-1^{560,816}, which differ in their mutations in the c-kit proto-oncogene. HMC-1^{560} cells have an amino acid exchange (Gly-560->Val) in the juxtamembrane region of the tyrosine kinase (TyrK) receptor c-kit. This exchange causes a permanent activation of the inner TyrK and thereby a ligand-independent proliferation of these cells. The mutation is common in various human malignant diseases, including gastrointestinal stromal tumor and cutaneous mastocytosis [2]. HMC-1^{560,816} cells carry another mutation, which leads to an amino acid exchange (Asp-816->Val) in the intracellular side of c-kit and modifies the conformation of the TyrK [3-5]. In the great majority (>90%) of adult cases with SM mutations in the activation loop of KIT (most frequently D816V) are detected in association with an aberrant CD25+ phenotype [6].

One of the most important proteins that regulate mast cell signaling is Protein kinase C (PKC). PKC plays an important role in a variety of cellular functions, specifically in mast cell activation [7]. PKC proteins are classified depending on their sensitivity to calcium (Ca^{2+}) and phorbol esters (PMA) in i) conventional or Ca^{2+}-dependent and PMA-activated PKCs (α, βI, βII, γ); ii) novel or Ca^{2+}-independent PKCs (δ, ε, η, θ), also activated by PMA; iii) atypical PKCs (ζ, λ, ι), which are Ca^{2+} and PMA-independent [8]; and iv) novel/atypical PKCμ. The relationship between PKC and apoptotic cell death has been described in several studies [9-12].

In the last decade major advances have been achieved in the field of molecular-targeted therapy, in which drugs are selected on the basis of specific molecular abnormalities causing individual diseases. Among the new drugs developed,

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Summary sentence: Apoptosis induced by Aurora Kinase inhibition is Protein Kinase C-dependent in the HMC-1^{560,816} cell line.

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the STI571 TyrK inhibitor (Imatinib mesylate or Gleevec®) is one of the most studied. *In vitro* studies have proven that imatinib inhibits wild type Kit (wtKit) and suppresses proliferation of the HMC-1^{560} cell line, while is ineffective on inhibiting the growth of HMC-$1^{560,816}$ cells [3, 13]. In line with *in vitro* results, significant clinical responses to imatinib have been shown in the absence of the D816V mutation, as well as in SM cases associated with other hematological diseases carrying another imatinib-target, such as SM-CEL with FIP1L1/PDGFRA gene rearrangements [14, 15]. In contrast, other KIT TK-targeting drugs such as dasatinib [2], PKC412 [16], and EXEL-0862 [17], among others, show *in vitro* activity against the D816V mutant form of KIT despite resistance to imatinib mesylate.

In the last few years, different Aurora kinase inhibitors were developed as potential new and effective anti-cancer drugs. The Aurora family of serine/threonine kinases is essential for chromosome alignment segregation, formation of mitotic spindle, maturation of centrosomes and cytokinesis during mitosis. Thus, Aurora kinases are key regulators of mitotic progression. There are three isoforms, Aurora kinase A, B and C that share the highest degree of sequence homology in their catalytic domains. Aurora A is elevated at the onset of mitosis and mainly implicated in chromosome maturation and spindle assembly [18, 19]. It associates with centrosomes during interphase and spindle poles and spindle microtubules during early mitosis [20, 21]. Aurora B is a chromosomal passenger protein found in a tight complex with inner centromere protein and survivin [22]. Depletion of one of these proteins leads to similar defects in chromosome segregation and cell division. Aurora kinase B is upregulated in the mitosis and regulates chromosome condensation, kinetochore assembly and chromosome attachment, coordinating chromosome segregation and cytokinesis [23]. Additionally, Aurora kinases A and B phosphorylate histone H3, a structural component of chromatin [24]. Aurora C is expressed in sperm cells and is localized to spindle poles later in mitosis and plays a crucial role in spermatogenesis [25, 26]. Over-expression of Aurora kinases leads to genetic instability, which is associated with tumorogenesis [27]. The validation of these kinases as a potential therapeutic target is commonly based on gene silencing experiments. Aurora inhibitors are specific for protein isoforms, for example MLN8054 is Aurora A selective, whereas AZD1152 and VX680 are Aurora B and pan-Aurora selective respectively. Their effectiveness in different cancer cell types *in vivo* and *in vitro* provides hope for new treatments [28-32]. The properties of CCT129202 (CCT), a representative of a structurally novel series of imidazopyridine small-molecule inhibitors of Aurora kinase activity, were reported a few years ago. This compound potentially inhibits Aurora kinase A, B and C in different human tumor cell lines, with IC_{50} values of 0.042 ± 0.022, 0.198 ± 0.05 and 0.227 ± 0.064 µmol/L respectively [33]. It has been reported that CCT is an ATP-competitive inhibitor of recombinant Aurora A kinase with a K_i of 49.8 nmol/L [33]. In tumor cell lines, apoptosis was induced after CCT treatment and causes accumulation of human tumor cells with a high level of DNA, leading to apoptosis. In addition, CCT decreased histone H3 phosphorylation and caused stabilisation of the tumor suppressor p53. In this present study, the effects of the Aurora kinase inhibitor CCT are checked in HMC-$1^{560,816}$ cells in order to discuss new potential ways of treating aggressive forms of mastocytosis.

METHODS

Chemicals

CCT129202 was kindly supplied by Centro de Investigaciones Biomédicas (Granada, Spain). Gö6976 and GF109203X were from Alexis Corporation (Läufelfingen, Switzerland). Phosphate buffered saline (PBS), EnzChek® Caspase-3 Assay Kit #1 and Caspase-8/FLICE colorimetric protease assay were from Invitrogen (Camarillo, CA, USA). Propidium iodide was from Beckman Coulter (Fullerton, CA, USA). PMA, 3-[4,5-dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and bovine serum albumin (BSA) were from Sigma-Aldrich (Madrid, Spain). Anti PKCδ was from BD Biosciences (Madrid, Spain). Anti Mouse IgG was purchased from GE Healthcare (Barcelona, Spain). Anti cPKC clone M110, anti Actin and polyvinylidene fluoride (PVDF) membrane were from Millipore (Temecula, CA, USA). Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were from BioRad (Barcelona, Spain). Complete Protease Inhibitor and 1X Phosphatase Inhibitor Cocktail were from Roche (Barcelona, Spain). Anti PKC (sc-17769), PKC siRNA (sc-29449) and siRNA control (sc-37007) were from Santa Cruz Biotechnology (CA, USA). GeneSilencer® was from Genlantis (San Diego, CA, USA). Ethanol was from Panreac (Barcelona, Spain).

Cell Culture

HMC-$1^{560,816}$ cells were kindly provided by Dr. Luis Escribano Mora with permission from Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and maintained in Iscove´s modified Dulbecco´s medium (IMDM) (Gibco, Invitrogen, Spain). They were supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Spain), 10,000 IU/ml penicillin and 10,000 µg/ml streptomycin at 37ºC in a humidified atmosphere containing CO_2. Cells were split weekly.

Cell Viability

After exposure to CCT, PMA, GF109203X and Gö6976 for 48 hours in culture medium, cells were centrifuged (1,500 r.p.m., 5 minutes, 4 ºC). The pellets were resuspended in saline solution with MTT (250 µg/ml) and incubated at 37 °C for 30 minutes in darkness. The composition of the saline solution was (mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2; glucose 1 g/l pH=7.2. After washing twice with saline solution cells were sonicated for 60 seconds. The coloured formazan salt was measured at 595 nm in a spectrophotometer plate reader (Synergy™ 4, BioTek Instruments, Vermont, USA).

Cell Cycle Analysis

Cells were treated with 1µM CCT for 48 hours in culture medium. After washing with PBS they were fixed with 70% ethanol for 2 hours. The cells were then washed with PBS and stained with propidium iodide (5µg/ml) for 30 minutes at room temperature and analysed in a flow cytometer.

Apoptosis Assay

Cells were treated and incubated for 48 hours in culture medium. Afterwards, they were washed with PBS (Gibco, Invitrogen, Spain) and prepared exactly following manufacturer's instructions of both kit–assays: EnzChek® Caspase-3 Assay Kit #1 and Caspase-8/FLICE Colorimetric Protease Assay (Invitrogen, Spain).

