UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

PAPEL DE LAS INMUNOFILINAS EN LA HOMEOSTASIS DEL ION CALCIO EN PLAQUETAS HUMANAS

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The Philosopher Mercedes Gómez Blázquez

ÍNDICE

ABREVIATURAS

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- $[Ca^{2+}]_c$: Concentración de Ca^{2+} citosólico
- $[Ca^{2+}]_{RE}$: Concentración de Ca^{2+} del retículo
- 4E-BP1: Proteína de unión a eIF-4E
- ACD: Anticoagulante citrato dextrosa
- ADN: Ácido desoxirribonucleico
- ADP: Adenosina difosfato
- AMP: Adenosina monofosfato
- AMPK: Cinasa activadora de AMP
- ATP: Adenosina trifosfato
- ATPasa: Enzima que hidroliza el ATP
- BSA: Albúmina de suero bovino
- Ca^{2+}: Calcio
- CaM: Calmodulina
- CaMK: Cinasa de la calmodulina
- CN, CaN: Calcineurina
- CaV: Caveolina
- CICR: Liberación de Ca^{2+} inducida por Ca^{2+}
- CIRB: Dominio de unión de los TRPC a IP_3R y a calmodulina
- CLD: Dominio de unión a ciclofilinas
- COX-I: Ciclooxigenasa I
- CPA: Ácido ciclopiazónico
- CRAC: Corrientes activadas por la liberación de Ca^{2+}
- CsA: Ciclosporina A
- CyP: Ciclofilina
- DAG: D-1,2-diacilglicerol
- DTT: Ditiotreitol
- ECC: Entrada Capacitativa de Ca^{2+}
- EDTA: Ácido etilendiaminotetraacético
- EF-hand: Dominio de unión a Ca^{2+}
- EGFR: Receptor del factor de crecimiento epidermal
- EGTA: Ácido etilenglicol tetraacético
- ERK: Cinasa reguladora de señal extracelular
- ERM: Dominio ezrina-radexina-moesina
- FKBD: Dominio de unión del FK506
- FKBP: Proteína diana del FK506
- FSC: Detector de dispersión frontal
- GAPDH: Enzima gliceraldehído-3- fosfato deshidorgenasa
- GTPase: Enzima guanosina trifosfato
- H^+: Protones
- H^+-ATPasa: Bomba de H^+ vacuolar
- HBS: Tampón hepes salino
- HEK293: Células embrionarias de riñón humano
- HRP: Peroxidasa de rábano picante
- IgG: Inmunoglobulina G
- IL-2: Interleucina-2
- IP_3: Inositol 1,4,5-trifosfato
- IP_3: Inositol 1,3,4,5-tetrakisfosfato
- IP_3R: Receptor de inositol 1,4,5-trifosfato
- Kd: Constante de disociación
- kDa: Kilo Daltons
- KO: Cepa carente de una proteína
- LB: Solución Laemmli
- MEG-01: Línea celular megacarioblástica
- MFI: Intensidad media de fluorescencia.
- Mn^{2+}: Manganeso

- mTOR: Proteína diana de la rapamicina
- Na^+: Sodio
- NAADP: Nicotinato de adenina dinucleótido fosfato
- NADP: Nicotinadenín dinucleótido fosfato

NCCE: Entrada No Capacitativa de Ca^{2+}

- NCX: Intercambiador Na^+/Ca^{2+}
- NFAT: Factor nuclear de activación de los linfocitos-T
- NMDA: Ácido N-metil-D-aspártico
- OAG: 1-oleoil-2-acetil-sn-glicerol
- PAR: Receptor activado por las proteasas
- PBS: Tampón fosfato salino
- PDGF: Factor de crecimiento derivado de las plaquetas
- PIKK: Cinasas relacionadas con la fosfatidilinositol cinasa
- PIP_2: Fosfatidil inositol 4,5-bifosfato
- PIP_3: Fosfatidil inositol 3,4,5-trifosfato
- PKC: Proteína cinasa C
- PLC: Fosfolipasa C
- PMCA: Bomba ATPasa de Ca^{2+} de la membrana plasmática
- PP2b: Fosfatasa 2b
- PPIases: Peptidilprolil isomerasas
- PPP: Plasma pobre en plaquetas
- PRP: Plasma rico en plaquetas
- RE: Retículo endoplásmico
- RHEB: Homólogo de Ras enriquecida en cerebro
- ROC: Canales operados por receptores
- RyR: Receptor de la rianodina
- SAM: Dominio alfa estéril
- SCaMPER: Canal liberador de Ca^{2+} sensible a esfingolípidos
- Ser/Thr: Serina/treonina
- SERCA: Bomba ATPasa de Ca^{2+} del retículo sarco/endoplásmico
- SMOC: Canales operados por segundos mensajeros
- SOAR: Región de STIM1 activadora de Orai1
- SPCA: ATPasa del aparato de Golgi
- SSC: Detector de dipersión lateral
- TBHQ: 2,5-di(ter-butil)-1,4-hidroquinona
- TBS: Solución de lavado
- TBST: Solución de lavado con Tween20
- TG: Tapsigargina
- TGF-βR: Receptor del factor de crecimiento transformante β
- Thr: Trombina
- TPC: Canales de dos dominios de poro
- TPR: Dominio tetratricopeptídico repetido de las FKBPs
- TRP: Receptor de potencial transitorio
- TRPC: Canal receptor de potencial transitorio
- TSC2: Complejo esclerosis tuberosa2
- VOC: Canales operados por voltaje
- vWF: Factor de Von Willebrand
- WT: Cepa control

INTRODUCCIÓN

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Las células son capaces de utilizar los cambios en la concentración citoplasmática de Ca^{2+} para regular multitud de rutas de señalización, con el fin de desempeñar diferentes funciones. En determinados modelos celulares la cantidad de Ca^{2+} almacenada en el interior celular no es suficiente para desempeñar dichas funciones, por lo tanto, las células activan mecanismos de entrada de Ca^{2+} desde el exterior celular. Estos mecanismos de entrada de Ca^{2+} requieren la participación de multitud canales que se encuentran regulados por diferentes proteínas accesorias. Estas proteínas van a modular la apertura o cierre de estos canales controlando así la homeostasis del Ca^{2+} intracelular. Recientemente se ha descrito que las inmunofilinas podrían también participar en la regulación de dichos canales, lo cual constituye el eje principal de los estudios realizados en la presente Tesis Doctoral.

Las inmunofilinas son una familia de proteínas diana de los principales fármacos inmunosupresores como el FK506, la rapamicina o la ciclosporina. Estas proteínas se encuentran expresadas en una amplia variedad de tejidos y con funciones biológicas muy diversas. Durante esta Tesis Doctoral no hemos centrado en las FKBPs (*FK506-binding proteins*), que son inmunofilinas que tienen la capacidad de unirse al FK506 y a la rapamicina inhibiendo así su actividad. Se conoce que estas proteínas se encuentran regulando canales de Ca^{2+} como el receptor de ryanodina o el receptor de IP_3, participando por lo tanto, en la homeostasis del Ca^{2+}.

Las FKBPs pueden ejercer su acción sobre los canales de manera directa a través de su unión con un dominio del canal, o mediante interacción con proteínas efectoras encargadas de controlar el estado de fosforilación de los canales. Esta manera indirecta de actuar se produce a través de la fosfatasa calcineurina o la cinasa mTOR, que se inhiben cuando las FKBPs se unen a la rapamicina o al FK506, o mediante unión directa de las inmunofilinas “per se”, tal y como ocurre con la FKBP38 y la mTOR.

La importancia biológica de las FKBPs reside además por su papel en la fisiopatología de un gran número de enfermedades. La alteración en la regulación de la actividad de las inmunofilinas provoca una perturbación en la homeostasis del Ca^{2+} que podría estar en el trasfondo o complicar aún más diversas patologías como enfermedades neurodegenerativas, arterioesclerosis y fallo cardíaco. Existen otras enfermedades como la diabetes mellitus tipo 2 (DM2) en el que se ha observado una

elevada hiperagregabilidad plaquetaria debida a una alteración en la homeostasis del Ca^{2+}. De ahí que un apartado de la presente Tesis Doctoral consista en el estudio del papel de las inmunofilinas en plaquetas de pacientes afectados de DM2.

Adicionalmente, el hecho de que las inmunofilinas son la diana molecular de algunos tratamientos inmunosupresores o cancerígenos, en los que se usan los fármacos everolimus, tacrolimus o sirolimus, implicaría que artificialmente podríamos estar alterando otras funciones u órganos diferentes del que tenemos por objetivo, consiguiéndose pues un empeoramiento de los pacientes.

Los resultados obtenidos en la presente Tesis Doctoral referente a las vías de regulación en que las inmunofilinas participan, podría servir para el diseño de nuevos fármacos cuyas dianas terapéuticas fueran las inmunofilinas y con ello, aplicar dichos tratamientos para curar ciertas enfermedades que hasta ahora se estaban contrarrestando con fármacos de más amplio espectro. Sin embargo, siempre hay que tener en cuenta que la cantidad de procesos fisiológicos en los que participan las inmunofilinas a la hora de comenzar un tratamiento con inhibidores de las inmunofilinas, y evaluar pues, todos aquellos posibles efectos secundarios derivados de la aplicación de agentes que alteren su funcionalidad.

INTRODUCTION

Cells are able to use the changes in cytoplasmic Ca^{2+} concentrations to regulate a number of intracellular pathways, with the purpose of playing different functions. In some cell types the amount of Ca^{2+} stored into the cells is not enough to fully activate certain functions, hence cells activate Ca^{2+} entry mechanisms from the extracellular medium. These Ca^{2+} entry mechanisms require the recruitment of channels that are regulated by accessory proteins that modulate Ca^{2+} channel gating, hence controlling intracellular Ca^{2+} homeostasis. Recently, it has been described that immunophilins might also participate in the regulation of these channels, which will be aimed in the present Thesis.

Immunophilin is a family of proteins which are intracellular targets for immunosuppressant such as FK506, rapamycin and cyclosporine. These proteins are expressed in a wide variety of tissues, and they have many different functions. In this Thesis we have focused in FKBPs (*FK506-binding proteins*), which are immunophilins able to bind FK506 and rapamycin, which inhibit their activity. It is known that these proteins are involved in the regulation of Ca^{2+} channels such as ryanodine or IP_3 receptors, and therefore, participating in Ca^{2+} homeostasis.

FKBPs interact with channels through direct binding to specific domains in the channels or by interacting with effector proteins involved in the regulation of the phosphorylation state of the channels. The FKBPs regulatory role on protein phosphorylation controls the permeability of the channels through regulating either phosphatase calcineurin or kinase mTOR activities. However, mTOR is inhibited when FKBPs bind to rapamycin or FK506, but this protein also can be inhibited by direct binding of FKBP38.

The biological relevance of FKBPs is further highlighted by their involvement in the pathophysiology of certain diseases. The alteration in the activity of FKBPs promotes a perturbation in Ca^{2+} homeostasis, which might contribute to different pathologies such as neurodegenerative diseases, heart failure and atherosclerosis. There are other pathologies such as diabetes mellitus type 2 (DM2) in which the alteration in the Ca^{2+} homeostasis triggers an enhanced hyperaggregability. The latter led us to further investigate the role of immunophilins in platelets from DM2 patients during this Thesis.

Furthermore, treatment with carcinogenic drugs or immunosuppressant (everolimus, tacrolimus and sirolimus) whose therapeutic targets are the immunophilins could alter the physiology of different cell types, resulting in patient worsening.

The results presented in this Thesis regarding the intracellular pathways regulated by immunophilins, might be the base for the design of new drugs. These new drugs might have as therapeutic targets the immunophilins; hence the new therapies would treat current disorders while replacing other treatments with broader spectrum.

ANTECEDENTES BIBLIOGRÁFICOS

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1. Homeostasis del Ca^{2+}.

La universalidad del Ca^{2+} como mensajero intracelular depende de su enorme versatilidad. Las células tienen una gran cantidad de mecanismos de señalización de Ca^{2+}, que van a crear una amplia gama de señales espaciales y temporales. Esta versatilidad es aprovechada para el control de procesos tan diversos como la fertilización, la proliferación, el desarrollo, la contracción, la secreción y la apoptosis entre otras (Berridge et al., 2000).

La célula puede controlar la concentración intracelular de Ca^{2+} modulando los flujos de entrada y salida desde el medio extracelular o desde depósitos intracelulares, de esta manera, es capaz de generar señales intracelulares. La señal de Ca^{2+} es activada por estímulos que generan diferentes señales movilizadoras de Ca^{2+}, esto provoca en su interior una descompartimentalización o una entrada externa del mismo, provocando un aumento de la concentración de Ca^{2+} citosólico ($[Ca^{2+}]_c$). Este hecho inicia el proceso fisiológico que va a tener lugar, tras el cual la célula debe recuperar la $[Ca^{2+}]_c$ propia de su estado de reposo denominada concentración basal.

La concentración de Ca^{2+} en el medio extracelular (1,2 mM) y la del interior de los depósitos (1 mM) es muy superior a la $[Ca^{2+}]_c$ que se mantiene aproximadamente entre 20 y 100 nM cuando la célula se encuentra en reposo. Esto produce un fuerte gradiente electroquímico que las células tienen que regular con el fin de evitar los efectos citotóxicos de una elevación prolongada (Berridge, 1997).

Cuando se produce la estimulación celular, se activan mecanismos que permiten la liberación del Ca^{2+} almacenado en los depósitos intracelulares o la entrada de Ca^{2+} extracelular a través de canales presentes en la membrana, que están cerrados cuando las células se encuentran en reposo, alcanzándose una concentración en el citosol de 500 a 1000 nM. A estos mecanismos se les denomina Ca^{2+} “on”.

Por el contrario, los mecanismos de Ca^{2+} “off” son aquellos que se activan para permitir a la célula retornar a su $[Ca^{2+}]_c$ basal, y se basan en la reintroducción de Ca^{2+} hacia los depósitos intracelulares y en la expulsión al exterior celular, mediante el uso de las ATPasas.

1.1. Mecanismos de Ca^{2+} ON.

Estos mecanismos se dividen en dos grupos que pueden ser utilizados por la célula separadamente o de manera combinada.

1.1.1. Liberación de Ca^{2+} desde los depósitos intracelulares.

El principal almacén de Ca^{2+} intracitoplasmático es el retículo endoplásmico (RE), aunque hay también evidencias de liberación desde otros orgánulos como el aparato de Golgi (Missiaen et al., 2007), las vesículas secretoras, lisosomas, melanosomas, endosomas y mitocondria (Colegrove et al., 2000).

El Ca^{2+} acumulado en el RE sale al citosol a través de canales intracelulares específicos sensibles a diferentes moléculas y a través de un mecanismo de salida pasiva de Ca^{2+} (leak).

- Receptor de inositol 1,4,5-trifosfato (IP_3R):

El IP_3R es un canal catiónico situado en el RE además de en la membrana nuclear, en el aparato de Golgi y en las vesículas secretoras. Es un tetrámero y cada subunidad posee un dominio que atraviesa seis veces la membrana formando el poro. El extremo N-terminal contiene los sitios de unión al inositol 1,4,5-trifosfato (IP_3) y a la calmodulina (CaM); a continuación está la región de acoplamiento, la región transmembrana y la cola C-terminal (Mikoshiba et al., 1994). Se encuentra regulado por un amplio grupo de ligandos como el IP_3, el Ca^{2+}, el ATP, la CaM o mediante mecanismos de fosforilación/defosforilación (DeSouza et al., 2002). Existen compuestos que lo bloquean como la heparina que compite con el IP_3 (Vazquez-Martinez et al., 2003), o compuestos como la xestospongina C cuyo contacto directo bloquea el IP_3R (Gafni et al., 1997).

El IP_3 se genera por activación de receptores acoplados a proteínas G, receptores de tirosinas cinasas. Éstos activan a su vez a una PLC, que hidroliza específicamente el fosfatidilinositol-4,5-bisfosfato (PIP_2) liberando IP_3 y DAG (Berridge and Irvine, 1984). La unión del IP_3 al dominio amino terminal induce un cambio conformacional en la proteína, provocando la apertura del canal. El papel del Ca^{2+} en la regulación de la actividad del canal es doble, ya que a bajas concentraciones (100-300

nM) estimula su apertura, mientras que tras el estímulo del mismo con el IP_3 las altas $[Ca^{2+}]_c$ reducen su actividad hasta llegar a inhibirla por completo.