Western Blotting

After exposure to CCT, PMA, GF109203X and Gö6976 for 48 hours in culture medium, cells were centrifuged and washed twice with saline solution. Afterwards cells were resuspended in a lysis buffer with the following composition: 50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 1%Triton X-100, 2 mM DTT, 1X Complete Protease Inhibitor and 1X Phosphatase Inhibitor Cocktail. Bradford assay was the method used to determine the sample's protein concentration and BSA was used as the protein standard. Samples were blotted to PVDF membrane by reduced SDS-PAGE. To determine the protein size and also to monitor the progress of an electrophoretic run, Precision Plus Protein™ Standards Kaleidoscope™ molecular weight marker was used. After blockage with 0.5% BSA, the membrane was incubated for 10 minutes with anti-PKCδ (1:1000) or anti cPKC (1:1000). After three washes with washing buffer (PBS + 0.1%Tween), the membrane was incubated for 10 minutes with secondary peroxidase-labelled antibody (GE Healthcare). After three washes, chemiluminescence was visualised with SuperSignal® West Pico (Pierce). Relative protein expression was calculated in relation to β-actin expression for each experiment. In order to check the effectiveness of the inhibition of PKC expression, HMC-1^{560,816} cell lysates were obtained 36 and 48 hours post-CCT treatment from transfected and non-transfected cells. In this case, an antibody recommended for detection of all PKC family isoforms (Anti PKC (A-3) (sc-17769)) was used during an incubation of 10 minutes at 1:1000 dilution. Secondary antibody and chemiluminescence procedures were the same as previously described. Experiments were carried out three times.

Transfection by Lipid-based Method (Genlantis)

GeneSilencer® (Genlantis) was composed by the transfection reagent and the diluent. HMC-1^{560,816} cells were incubated at 2x10^{6}cls/ml in FBS- and antibiotics-free IMDM. Manufacturer's instructions were followed carefully. On the day of transfection two solutions were prepared: solution A (composed by diluent, IMDM medium and PKC siRNA) and solution B (composed by transfection reagent diluted in IMDM medium). Solutions A and B were mixed and incubated for 5 minutes at room temperature. HMC-1^{560,816} cells were incubated in a total volume of 500µl in a FBS- and antibiotic-free IMDM. After 5 hours of incubation, 500 µl of complete medium was added. Afterwards, cells were allowed to recover at 37ºC for 19 hours and were subsequently stimulated with 1 µM CCT, 50 nM Gö6976, 500 nM GF102903X for 48 hours. Control siRNA (sc-37007) was used as a negative control for evaluating RNAi off-target effects.

Statistical Analysis

Results were analysed using the Student's t-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

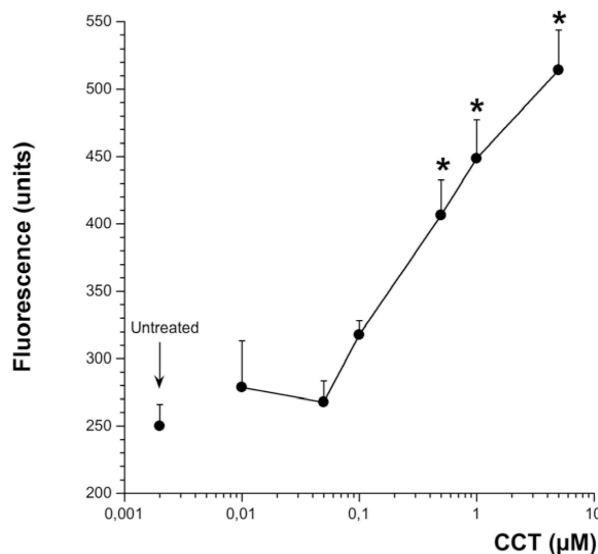


Fig. (1). Effect of Aurora kinase inhibition on caspase-3 activity in HMC-1^{560,816} cells. Caspase-3 activation of HMC-1^{560,816} cells was measured with EnzChek® Caspase-3 Assay Kit after 48 hours of incubation at 37°C in culture medium with CCT. Mean ± SEM of three experiments. (*) Significant differences between control and CCT-treated cells.

RESULTS

First of all, the effect of the Aurora kinase inhibitor CCT was checked on HMC-1^{560,816} cells by studying caspase activation in the presence of the drug. Caspases are essential for the execution of programmed cell death. They exist as an inactive proenzyme and activate themselves or other caspases upon apoptotic stimulation. The signalling pathway is cell type and stimuli-dependent. DNA-damaging agents usually produce mitochondrial cytochrome C release, which facilitates the interaction of apoptotic protease activation factor (Apaf-1) with procaspase-9. This leads to the stimulation of downstream effector caspases like caspase-3 or -7 and causes cell death [34]. HMC-1^{560,816} cells were incubated with Aurora kinase inhibitor CCT and caspase-3 activation levels were determined. In these experiments, 0.01, 0.05, 0.1, 0.5, 1 and 5 μM of CCT were used. Fig. (**1**) shows that CCT significantly induces caspase-3 activation in HMC-1^{560,816} cells after 48 hours of incubation. This effect is dose-dependent and is statistically significant at the three highest concentrations: 0.5, 1 and 5 μM. Another method was used in order to clarify CCT apoptotic effect in HMC-1^{560,816} cells. In this sense, apoptotic population was also determined with propidium iodide staining using a flow cytometer equipment (Fig. **2**). Figures 2A and 2B show a representative experiment of each condition. CCT induces a significant decrease in G_0/G_1 population (about 20%). On the other hand, CCT increases apoptotic population, since approximately 10% of the cells are in apoptosis. Aside from this, Aurora kinase inhibitor induces a mitotic arrest (about 10%). The S-phase was identical among treatments. In order to know if CCT has any effect on cell viability, the cytotoxic effect of Aurora kinase inhibitor in HMC-1^{560,816} cells was the checked. Fig. (**3**) shows that Aurora kinase inhibitor induces a decrease in cell viability at all concentrations checked after 48 hours of incubation. This decrease is dose dependent, since cell viability decreases by 20% with 0.01 μM, 40% with 0.05, 0.1 and 0.5 μM CCT, 45% with 1 μM CCT and 50% with 5 μM CCT. From these experiments 1 μM CCT was the concentration selected for the following assays.

PKC has been related with apoptosis cell death [35]. Specifically, PKC activation induces apoptosis through the caspase-3 pathway in SNU-16 gastric adenocarcinoma cells [36]. Since PKC isoforms play an important role in HMC-1 activation [37-39], we studied the combination of CCT and PKC pathways. HMC-1^{560,816} cells were incubated in the presence of 1 μM CCT, 50 nM Ca^{2+}-dependent PKCs inhibitor Gö6976 and with 500 nM of PKC inhibitor GF102903X, which inhibits Ca^{2+}-independent PKC isoforms. As (Fig. **4A**) shows, GF102903X by itself does not modify caspase-3 activation but it significantly reduces the effect induced by 1 μM of CCT. However, PKC inhibitor Gö6976 which inhibits various Ca^{2+}-dependent PKCs but not PKCδ, ε and θ [40], significantly increases caspase-3 activation induced by 1 μM CCT. The compounds alone (Gö6976 or GF109203X) do not show any effect in caspase-3 activation. Since CCT has a cytotoxic effect, the next step was to determine the effect of PKC modulation on the CCT-viability effect. As (Fig. **4B**) shows, 1 μM CCT induces a significant decrease in the percentage of viable cells (around 40%), whereas GF102903X (500 nM) and Gö6976 (50 nM) treatments do not modify the percentage of cell viability. In contrast, the same figure shows that the treatments with CCT+GF102903X and CCT+Gö6976 induce a significant decrease in cell viability (around a 40% decrease in all treatments). On the other hand, PKC activator (100ng/ml PMA) does not modify cell viability by itself. However, in combination with CCT the decrease of viability is similar than with CCT alone (data not shown). According to these results CCT cytotoxic effect and caspase-3 activation due to CCT seems to occur by different pathways, since kinase modulation does not affect cell death due to CCT.

It was demonstrated in previous studies that the catalytic fragment of PKCδ is directly associated with apoptotic cell death [41-43]. The role of PKCδ in apoptosis was usually associated with translocation of the kinase from cytosol to mitochondria or nucleous where it has its target sites [44]. In addition, the inhibition of PKCδ, more than any other PKC Ca^{2+}-independent isoform, modulates HMC-1^{560,816} activation [37, 45]. Therefore, the relationship between PKCδ (an isoform which is Ca^{2+}-independent and inhibited by GF109203X) and CCT was studied. PKCδ cytosolic expression after 48 hours of incubation with CCT (1 μM), PMA (100 ng/ml) and GF102903X (500 nM) has been checked. As (Fig. **5A**) shows, the Aurora kinase inhibitor CCT does not modify cytosolic PKCδ expression in HMC-1^{560,816} cells after 48 hours of incubation. However, the treatment with PMA induces a significant decrease in cytosolic PKCδ expression, this effect was also observed when the PKC activator was incubated with CCT (Fig. **5B**). Moreover, the treatment with GF102903X does not modify the expression of this protein and no effect on cytosolic PKCδ expression was observed with the co-treatment CCT+GF109203X. As was shown in (Fig. **4A**), Ca^{2+}-dependent isoforms are related with CCT-induced apoptotic cell death, since the Ca^{2+}-dependent PKC inhibitor Gö6976 potentiates the apoptotic effect of CCT. Therefore, cytosolic Ca^{2+}-dependent PKC (cPKC) levels were determined. Fig. (**5D**) shows that CCT treatment alone, as well as Gö6976, does not modify cytosolic cPKC levels. As was previously described in another study [46], PMA treatment induces a significant decrease in cytosolic cPKC levels (from 1.5 to 0.45 relative expression units). Moreover, co-treatment with CCT and PMA induces a decrease in cytosolic cPKC levels, even higher than observed with PMA alone (from 1.5 to 0.2 relative expression units). However, CCT+Gö6976 co-treatment, as well as the treatments with CCT and Gö6976 alone do not modify cytosolic cPKC levels. Therefore, Ca^{2+}-dependent PKC isoforms are interrelated with cytotoxic and CCT-induced caspase-3 activation.

Two major pathways have been implicated in apoptosis activation: intrinsic and extrinsic pathways. In the first one, factors such as stress, DNA damage, chemotherapy or UV exposure trigger mitochondrial damage and release of apoptogenic proteins, like cytochrome C and smac/DIABLO. The next step in this study was to determine if CCT effect is related with the apoptotic extrinsic pathway. Caspase-8, also known as MACH, Mch5 and FLICE, is a member of the interleukin-1β converting enzyme (ICE) family of cysteine proteases (caspases). Caspase-8, which is the most important caspase that takes part in the apoptosis extrinsic pathway, provides a direct link between cell death receptors and the caspases. The downstream substrates of caspase-8 include

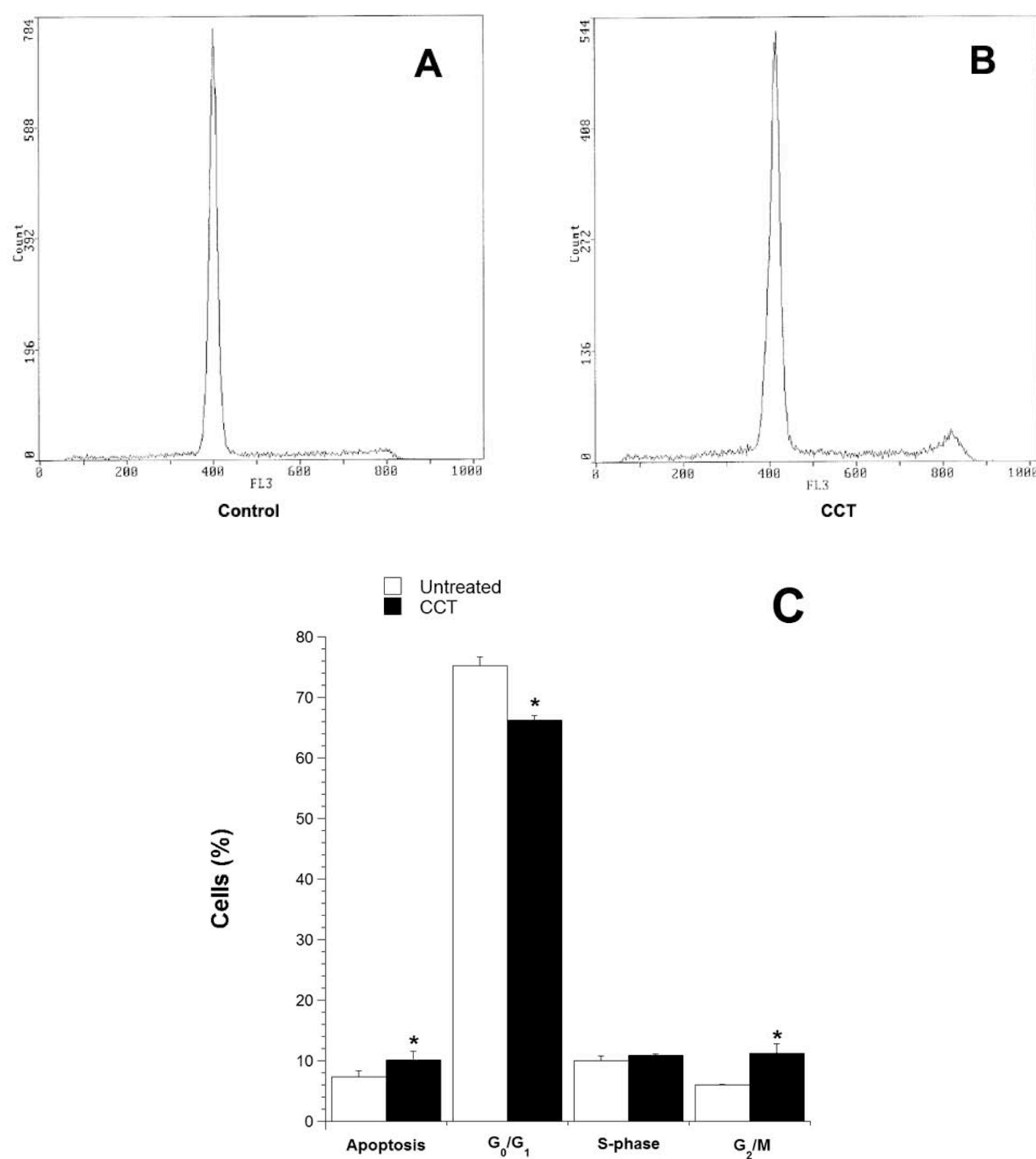


Fig. (2). Effect of Aurora kinase inhibition on cell cycle in HMC-1^{560,816} cells. Cells were incubated with 1 µM CCT. Cell cycle was analysed by flow cytometry after 48 hours at 37°C in culture medium. **A)** Representative experiment of the condition of untreated cells. **B)** Representative experiment of the condition of CCT treated cells. **C)** Distribution of the four populations (Apoptotic, G_0/G_1, S-phase and G_2/M). Mean ± SEM of three experiments (*) Significant differences between control and CCT-treated cells.

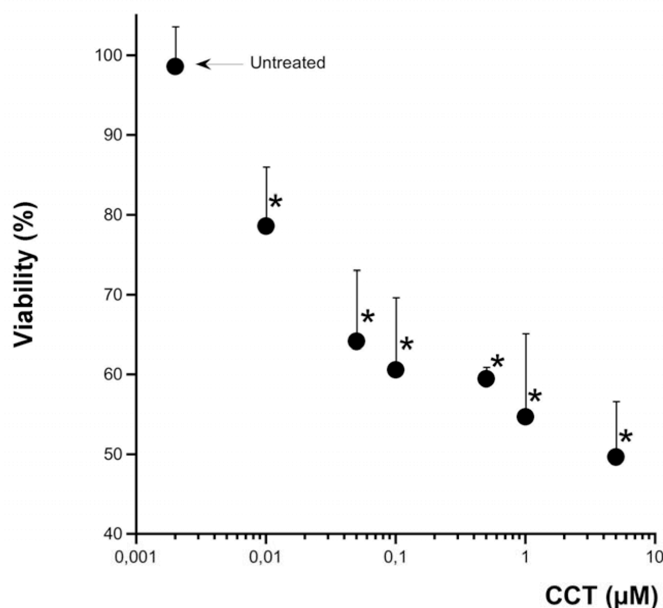


Fig. (3). Effect of Aurora kinase inhibition on cell viability in HMC-1^{560,816} cells. Cell viability was tested by MTT after 48 hours of incubation at 37ºC with CCT. Mean ± SEM of three experiments. (*) Significant differences between control and CCT-treated cells.