- Receptor de rianodina (RyR):

Se encuentra en el RE y se trata de un tetrámero con sitios de unión al Ca^{2+}, CaM y ATP en el extremo N-terminal. Los RyR se activan por la rianodina (a concentraciones inferiores a 10 μM), la cafeína, la ADP ribosa. La CaM actúa como modulador de este canal, de tal modo que, a $[Ca^{2+}]_c$ nanomolares actúa como agonista del receptor, mientras que a altas concentraciones actúa como inhibidor. El Ca^{2+} por sí sólo también induce la apertura del canal a concentraciones del orden de micromolar, participando en un mecanismo de señalización de $[Ca^{2+}]_c$ conocido como CICR (*calcium-induced calcium release*); por otro lado, altas $[Ca^{2+}]_c$ y bajas $[Ca^{2+}]$ en el interior del RE ($[Ca^{2+}]_{RE}$) inhiben su apertura (Wagenknecht and Radermacher, 1997).

Aún no han sido descritos en plaquetas humanas ni en megacariocitos pero sí en células de cultivo MEG-01 (Hosoi et al., 2001).

- Canal liberador de Ca^{2+} sensible a esfingolípidos (SCaMPER):

Este canal libera Ca^{2+} intracitoplasmático, respondiendo a lípidos derivados de la esfingosina. La esfingosina 1-fosfato actúa uniéndose a proteínas situadas en el RE que secuestran el Ca^{2+} en su estructura haciendo que lo liberen a través de este canal (Mao et al., 1996).

En plaquetas se han identificado dos tipos diferentes de receptores relacionados con estos mensajeros, uno es sensible al ácido lisofosfatídico y el otro lo es a la esfingosina 1-fosfato (Motohashi et al., 2000).

- Salida pasiva de Ca^{2+} o *leak*:

La salida pasiva de calcio del RE es un flujo pasivo que se pone de manifiesto como un descenso paulatino de la $[Ca^{2+}]_{RE}$. El *leak* de Ca^{2+} es compensado mediante el flujo de entrada de Ca^{2+} al lumen causado por un bombeo activo de la SERCA, y el balance entre ambos mecanismos es el responsable de la $[Ca^{2+}]_{RE}$ que se alcanza en el estado basal. El *leak* puede evidenciarse en presencia de inhibidores de SERCA (Camello et al., 2002).

La proteína responsable de esta salida pasiva de Ca^{2+} es el translocón, ya que se ha demostrado que moléculas polares pueden atravesar la membrana del RE a través de esta proteína (Simon and Blobel, 1991), y por lo tanto, puede ser permeable al Ca^{2+}.

- Salida de Ca^{2+} de otros orgánulos:

El NAADP (nicotinato de adenina dinucleótido fosfato), un derivado desaminado de la nicotinamida adenina dinucleótido fosfato (NADP) actúa como un mensajero capaz de liberar Ca^{2+} de depósitos internos (endosomas, lisosomas y vesículas secretoras). El NAADP induce la liberación de Ca^{2+} de forma independiente del IP_3 y el ADP y, a diferencia de ellos, su receptor no es activado por Ca^{2+}, siendo sensible únicamente a su agonista, una proteína de bajo peso molecular regulada por el NAADP (Lin-Moshier et al., 2012; Walseth et al., 2012). Unos de los canales cuya activación está mediada por este mensajero son los two pore channels (TPCs) localizados en los depósitos acídicos regulando la excitabilidad de la membrana plasmática mediante el intercambio de iones (Zhu et al., 2010).

En plaquetas humanas los principales reservorios de Ca^{2+} son el sistema tubular denso, que se comporta como el RE de otras células de mamífero, y los depósitos acídicos (lisosomas y orgánulos de secreción). Ambos compartimentos se diferencia por su naturaleza acídica y por su sensibilidad a tapsigargina y TBHQ (*2,5-di-(t-butyl)-1,4-hydroquinone*), un inhibidor selectivo de la isoforma SERCA3, lo cual nos indica la presencia de dos isoformas diferentes de SERCA en cada uno de ellos (Kovacs et al., 1997). Además se conoce que la acumulación de Ca^{2+} en estos depósitos acídicos está regulada por Ca^{2+} y por una bomba H^+-ATPasa (Lopez et al., 2005).

Otros orgánulos como mitocondrias y aparato de Golgi actúan como reservorios de Ca^{2+}. Se ha identificado la presencia de IP_3R en la membrana del aparato de Golgi, contribuyendo éste a la modulación de la homeostasis del Ca^{2+} (Pinton et al., 1998).

1.1.2. Entrada de Ca^{2+} desde el medio extracelular.

Al activarse ciertas funciones celulares las células requieren que se llegue a una $[Ca^{2+}]_c$ sostenida y prolongada, y en muchos casos no es suficiente con el Ca^{2+} acumulado en los depósitos para completar este requerimiento. Es por ello que la

entrada de Ca^{2+} desde el medio extracelular a través de canales de membrana es esencial. Según el tipo celular, existen distintos mecanismos para la entrada de Ca^{2+}.

- Entrada de Ca^{2+} activada por voltaje (VOC):

Se produce a través de canales que han sido descritos principalmente en células excitables como neuronas, células musculares, etc. La transmisión del potencial de acción provoca una despolarización de las membranas que va a inducir un cambio conformacional del canal adoptando un estado abierto transitorio, de modo que el Ca^{2+} puede entrar a favor de gradiente (McCleskey, 1994).

En plaquetas, tan solo se ha demostrado la presencia de un canal de K^+ dependiente de voltaje que permite la entrada de Ca^{2+} al citosol (Mahaut-Smith, 1995).

- Entrada de Ca^{2+} activada por segundos mensajeros (SMOC):

Fisiológicamente, algunos segundos mensajeros inducen la apertura de canales sensibles a moléculas producidas tras la activación de un receptor en la membrana, que permiten el flujo de cationes monovalentes o divalentes. Estos canales han sido principalmente descritos en células no excitables. Los segundos mensajeros que se han visto implicados en este proceso son nucleótidos cíclicos, diacilglicerol (DAG), IP_3, IP_4 e incluso el propio Ca^{2+}. En plaquetas se ha demostrado un modelo de entrada no capacitativa de Ca^{2+} (NCCE) estimulada por trombina, en el que este agonista, al generar la formación de DAG, activa la PKC, que a su vez activa los canales TRPC de la membrana (Rosado and Sage, 2000b) . Este mecanismo se desarrolla más detenidamente en el apartado 1.3.

- Entrada de Ca^{2+} activada por receptores (ROC):

Estos canales operados por receptores son muy ubicuos, y se sitúan principalmente en células secretoras y en las terminaciones nerviosas. Son activados por la unión de un agonista al dominio extracelular del receptor. Un ejemplo de ROCs son los receptores del ácido N-metil-D-aspártico (NMDA) que se abren en respuesta al glutamato, provocando la entrada de Ca^{2+} desde el medio extracelular. Han sido descritos en plaquetas, cuyo receptor $P2X_1$ se activa en presencia de ADP y ATP (Sargeant and Sage, 1994).

- Entrada de Ca^{2+} activada por el vaciamiento de los depósitos intracelulares (SOC):

Esta entrada se produce tras el vaciamiento de los depósitos intracelulares de Ca^{2+} y es uno de los principales mecanismos de entrada de Ca^{2+} en células no excitables, estando regulado por el estado de relleno de los almacenes intracelulares de Ca^{2+}. Este mecanismo se detallará más detenidamente en el apartado 1.3.

1.2. Mecanismos de Ca^{2+} OFF.

Las elevaciones sostenidas de $[Ca^{2+}]_c$ son incompatibles con la vida celular, por lo que existen mecanismos que revierten estas concentraciones hasta sus niveles basales, trabajando en contra de gradiente.

Las bombas e intercambiadores se ocupan del control de la homeostasis, generando gradientes de Ca^{2+}. De este modo, mantienen la $[Ca^{2+}]_c$ mediante la reintroducción de Ca^{2+} en los depósitos o mediante su extrusión al exterior celular.

1.2.1. Secuestro de Ca^{2+} en el interior de los orgánulos intracelulares:

- Bomba ATPasa de Ca^{2+} del retículo sarco/endoplásmico (SERCA):

La función principal de SERCA es la de introducir Ca^{2+} en contra de gradiente en los depósitos. En mamíferos existen tres genes que codifican para las diferentes isoformas.

Su estructura está formada por diez u once hélices de transmembrana (M1-M11), que genera tres dominios citoplasmáticos, un dominio A de unión al Ca^{2+}, un dominio P (fosforilación) y un domino N (nucleótido) donde se une el ATP. Para activarse es necesaria la transferencia del fosfato terminal del ATP a un residuo de aspartato de su domino catalítico, resultando en un cambio conformacional reversible de la hélices de los dominios transmembrana, de manera que el acceso del Ca^{2+} al lado citosólico se pierde y aparece accesible el lumen, permitiendo el movimiento de los iones a través de la membrana. La disociación de los iones de Ca^{2+} causará la hidrólisis del fósforo en el residuo de aspartato, de forma que se produce un nuevo cambio en la estructura proteica, en la que el dominio A volverá a situarse en el citoplasma (Toyoshima, 2008).

Por cada molécula de ATP hidrolizada, SERCA intercambia dos iones Ca^{2+} por dos H^+. Requiere Mg^{2+} en el lado citosólico, aunque éste puede tener propiedades inhibitorias a una concentración elevada, y la propia $[Ca^{2+}]_c$ modula su actividad.

Existen inhibidores selectivos de SERCA como la tapsigargina, que se une estequiométricamente a su estructura causando un bloqueo irreversible, impidiendo que continúe bombeando Ca^{2+} al interior, resultando por lo tanto muy útil para analizar el efecto que tiene en la célula el vaciamiento del RE sin interferir con ninguna otra ruta (Wictome et al., 1992). Otra droga inhibitoria es la di(ter-butil)-1,4-hidroquinona (TBHQ), con un mecanismo de actuación similar al de la tapsigargina, pero con menor potencia a la hora de inhibir las distintas isoformas de SERCA (Cavallini et al., 1995). Existen otras drogas inhibidoras de SERCA como el ácido ciclopiazónico (CPA) que posee un carácter inhibitorio reversible y la curcumina. Su actividad se regula por la $[Ca^{2+}]_c$, los niveles de ATP, ADP, fosfato inorgánico, pH y pequeñas proteínas como el fosfolamban, la sarcopilina o el complejo calnexina/calreticulina. Estos factores intervienen bien modificando su afinidad por el Ca^{2+} y/o alterando el grado de fosforilación (Vangheluwe et al., 2005). Recientemente se ha descrito que SERCA podría actuar como un modulador de la ECC, lo cual indicaría la existencia de microdominios donde se incluirían no sólo STIM, Orai y TRPCs sino también isoformas de SERCA que bombearían el Ca^{2+} que entra en la célula al interior del RE, limitando la progresión de la señal de Ca^{2+}, rellenando los depósitos y modulando la ECC (Redondo et al., 2008b).

En plaquetas se han identificado varias isoformas de SERCA, siendo SERCA2b de 100 kDa y SERCA3 de 97 kDa las más representativas (Kovacs et al., 1997). Estas isoformas tienen diferente sensibilidad a sus inhibidores, algo que resultó ser útil para la identificación de los dos principales depósitos de calcio en plaquetas. La tapsigargina inhibe SERCA2b a bajas concentraciones, mientras que se necesitan concentraciones muy elevadas para inhibir la otra isoforma. En cambio, la TBHQ sólo inhibe SERCA3 (Lopez et al., 2005).

- ATPasa del aparato de Golgi (SPCA):

El aparato de Golgi puede funcionar como un depósito de Ca^{2+} ya que posee en su membrana la bomba SERCA y una Ca^{2+}-ATPasa denominada SPCA. Esta última tiene la capacidad de transportar tanto Ca^{2+} como Mn^{2+} al interior del lumen, modulando así las concentraciones citosólicas y luminares de ambos iones en la célula. Esta proteína está formada por diez dominios transmembrana entre los que se encuentra el sitio de unión del Ca^{2+}. Se considera que este transportador tiene la misma afinidad por

ambos iones y además compiten por el mismo sitio de unión. Por cada hidrólisis de ATP se transporta sólo un Ca^{2+} o un Mn^{2+} (He and Hu, 2012).

- Mitocondria:

Se sabe que la mitocondria es capaz de almacenar Ca^{2+} en su interior. La velocidad y magnitud de esta acumulación dependen de la $[Ca^{2+}]_c$, de la fuente de Ca^{2+} y del mecanismo a través del cual se produce dicho aumento. Por lo tanto, este orgánulo actúa como modulador de la señalización citosólica del Ca^{2+} (Camello-Almaraz et al., 2002), presentando una coordinación espacio-temporal que le permiten responder a cambios en la $[Ca^{2+}]_c$ y establecer comunicación con regiones adyacentes del RE (Rutter and Rizzuto, 2000) y de la membrana plasmática (Gilabert and Parekh, 2000). Los mecanismos implicados en la dinámica del Ca^{2+} mitocondrial son: el uniportador de Ca^{2+} mitocondrial, que es un canal iónico selectivo al Ca^{2+} y dependiente del potencial de membrana generado por la cadena respiratoria (Rizzuto et al., 2000); el intercambiador Na^+/Ca^{2+} mitocondrial, que es el principal responsable de la salida de Ca^{2+} en contra de gradiente electroquímico; el intercambiador H^+/Ca^{2+} mitocondrial; y el poro de transición de permeabilidad mitocondrial, que se trata de canal no selectivo de alta conductancia dependiente de voltaje.

El papel de las mitocondrias en plaquetas no está definitivamente establecido, y parece que no tienen una función relevante en la ECC en plaquetas (Redondo et al., 2004), posiblemente debido a su escasa abundancia (unas 7 mitocondrias por plaqueta) (Beutler and Williams, 2001).

- Bomba ATPasa vacuolar de protones (H^+-ATPasa).

Se trata de bombas de protones situadas en los lisosomas y otros orgánulos acídicos de las células, encargadas de reintroducir H^+ al interior de lumen. Este mecanismo consigue mantener en el interior del orgánulo un pH muy bajo, entre 4-5. Gracias al mantenimiento de este gradiente de H^+ se produce la acumulación de Ca^{2+} en su interior (Christensen et al., 2002). El uso de bloqueantes, como la bafilomicina A1, provoca un aumento del pH en el interior de estos orgánulos, favoreciendo la salida de Ca^{2+} al citosol.

En plaquetas se diferencian dos principales depósitos de Ca^{2+} en función de la expresión de las isoformas de SERCA o de la presencia de la H^+-ATPasa, el sistema tubular denso y los orgánulos acídicos (Rosado, 2011).

1.2.2. Mecanismos de expulsión de Ca^{2+} al exterior celular:

- Bomba ATPasa de Ca^{2+} de la membrana plasmática (PMCA):

Tiene una estructura molecular formada por diez segmentos que atraviesan la membrana y cinco dominios extracelulares mientras que sus extremos amino y carboxilo están orientados al interior de la célula (Strehler and Zacharias, 2001). Han sido descritas hasta cuatro isoformas en humanos.

La actividad de esta bomba está regulada por segundos mensajeros que activan proteínas como la Ca^{2+}/Calmodulina, proteínas tirosinas cinasas, PIPs, proteínas serina/treonina cinasas como la PKA y PKC, y por proteasas como la calpaína (Brown and Dean, 2007; Strehler and Zacharias, 2001). Los incrementos en la $[Ca^{2+}]_c$ inducidas por los estímulos de agonistas, provoca la síntesis o activación de estos mensajeros secundarios, incrementando o inhibiendo la actividad de la PMCA (Rosado and Sage, 2000c).

En plaquetas juega un papel esencial en el mantenimiento de bajas $[Ca^{2+}]_c$. En condiciones de reposo se encuentra distribuida por toda la membrana plasmática, pero tras la activación de las plaquetas con trombina, migra hacia los pseudópodos que emite la célula, cerca del citoesqueleto de actina. Esta acción favorece la disminución de la $[Ca^{2+}]_c$ de esta zona favoreciendo su retracción (Dean and Whiteheart, 2004). Las isoformas identificadas en plaquetas son PMCA1b, 4 y 4b (Bobe et al., 2005).