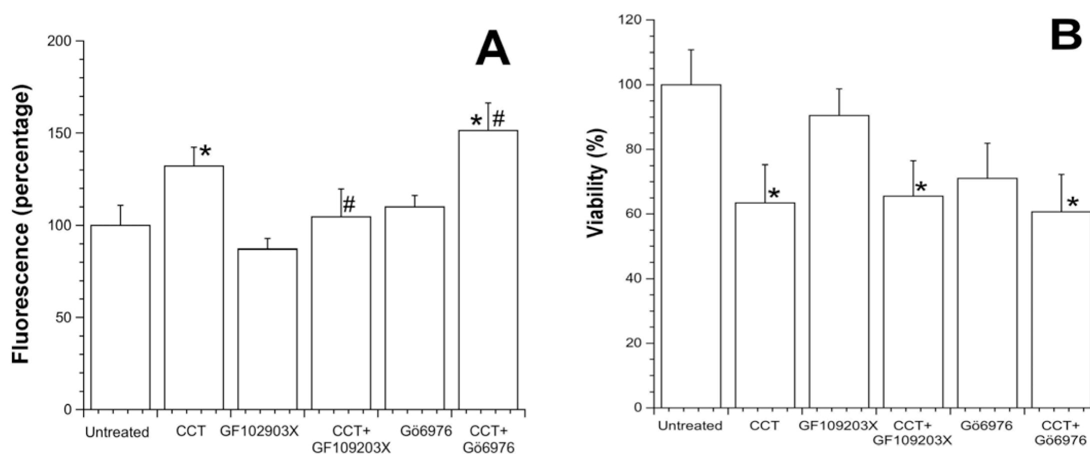


Fig. (4). Effect of Aurora kinase inhibition and PKC modulation on caspase-3 activity and cell viability in HMC-1^{560,816} cells. A) Caspase-3 activation was measured in cells that were treated with PKC inhibitors 500 nM GF109203X or 50 nM Gö6976 alone or in combination with 1 µM CCT. **B**) Cell viability was tested by MTT after 48 hours of incubation at 37ºC with 1 µM CCT, 500 nM GF109203X and 50 nM Gö6976. Mean ± SEM of three experiments. (*) Significant differences between control and drug treated cells. (#) Significant differences between CCT alone or in combination with GF109203X or Gö6976 treatments.

caspase-3, -7, -13 (ERICE) and PARP. For this, caspase-8 activity has been determined in the presence of 1 µM CCT and two PKC pathway modulators (GF109203X and Gö6976). As (Fig. **6**) shows, treatment with 1 µM CCT for 48 hours induces a significant increase in caspase-8 activity from 100±7.53 (untreated cells) to 117.26±7.92 (CCT treated cells), whereas GF109203X and Gö6976 treatments do not modify caspase-8 activity. Moreover, PKC modulation affects the caspase-8 increase due to CCT. In this way, Ca^{2+}-independent PKC inhibition (GF109203X treatment) induces

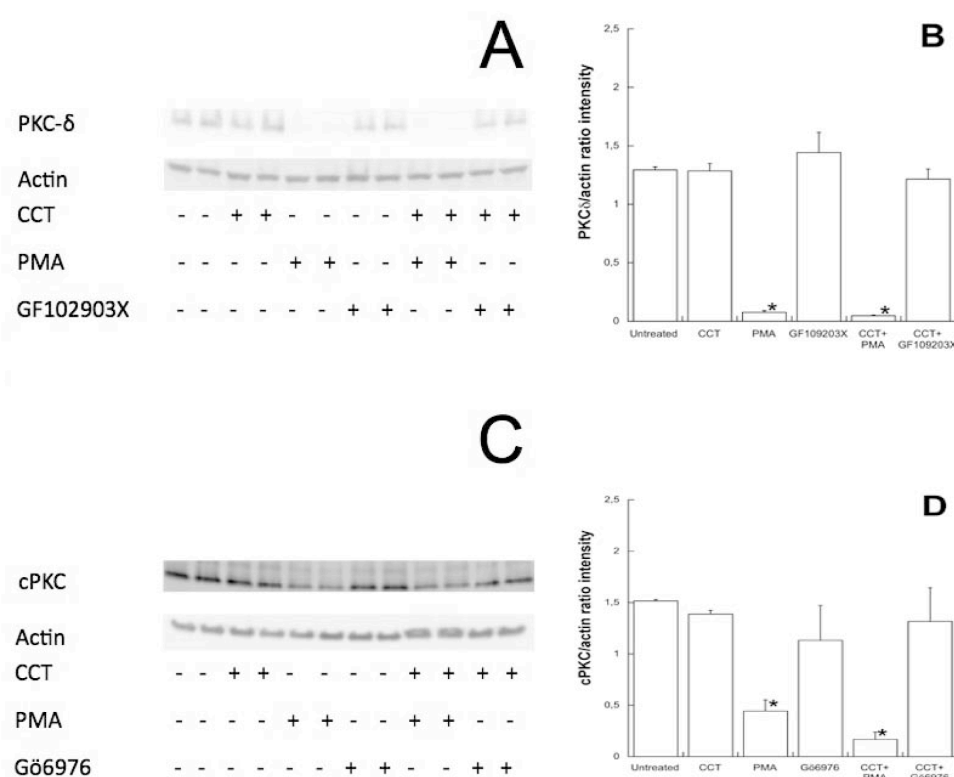


Fig. (5). Effect of Aurora kinase inhibition and PKC modulation on cytosolic PKCδ and cPKC expression in HMC-1^{560,816} cells. Cells were treated with 1 µM CCT, 100 ng/ml PMA, 500 nM GF109203X and 50 nM Gö6976 for 48 hours at 37°C. **A**) PKCδ and β-actin expression, as loading control, were quantified by Western Blot analysis. **B**) Mean of cytosolic PKCδ expression obtained in the three experiments. **C**) cPKC and β-actin expression, as loading control, were quantified by Western Blot analysis. **D**) Mean of cytosolic cPKC expression obtained in the three experiments. Mean ± SEM of three experiments. (*) Significant differences between control and treated cells.

an increase in the CCT apoptotic effect. The percentage of caspase-8 activity increases from 117.26±7.92 (CCT treated cells) to 137.13±7.71 (CCT+GF109203X treated cells), whereas the inhibition of Ca^{2+}-dependent PKCs (Gö6976 treatment) induces a statistical inhibition of the CCT effect, the percentage of caspase-8 activity falls down from 117.26±7.92 (CCT treated cells) to 74.49±5.97 (CCT+Gö6976 treated cells). Therefore, the cytotoxic effect on the HMC-1^{560,816} cell line induced by Aurora kinase inhibition seems to be independent of PKC modulation. However, the PKC pathway appears to be related with CCT-dependent caspase-3/caspase-8 activation.

To confirm PKC dependence of Aurora kinase inhibitor in caspase-3 induced cell death, PKC protein was silenced in HMC-1^{560,816} cells. For this, HMC-1^{560,816} cells were transfected with siRNA PKC and then incubated with CCT, as well as with Gö6976 and GF109203X for 48 hours in order to check the activity of caspase-3 when PKC is silenced. The first step was to evaluate the effectiveness of transfection; for this, PKC expression has been determined in non-transfected and transfected cells at 36 and 48 hours post-CCT treatment. A representative experiment and PKC/β actin ratio intensity is represented in (Figs. **7A** and **B**) respectively. As (Fig. **7B**) shows, the expression of this protein significantly decreases at 36 hours post-CCT treatment, since relative PKC expression falls down from 1.1±0.07 to 0.59±0.09. Alongside, this sharp decrease on PKC expression is observed at 48 hours post-CCT treatment. At this time PKC expression in transfected cells is significantly lower ($0.06 \pm 4x10^{-6}$) than in non-transfected cells (1.1±0.001). Therefore, it can be confirmed that the effectiveness of PKC silencing is around 45,5% and 94.2% at 36 or 48 hours respectively. Having demonstrated that PKC expression is significantly inhibited in HMC-1^{560,816} cells, caspase-3 activity was determined in PKC silenced cells treated with 1 µM CCT, 50nM Gö6976 and 500nM GF109203X (Fig. **8**). It can be observed in figure 8 that CCT treatment implies a decrease (not significant) in caspase-3 activity, since fluorescence levels decreases from 100±18.92 to 67.72±23.38. Also, neither Gö6976 nor GF109203X treatments alone or in combination with CCT modify caspase-3 activity. Any effect on caspase-3 activity was observed when HMC-1^{560,816} cells were incubated with control siRNA.

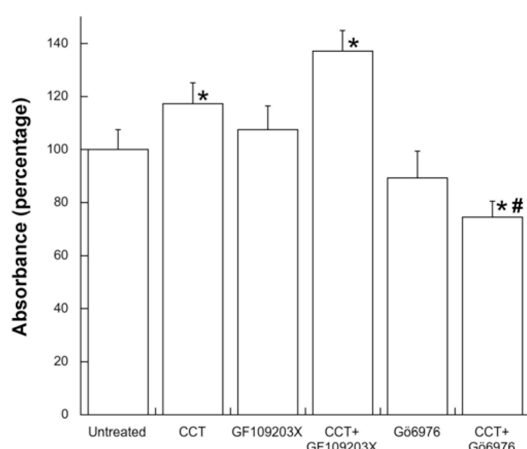


Fig. (6). Effect of Aurora kinase inhibition and PKC modulation on caspase-8 activity in HMC-1^{560,816} cells. Caspase-8 activation of HMC-1^{560,816} cells was measured with Caspase-8/FLICE colorimetric protease assay after 48 hours of incubation at 37°C in culture medium with 1 µM CCT, 500 nM GF109203X and 50 nM Gö6976. Mean ± SEM of three experiments. (*) Significant differences between control and drug treated cells. (#) Significant differences between CCT alone or plus Gö6976.

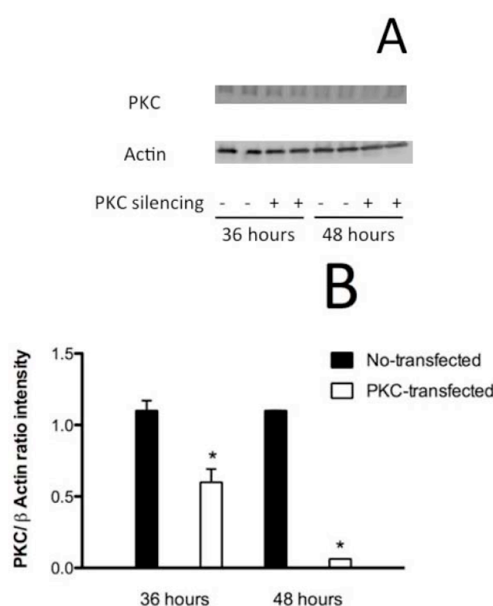


Fig. (7). Determination of PKC expression in non-transfected and PKC-silenced HMC-1^{560,816} cells. A) Cells were lysed at 36 and 48 hours post-CCT treatment and PKC expression was determined by Western Blot. β actin levels were used in order to obtain relative protein expression. **B)** Mean ± SEM of cytosolic PKC expression obtained in the three experiments. (*) Significant differences between non-transfected and transfected cells.

DISCUSSION

CCT is one of the novel small-molecule Aurora kinase inhibitors [33]. Its success *in vitro* and *in vivo* on different human tumor cell lines and a cancer mouse model encourages its potential use in the treatment of malignant diseases such as aggressive forms of mastocytosis. HMC-1^{560,816} cells were isolated from a patient with mast cell leukemia. Aurora kinase activity might be altered in different tumor cell lines, and the results obtained in this paper demonstrate that CCT increases caspase-3 and caspase-8 activity in HMC-1^{560,816} cells. Furthermore, the effect elicited by this Aurora kinase inhibitor is cytotoxic in this cell line. Therefore, the higher CCT concentration used, 5 µM, induces the death in half the population. In addition, Aurora kinase inhibition increases the sub G_0/G_1 population, an indicator of apoptosis, as well as caspase-3 activation. Aurora kinase inhibition causes the inactivation of mitotic checkpoint proteins and cells thereby accumulate abnormal mitotic spindles and can exit mitosis without undergoing cytokinesis [31]. These events are shown in HMC-1^{560,816} cells as an increase of cells arresting in mitosis after CCT treatment. This might lead to DNA damage and nucleous disruption.

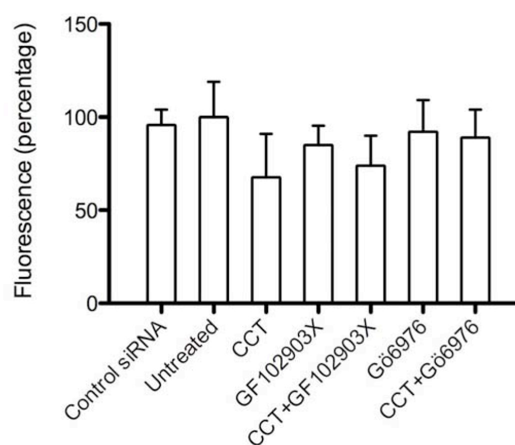
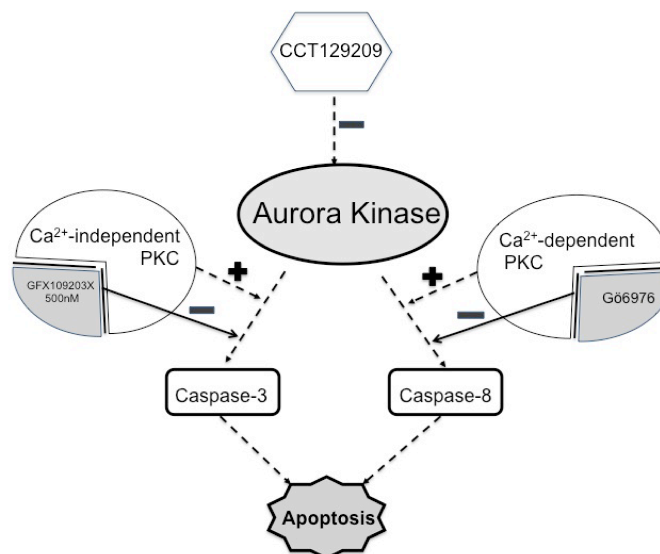


Fig. (8). Effect of Aurora kinase inhibition and PKC modulation on caspase-3 activity in PKC-silenced HMC-1^{560,816} cells. Caspase-3 activation of HMC-1^{560,816} cells was measured with EnzChek® Caspase-3 Assay Kit after 48 hours of incubation at 37°C in culture medium with 1 µM CCT, 500 nM GF109203X and 50 nM Gö6976. Mean ± SEM of three experiments.

Although the intrinsic and extrinsic apoptotic pathways are distinct, they are also interrelated, since caspase-8 cleaves the BH3-only domain Bcl-2 family member Bid, which translocates to the mitochondria and induces cytochrome c release. Results shown in this paper indicates that CCT-apoptotic effect seems to be triggered with caspase-8 activation and then the caspase-3 activating effect takes place, since caspase-3 is a substrate of caspase-8. For this, it can be stated that in HMC-1^{560,816} cells, the Aurora kinase inhibitor CCT acts first through the extrinsic pathway and then the intrinsic pathway (with the caspase-3 activation ef-



Scheme (1). Apoptotic pathways stimulated by Aurora kinase inhibition. Aurora kinase inhibitor CCT can induce apoptosis in HMC-$1^{560,816}$ cells through caspase-3 and caspase-8 activation. While Ca^{2+}-independent PKCs are necessary for caspase-3 dependent apoptotic cell death, Ca^{2+}-dependent PKCs are essential in the case of the caspase-8 apoptotic pathway. When Ca^{2+}-independent PKCs are inhibited (GF109203X 500nM) the CCT enhancer effect over caspase-3 activity is blocked. However, the apoptotic cell death takes place following the caspase-8 pathway. On the other hand, after Gö6976 treatment, Ca^{2+}-dependent PKCs are inhibited and caspase-8 activity are not increased. In this case, CCT is able to induce an increase in caspase-3 activity which leads to final apoptotic cell death.

fect) taking part in the CCT mechanistic effect. In HMC-$1^{560,816}$ cells, Ca^{2+}-independent PKCs are related with the CCT-effect on the caspase-3 pathway, since when this group of PKCs is inhibited (by GF109203X treatment) the CCT effect disappears. On the other hand, Ca^{2+}-dependent PKCs seem to be related with the Aurora kinase inhibition-effect on the caspase-8 pathway, since when they are inhibited (by Gö6976 treatment) the Aurora kinase inhibition-effect is blocked. Hence, Aurora kinase inhibitor modulates caspase-3 and caspase-8 pathways by independent routes (Scheme **1**).