- Intercambiador Na^+/Ca^{2+} (NCX):

El intercambiador Na^+/Ca^{2+}, que se encuentra en la membrana plasmática, utiliza la energía almacenada en forma de gradiente electroquímico del Na^+ para extraer al medio extracelular 1 ion de Ca^{2+} por cada 3 de Na^+ que entran en la célula (Philipson and Nicoll, 1992). Está constituido por un péptido inicial (M0), seguido de 5 regiones transmembrana (M1-M5), un bucle intracelular muy grande, donde se localizan tanto el sitio de unión a Ca^{2+} como el sitio de inactivación de Na^+, y 6 regiones transmembrana más (M6-M11) con el extremo citosólico. Se regula por proteínas cinasas de la familia de la PKA y PKC (Blaustein et al., 1999). El intercambiador Na^+/Ca^{2+} en ciertas circunstancias, puede a veces operar en dirección reversa causando la entrada de Ca^{2+}, como ocurre durante el potencial de acción cardíaco, donde un incremento transitorio en la concentración de Na^+ bajo la membrana plasmática revierte la dirección del flujo del intercambiador, dando lugar a una rápida entrada de Ca^{2+} (Lipp and Niggli, 1994).

1.2.3. Proteínas secuestradoras de Ca^{2+}:

Tanto en el interior del RE como en el citoplasma existen una gran cantidad de proteínas capaces de unir Ca^{2+}, actuando como sensores, efectores y proteínas tamponantes que inician, ejecutan o terminan determinadas funciones celulares dependientes de Ca^{2+}.

La mayoría de las moléculas secuestradoras de Ca^{2+} actúan como tampones de la concentración de Ca^{2+} intracelular, manteniendo su concentración muy baja en condiciones de reposo, de modo que mantienen un equilibrio entre el Ca^{2+} libre y unido a proteínas. Aproximadamente el 98-99 % de Ca^{2+} existente en el citoplasma está unido a proteínas (Augustine and Neher, 1992).

Entre las proteínas capaces de unir Ca^{2+} en el citoplasma celular destacamos: la calretinina, la calbindina, la parvalbumina y la calmodulina. Esta última, es una de las más importantes ya que su subunidad reguladora es capaz de unir dos moléculas de Ca^{2+}, liberándose en estas condiciones la subunidad catalítica activa. Esta subunidad catalítica activa llevará a cabo funciones como la de activar a la CaMK que a su vez puede fosforilar entre otras proteínas al IP_3R, a la PKC o incluso a los TRPC (Tang et al., 2001).

Las proteínas encargadas de unir Ca^{2+} dentro de los depósitos, permiten a la célula retener el Ca^{2+} en su interior para que exista la diferencia de concentración con el citoplasma que le permite mantener la integridad celular. Existen dos tipos de proteínas secuestradoras de Ca^{2+} dentro del RE: las que se encuentran insertas en la membrana del RE como la calnexina y STIM1, y las que se encuentran libres en el lumen como la calreticulina, calsecuestrina, PD1, Bip (GRP78), endoplasmina (GRP94), calstorina, reticulocalbina, etc (Villa et al., 1993).

1.3. Mecanismos SOC y SMOC de entrada de Ca^{2+} en plaquetas.

En plaquetas, los principales mecanismos de entrada de Ca^{2+} se producen a través de la generación de segundos mensajeros que activan canales presentes en la membrana, lo que conocemos como entrada no capacitativa de Ca^{2+} (NCCE), o bien a través del mecanismo que se activa por el vaciamiento de los depósitos intracelulares, también llamada entrada capacitativa de Ca^{2+} (ECC).

1.3.1. Proteínas que participan en SOC y SMOC.

Se trata de un mecanismo muy complejo, en el que se encuentran involucradas una gran variedad de proteínas que interactúan entre sí. Entre ellas destacamos:

- **STIM1** (Stromal Interaction Molecule 1): es una proteína localizada tanto en la membrana del RE como en la membrana plasmática. Se identificó como el sensor de Ca^{2+} del RE en 2005 (Liou et al., 2005; Roos et al., 2005). Atraviesa la membrana del RE a través de un único dominio transmembrana con su extremo N-terminal orientado hacia el lumen que contiene un dominio EF-*hand* y un dominio SAM (*sterile alpha-motiv*). El dominio EF-*hand* es el encargado de unir Ca^{2+}. La afinidad de este dominio por el Ca^{2+} es muy baja (0,5-1 mM) (Stathopulos et al., 2009), de forma que, con los depósitos llenos, la alta concentración de Ca^{2+} en el interior de los mismos favorece la unión, pero una vez que se produce el vaciamiento de estos depósitos y la concentración de Ca^{2+} disminuye en el RE, queda libre el dominio EF-*hand*. Cuando esto ocurre, STIM1 sufre un cambio conformacional que le permitirá unirse y activar a los canales en la membrana plasmática que participan en la ECC (Liou et al., 2005). Además es capaz de inhibir canales de Ca^{2+} dependientes de voltaje como el $Ca_V1.2$. (Park et al., 2010). Los dominios SAM son habitualmente zonas de interacción proteína-proteína implicados en la formación de dímeros u oligómeros. Por otro lado, el extremo C-terminal se orienta hacia el lado citosólico y está compuesto por varias doble-alfa hélices (dominios *coiled-coil*) y un dominio ERM (ezrina-radexina-moesina) que estarían involucrados en la asociación de STIM al citoesqueleto y en la oligomerización. En esta zona existe una región que media la asociación de STIM1 con Orai conocida como SOAR (*STIM1-Orai1 activating region*) que tiene la capacidad de activar la ECC sin tener que vaciar los depósitos (Yuan et al., 2009). A continuación aparece una zona rica en residuos de serina y prolina y un extremo policatiónico rico en lisina (Soboloff et al., 2006).

- **Orai1**: se ha descrito como una proteína de membrana plasmática capaz de asociarse en tetrámeros durante la activación celular (Feske et al., 2006) para formar un canal permeable al Ca^{2+}. Cada monómero posee cuatro dominios transmembrana formados por cuatro hélices alfa que participan en la oligomeración, un extremo C-terminal responsable de la interacción con STIM1 a través de una doble hélice alfa, y un extremo N-terminal encargado de la apertura del canal, ambos extremos hacia el lado citosólico (Muik et al., 2008). La selectividad del poro para los diferentes cationes viene dada por residuos acídicos en los dominios transmembrana M1 y M3 y en el primer bucle (Prakriya et al., 2006).Se ha propuesto que esta proteína forma parte del poro del

canal que media la corriente I_{CRAC}, una corriente no activada por cambios en el voltaje y altamente selectiva para Ca^{2+} (Parekh and Putney, 2005). Orai1 forma complejos multiméricos en la membrana celular. La estructura multimérica del canal responsable de I_{CRAC} consistiría en un tetrámero, en el que los residuos cargados de las cuatro subunidades individuales de Orai1, esenciales para la selectividad al Ca^{2+}, se encuentran organizados para formar la estructura tetramérica del poro del canal (Mignen et al., 2008). El canal formado por Orai1 está regulado por el vaciamiento de los depósitos intracelulares de Ca^{2+} con la participación del sensor intraluminal de Ca^{2+}, STIM1. Aunque una fracción de Orai1 se encuentra localizada constitutivamente en la membrana celular, se ha demostrado que el vaciamiento de los depósitos intracelulares de Ca^{2+} incrementa el reclutamiento de Orai1 en la membrana plasmática (Woodard et al., 2008), mediante un modelo que implicaría la reorganización de los microtúbulos y la localización de Orai1 en regiones puntuales de la membrana conocidas como “cluster” (Smyth et al., 2009; Xu et al., 2006).

- **TRPCs** (Transient receptor potencial channels): Los TRP son canales descubiertos en las moscas *Drosophila melanogaster* donde una mutación daba lugar a que la luz provocara un potencial receptor transitorio, en lugar de sostenido, debido a la entrada de Na^{+} y Ca^{2+} a través de los canales existentes en la membrana (Cosens and Manning, 1969). Hoy día se conoce la existencia de canales homólogos en células de mamíferos y se agrupan en distintas subfamilias de TRP: TRPC, TRPA, TRPV, TRPM, TRPML, TRPP; y cada subfamilia está constituida por distintas isoformas. Los principales canales TRPC descritos en plaquetas son: TRPC1, TRPC3 y TRPC6.

El **TRPC1** fue el primer homólogo mamífero del TRP que se identificó en humanos (Wes et al., 1995; Zhu et al., 1995). La estructura del TRPC1 es muy parecida a la del resto de la familia de los TRPC. En su extremo N-terminal citosólico posee 4 dominios repetidos de ankirina, necesarios para formar la unidad heteromérica con otros TRPCs, y un dominio *coiled-coil*, necesario para la unión entre TRPC1. Asimismo, tiene un sitio de unión para caveolina1 (Cav1), que es una proteína adaptadora y reguladora que actúa como nexo entre las interacciones de las proteínas en la membrana plasmática y los lípidos (Cohen et al., 2004). El TRPC1 atraviesa la membrana 6 veces (6 dominios transmembrana), con el poro para permitir el paso de cationes entre el 5º y el 6º dominio. En su extremo citosólico también, el TRPC1 tiene la secuencia característica de los TRP (EWKFAR), además de un dominio rico en prolina altamente conservado y otro sitio de unión para Homer1, una proteína de soporte que facilitará la interacción entre diversas proteínas (Jardin et al., 2012). Por último, posee también un

dominio de unión a CaM e IP_3R llamado CIRB, el cual modulará la activación del TRPC1 en función de la $[Ca^{2+}]_c$ (Tang et al., 2001) y dos dominios *coiled-coil*, donde se encuentra insertado un segundo dominio de unión a CaM, involucrado en la inactivación del TRPC1 dependiente de Ca^{2+} (Singh et al., 2002). El TRPC1 presenta selectividad Na^+/Ca^{2+} 1:1. Este TRPC1 forma también diferentes canales con distinta selectividad para el Ca^{2+}, desde algo selectivo hasta nada selectivo (para ello se mide la permeabilidad del Ca^{2+} frente a la del Na^+). Esta variación en la selectividad del canal se debe a que los monómeros de TRPC1 forman heterotetrámeros, por lo general dos TRPC1 y dos TRPCs, variando la selectividad en función del heterotetrámero formado (Liu et al., 2005). Hasta ahora, se ha comprobado que el TRPC1 constituye canales con TRPC4/TRPC5 (Plant and Schaefer, 2005) o con TRPC3/TRPC7 (Zagranichnaya et al., 2005). Sin embargo, no existen pruebas concluyentes de que forme un canal homotetramérico.

El **TRPC3** está situado en la membrana plasmática y participa fundamentalmente en la entrada no capacitativa de Ca^{2+} (NCCE). Estructuralmente es muy parecido al TRPC1, pero tiene algunas particularidades. Ambos extremos se sitúan en el citoplasma y en su extremo N-terminal además tiene un sitio de unión a la PLCγ en el primer dominio de ankirina y una unión para VAMP2 entre los dominios de ankirina 3 y 4. No posee ningún dominio donde se inserta Homer1, y el dominio CIRB termina en solo un dominio *coiled-coil* (Eder et al., 2007). El TRPC3 presenta selectividad Na^+/Ca^{2+} 1:1,5. Al igual que otros TRPCs, forma heterotetrámeros, e interviene en la NCCE activada por agonistas, cuyos receptores están acoplados a proteínas G, que ponen en marcha la maquinaria de hidrólisis de PIP_2 generando DAG, que activará el canal. Se ha demostrado que en algunos tipos celulares el TRPC3 puede activarse por el vaciamiento de los depósitos (Trebak et al., 2003).

TRPC6 también presenta los dos extremos en el citoplasma. A diferencia de TRPC1 y TRPC3 no posee sitios de unión para la Cav1 y una selectividad Na^+/Ca^{2+} 1:5. Este canal está regulado por muchas señales, incluyendo el DAG, la fosforilación en residuos de serina o tirosina, el PIP_2 e incluso el vaciamiento de los almacenes de Ca^{2+} intracelulares (Jardin et al., 2008b).

1.3.2. Actualización del mecanismo de activación de la entrada de Ca^{2+}.

El mecanismo comienza con el agonista que se une a su receptor de membrana acoplado a proteínas G, produciéndose la activación de la PLC, que hidroliza PIP_2 y lo transforma en IP_3 y DAG. El IP_3, por su parte, se une a su receptor en el RE, lo cual hace que éste sufra un cambio conformacional que permitirá la salida de Ca^{2+} al citoplasma, desencadenándose así la activación de varias rutas (Berridge, 1995). La liberación de Ca^{2+} en los depósitos provoca que el Ca^{2+} que se encuentra unido al dominio EF-hand de STIM1 se libere, por lo que STIM1 sufrirá un cambio conformacional que activará a los canales de Ca^{2+} de la membrana plasmática. En reposo, STIM1 tiene ocultos los dominios *coiled-coil* por los que se activa a Orai1 y el dominio rico en prolina que activa el TRPC1. El cambio conformacional sufrido por STIM1 le permite, en primer lugar, formar homodímeros u homotetrámeros con otras moléculas de STIM1. Estas uniones se formarán mediante el dominio SAM, situado en el extremo N-terminal de las STIMs (Muik et al., 2011). Una vez que se han vaciado los reservorios de Ca^{2+}, se ha visto que parte de STIM1 se transloca a la membrana (Zhang et al., 2005). Además, se produce una estrecha aproximación entre la membrana plasmática y la membrana del RE que se manifiesta en zonas de agregados de estas proteínas con alto contenido en colesterol conocidas como balsas lipídicas que facilitan el reclutamiento y el anclaje de STIM1 con otros canales. Se conoce que estas balsas lipídicas son necesarias para la activación de la ECC, pero no para el mantenimiento de ésta. Si se produce una disrupción de las balsas lipídicas antes del vaciamiento de los depósitos, la ECC se inhibe, pero una vez vaciados los depósitos, la ECC no se altera (Galan et al., 2010). Estas zonas, también sirven como soporte para la interacción entre Orai1 y STIM de la membrana, que en presencia de Ca^{2+} extracelular protege a la célula de una sobrecarga de Ca^{2+} (Dionisio et al., 2011). Además, existen proteínas como la Cav1 que actúan como proteínas de anclaje de otras proteínas como TRPC1, involucradas en la regulación de la ECC (Pani et al., 2008).

La unión entre STIM1 y Orai1 se produce mediante los dominios *coiled-coil* de los extremos C-terminal de ambas proteínas, y el dominio SOAR de STIM1 activa Orai1, permitiendo la entrada de Ca^{2+} desde el exterior (Yuan et al., 2009). Asimismo, STIM1 se une a la membrana por su dominio rico en lisinas en el extremo C-terminal, de forma que estabiliza las uniones con las proteínas de membrana y activa el canal TRPC1 (Yuan et al., 2007). En HEK293 se ha demostrado la importancia del citoesqueleto en la modulación de estas asociaciones de manera directa y a través de otras moléculas como la CaM (Galan et al., 2011).

Tanto TRPC1 como Orai1 van a ser activados por STIM1 tras la depleción de los depósitos, sin embargo, van a formar dos canales distintos con contribuciones funcionales diferentes. Por ejemplo en leucocitos se ha descrito que el canal TRPC1/STIM1 media una corriente de cationes no selectiva cuya principal función es la activación del factor nuclear de transcripción NFκB (*nuclear factor kappa-light-chain B cells*), mientras que el canal Orai1/STIM1 media corrientes I_{CRAC} que activan la ruta de activación del factor nuclear de activación de los linfocitos-T (NFAT) (Cheng et al., 2011a).

En el caso de las plaquetas humanas, que expresan endógenamente STIM1, Orai1 y TRPC1, la electrotransfección con el anticuerpo anti-STIM1, específico para el dominio de EF-*hand*, reduce tanto la interacción de STIM1 con TRPC1 como la ECC (Lopez et al., 2006) . En estas células se ha demostrado la interacción funcional entre STIM1, Orai1 y TRPC1 en la activación de la ECC. La inhibición de la interacción entre STIM1 y Orai1, y por tanto entre STIM1 y TRPC1, modifica el comportamiento del TRPC1 pasando de participar en la ECC a formar parte de un canal no capacitativo activado por DAG (Jardin et al., 2008a). De modo similar, se ha comprobado en plaquetas humanas que la participación de TRPC6 en la ECC o en la entrada de Ca^{2+} activada por receptor está regulada por su interacción con el complejo Orai1-STIM1 o con el hTRPC3, respectivamente (Jardin et al., 2009).