PKC plays an important role in several intracellular pathways in mast cells, finally affecting degranulation [47-49]. The most important PKC isoform related with apoptosis, PKCδ, was analysed in this work in HMC-$1^{560,816}$ cells for the first time. It is well known that PKCδ can be involved in DNA-damage induced apoptosis [50, 51]. The isoform is cleaved by caspase-3 and the activated catalytic fragment translocates to the nucleus or the mitochondrion, where its target sites are located. PKCδ and PKCθ are substrates for caspase-3 and apoptotic morphology can be induced for the catalytic fragment of these PKCs [52, 53]. Results shown in this study demonstrate that in HMC-$1^{560,816}$ cells, PKCδ is not related with the apoptotic effect of CCT, since the Aurora kinase inhibitor does not modify cytosolic PKCδ levels, and thus it seems that this isoform is not activated. However, the activation of PKCδ with PMA treatment induces their translocation, since cytosolic levels of this isoform after activation are undetectable [54-56]. As for PKCδ isoform, PMA treatment also induces the translocation of Ca^{2+}-dependent isoforms when they are activated in HMC-$1^{560,816}$ cells [46]. It is shown that either cPKC or PKCδ translocations have not been affected by Aurora kinase inhibiton, since cytosolic expression of cPKC and PKCδ is not modified by CCT+PMA co-treatment. Also, results support that Ca^{2+}-independent isoforms inhibition (treatment with GF109203X) as well as Ca^{2+}-dependent isoforms inhibition (treatment with Gö6976) do not modify cytosolic expression of PKCδ or cPKC respectively. Moreover, no effect on PKCδ or cPKC cytosolic levels were observed when Aurora kinase and PKCs isoforms were inhibited, which indicates that in HMC-$1^{560,816}$ cells PKC translocation directly depends on its activation. Aurora kinase inhibition does not affect PKCδ or cPKC translocation. However, PKC is essential in apoptosis due to Aurora kinase inhibition.

Proteins such as Bin (tumor suppressor) or MCL-1 (member of the Bcl-2 family) were silenced in HMC-$1^{560,816}$ cells in previously studies [57-59]. However, the PKC protein in HMC-$1^{560,816}$ cells had never been silenced before. In this study, a successful method of PKC protein silencing in HMC-$1^{560,816}$ cells is described for first time. Transfection by a lipid-based method was employed in our experiments and the efficiency of PKC silencing was high. In this case, PKC has been knocked down and the percentage of protein expression decreased by 95%. Furthermore, all PKCs isoforms have been silenced in order to prevent any interrelation between subfamilies, since the apoptotic CCT effect takes place through different pathways (caspase-3 or 8) depending on the PKCs activated (Ca^{2+}-independent or Ca^{2+}-dependent

respectively). Our results demonstrate that PKC is an essential link in the CCT apoptotic effect through the caspase-3 pathway, as when this protein is silenced the enhancer CCT effect over caspase-3 disappears.

Finally, the Aurora kinase and PKC relationship that is described in this study is in accordance with the therapeutic role which both proteins have in cancer therapy. It was determined that in eukaryotes there are several networks of genes closely linked to cell death. Specifically, Aurora kinase and PKC take part in one of these networks as well as the widely known cancer therapeutic target epidermal growth factor receptor (EGFR) [60]. Therefore, in the tumoral cell line HMC-1^{560,816}, an apoptotic induced network composed by Aurora kinase and PKC might be present, bearing in mind that the apoptotic CCT effect through caspase-3 pro-enzyme is clearly PKC-dependent. In order to improve cancer therapies it is necessary to deepen the role of both proteins linked in apoptotic cell death induced by Aurora kinase inhibitors (such as CCT or PMA-739358). From these results, besides its application in solid cancers, Aurora inhibitors might be a promising tool in the treatment of aggressive forms of mastocytosis.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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AUTHORSHIP

A.T. performed all the experiments with assistance from A.F. and E.A. A.A. and L.M.B. assisted in data interpretation and in the design of the paper while A.T. wrote the paper.

ABBREVIATIONS

CCT = CCT129202

HMC-1 = Human Mast Cell Line

PKC = Protein kinase C

TyrK = Tyrosine kinase

PMA = Phorbol 12-myristate 13-acetate

MTT = 3-[4,5-dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide

FBS = Fetal bovine serum

BSA = Bovine serum albumin

Ca^{2+} = Calcium

PBS = Phosphate buffered saline

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5. Discusión

La PKC posee un papel fundamental en la exocitosis de mastocitos de tal forma que su activación es indispensable para que esta tenga lugar [33]. Los resultados obtenidos en la presente tesis doctoral ponen de manifiesto el papel fundamental de la PKC sobre la desgranulación en la línea celular HMC-1, al igual que se había descrito en mastocitos normales. En células HMC-1, la activación de la PKC estimula la liberación espontánea de histamina en las líneas celulares HMC-1^{560} y HMC-1^{560,816}. La activación de esta proteína también incrementa la exocitosis inducida por alcalinización intracelular en mastocitos normales y en ambas líneas celulares HMC-1 [194,282]. Por otro lado, la inhibición de la PKC bloquea la exocitosis en mastocitos nomales, mientras que en la línea celular HMC-1^{560} no se observa tal efecto [195,196]. La PKC no solo regula la liberación espontánea de histamina y la inducida por alcalinización, sino también la liberación por fármacos básicos como el compuesto 48/80, siendo este un efecto dependiente de Ca^{2+} [187], lo que indica que probablemente las isoformas de PKC responsables de este efecto sean las clásicas. En el presente trabajo se ha comprobado que la PKC posee un papel esencial en la liberación de histamina inducida por ionomicina en las líneas celulares HMC-1. Por un lado la PKC estimula la liberación de histamina inducida por ionomicina en células HMC-1^{560}, al igual que ocurre en mastocitos no tumorales [283]. Sin embargo, en células HMC-1^{560,816}, la activación de la PKC bloquea esta liberación de histamina. Por lo tanto se puede afirmar que la PKC actúa regulando negativamente la liberación de histamina inducida por ionomicina en esta línea celular, al contrario de lo que ocurre en la línea celular HMC-1^{560}. Además, cabe destacar que tanto en las células HMC-1^{560} como en HMC-1^{560,816} este efecto modulador de la PKC sobre la liberación de histamina está mediado fundamentalmente por las isoformas de PKC clásicas y nuevas, exceptuando la PKCδ, ya que el tratamiento con roterelina, inhibidor específico de esta isoforma, no modifica el efecto producido por la activación de la PKC. Además, la regulación negativa ejercida por la PKC en la línea celular HMC-1^{560,816} podría estar relacionada con la inhibición del vaciado de reservorios intracelulares de Ca^{2+} observada en estas condiciones. Se trata por tanto de un nuevo ejemplo de retrocontrol de la PKC sobre los niveles citosólicos de Ca^{2+} descrito con anterioridad [187,284]. Este efecto sobre el vaciado de reservorios de Ca^{2+} indica que la PKC no solo modula la liberación de histamina inducida por ionomicina sino que posee además la capacidad de regular los niveles citosólicos de Ca^{2+}. En células HMC-1^{560,816}, la activación de la PKC provoca un aumento de la entrada de Ca^{2+} desde el

medio extracelular inducido por ionomicina tanto a concentraciones bajas (0.1 µM) como altas (10 µM) del iónóforo. Sin embargo, en células HMC-1^{560}, este efecto solo se observa con la concentración más baja de ionomicina, lo que podría indicar que en esta línea celular se produce una saturación de Ca^{2+} citosólico, que evitaría la entrada de Ca^{2+} desde el exterior.