El vaciamiento de los depósitos por IP_3 y el consiguiente incremento en la $[Ca^{2+}]_c$ desencadenan la apertura de otros canales de Ca^{2+} de los depósitos, como los receptores de rianodina, mecanismo que se denomina liberación de Ca^{2+} inducida por Ca^{2+} (CICR) (Kiselyov et al., 2001).

Por otra parte, el DAG actuará como segundo mensajero, activando canales permeables a Ca^{2+}, entre los que destacan TRPC3 y TRPC6. Es un mecanismo de entrada de Ca^{2+} no capacitativo (NCCE) que contribuye al incremento de $[Ca^{2+}]_c$ necesario para la función celular.

En plaquetas se considera que la ECC es el mayor componente de entrada de Ca^{2+} cuyo principal papel es la amplificación de estímulos más débiles, mientras que el componente no capacitativo se encuentra favoreciendo la actividad procoagulante (Harper and Poole, 2011). Se conoce que el mecanismo de NCCE en plaquetas estimulada por OAG provoca la disociación del TRPC6 de Orai y STIM1 y promueve su interacción con TRPC3, de ahí que consideremos estos canales como no capacitativos (Jardin et al., 2009).

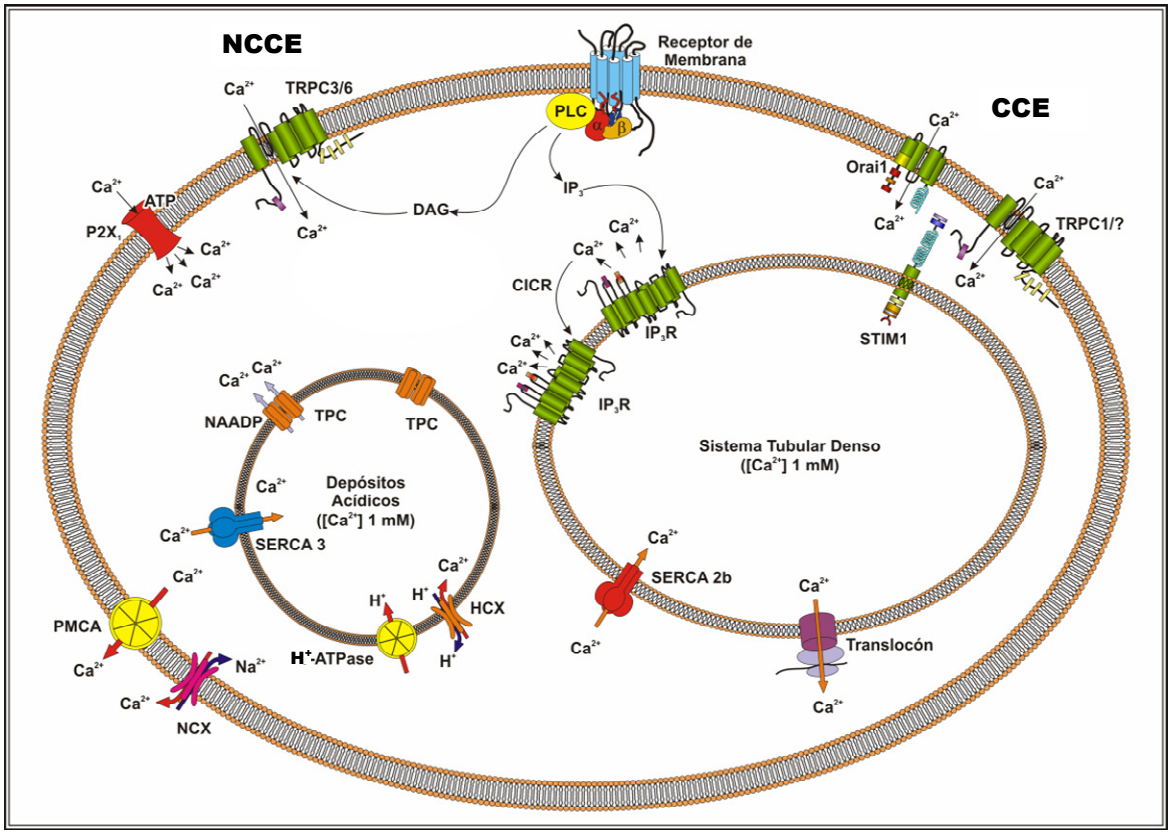

Ilustración 1: Homeostasis de Ca^{2+} en plaquetas humanas. (Autor: Isaac Jardín Polo)

2. Inmunofilinas.

Las inmunofilinas son un grupo de enzimas citosólicas que catalizan la reacción de isomerización *cis/trans* de las proteínas. Se denominan también peptidilprolil isomerasas (PPIases) o chaperonas, y su principal función es facilitar el correcto plegamiento de las proteínas. Se encuentran ampliamente distribuidas y abundantemente expresadas, lo que sugiere que jueguen un papel muy importante en la biología celular.

Las proteínas una vez plegadas, pueden adquirir dos conformaciones diferentes, en función de la orientación de su grupo funcional, la conformación *cis* o *trans*. La interconversión *cis/trans* se produce por la unión de la proteína sustrato al dominio catalítico de la PPIase, de tal manera que tiene lugar un giro tridimensional en el residuo prolina, estabilizándola a través de puentes de hidrógeno con el aminoácido localizado en el sitio de unión (Galat, 2003).

Las inmunofilinas se identificaron inicialmente como las dianas moleculares de las drogas inmunosupresoras ciclosporina A (CsA), FK506 y rapamicina. Estos compuestos son macrólidos producidos en hongos y aislados de unas sustancias que presentaban propiedades antibióticas, que después se descubrió ser unos potentes inhibidores de la activación de los linfocitos-T.

Según esto, se clasificaron en función al inhibidor al que se unían, por lo tanto, las FKBPs (*FK506-binding proteins*) son las principales dianas intracelulares para el FK506 y la rapamicina, mientras que la CsA se une a las ciclofilinas (CyP). La formación de los complejos FKBP/FK506 y CyP/CsA inhiben no solo la actividad PPIase de sus proteínas diana, sino también la actividad fosfatasa de la calcineurina (CaN) evitando de ese modo la fosforilación del factor nuclear de activación de los linfocitos-T (NFAT), y por consiguiente la transcripción de genes que codifican las citocinas, como la interleucina-2 (IL-2). Por otra parte, el complejo FKBP/rapamicina regula la proteína mTOR (*mammalian target of rapamycin*) y ejerce su acción inmunosupresora sin alterar la ruta de la CaN (Kang et al., 2008).

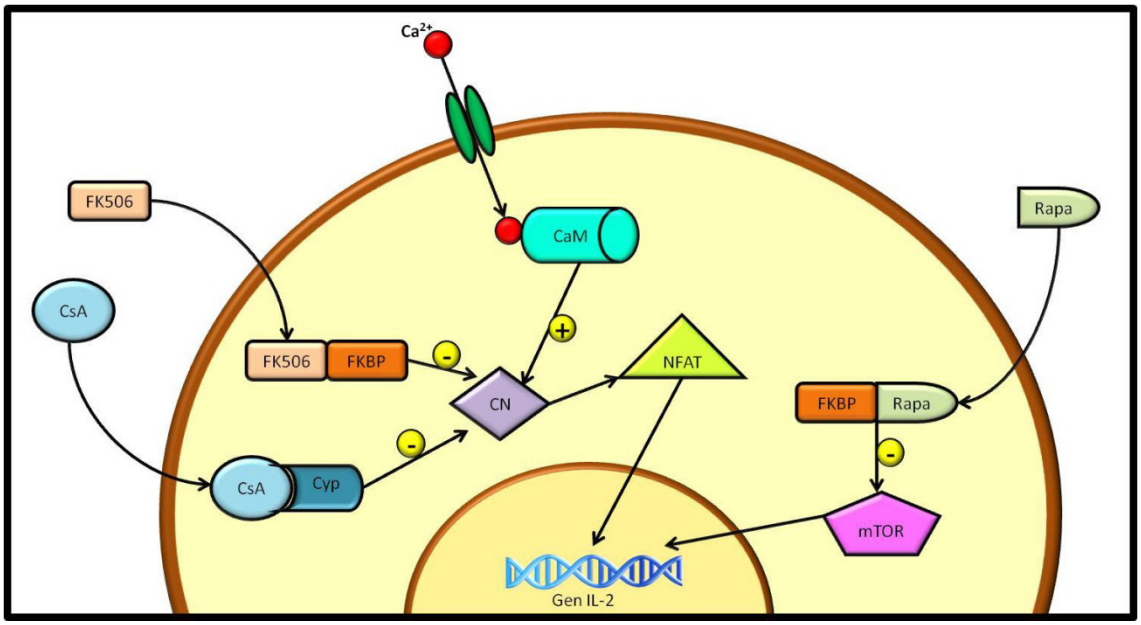

Ilustración 2: Rutas de señalización descritas en leucocitos tras la unión de las inmunofilinas a su inmunosupresor (Modificado Kang et al., 2008)

Las parvulinas son el tercer miembro de las inmunofilinas, cuyo inhibidor, la juglona (*5-hydroxy-1,4-naphtoquinone*), ha sido descubierto recientemente e inhibe su actividad PPIase. La única identificada en humanos en la Pin1 y parece tener una importante función en el proceso de mitosis (Gothel and Marahiel, 1999) y en la fosforilación de la proteína Tau que se encuentra involucrada en la enfermedad de Alzheimer (Zhou et al., 2000).

2.1. Ciclofilinas (CyP).

Las ciclofilinas han sido identificadas en mamíferos, plantas, insectos, hongos y bacterias. Poseen una estructura altamente conservada a lo largo de la evolución y todas ellas tienen actividad PPIase. En humanos son siete las principales ciclofilinas encontradas: CyPA (también llamada CyP18), CyPB (CyP22), CyPC, CyPD, CyPE, CyP40 y CyPNK (Wang and Heitman, 2005).

Todas las ciclofilinas tienen en común en su estructura un dominio de aproximadamente 109 aminoácidos que se conoce con el nombre de cyclophilin-like domain (CLD). En este dominio se encuentran una serie de residuos hidrofóbicos que conforman el sitio de unión para la CsA. A su alrededor se situarán los dominios propios de cada miembro de la familia, que estarán relacionados con su compartimentalización

subcelular y su especialización funcional. Según esto, se pueden encontrar en el citosol, en el RE, en la mitocondria o en el núcleo (Wang and Heitman, 2005).

La CyPA fue la primera ciclofilina descubierta. Se encuentra en todos los tejidos de mamíferos y su localización subcelular puede ser citosólica o nuclear (Arevalo-Rodriguez and Heitman, 2005). Estructuralmente es la ciclofilina más simple, ya que consta sólo del dominio CLD y posee 18 kDa. Entre sus principales funciones destaca su papel en la apoptosis, en la que presenta una cierta actividad nucleasa siendo capaz de degradar una amplia variedad de sustratos de ADN (Montague et al., 1997). También puede funcionar como mediadora de comunicaciones intercelulares en procesos inflamatorios ya que se ha demostrado que produce quimiotaxis de linfocitos (Xu et al., 1992). La CyPA además puede activar una ruta de señalización intracelular muy importante como es la de las cinasas ERK1/2. Posee muchas implicaciones funcionales involucradas en cáncer y en infección viral. Tiene un papel muy importante en la homeostasis de Ca^{2+} participando en la expresión del intercambiador Na^{+}/Ca^{2+} (Elbaz et al., 2010), en la entrada de Ca^{2+} modulando STIM1, Orai1 (Elvers et al., 2012) y SERCA2b (Rosado et al., 2010).

La CyPB también participa como mediadora de multitud de procesos celulares, pero debido a su localización en el RE y en el aparato de Golgi su principal función es la de llevar a cabo el plegamiento inicial o tardío de las proteínas que se dan en ambos compartimentos (Zhang and Herscovitz, 2003). La presencia de esta ciclofilinas en el plasma sanguíneo ha servido para demostrar su papel en la fisiología plaquetaria, aumenta su adhesión al colágeno y activando canales de Ca^{2+} presentes en la membrana (Allain et al., 1999).

La CyPD está considerada un componente esencial de la maquinaria de plegamiento de proteínas de la mitocondria y regula la apertura del poro de transición mitocondrial mediante la unión directa a las proteínas que lo constituyen, por lo que su inhibición va a disminuir la probabilidad de apertura del poro (Elrod and Molkentin, 2013). La ECC se ha visto relacionada con la enfermedad de Alzheimer mediante esta ciclofilina, de tal forma que su inhibidor, la CsA podría ser usada como estrategia terapéutica (Ma et al., 2012).

Otras ciclofilinas como la CyPE tienen un dominio de unión de ARN localizado en el núcleo, o la CyP40 también conocida como CyPNK (CyP Natural Killers) localizada en el citosol.

2.2. FKBPs.

Las FKBPs pertenecen también a la superfamilia de las peptidilprolil isomerasas aunque no poseen ninguna similitud en sus secuencias con las ciclofilinas. En comparación con las ciclofilinas, su actividad PPIase es moderada, menor o en ocasiones indetectable. La mayoría, tienen la capacidad de unirse al FK506 o a la rapamicina. Han sido identificadas más de 20 en multitud de organismos, desde levaduras hasta humanos. En humanos se conocen hasta diez de ellas. Los miembros de la familia se denominan según su peso molecular, de manera que la más pequeña es FKBP12 y la mayor FKBP135.

El FK506 y la rapamicina son ligandos de las FKBPs que se usan en clínica para inmunodeprimir a pacientes transplantados y evitar el rechazo del órgano transplantado. El FK506 fue aislado de *Streptomyces tsukubaensis* y es el que da el nombre a esta familia. A partir de este compuesto se han aislado otros como el ascomycin (FK520) también con grandes efectos inmunosupresores, o se han desarrollado análogos como el pimecrolimus, usado en enfermedades cutáneas. La rapamicina (también conocida como sirolimus) fue aislada de *Streptomyces hygroscopicus* y se encuentra inhibiendo mTOR a través del complejo formado con las FKBPs. Una de las mayores limitaciones de este compuesto es su pobre solubilidad, para mejorar esta propiedad se han desarrollado análogos con grupos hidrofílicos como: el temsirolimus, usado en gran variedad de tipos de cáncer; el everolimus, usado en pacientes transplantados de riñón; el deferolimus, usado en cáncer con metástasis en tejidos blandos; el zotarolimus, el biolimus y el myolimus, inhibiendo la proliferación celular en las estenosis coronarias. Existen otros derivados semisintéticos de la rapamicina como el WYE-592, ILS-920 y WAY-124,466 usados en enfermedades neurodegerativas (Gaali et al., 2011).

2.2.1. FKBP12.

La primera vez que se aisló esta proteína fue en 1989 de timo de ternero y en el bazo de humano (Harding et al., 1989) y la secuencia completa se completó en 1990 mediante secuenciación de cDNA de células Jurkat (Maki et al., 1990).

FKBP12 es el prototipo de la familia y se encuentra ampliamente caracterizado. Contiene únicamente el dominio conservado de las FKBPs, el FKBD (FK506-binding domain) que está formado por 108 aminoácidos. La estructura del resto de miembros de la familia consiste en un único dominio FKBD seguido por diferentes unidades

funcionales. La estructura tridimensional de este dominio está compuesta por cinco láminas-β unidas. Estas láminas envuelven a una pequeña hélice-α que se encuentra entre los residuos 57-65. Entre los residuos 39-45 se genera un saliente que podría ser el sitio de inserción o reconocimiento de diferentes secuencias (Braun et al., 1995). FK506 y la rapamicina tienen un dominio de unión común, por lo tanto ambas se unen a una profunda hendidura de carácter hidrofóbico que incluye los residuos Tyr26, Phe46, Phe99, Val55-Ile56 y TRP59. La rapamicina apenas sufre cambio conformacional cuando se une a la FKBP12, en cambio el FK506 que en solución se encuentra tanto en conformación *cis* como *trans*, tras unirse a la inmunofilina tiene que adoptar exclusivamente la conformación *trans* (Galat, 1993).

En humanos, el gen que codifica para FKBP12 tiene un tamaño de 24 Kb y cinco exones, cada uno de ellos relacionado con las características estructurales de las láminas-β y las hélice-α (DiLella and Craig, 1991).

En humanos, FKBP12 interacciona con FK506 con una K_D de 0,4 nM y con la rapamicina con una K_D de 0,2 nM (Gothel and Marahiel, 1999). La formación de complejos entre las FKBP y el ligando, conlleva un aumento en la estabilidad de la FKBP haciéndola más resistente a la proteólisis y creando una superficie de unión apropiada para CaN y mTOR respectivamente (Harrar et al., 2001). En cambio, en ausencia de FK506, FKBP12 se une a dianas intracelulares y modula proteínas como RyR, IP_3R, TGF-βR (*transforming growth factor-β receptor*), EGFR (*epidermal growth factor receptor*) (Mathea et al., 2011).

2.2.2. FKBP52.

FKBP52 fue descubierta no sólo como una PPIase, si no como un componente de los heterocomplejos de los receptores esteroideos no ligados, una interacción que ocurre a través de la proteína Hsp90 (*heat shock protein 90*). Gracias a esto, un amplio número de inmunofilinas se han identificado (FKBP51, Cyp40) por su capacidad para unirse a Hsp90 a través del dominio TPR (*tetratricopeptide repeat*).

Esta inmunofilina está compuesta por dos dominios que corresponden al dominio catalítico FKBD, seguido de tres dominios TPR y en el extremo C-terminal posee un sitio de unión para CaM. El FK506 se une al primer dominio FKBD, sin embargo, pero a diferencia de lo que ocurre con la FKBP12, esta inhibe no inhibe la CaN. Ello es debido a la diferencia estructural de un solo aminoácido, el Lys121 corresponde al Ile90 de la

FKBP12 (Li et al., 2003). Además, a este primer dominio se unirán las Hsp90, los receptores esteroideos y además formará complejos con otras FKBP52. El segundo dominio FKBD apenas tiene actividad PPIase y no se conoce que se una el FK506, sin embargo, tiene una secuencia de aminoácidos de unión al ATP/GTP. Los dominios TPR son necesarios para la unión con receptores esteroideos y además median las interacciones entre proteínas (Goebl and Yanagida, 1991). En el extremo C-terminal, a continuación del dominio de unión a la CaM, FKBP52 contiene una secuencia denominada PEST que favorece la unión entre esta molécula y Hsp90 (Cheung-Flynn et al., 2003).

Se conoce que FKBP52 se asocia con hormonas esteroideas y otras proteínas, siendo solo este complejo estable si la proteína Hsp90 está presente. Este complejo formado, tiene como finalidad la translocación de los receptores esteroideos desde el citoplasma hasta el núcleo (Davies et al., 2002). Entre otras funciones FKBP52 también participa en la enlogación del axón en neuronas, la inhibición de la replicación del genoma viral y modulando la fosforilación de los linfocitos-T (Davies and Sanchez, 2005).

2.2.3. FKBP38.

FKBP38 es una proteína multifuncional que contiene un dominio FKBD, tres dominios TPR separados, un dominio para CaM y en su extremo C-terminal posee una región transmembrana. A pesar de la similitud del dominio FKBD con el de otros miembros de las FKBPs, los aminoácidos principalmente conservados para la unión del FK506 y para su actividad PPIase, no son exactamente iguales, por lo que se cree que esta proteína no tiene una completa actividad PPIase ni capacidad para unirse al FK506 (Lam et al., 1995). Sólo ejerce su actividad enzimática en un complejo con CaM-Ca^{2+} y es sólo en este macrocomplejo donde tiene afinidad por el FK506 (Edlich et al., 2005). FKBP38 aparece con una estructura secundaria similar a la de una FKBP inactiva. Para investigar el papel de esta proteína se han desarrollado inhibidores específicos como el N-(N',N'-dimethylcarboxamidomethyl)cycloheximide con mayor afinidad por el complejo FKBP38-CaM-Ca^{2+} que el FK506 y la rapamicina (Edlich et al., 2006).

La función antiapoptótica de FKBP38 se debe a la interacción con las proteínas Bcl-2 y Bcl-X_L localizadas en la membrana de la mitocondria. FKBP38 se une a Bcl-2 y la protege de la degradación, con lo cual en células con esta proteína silenciada, los

niveles de Bcl-2 se reducen significativamente (Kang et al., 2005). La interacción entre FKBP38 y Hsp90, no solo inhibe la actividad PPIase de la inmunofilina, sino también su unión con Bcl-2. Esta interacción es dependiente de CaM y de elevadas $[Ca^{2+}]_c$ (Edlich et al., 2007). FKBP38 se considera un inhibidor endógeno de la CaN, ya que se une a ella y la inhibe en ausencia de FK506 (Kang et al., 2008). Se conoce también que, fosfatasas como Rheb o Ras, en respuesta a factores de crecimiento o nutrientes, interactúan directamente con FKBP38 en ausencia de rapamicina e inhibe su asociación con mTOR. Esto sugiere que FKBP38 es también un inhibidor endógeno de mTOR (Bai et al., 2007).

2.2.4. FKBP25.

FKBP25 fue por primera vez detectada como una proteína en linfocitos-T humanos y en timo de ternero, la cual se une con mayor afinidad a la rapamicina que al FK506 (Galat et al., 1992). Su peso molecular es de 25,2 kDa con 224 aminoácidos. Esta proteína posee en su mitad C-terminal el dominio FKBD y su extremo N-terminal tiene un alto carácter hidrofílico que impide que interactúe con otras proteínas. El dominio FKBD difiere del de otras FKBPs en que posee una secuencia adicional de siete aminoácidos (incluye secuencia KKKK) insertados dentro de un loop que separa dos láminas-β. Esta zona forma parte del sitio de unión del FK506, lo cual puede explicar la disminución de su afinidad (Kay, 1996).

La secuencia de aminoácidos Lys presente en el domino FKBD podría ser la responsable de su localización nuclear, como se demuestra en linfocitos-T de humanos y ratón, y de su unión al ADN (Riviere et al., 1993). En su localización nuclear actúa como factor capaz de interaccionar con proteínas ribosomales afectando a la síntesis proteica (Gudavicius et al., 2014). FKBP25 también contiene secuencias consenso para la fosforilación de la proteína caseína cinasa II, una cinasa presente tanto en el núcleo como en el citosol y que se transloca de un sitio a otro para fosforilar sustratos citosólicos o nucleares. Esta inmunofilina también se acompleja a la glutatión S-transferasa en el núcleo y, este complejo también puede ser fosforilado por la caseína cinasa II participando en la regulación del crecimiento celular (Jin and Burakoff, 1993).

2.2.5. FKBP13.

Se trata de la segunda FKBP identificada, después de la FKBP12, purificada de timo de bovino. La proteína madura contiene 140 aminoácidos y una masa molecular de 13,2 kDa (Jin et al., 1991). Presenta una mayor afinidad de unión por el FK506 que por la rapamicina, en ambos casos mayores que las que presenta la FKBP12. En su extremo N-terminal contiene un péptido señal de 21 aminoácidos que parece ser el causante de que esta proteína se localice en el lumen del RE (Nigam et al., 1993). En el extremo C-terminal se encuentra el dominio FKBD casi idéntico a FKBP12, pero contiene cinco aminoácidos adicionales cuya secuencia es Arg-Thr-Glu-Leu. Esta secuencia es reconocida por un receptor de membrana del RE lo que permite que esta proteína quede retenida en el lumen del RE (Pelham, 1990).

Por su situación, se cree que esta proteína está involucrada en plegamiento de las proteínas que se encuentran en el RE y en la regulación de las señales de Ca^{2+} del lumen. En presencia de FK506, FKBP13 no presenta ningún cambio en su distribución, esto puede deberse a que el fármaco ejerce su efecto inmunosupresor mediante su unión a la inmunofilina dentro del lumen, donde el procesamiento y la presentación de los antígenos del sistema inmune se llevará a cabo (Nigam et al., 1993).

2.2.6. FKBP51.

Posee una estructura similar a la de FKBP52, con dos dominios FKBD en su extremo N-terminal, el primero de ellos donde se une el FK506. En la región C-terminal contiene tres dominios TPR que participan en la interacción entre proteínas. Fue principalmente descubierta como miembro del complejo del receptor de progesterona en el que también se encuentra involucrada la proteína Hsp90.

FKBP51 actúa como un potente modulador negativo del receptor de glucocorticoides y positivo del receptor androgénico, regulando la biología de las diferentes hormonas (Stechschulte and Sanchez, 2011).

FKBP51 y 52 comparten una elevada homología en la secuencia de sus aminoácidos así como una baja afinidad por el FK506 y la rapamicina. Para conocer mejor la biología de estas proteínas se han desarrollado compuestos análogos a estos mediante la adición de diferentes grupos funcionales. Así conocemos los análogos 1a,

1b y 1c de la rapamicina y, 2a y 2b del FK506, que usados a diferentes concentraciones pueden inhibir diferentes inmunofilinas (Kozany et al., 2009).

2.2.7. Otras FKBPs en humanos.

Se han encontrado inmunofilinas que también poseen dominios *EF-hand* en su estructura. FKBP22 y FKBP23 contienen un dominio FKBD y dos *EF-hand*, y FKBP60 y 65 (también conocida como FKBP10) contienen cuatro dominios FKBD y dos dominios *EF-hand*. Todas estas inmunofilinas contienen en su extremo C-terminal un péptido señal que les condiciona a quedar retenidos dentro del RE. El ambiente oxidativo del RE obliga a la proteínas que se encuentran en su interior a estabilizarse mediante puentes disulfuro gracias a los aminoácidos cisteína que contiene su estructura (Boudko et al., 2014). La unión de Ca^{2+} al dominio EF-*hand* es importante para la estabilidad y la función de estas proteínas. FKBP23 se ha demostrado que se une al complejo GRP78/BiP regulando su actividad de una manera dependiente de Ca^{2+} (Wang et al., 2007). El estrés reticular provoca cambios en los depósitos de Ca^{2+} lo cual induce una rápida proteólisis de FKBP65, una chaperona que se encarga de mediar el plegamiento del colágeno y la tropoelastina (Murphy et al., 2011). La disociación de Ca^{2+} de la FKBP22 afecta a su estructura y por lo tanto a su interacción con otras proteínas. FKBP19 es otra inmunofilina localizada en el RE pero que no posee dominio *EF-hand* (Nigam et al., 1993).

FKBP36 está expresada predominantemente en testículos y juega un papel importante en la espermatogénesis. Contiene un dominio FKBD y tres TPR. Interacciona con Hsp90 a través de los dominios TPR y forma complejo con la GAPDH (*Glyderaldehyde-3-phosphate Dehydrogenase*) a la cual inhibe (Jarczowski et al., 2009).

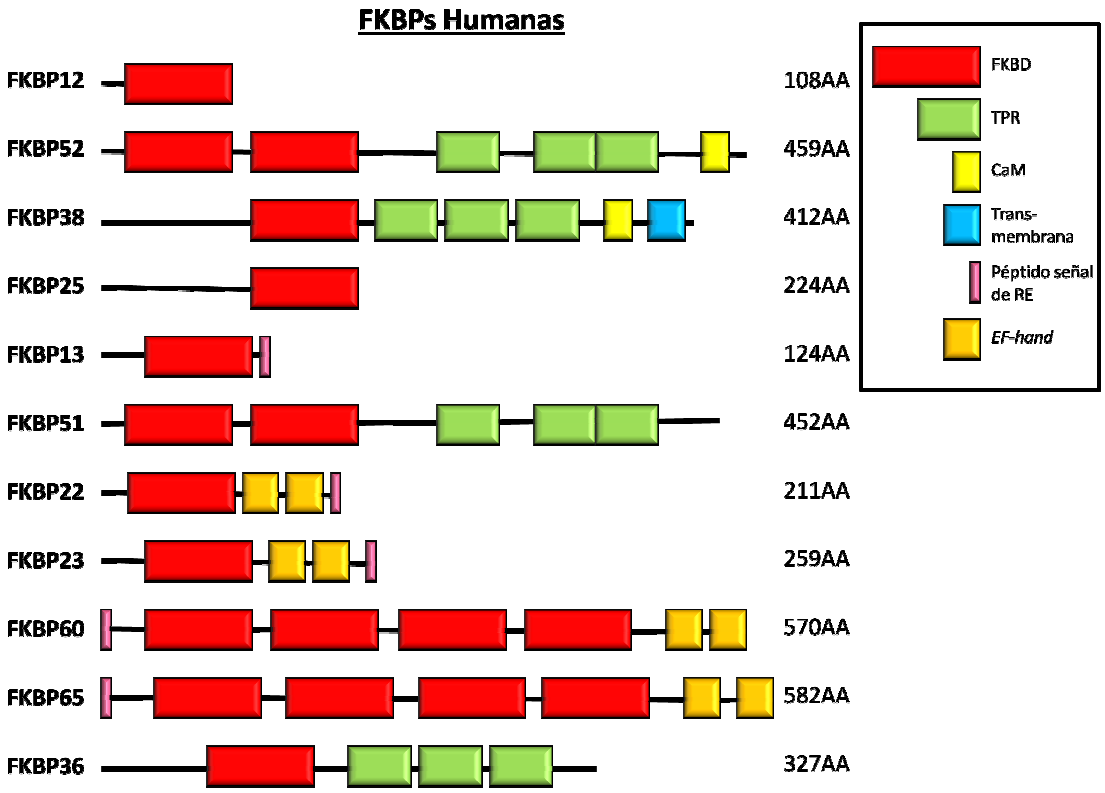

Ilustración 3: Dominios estructurales de las FKBPs humanas.

3. Participación de las FKBPs en la homeostasis del Ca^{2+}.

El uso de inhibidores de las inmunofilinas demostró que se producían alteraciones en la homeostasis del Ca^{2+} en varios tipos celulares, sugiriendo la participación de estas proteínas como moduladoras de la señal de Ca^{2+}. La primera observación que se hizo era que la FKBP12 participaba en la regulación de la salida de Ca^{2+} desde el RE debido a su interacción con el RyR. FKBP12 aparece unida al RyR con alta afinidad, facilitando la defosforilación por la fofatasa 2b (PP2b) y estabilizando el estado cerrado del canal (Ahern et al., 1994). La separación de la FKBP12 del complejo, mediante la adición de uno de los fármacos inmunosupresores, incrementa la probabilidad y el tiempo medio de apertura del canal.

La interacción funcional de FKBP12 con el IP_3R también se encuentra alterada en presencia de los inhibidores de las inmunofilinas. FKBP12 puede regular directa o indirectamente, a través de la CaN, la apertura del canal. La disociación de FKBP12 de IP_3R por la adición de FK506, resulta en la formación del complejo FK506-FKBP12 que

inhibe a la CaN. Esta asociación altera el estado de fosforilación del canal por inhibición de la fosfatasa, provocando un aumento de la salida de Ca^{2+} (Cameron et al., 1997).

Las FKBPs también se han visto asociadas a proteínas clave en la regulación de la entrada de Ca^{2+} como son los TRPCs. Se cree que actúan como proteínas accesorias en la activación de estos canales tras su estimulación (Sinkins et al., 2004). FKBP12 y 52 catalizan la isomerización de regiones de TRPC1 implicadas en el control de la apertura del canal (Shim et al., 2009). Como se ha sugerido que TRPC1 es un componente del canal capacitativo, las inmunofilinas podrían estar modulando la entrada capacitativa de Ca^{2+}, que es uno de los objetivos a desarrollar en esta Tesis.

La participación de las FKBPs en la recaptación de Ca^{2+} al interior de los orgánulos también ha sido estudiada. Se conoce que la rapamicina y el FK506 inhiben la actividad de SERCA. Elevadas concentraciones de FK506 inhiben la recaptación de Ca^{2+} mediante SERCA sin afectar la salida de Ca^{2+} mediada por IP_3R en músculo liso y células de neuroblastoma (Bultynck et al., 2000).

En la expulsión de Ca^{2+}, las únicas evidencias que relacionan las FKBPs con la PMCA parece ser a través de la inhibición de la CaN en presencia del FK506 (Sasamura et al., 2002).

4. Participación de las FKBPs en procesos patológicos.

La importancia biológica de estas proteínas se demuestra aún más por su implicación en la fisiopatología de un número de enfermedades, incluyendo enfermedades neurodegenerativas, alteraciones cardíacas y vasculares como la arterioesclerosis.