La localización de la PKC una vez activada difiere claramente entre las dos líneas celulares en un medio sin Ca^{2+}, puesto que su traslocación al núcleo se produce tan solo en células HMC-$1^{560,816}$ y no en HMC-1^{560}. Esta traslocación nuclear presente en HMC-$1^{560,816}$ podría estar relacionada con la modulación negativa sobre la liberación de histamina previamente descrita sobre esta línea celular, ya que ambos efectos tienen lugar en un medio sin Ca^{2+}. Por otro lado, cuando hay Ca^{2+} en el medio, la PKC migra desde citosol hacia la membrana plasmática en HMC-$1^{560,816}$, localización habitual de la proteína tras su activación [208,285]. Esta migración a la membrana plasmática está relacionada con la actividad del receptor c-kit, ya que la inhibición del mismo causa un bloqueo de la traslocación. Por el contrario en células HMC-1^{560} no se observa la traslocación de esta proteína en ninguna ocasión, incluso tras la estimulación con PMA durante 48 horas. Además, la ionomicina inhibe la expresión de la PKC en células HMC-$1^{560,816}$, mientras que no tiene ningún efecto en HMC-1^{560}. La ausencia de efecto de ambos compuestos, PMA e ionomicina, sobre la línea celular HMC-1^{560} indica que no ejercen ningún tipo de regulación sobre la expresión de esta proteína en estas condiciones.

Las técnicas de determinación de los niveles citosólicos de Ca^{2+} y de expresión proteica fueron puestas a punto con el compuesto YTX. Observándose un aumento del vaciamiento de los reservorios intracelulares de Ca^{2+} inducidos por el fármaco y una posterior entrada capacitativa de Ca^{2+} del medio extracelular. Esta entrada de Ca^{2+} puede ser regulada modulando el potencial de membrana mitocondrial y la expresión de proteínas quinasas citosólicas.

La activación de la PKC durante un largo periodo de incubación provoca una retro-regulación de la isoforma PKCδ en células HMC-$1^{560,816}$, sin embargo este efecto no se observa en HMC-1^{560}, demostrando una diferencia clara en la regulación de esta proteína entre ambas líneas celulares. Este efecto de retro-regulación de la PKC se ha descrito con anterioridad en la línea de basófilos de rata

RBL-2H3, sin aclarar qué isoforma/s presenta/n esta regulación [286]. La inhibición del c-kit causa el bloqueo de esta retro-regulación en el citosol y en el núcleo pero no en la membrana plasmática, lo que parece indicar la existencia de una conexión reguladora entre el c-kit y la PKC en el espacio intracelular, sin afectar a la membrana. Otro de los efectos de la activación prolongada de la PKC es la muerte celular por apoptosis en las líneas celulares HMC-1^{560} y HMC-$1^{560,816}$. El efecto de los ésteres de forbol sobre la muerte celular es controvertido, ya que en algunas ocasiones se ha descrito un efecto citoprotector [287-291], mientras que en otras, como ocurre en la línea celular HMC-1 posee un claro efecto citotóxico [292,293]. Además, cabe destacar que la apoptosis inducida por una exposición prolongada al PMA es dependiente de la isoforma PKCδ. La relación de esta isoforma con el proceso de apoptosis celular ha sido ampliamente descrita [203,209,292-294], mientras que otros estudios destacan el papel de esta isoforma como estimuladora del crecimiento celular [295,296], lo que indica que la actividad de la PKCδ varía según el tipo celular. Los resultados obtenidos en la presente tesis doctoral demuestran que la relación de esta isoforma con la apoptosis celular provocada por su activación prolongada es clara ya que la silenciación de la PKCδ provoca la desaparición de este efecto apoptótico en la línea celular HMC-1.

Los inhibidores de tirosina quinasas se han convertido en el principal grupo de fármacos en el tratamiento de la mastocitosis. En este estudio se ha comprobado que la PKC, además de modular el proceso de activación de las líneas celulares HMC-1^{560} y HMC-$1^{560,816}$, juega un papel fundamental en el mecanismo de acción de este tipo de fármacos. En concreto, se ha observado que la isoforma PKCδ, al igual que ocurría en la apoptosis mediada por una exposición prolongada al PMA, tiene un papel importante en la muerte apoptótica inducida por el inhibidor de tirosina quinasas STI571. De esta forma la presencia de la PKCδ en la célula y su traslocación al núcleo es requisito imprescindible para que el efecto apoptótico del STI571 tenga lugar en células HMC-1^{560}. Esta traslocación al núcleo de la PKCδ, relacionada con el efecto pro-apoptótico de esta isoforma, se ha descrito anteriormente [203,204,206,209]. Además, nuestros resultados demuestran que el traslado de la isoforma desde el citosol al núcleo tiene lugar a través de citoesqueleto de actina, ya que la presencia de un citoesqueleto de actina desintegrado bloquea esta traslocación. Cabe destacar que en células de adenocarcinoma de colon el tratamiento con STI571 causa una inestabilidad en los filamentos de actina [297]. Este efecto no ha sido estudiado en la línea celular

HMC-1, sin embargo en el caso de que ocurra algo semejante esta alteración no es lo suficientemente grave como para evitar la traslocación de la PKCδ. Además, el efecto apoptótico del STI571 persiste aún cuando la PKC está activada en la línea celular HMC-1^{560}, lo que indica el efecto predominante de esta ruta. Por lo tanto, los resultados obtenidos en este trabajo nos permiten definir a la proteína PKCδ como imprescindible en el mecanismo de acción del fármaco STI571, en el cual se encuentran involucradas otras proteínas como la caspasa-3, las MAPKs y la PARP [298,299]. Después de comprobar que tanto el STI571 como la activación prolongada de la PKC provocan muerte celular por apoptosis en la línea celular HMC-1^{560} se estudió el efecto combinado de ambos. Los resultados obtenidos revelan un efecto no descrito hasta ahora ya que indican que la activación de la PKC potencia el efecto citotóxico del STI571 produciendo un aumento del porcentaje de células que mueren por necrosis y disminuyendo el número de células apoptóticas. El efecto potenciador observado tras la modulación de las tirosina quinasas y PKC es muy importante ya que no se han descrito efectos sumatorios entre los inhibidores de tirosina quinasa en la línea celular HMC-1^{560} [140].

El STI571, aunque no provoca la muerte en la línea celular HMC-1^{560,816} sí modifica los niveles citosólicos de la PKC además de bloquear, tal y como se ha descrito anteriormente, la traslocación de las isoformas clásicas a la membrana tras su activación. En concreto, la inhibición de c-kit está relacionada con los niveles de PKC clásicas. Esta relación entre las isoformas de PKC dependientes de Ca^{2+} y el STI571 ha sido descrita en un estudio anterior realizado en células T98G [129]. Además de afectar a los niveles citosólicos de PKC se ha comprobado que el STI571 actúa inhibiendo la liberación espontánea de histamina en células HMC-1^{560,816} [282], por lo que ambos efectos podrían estar relacionados. En la línea celular HMC-1^{560} el hecho de que el STI571 solo tenga efecto sobre las isoformas clásicas de la PKC cuando el citoesqueleto de actina presenta alteraciones implica que estas isoformas son menos sensibles al STI571 que en células HMC-1^{560,816}, sin embargo, en lo que se refiere a efecto citotóxico, la respuesta observada es opuesta, ya que la línea celular HMC-1^{560} es mucho más sensible al fármaco. Esto indica que el mecanismo de acción del STI571 en ambas líneas celulares es diferente, de ahí las diferencias obtenidas tanto en el efecto final como en la modulación ejercida sobre los niveles celulares de PKC.