Las FKBPs presentan unos altos niveles de expresión en el sistema nervioso y regulan vías de señalización involucradas en la plasticidad sináptica. Consecuentemente con esta expresión, las FKBPs se han visto implicadas tanto en procesos de neuroprotección (Bavetta et al., 1999) como de neurodegeneración (Gant et al., 2011). El desarrollo y plasticidad neuronal del sistema nervioso se encuentra regulado por la actividad del RyR y el IP_3R. Se conoce que la expresión de FKBP12 disminuye en los estadíos tempranos de la degeneración de neuronas motoras provocando un desequilibrio entre ambas proteínas, lo que conlleva a una desregulación del canal con alteraciones en la $[Ca^{2+}]_c$ (Kihira et al., 2005). El uso de ratones deficientes

en la proteína FKBP12 provoca alteraciones en la regulación de la señal de mTOR, alteraciones en la memoria y comportamientos perseverantes y repetitivos (Hoeffer et al., 2008), características presentes en varios desórdenes cognitivos como: comportamientos autistas, desórdenes obsesivos/compulsivos, esquizofrenia y alteraciones neurodegenerativas.

La alteración en la unión entre el isotipo FKBP12.6 y el RyR2 ha sido propuesta como la causante de las alteraciones cardíacas. Por ejemplo, en el fallo cardíaco o cardiomiopatías la disociación de FKBP12.6 del RyR provoca un aumento la $[Ca^{2+}]_c$ que puede desencadenar arritmias. En estas enfermedades la expresión de la inmunofilina se encuentra disminuida, lo cual provoca el desequilibrio entre ambas proteínas, aumentando el número de canales abiertos (Hu et al., 2010). Gracias a estos descubrimientos, FKBP12 se ha considerado un biomarcador para la predisposición de arritmias (Lehnart et al., 2004).

La alteración entre ambos canales también afecta al músculo liso. En el caso de las enfermedades cerebrovasculares, la correlación es al revés, se encuentra un aumento de la expresión de FKBP12.6 y una disminución de los canales RyR, lo cual conlleva a una disminución en la señal de Ca^{2+} dando lugar a una constricción arterial (Koide et al., 2011).

En humanos, una mutación en el gen que codifica para la FKBP65 (FKBP10), da lugar a una enfermedad que se conoce como osteogénesis imperfecta. Esta enfermedad se caracteriza por presentar defectos en la matriz ósea, generando huesos muy frágiles debido a defectos en el plegamiento del colágeno y la tropelastina (Alanay et al., 2010).

La primera evidencia de que los inhibidores de la inmunofilinas alteraban la funcionalidad plaquetaria se vio en pacientes transplantados de riñon y pulmón, que presentaban un aumento de la actividad plaquetaria debido a un aumento en el torrente circulatorio del factor de von Willebrand y fibrinógeno (Taylor et al., 1999). La administración de FK506 en estos pacientes provoca trombopatías y trombopenias, dando lugar a enfermedades como: trombocitopenia púrpura trombótica, síndrome urémico hemolítico o microangiopatías trombóticas (Boctor, 2006). Estas anomalías se producen debido al elevado número de rutas de señalización que alteran estos inhibidores. El aumento en la activación plaquetaria de pacientes tratados con rapamicina y FK506 puede deberse a una alteración en la producción de tromboxano o de otros factores de secreción autocrinos, mientras que en la disminución del número de

plaquetas podría estar involucrado mTOR participando en la producción plaquetaria (Lopez et al., 2011).

5. mTOR.

Otra proteína muy relacionada con las inmunofilinas es la proteína mTOR (*mammalian target of rapamycin*). mTOR es una serina/treonina cinasa que pertenece a la familia de las PIKK (*phosphatidylinositol kinase-related kinase*) con un peso molecular aproximado de 290 kDa. Tiene un papel muy importante en la regulación del metabolismo celular, la síntesis de proteínas, el crecimiento celular y la proliferación. La rapamicina fue inicialmente identificada como un fungicida natural y en la búsqueda de dianas moleculares que se encontraban afectadas por este compuesto, se identificó TOR. En levaduras se encontraron dos genes, TOR1 y TOR2, cuyas mutaciones conferían resistencia a la rapamicina (Heitman et al., 1991). Como consecuencia, se descubrieron rutas de señalización de estas proteínas en varios organismos y se llegó identificar su homólogo en mamíferos.

Esta proteína presenta en su extremo N-terminal dos dominios HEAT formados por 20 secuencias repetidas que favorecen la interacción entre proteínas. A continuación se encuentra el dominio conservado de las PIKK, el dominio FAT (comprende los subdominios FRAP, ATM y TRRAP), seguido del sitio de unión del complejo FKBP12-rapamicina, conocido como dominio FRB. El complejo FKBP-rapamicina se une a este dominio, el cual está situado al lado de su dominio catalítico, lo que implica que se restrinja el acceso de mTOR a sus sustratos (Yang et al., 2013). El dominio catalítico comprende unos 300 residuos y es el encargado de desarrollar la actividad cinasa de la proteína. Y por último, en extremo C-terminal se encuentra el dominio FATC (FAT C-terminal). Raptor y rictor se unen a la región N-terminal del dominio FAT (Yang et al., 2013).

mTOR es el centro catalítico de dos complejos diferentes de proteínas que tienen rutas efectoras dispares. El complejo 1 de mTOR (mTOR1) está compuesto por la cinasa mTOR unida a su subunidad raptor y a otras proteínas accesorias como mLST8, deptor y PRAS40. Este complejo modula señales provenientes de nutrientes, insulina, factores de crecimiento y niveles de energía, activando rutas para el control del metabolismo y el crecimiento celular. El complejo 2 de mTOR (mTOR2) contiene la proteína mTOR unida a su subunidad rictor además de otras accesorias como son

mLST8, mSIN1, protor y deptor. A diferencia de mTOR1, mTOR2 sólo se encuentra regulada por factores de crecimiento y es insensible a la rapamicina (Soliman, 2013).

mTOR va regular el crecimiento celular a través de factores de crecimiento que activan la ruta de señalización de la PI3K, que permite la generación de segundos mensajeros como el PIP_3 (*phophatidylinositol-3,4,5-triphosphate*). El PIP_3 provoca la translocación del Akt a la membrana, donde se fosforila. A su vez, AKT fosforila e inactiva a la proteína TSC2 (*tuberous sclerosis complex 2*), proteína que se encuentra inhibiendo el complejo mTOR1. Cuando existen mutaciones en esta proteína, se produce un crecimiento descontrolado de la células dando lugar a la enfermedad conocida como esclerosis tuberosa (Yang and Guan, 2007). La proteína TSC2 va activar a una GTPase conocida con el nombre de RHEB (*Ras homolog enriched in brain*) (Inoki et al., 2003). Esta unión provoca la disociación de la proteína PRAS40 de raptor, que actúa como inhibidora del complejo mTORC1 (Sancak et al., 2007), produciéndose la activación del dominio catalítico de mTOR. Una vez que mTOR1 se encuentra activa, va a tener como principales substratos la proteína ribosomal S6K1 y la proteína 4E-BP1 (*eIF-4E binding protein*), reguladores positivos y negativos de la síntesis de proteínas, respectivamente. La fosforilación de 4E-BP1 controla el comienzo de la transcripción del ARN mesajero a proteínas, mientras que la fosforilación de S6K1 continúa con el proceso de elongación (Ma and Blenis, 2009).

mTOR también puede ser regulada de manera independiente de AKT, mediante la cinasa AMPK (*AMP-activated protein kinase*) en condiciones de ausencia de nutrientes. Los bajos niveles de ATP activan a esta proteína que fosforila TSC2, alterando la unión de RHEB con mTOR y en consencuencia la síntesis de proteína (Weber and Gutmann, 2012).

mTOR2 tiene la capacidad de fosforilar directamente cinasas tan importantes como Akt, PKC y SGK1, activando sus dominios catalíticos. Además mTOR2 tiene la capacidad de regular la reorganización del citoesqueleto de actina (Jacinto et al., 2004).

La presencia de mTOR ha sido descrita en plaquetas mediante el efecto de la rapamicina. Se ha descrito que su inhibición que disminuye el recuento plaquetario, la agregación y la retracción del coágulo formado (Weyrich et al., 2007).

La alteración de la regulación de mTOR provoca multitud de patologías incluidas cáncer, obesidad, diabetes mellitus tipo 2 y neurodegeneración, de ahí que esta

proteína sirva de diana molecular contra estas enfermedades (Laplante and Sabatini, 2012).

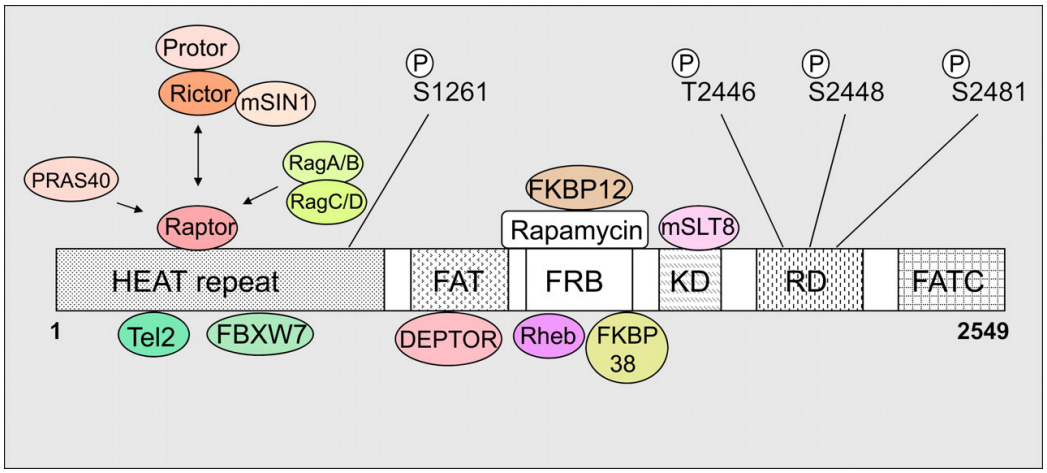

Ilustración 4: Estructura mTOR (Watanabe et al., 2011).

OBJETIVOS

OBJETIVOS

- Determinar el papel de las FKBPs en la entrada capacitativa de Ca^{2+} en plaquetas humanas.
- Analizar si la administración prolongada de inhibidores de las inmunofilinas provoca alteraciones en la homeostasis del Ca^{2+} en plaquetas humanas.
- Investigar el posible papel de las inmunofilinas en las alteraciones observadas en la homeostasis del Ca^{2+} en plaquetas de pacientes diabéticos tipo 2.
- Establecer la participación de las FKBPs en el mecanismo de entrada no capacitativa de Ca^{2+}.

OBJECTIVES

- To determine the role of FKBPs in capacitative calcium entry in human platelets.
- To analyze whether long-term immunophilin inhibitors administration might alter calcium homeostasis in human platelets.
- To investigate whether immunophilins are involved in the disorder of calcium homeostasis observed in patients with type 2 diabetes mellitus.
- To establish the role of FKBPs in the mechanism of non capacitative calcium entry.

MATERIALES
Y
MÉTODOS

MATERIALES Y MÉTODOS

6. Reactivos.

- ADP. *Sigma (Madrid, España).*
- Albúmina de suero bovino (BSA). *Sigma (Madrid, España).*
- Anticuerpo anti-α-actina. *Sigma (Madrid, España).*
- Anticuerpo anti-calcineurina. *Santa Cruz Biotechnology (Santa Cruz, Estados Unidos).*
- Anticuerpo anti-CD41-a PerCP (clone HIP8). *Becton Dickinson Transduction Laboratories (Madrid, España).*
- Anticuerpo anti-CD62P-PE. *Becton Dickinson Transduction Laboratories (Madrid, España).*
- Anticuerpo anti-PE (Isotipo). *Becton Dickinson Transduction Laboratories (Madrid, España).*
- Anticuerpo anti-phospho-mTOR (Ser 2481). *Millipore (Hayward, Estados Unidos).*
- Anticuerpo anti-phospho-raptor (Ser 722). *Millipore (Hayward, Estados Unidos).*
- Anticuerpo anti-phospho-Akt (Thr 308). *Cell Signalling (Beverly, Estados Unidos).*
- Anticuerpo anti-FKBP12. *Santa Cruz Biotechnology (Santa Cruz, Estados Unidos).*
- Anticuerpo anti-FKBP52. *Santa Cruz Biotechnology (Santa Cruz, Estados Unidos).*
- Anticuerpo anti-hTRPC1 C-terminal. *Alomone (Jerusalén, Israel).*
- Anticuerpo anti-hTRPC3. *Abcam (Cambridge, Reino Unido).*
- Anticuerpo anti-hTRPC6. *Alomone (Jerusalén, Israel).*
- Anticuerpo antiinmunoglobulina G de cabra conjugado con peroxidasa de rábano.
- Anticuerpo antiinmunoglobulina G de conejo conjugado con peroxidasa de rábano. *GE Healthcare (Madrid, España).*
- Anticuerpo antiinmunoglobulina G de ratón conjugado con peroxidasa de rábano. *GE Healthcare (Madrid, España).*
- Anticuerpo anti-IP_3Rs tipo II. *Santa Cruz Biotechnology (Santa Cruz, Estados Unidos).*
- Anticuerpo anti-Orai1 C-terminal. *Sigma (Madrid, España).*
- Anticuerpo anti-STIM1. *BD Transduction Laboratories (Franklin Lakes, Estados Unidos).*
- Apirasa (grado VII). *Sigma (Madrid, España).*
- Aspirina. *Sigma (Madrid, España).*
- Ciclosporina A. *Selleck Chemical (Huston, Texas, Estados Unidos).*
- Cipermetrina. *Sigma (Madrid, España).*
- Detergente iónico Tween 20. *Sigma (Madrid, España).*
- Dodecilsulfato sódico (SDS). *Sigma (Madrid, España).*
- Dithiothreitol (DTT). *Sigma (Madrid, España).*
- FK506. *Selleck Chemical (Huston, Texas, Estados Unidos).*
- Fura-2-acetoxymetil éster (fura-2/AM). *Invitrogen (Madrid, España).*

- Hiperfilms ECL. *Amersham (Buckinghamshire, Reino Unido).*
- Hyp 9. *Sigma (Madrid, España).*
- Kit-C nucleotransfección Amaxa. *Lonza (Cologne A.G., Alemania)*
- Marcador de peso molecular. *Bio-Rad (Madrid, España).*
- Medio de Eagle modificado por Dulbecco (DMEM). *Lonza (Cologne A.G., Alemania)*
- Nonidet P-40. *Sigma (Madrid, España).*
- OAG. *Calbiochem. (Darmstadt, Alemania)*
- Papel de transferencia (blotting paper). *GE Healthcare (Madrid, España).*
- Probenecid. *Sigma (Madrid, España).*
- Proteína A agarosa. *Upstate Biotechnology Inc. (Waltham, Estados Unidos).*
- Quinacrine. *Sigma (Madrid, España).*
- Rapamicina. *Selleck Chemical (Huston, Texas, Estados Unidos).*
- Reactivos de revelado quimioluminiscente. *Pierce (Rockford, Estados Unidos).*
- RPMI medio. *Lonza (Cologne A.G., Alemania)*
- Suero fetal bovino inactivado por el calor. *Sanex (Badajoz, España).*
- siRNA FKBP25. *Santa Cruz Biotechnology (Santa Cruz, Estados Unidos).*
- siRNA FKBP38. *Santa Cruz Biotechnology (Santa Cruz, Estados Unidos).*
- siRNA FKBP52. *Santa Cruz Biotechnology (Santa Cruz, Estados Unidos).*
- shRNA de TRPC1. *Proporcionado por Dr. Ambudkar.* (*National Institutes of Health, Bethesda, EEUU*)
- Tapsigargina (TG). *Sigma (Madrid, España).*
- TBHQ. *Alexis (Nottingham, Reino Unido)*
- Transfectin. *Bio-Rad (Madrid, España).*
- Tritón X-100. *Sigma (Madrid, España).*
- Trombina. *Sigma (Madrid, España).*

El resto de reactivos utilizados son de grado analítico.

7. Células empleadas.

7.1. Plaquetas humanas.

7.1.1. Selección de los individuos susceptibles de investigación.

El principal modelo celular empleado son las plaquetas humanas. Las plaquetas de donantes sanos fueron obtenidas mediante extracciones de sangre gracias a las donaciones voluntarias realizadas por pacientes que no tomaban ninguna medicación.

Las muestras de sangre diabética se obtuvieron de pacientes diagnosticados de enfermedad diabetes mellitus tipo 2 (DM2), que presentan una elevada concentración de glucosa en sangre, entre 180 y 240 mg/dL, y sin ninguna otra enfermedad

diagnosticada. Los individuos fueron seleccionados en el Hospital Clínico "San Pedro de Alcántara" y en el laboratorio de análisis clínico del Dr. Juan Manuel Hernández Cruz en Cáceres.