Los inhibidores de tirosina quinasas se caracterizan por bloquear la fosforilación de la proteína al unirse al sitio de anclaje de ATP, anulando así la cascada de señales de transducción de las células tumorales [148]. En la presente tesis doctoral se ha estudiado el efecto de tres compuestos con actividad citotóxica frente a las líneas celulares HMC-1^{560} y HMC-1^{560,816}, el dasatinib, el nilotinib y la midostaurina. De todos ellos, el dasatinib ha resultado ser el más potente teniendo en cuenta los resultados obtenidos en las dos líneas celulares. El dasatinib provoca apoptosis en ambas líneas celulares. Sin embargo, a diferencia de lo observado con el STI571, la activación de la PKC potencia el efecto citotóxico del dasatinib tan solo en células HMC-1^{560,816} y no en HMC-1^{560}. A pesar de esta diferencia, el mecanismo de potenciación parece ser semejante en ambos casos, ya que tiene como resultado un aumento de la población necrótica que coincide además con la traslocación al núcleo de la isofoma PKCδ en ambos tratamientos, con STI571 y dasatinib. Además, se observa que el dasatinib estimula la liberación espontánea de histamina en ambas líneas celulares. Se trata de un efecto sorprendente ya que hasta el momento se había descrito que los inhibidores de tirosina quinasas, como es el caso del STI571, nilotinib y la midostaurina, no modifican y en algunos casos reducen la liberación espontánea de histamina [282,300,301]. El efecto del dasatinib sobre la liberación espontánea de histamina puede estar relacionado con su proteína diana Lyn, ya que en células HMC-1 esta se encuentra permanentemente activada [200]. Este efecto del dasatinib se ha observado también en basófilos [302,303], con mayor presencia en pacientes con edema y otras reacciones cutáneas [302]. La actividad del dasatinib puede justificar la aparición de reacciones cutáneas, consideradas uno de los principales efectos secundarios descrito para este grupo de fármacos [148,302].

A pesar de que el dasatinib provoca la muerte por apoptosis tanto en HMC-1^{560} como en HMC-1^{560,816}, el compuesto no posee exactamente el mismo mecanismo de acción en las dos líneas celulares. Al igual que el STI571, el dasatinib afecta a la expresión de la PKCδ. Por un lado la isoforma PKCδ se trasloca hacia el núcleo en respuesta al estímulo apoptótico en células HMC-1^{560,816}. Esta traslocación ha sido descrita con anterioridad produciéndose la muerte celular tras la interacción de la isoforma PKCδ con varios mediadores apoptóticos nucleares [203,210,294]. En el caso del dasatinib, y también del STI571, la traslocación nuclear de la PKCδ es imprescindible para que el efecto apoptótico tenga lugar. Es por tanto esencial tener en cuenta que la inhibición de esta

isoforma, bien provocada por fármacos inhibidores de PKC empleados en el tratamiento de tumores o por una causa indirecta, va a reducir notablemente el éxito del tratamiento de las mastocitosis, por lo que es necesario considerar esta posible interacción. De este modo, al conocer mejor las rutas intracelulares moduladas por estos fármacos se pueden evitar combinaciones inadecuadas con otros compuestos y lograr una mayor eficacia en el tratamiento de esta enfermedad. La regulación que posee el dasatinib sobre la PKCδ en la línea celular HMC-1^{560} es completamente distinta a la presente en HMC-1^{560,816}. En células HMC-1^{560} los niveles de PKCδ disminuyen en el citosol y en núcleo pero no en la membrana plasmática, lo que parece indicar que se está produciendo una retro-regulación de la misma [159,170]. En estudios anteriores se ha descrito que la asociación de la PKC a la membrana es un requisito fundamental para que tenga lugar la retro-regulación, de ahí que se considere esta posibilidad. Este modelo de regulación ejercido por el dasatinib difiere del provocado por la activación prolongada de la PKC descrito anteriormente en células HMC-1^{560,816}, ya que en este caso también se observa un descenso de los niveles de PKCδ en la membrana plasmática. Esto pone de manifiesto que la retro-regulación de la PKCδ tiene lugar de forma distinta dependiendo de la línea celular y del estímulo. El dasatinib no solo afecta de manera diferente a la PKCδ en ambas líneas celulares, sino también a las isoformas clásicas de la PKC. Por un lado, en células HMC-1^{560}, el fármaco provoca una potente inhibición de los niveles de estas isoformas, mientras que en células HMC-1^{560,816} el dasatinib estimula la traslocación de las mismas hacia la membrana plasmática. Por lo tanto, puede afirmarse que el dasatinib tiene un potente efecto inhibitorio sobre las isoformas clásicas en células HMC-1^{560}, mientras que se considera activador en la línea celular HMC-1^{560,816}, ya que la traslocación a la membrana se describe como un paso previo a la activación de la PKC [161,162,304,305].

El estudio de la inhibición de la aurora quinasa realizado en esta memoria de tesis es muy importante ya que pone de manifiesto por primera vez la estrecha relación entre la PKC y la aurora quinasa. El inhibidor de la aurora quinasa CCT129202 tiene la capacidad de provocar apoptosis en la línea celular HMC-1^{560,816} mediante la activación de dos de las proteínas relacionadas con la cascada apoptótica, la caspasa 8 y la 3. La interacción de este fármaco con la PKC se observa después de que desaparezca el efecto apoptótico del CCT129202 al silenciar la PKC, lo que indica que el mecanismo de acción de este compuesto se

produce a través de esta proteína. Además, tanto las isoformas clásicas como las nuevas tienen la capacidad de regular la actividad del compuesto CCT129202. De esta manera las isoformas clásicas son imprescindibles para que el efecto apoptótico del CCT129202 tenga lugar a través de la activación de la caspasa-8, ya que cuando estas isoformas están inhibidas el fármaco no posee la capacidad de activar esta proteína apoptótica. Por otro lado, el efecto estimulador del CCT129202 sobre la caspasa-3 no está regulado por las isofomas de PKC clásicas, sino por las nuevas. De tal forma que la inhibición de estas isoformas bloquea el efecto del CCT129202 sobre la caspasa-3. Sin embargo, aunque la ruta de la caspasa 8 ó 3 esté bloqueada por la inhibición de las PKC clásicas o nuevas respectivamente, el CCT129202 causa igualmente mortalidad celular, al ser capaz de redirigir su efecto a través de la ruta no inhibida. Por lo tanto, al estar inhibida la caspasa-8, la apoptosis puede tener lugar a través de la vía intrínseca de la misma, en la cual se produce la liberación del citocromo c mitocondrial encargado de activar el APAF-1 y este a su vez estimular a la caspasa-3. Mientras que si está inhibida la ruta de la caspasa-3, el proceso apoptótico puede tener lugar a través de las caspasas 6 ó 7, sustratos de la caspasa-8 al igual que la 3. Los resultados obtenidos en este estudio nos han permitido clarificar el mecanismo de acción del fármaco CCT129202 y la función esencial que posee la PKC en el mismo. Además, pone de manifiesto la necesidad de controlar la modulación de la PKC durante el tratamiento con CCT129202, con el fin de evitar la anulación del efecto antitumoral que posee este compuesto. Por lo tanto, la inhibición de la ruta de la aurora quinasa, además de su eficacia ya contrastada en el tratamiento de tumores sólidos puede ser una opción terapéutica en el tratamiento de la mastocitosis. En resumen, el presente estudio aclara el papel de varias isoformas de la PKC y de la aurora quinasa en la activación de las células HMC-1 y demuestra el papel de las tirosina quinasas y de la isoforma PKCδ en la muerte celular.

6. Conclusiones

1. La activación de la proteína quinasa C estimula la liberación de histamina en las células HMC-1.

2. La mutación en la posición 816 del c-kit hace que la proteína quinasa C adquiera el efecto inhibidor sobre el vaciado de reservorios intracelulares de Ca^{2+} inducido por ionomicina.

3. En las células HMC-1^{560,816} las isoformas clásicas de la proteína quinasa C se traslocan desde el citosol. En condiciones normales esta traslocación se produce a la membrana de forma dependiente de la activación del receptor c-kit. En ausencia de Ca^{2+} la traslocación se produce al núcleo.

4. La isoforma proteína quinasa C δ es la responsable de la apoptosis inducida por la activación prolongada de la proteína quinasa C y por la inhibición del c-kit en las líneas celulares HMC-1^{560} y HMC-1^{560,816}.

5. El inhibidor de c-kit dasatinib presenta un mayor efecto citotóxico que los inhibidores nilotinib y midostaurina en células HMC-1.

6. El dasatinib aumenta la liberación de espontánea de histamina en las dos líneas celulares HMC-1.

7. La modulación simultánea de la proteína quinasa C y de las tirosina quinasas provoca un aumento de la muerte celular por necrosis en las células HMC-1^{560} y HMC-1^{560,816}.

8. El mecanismo de acción del inhibidor de la aurora quinasa CCT129202 es dependiente de las isoformas clásicas y nuevas de la PKC. El efecto de este compuesto sobre la caspasa 8 está mediado por las isoformas de PKC clásicas, mientras que sobre la caspasa 3 está mediado por las isoformas de PKC nuevas.

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