Las muestras de pacientes trasplantados de riñón fueron seleccionadas en el Hospital Infanta Cristina de Badajoz. Se analizaron 35 pacientes, 21 de ellos tratados con sirolimus (Rapamune® administrado a 1,88 ± 0,5 mg/12 hr), 8 pacientes tratados con everolimus (Certican® administrado a 1,81 ± 0,3 mg/24 hr) y 6 donantes sanos. A los individuos trasplantados además se les administraba prednisona (10 mg). Estos pacientes no mostraban ninguna alteración vascular evidente en el momento de la extacción

Todas las muestras se trataron de forma anónima para preservar la identidad del donante y fueron tratadas de acuerdo con los principios de la Declaración de Helsinki, y los Comités de Bioética del Hospital y de la Universidad de Extremadura.

7.1.2. Preparación de la suspensión de plaquetas.

La sangre se extrajo mediante punción venosa de la vena humeral del brazo y se mezcló con una solución anticoagulante de dextrosa citrato ácida (ACD), (1/6 del volumen total), cuya composición se muestra a continuación (Tabla 1):

REACTIVOS	Concentración (mM)	(g/L)
Ácido cítrico	85	12,5
Citrato sódico	78	7,5
Glucosa	111	10

Tabla 1: ACD (pH=5)

En el caso de los pacientes trasplantados, la sangre se extrajo en un vacutainer con 6.3 mg EDTA-K3 como anticoagulante, el cual se ha demostrado no modifica la actividad celular (Lippi et al., 1987).

Para separar las diferentes fracciones de la sangre se realizó una primera centrifugación a 700 *g* durante 5 min a 25ºC. Como resultado de esta centrifugación se

obtuvieron tres fracciones bien diferenciadas: en el fondo, los eritrocitos; a continuación un halo blanquecino correspondiente a los glóbulos blancos; y en la parte superior, el plasma rico en plaquetas (PRP). Con ayuda de una pipeta Pasteur, el PRP se separa del resto de componentes y se mezcla con apirasa (40 µg/mL) que hidroliza el ADP y ATP secretado por las plaquetas a través de su actividad di-trifosfatasa, y con ácido acetil salicílico (100 µM) que inhibe la COX-I evitando la liberación plaquetaria de prostaglandinas para evitar la activación y agregación plaquetaria inducida por la manipulación de la muestra antes del experimento. En el caso de los experimentos de agregación, no se le añade aspirina.

Se realiza una segunda centrifugación a 350 *g* durante 20 min a 25ºC, obteniéndose un pellet de color blanco que es separado del plasma pobre en plaquetas (PPP). Posteriormente, se resuspende el pellet de plaquetas en un tampón HEPES salino (HBS) de pH 7,4 (Tabla 2), dejando las células un nuevo período de tiempo de 15 min en reposo antes de comenzar los experimentos.

REACTIVOS	Concentración (mM)	(g/L)
HEPES	10	1,19
Cloruro sódico	145	4,23
Cloruro potásico	5	0,19
Sulfato de magnesio	1	0,13
Glucosa	20	1,8
BSA	1mg/mL	1,0

Tabla 2: HBS (pH=7,4)

7.2. Plaquetas de ratón.

Las dos cepas de ratones con las que trabajamos, WT (*wild type*) y TRPC6 KO (*knock-out*) fueron adquiridos de Deltagen, Inc. (San Mateo, CA, USA) a través de la red European Mouse Mutant Archive (EMMA).

Todos los experimentos fueron realizados en ratones de entre 8 y 12 semanas de edad, los cuales fueron sangrados bajo el efecto anestésico del isoflourano. La

extracción se realizó con un capilar de unos 2 cm de longitud que se introduce en el plexo venoso retroorbital del animal, siempre siguiendo las recomendaciones del Comité Ético de Experimentación Animal. La sangre fue recogida en tubos con 300 µl de ACD y centrifugada a 300 *g* durante 5 min. Se separó el PRP, se le añadió aspirina (100 µM) y apirasa (40 µg/mL) y se volvió a centrifugar a 600 *g* durante 5 min. El pellet de plaquetas se resuspendió en una solución conocida con el nombre de Tyrode´s (Tabla 3) con apirasa (40 µg/mL).

REACTIVOS	Concentración (mM)	(g/L)
HEPES	5	5,2
Cloruro sódico	137	32
Cloruro potásico	2,7	0,80
$NaHCO_3$	12	4,04
NaH_2PO_4	0,43	0,20
$MgCl_2$	1	0,82
BSA	0,35 %	3,5
Glucosa	0,1 %	1

Tabla 3: Solución Tyrode´s (pH=7,13)

7.3. Cultivo celular.

Se usó una línea celular megacarioblástica, células MEG-01, como precursoras de plaquetas. Fueron adquiridas de la empresa ATCC (Manassas, VA, USA) y cultivadas a 37ºC con 5 % CO_2 en medio RPMI con L-glutamina, suplementado con un 10 % de suero bovino fetal inactivado a altas temperaturas y con antibióticos como estreptomicina y penicilina.

Para la realización de la técnica *Patch Clamp* se usó una línea celular HEK293 (*Human Embryonic Kidney 293 cells*), que son células derivadas de células embrionarias de riñón humano. Estas células sobreexpresaban de manera estable la proteína TRPC6 lo cual hace mucho más simple su estudio. Se cultivaban a 37ºC con un 5 % de CO_2 en medio de Eagle modificado por Dulbecco, suplementado con un 10 %

de suero fetal bovino inactivado más penicilina y estreptomicina. Como antibiótico de selección de las células transfectadas, se le añadía G418 (Sigma®) 8 µl/mL. Las células se cultivan en placas multipocillos con cubreobjetos en su interior, hasta conseguir una confluencia del 70 % aproximadamente.

8. Transfección celular.

Las células MEG-01 fueron transfectadas usando plásmidos siRNA para silenciar FKBP52, FKBP25, FKBP38 (*Santa Cruz Biotechnology*®) y otro shRNA para silenciar TRPC1, cedido por la doctora Ambudkar (*National Institutes of Health, Bethesda, EEUU*). Para ello se usó el Kit-C del sistema Amaxa Nucleofection®. Se partió de una concentración de 10^6 células/mL que se resuspendieron en 100 µL de la solución C del Kit comercial junto con 2 µg del plásmido. Las células fueron sometidas a un campo eléctrico que favorece la penetración del plásmido en el interior celular. Después de 10 min tras la electroporación, se le añadió medio RPMI y se cultivaron durante 72 horas.

La secuencia del shRNA para el TRPC1 humano fue: 5'-CACCGGGTGACTTTATATGGTTCGAAAACCATATAATAGTCACCC-3' y la secuencia antisentido fue: 5'-AAAAGGGTGACTATTATATGGTTTTCGAACCATATAATAGTCACCC-3'.

Las células HEK293 se transfectaron cuando alcanzaron una confluencia entre el 70–90 %, con 7 µg/mL de plásmidos siFKBP25 y siFKBP38 (*Santa Cruz Biotechnology*®). Para ello, se mezclaron en 250 µL de medio DMEM sin reconstituir el plásmido y 5 µL de Transfectin (Bio-Rad®), dejando la mezcla durante 20 minutos a temperatura ambiente. Pasado este tiempo, se retiraron 250 µL de medio de las placas de siembra y se añadió la mezcla por toda la placa, moviéndose en círculos suavemente. Las células junto con la mezcla se mantuvieron en el incubador durante 5 horas tras la transfección, pasadas las cuales se les cambió el medio por medio DMEM nuevo reconstituido. Las células fueron usadas para los experimentos 72 horas después de la transfección.

9. Determinación de la concentración de Ca^{2+} intracelular mediante fluorimetría.

Para la determinación de la $[Ca^{2+}]_c$ en plaquetas y MEG-01 se utiliza una sonda fluorescente denominada fura-2/AM. El fura-2/AM es un fluoróforo de Ca^{2+} permeable a la membrana plasmática que una vez se introduce en el interior celular sufre la acción de esterasas citosólicas que liberan fura-2, incapaz de atravesar la membrana. Este fluoróforo presenta una K_d para el Ca^{2+} de 120-250 nM, en función de la temperatura, lo que permite determinar concentraciones de Ca^{2+} hasta el rango de nanomolar (Grynkiewicz et al., 1985).

Presenta dos longitudes de onda de excitación 340 y 380 nm que nos permiten observar los máximos y mínimos de emisión a 505 nm según tenga unido Ca^{2+} o éste esté disociado, tal como indica el espectro que se muestra en la Imagen 4. La ratio de fluorescencia emitida (340/380) es proporcional a las variaciones en la concentración de Ca^{2+} libre citosólico.

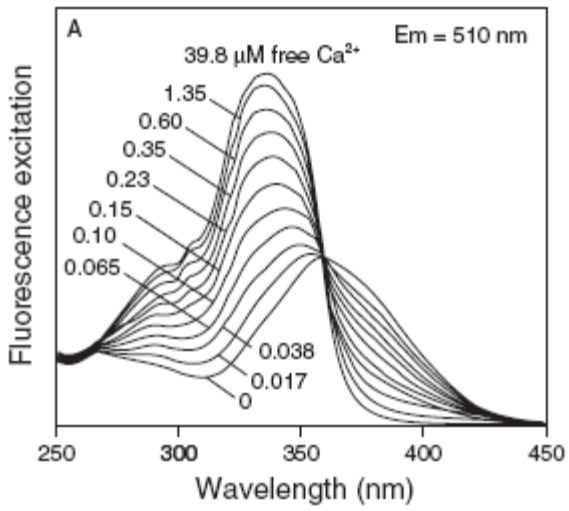

Imagen 1: Espectro de fluorescencia del fura-2.

En las mediciones de la $[Ca^{2+}]_c$ en HEK293 se utilizó una sonda diferente, el fluo-4/AM. Esta sonda, al igual que el fura-2, presenta también un grupo éster que facilita la penetración y el reclutamiento en el interior de la célula. No se trata de una sonda ratiométrica, ya que tiene solo una longitud de onda de excitación a 485 nm, registrándose una emisión a 525 nm. Estos niveles de emisión dependen de la cantidad de Ca^{2+} unido, a más Ca^{2+} unido, más intensidad de señal. Además, presenta una menor afinidad por el Ca^{2+} que el fura-2 ya que su K_d = 345 nm.

9.1. Incubación con el fluoróforo.

El PRP obtenido tras la primera centrifugación de la sangre, fue incubado con 2 µM fura-2/AM, durante 45 minutos a 37ºC. Tras la incubación, las plaquetas se aislaron mediante centrifugación a 350 *g* durante 20 min y el pellet se resuspendió en HBS más apirasa (40 µg/mL).

En el caso de los estudios realizados con MEG-01, éstas se incubaron con fura-2/AM a una concentración de 2 µM durante 30 minutos a temperatura ambiente y en oscuridad. Una vez transcurrida la incubación, las células se centrifugaron a 100 *g* durante 5 min, resuspendiendo el pellet en HBS con 50 µM de $CaCl_2$.

Las HEK293 se transfieren a una placa de 96 pocillos con una concentración de $5x10^5$ células/pocillo. Se incuban en medio SBS (Tabla 4) con Ca^{2+} en presencia de 1mg/mL de fluor-4 AM, 500 mM de probenecid y 10 % de ácido plurónico, durante una hora a 37ºC. Tras la incubación, las células quedan adheridas al fondo de la placa y se lavan con SBS tres veces retirando la solución con una pipeta multicanal.

En otra placa multipocillos se preparan las diluciones de los agonistas, en una concentración 5x, para que al transferirse a la placa de las células se alcance la concentración deseada.

REACTIVOS	Concentración (mM)	(g/L)
NaCl	134,3	7,85
KCl	5	0,37
$MgCl_2$	1,2	1,2 mL de 1 M
$CaCl_2$	1,5	1,5 mL de 1 M
Hepes	10	2,38
Glucosa	8	1,44

Tabla 4: SBS.

9.2. Determinación de la concentración de Ca^{2+} en plaquetas.

La determinación de la $[Ca^{2+}]_c$ en plaquetas se realizó mediante técnicas fluorimétricas. La cuantificación de la fluorescencia se realizó a partir de alícuotas de 2 mL de suspensión de plaquetas depositadas en cubetas de cuarzo mediante un espectrofluorímetro modelo Cary Elipse de Varian®. El sistema está provisto de un dispositivo de microagitación y termostatización que permitió mantener las células en suspensión y atemperadas a 37°C. La suspensión celular se excitó alternativamente a longitudes de onda de 340/380 nm y la fluorescencia emitida se registró a 505 nm. Todos los agentes aplicados durante la realización de los experimentos se adicionaron directamente en la cubeta del espectrofluorímetro en función del volumen de la suspensión celular para poder obtener así la concentración final deseada.

Las variaciones en la $[Ca^{2+}]_c$ se monitorizaron y se expresaron como ratio de las fluorescencias emitidas a 505 nm por el fura-2 tras ser excitado a 340 y 380 nm. Tras realizar el calibrado de cada uno de los experimentos, como se detalla posteriormente, la transformación de los valores de ratio de fluorescencia a concentración de calcio intracelular y el posterior calibrado se realizó mediante el método desarrollado por Grynkiewicz en 1985, aplicando para ello la fórmula siguiente:

$$[\mathrm{Ca}^{2+}]_c = \mathrm{Kd} * \frac{\mathrm{R} - \mathrm{Rmin}}{\mathrm{R} - \mathrm{Rmax}} * \frac{Sf}{Sb}$$

Donde:

-Kd = Constante de disociación del fura-2 y el Ca^{2+} (214 nM a 37°C).
-R = ratio de fluorescencia de la muestra.
-Rmin = ratio mínimo de fluorescencia.
-Rmax = ratio máximo de fluorescencia.
-Sf = S free (emisión del fura-2 libre de Ca^{2+} al ser excitado a 380 nm).
-Sb = S bound (emisión del fura-2 unido a Ca^{2+} al ser excitado a 380 nm).

Se analizó tanto el incremento en la $[Ca^{2+}]_c$ como la velocidad de retorno de la $[Ca^{2+}]_c$ a valores basales. Alcanzados los niveles basales después del estímulo,

realizamos la valoración de la ECC mediante la adición de Ca^{2+}, midiéndose el comportamiento del ratio de fluorescencia durante 3 minutos.

El siguiente paso consistió en la calibración del experimento mediante la adición de 5 mM de Ca^{2+} para saturar todo el fura-2. La lisis celular se realizó con tritón X-100 al 0,01 %, provocando la solubilización de los lípidos de la membrana plasmática de las células, desestabilizando la integridad de éstas y provocando la salida del Ca^{2+} y el fura-2 retenidos en el citosol al exterior, obteniendo el valor de ratio máximo del experimento. Al minuto de la lisis celular se adicionó 5 mM de EGTA que queló todo el Ca^{2+} presente en la solución, obteniéndose el ratio mínimo. Junto con EGTA se añadió Tris 300 mM para mantener el pH ligeramente alcalino, dado que la unión de Ca^{2+} al EGTA libera H^+ que podrían alterar la fluorescencia del fura-2.

Los resultados obtenidos se analizaron comparando las integrales bajo la curva, de tal manera que se compararon las áreas obtenidas tras la adición del estímulo (liberación de Ca^{2+} desde los depósitos) y tras la adición de Ca^{2+} (entrada de Ca^{2+} al interior de la célula). Los resultados se muestran en histogramas en los que se compara el área con el control.

9.3. Determinación de la concentración de Ca^{2+} en células aisladas.

Las células de cultivo se transfirieron a una cámara de perfusión adherida a unos cubreobjetos embebidos en polylisina para favorecer la inmovilización de las células. Los cambios en la fluorescencia del fura-2 fueron monitorizados mediante la utilización de un microscopio invertido usando una configuración que detecta los cambios de $[Ca^{2+}]_c$ en células individuales. Las células se excitaron alternativamente a longitudes de onda de 340/380 nm y la fluorescencia emitida se registró a 505 nm. Los valores se registraron y procesaron usando el software Aquacosmos (Hamamatsu®).

Los estímulos son añadidos mediante perfusión de las soluciones diluidas en medio HBS y retiradas mediante un sistema aspiración de vacío, de tal manera que las células están siendo continuamente lavadas. Los registros se hacen a 37ºC mediante un sistema de termostatización que atempera tanto la cámara como las soluciones perfundidas.

Los datos se expresan como cambios en la fluorescencia después de la adición del estímulo (Fn) dividido entre la fluorescencia de las células en condiciones basales

(Fo). También se hace la comparación entre tratamientos calculando las integrales bajo la curva.

9.4. Determinación de la concentración de Ca^{2+} mediante el sistema Flexstation.

El aparato Flexstation cuenta con tres plataformas: una para las células cargadas con la sonda, otra para los estímulos y otras para las puntas de pipeta. Su interior está provisto de una pipeta multicanal automática que transfiere la solución estímulo a la placa de las células.

Todos los pocillos se excitaron simultáneamente a 485 nm, recogiéndose una emisión a 525 nm. Los cambios en la fluorescencia que se observan, se deben a un aumento en el número de moléculas de fluo-4 unidas a iones Ca^{2+}. Estos experimentos fueron realizados a temperatura ambiente (24ºC).

9.5. Determinación de la entrada de Mn^{2+}.

El Mn^{2+}, que compite con el Ca^{2+} para entrar en las células, se utiliza habitualmente para monitorizar la entrada de cationes divalentes, dada su capacidad para fijarse a la sonda fura-2/AM, cuyo punto isosbéstico a 360 nm permite apreciar la pérdida de fluorescencia cuando se une a cationes diferentes al Ca^{2+}. Los movimientos del Mn^{2+} en las plaquetas se registraron en un espectrofotómetro Cary Eclipse, utilizando alícuotas de 1 mL de la suspensión de plaquetas (2×10^8 células/mL) a 37ºC y en agitación. La entrada de Mn^{2+} se calculó mediante la comparación de la disminución de la fluorescencia del fura-2 cuando las plaquetas se sometieron a diferentes tratamientos, las curvas se ajustaron a la ecuación y = Ax+B, donde A es la pendiente y B es la fluorescencia del fura-2 al inicio del experimento.

10. Citometría de flujo.

La citometría de flujo es un método que permite analizar múltiples parámetros celulares, como tamaño (*FSC, Forward scattering complexity*) y complejidad (*SSC, Side scattering complexity*), y cualquier componente celular o función susceptible de ser

marcado con un fluorocromo, en células o partículas suspendidas en líquido, que pasan alineadas y de una en una, por delante de un haz luminoso. Proporciona dos tipos fundamentales de información: la generada por la dispersión de la luz, y la relacionada con la emisión de luz por los fluorocromos, presentes en la célula o partícula, al ser excitados por el rayo luminoso. Las señales luminosas detectadas se transforman en impulsos eléctricos que se amplifican y se convierten en señales digitales, que son procesadas en un ordenador.

Se utilizaron sondas de fluorescencia específicas para detectar los gránulos α y densos, y su secreción se determinó mediante el registro de los cambios de fluorescencia en plaquetas seleccionadas en un citómetro de flujo (FASCcan cytometer, Becton-Dickinson, CA). Aproximadamente $1x10^6$ plaquetas lavadas, obtenidas tal y como se describe en el apartado 7.1.2, se suspendieron en 50 µL HBS y se marcaron con anti-CD41, anticuerpos anti P-selectina (CD62P) y 10 µM de la sonda de fluorescencia mepacrine® a 37º C durante 30 minutos. Posteriormente fueron estimuladas con trombina. La reacción se detuvo 10 min después de la estimulación de las plaquetas con PBS (1x) (Tabla 5) a 4ºC, y las muestras se analizaron por FACS en las 3 horas siguientes. Las plaquetas se seleccionaron primero por tamaño (FSC) y complejidad (SSC) y posteriormente por la presencia de CD41 en la superficie de las células, ya que este antígeno solo se expresa en megacariocitos y plaquetas, por lo que es ampliamente utilizado como marcador específico de la línea megacariocítica (Michelson, 2007).

REACTIVOS	Concentración (mM)	(g/L)
NaCl	137	8,00
KCl	2,70	0,20
Na_2HPO_4	5,62	2,01
NaH_2PO_4	1,09	0,17
KH_2PO_4	1,47	0,20
H_2O	1 L	

Tabla 5: PBS 1x (pH=7,4)

La secreción de los gránulos densos se evaluó mediante el registro de la fluorescencia del mepacrine, un fluoróforo que se incorpora a estos gránulos. La

disminución de la fluorescencia con mepacrine en plaquetas es indicativa de la secreción de los gránulos densos, y se expresa como intensidad de fluorescencia media o MFI (*mean fluorescence intensity*), que es el resultado del cálculo: MFI = fluorescencia de plaquetas marcadas con mepacrine menos fluorescencia endógena.

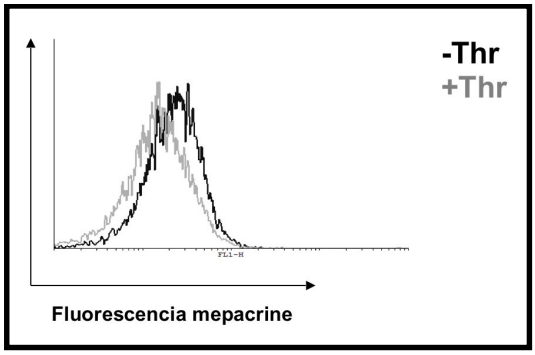

Figura 1: Quinacrine

La secreción de los gránulos α se detectó mediante el empleo de un anticuerpo específico anti-P-selectina (anti-CD62P-PE), que detecta únicamente la P-selectina expresada en la membrana externa de la plaqueta. Es una medida indirecta de la secreción de los gránulos α, ya que la P-selectina se expresa en la membrana de estos gránulos en plaquetas en reposo, pero cuando se activan, los gránulos α liberan su contenido y su membrana se fusiona con la membrana externa plaquetaria, que desde ese momento expresará P-selectina.

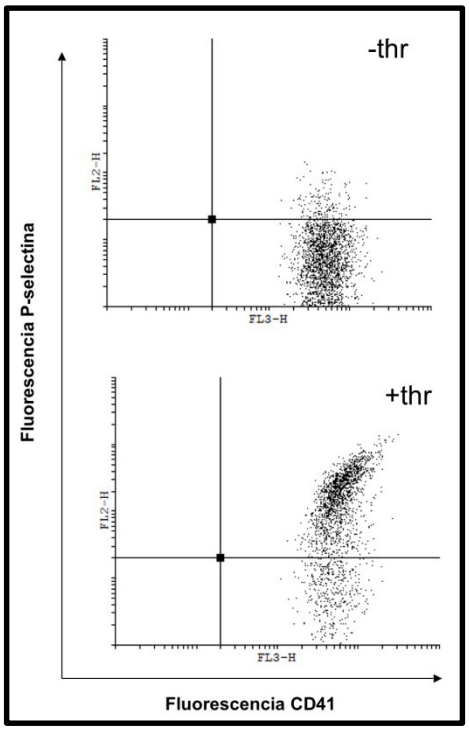

Figura 2: p-selectina

11. Agregación plaquetaria.

En estos estudios también hemos considerado valorar el papel fisiológico de las plaquetas mediante agregación. Para ello, al PRP obtenido tras la primera centrifugación sólo se le añadió aspirina sin apirasa, para no alterar el proceso de la

agregación. Las plaquetas se centrifugaron a 350 *g* durante 20 min y se resuspendieron en alícuotas de 400 µL de HBS. La agregación plaquetaria se midió en un agregómetro Chronolog (Havertown®, Pa, USA) a 37ºC y en agitación a 1200 r.p.m. Durante la medición, los agonistas utilizados (Thr, ADP) se añadieron en presencia de Ca^{2+} (1 mM) directamente a la cubeta del agregómetro. Este aparato mide el tiempo de reacción, el porcentaje de plaquetas que se agregan, la velocidad y el grado en que las plaquetas dispersas en una muestra forman los agregados plaquetarios durante la estimulación con estos agonistas, basándose en la observación de las variaciones de la densidad óptica de la muestra, de forma que, cuando se produce la agregación, la turbidez que se origina por la presencia de plaquetas en suspensión disminuye por la fuerte interacción entre las mismas, incrementándose la luz que pasa a través de la muestra y provocando así un aumento de la transmitancia. Para determinar esto, se utilizó un control (cubeta con HBS), de forma que el porcentaje de agregación (o amplitud) se estimó como el porcentaje de la diferencia en la transmisión de la luz entre la suspensión plaquetaria y el HBS. La pendiente de la agregación obtenida es, por tanto, el cambio en el porcentaje de agregación por minuto.

12. Patch clamp.

El *patch clamp* es una técnica usada en electrofisiología que nos permite medir las propiedades eléctricas de las células aisladas. En este caso concreto, analizaremos las corrientes de Ca^{2+} que tienen lugar cuando estimulamos los canales iónicos TRPC6.

La técnica se fundamenta en la capacidad conductora de las células cuando hay variaciones en el potencial de membrana. De esta forma, cuando las células reciben un estímulo eléctrico son capaces de conducir corriente, pues la membrana facilita la entrada o salida de iones. Los principios de electrofisiología se basan principalmente en la ley de Ohm:

$$I = G \cdot V = \frac{V}{R}$$

I = Intensidad de corriente (Amperios)
G = Conductancia (Siemens)
V = Diferencia de potencial (Voltios)
R = Resistencia (Ohmios, Ω)

Según esta ley, si fijamos un voltaje determinado, podremos medir la corriente iónica que fluye a dicho potencial; o por el contrario, si fijamos un valor de corriente determinado, podremos analizar la variación de voltaje, es decir, se pueden estudiar los potenciales de acción.

La técnica básicamente consiste en la formación de un sello de alta resistencia entre la membrana de la célula y una micropipeta rellena de una solución electrolítica. A su vez la pipeta va conectada a un amperímetro lo que permite la fijación del voltaje en la porción de membrana deseada. De este modo, se pueden determinar las corrientes iónicas, puesto que además del voltaje, también se conoce la resistencia que opone la pipeta al paso de la corriente, en la porción de membrana donde se ha formado el sello.

El registro de las corrientes de membrana se puede efectuar a partir de distintas configuraciones; *on-cell patch* (registro de la corriente sólo en la zona delimitada por el parche), *whole-cell* (registro de la corriente en toda la célula, la pipeta abre un orificio en la membrana), *perforated-patch* (registro de la corriente en toda la célula, la pipeta contiene una sustancia capaz de abrir pequeños poros en la membrana), *inside-out y outside-out* (registro de la corriente de una vesícula previamente extraída de la membrana dejando el interior celular o el exterior en el lado externo de la pipeta, respectivamente). El elegido en nuestro caso es el *whole-cell.*

12.1. Preparación de las micropipetas.

Uno de los componentes esenciales del equipo es el electrodo. Como electrodo se usan pipetas de cristal de borosilocato que se fabrican a partir de capilares de hematocrito de 1,5 cm de diámetro. Para la fabricación de las pipetas se usa un estirapipetas vertical, PP-830 Puller (Narishige®, Tokio, Japón) que se programa para que la punta que se genera presente una resistencia de entre 2-5 MΩ al introducirla en la solución. Posteriormente, la punta debe ser calentada acercándola a una resistencia, con el fin de pulirla para producir una superficie lisa que favorezca el sello de alta resistencia con la membrana.

El interior de la pipeta se rellena de una solución iónica muy parecida a la composición citoplasmática.

REACTIVOS	Concentración (mM)	(g/L)
CsOH	48	7,20
L-Ácido glutámico	48	7,06
Hepes	10	2,38
Na_2ATP	1	0,55
NaCl	8	0,47
$MgCl_2$	2	2 mL de 1 M
$CaCl_2$	17	1,89
EGTA	40	15,21

Tabla 5: Solución de la pipeta. (pH=7,2. Osm=300 mOsm)

12.2. Equipo utilizado.

Las pipetas fueron montadas en un soporte CV-4 (Molecular Devices, Sunnyvale, CA, USA), en las cuales se introduce un alambre de plata recubierto de cloruro de plata (Ag-AgCl). Gracias a un manipulador podemos redirigir el soporte en las tres direcciones, primero de una manera más burda y después con un micromanipulador (Mitotuyo, Japón) para contactar con la célula.

El sistema óptico utilizado fue un microscopio óptico invertido modelo Eclipse TS100 (Nikon®), al cual va acoplado la cámara y el equipo de perfusión, todo ello colocado sobre una mesa antivibratoria.

Las señales fueron amplificadas y registradas usando un amplificador modelo Axopatch 200A y un software pCLAMP10.2 (Molecular Devices). El amplificador cuenta con un botón de corrección de voltaje de *offset*, que nos permite eliminar todos los potenciales asociados a la técnica de medición, quedándonos solo con el potencial de membrana que deseamos registrar. Se encuentra conectado, a su vez, a un osciloscopio digital. Este osciloscopio nos va a permitir visualizar los potenciales de acción repetidos en tiempo real. Y por último, se conecta al ordenador un convertidor Digidata 1322A (Molecular Devices) que nos transforma la señal analógica que recibe a digital para poder visualizarla, almacenarla y analizarla posteriormente.

12.3. Configuración utilizada.

La configuración utilizada fue la de *whole-cell* o célula entera, en la que el interior de la pipeta queda en contacto directo con el citoplasma, permitiendo estudiar la corriente que pasa a través de toda la membrana celular.

El primer paso para la obtención de la citada configuración consiste en aproximar la punta de la pipeta a la superficie de la membrana. A la pipeta se le aplica cierta presión positiva para evitar el intercambio iónico con la solución extracelular, y para que al eliminar esa presión positiva, se favorezca el sello entre la punta de la pipeta y la membrana de la célula. Primero se aproxima con el manipulador y luego con el micromanipulador se deja lo más cerca posible de la célula, para que al liberar esa presión positiva se forme el sello de alta resistencia o *giga-seal* (superior a 1 GΩ).

Una vez formado el *giga-seal*, es necesario aplicar una leve succión para conseguir que la membrana se rompa y que el interior de la pipeta entre en contacto con el citoplasma. En este caso, la corriente registrada es la corriente que pasa a través de todos los canales de la membrana, y por tanto, dependerá del número de canales presentes, de la corriente unitaria y de su probabilidad de apertura. Esta configuración permite el aislamiento de corrientes iónicas ya que es posible la modificación del medio extra e intracelular. Para ello, simplemente será necesario el uso de bloqueantes específicos del resto de los canales o bien la sustitución de los iones que no interesen por otros incapaces de atravesar la membrana celular.

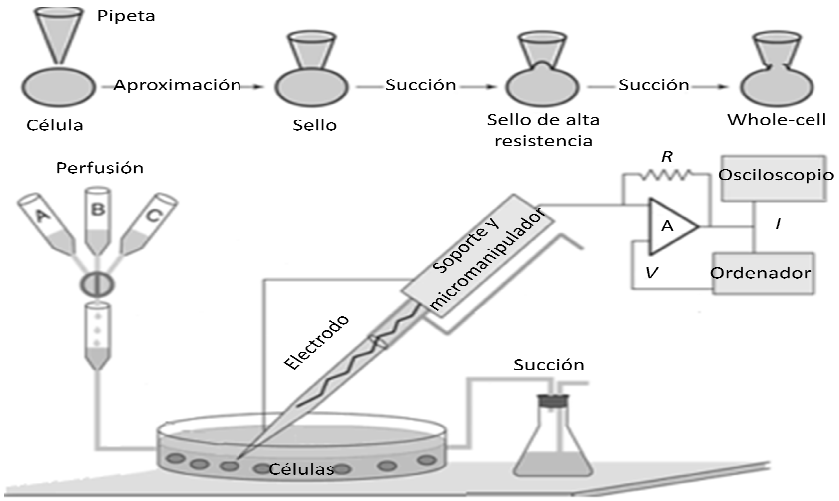

Imagen 2: Esquema de patch clamp (Modificado: http://ib-biology201012.wikispaces.com/Brain+anatomy,+neuron+structure+and+function).

12.4. Protocolo aplicado.

Los cubreobjetos se montan en la cubeta y se perfunden con la siguiente solución:

REACTIVOS	Concentración (mM)	(g/L)
NaCl	140	8,18
KCl	5	0,37
$MgCl_2$	1,2	1,2 mL de 1 M
$BaCl_2$	1	0,24
Hepes	10	2,38
Glucosa	10	1,80

Tabla 6: Solución experimental. (pH=7,4. Osm=290 mOsm)

Tras la obtención de la configuración *whole-cell* se le aplica un protocolo de diferentes voltajes en rampa que va desde los -100 mV hasta los +100 mV en 200 milisegundos que se repite en intervalos de 10 segundos. En el programa nos va apareciendo un registro de intensidad de corriente cada vez que se aplica el voltaje.

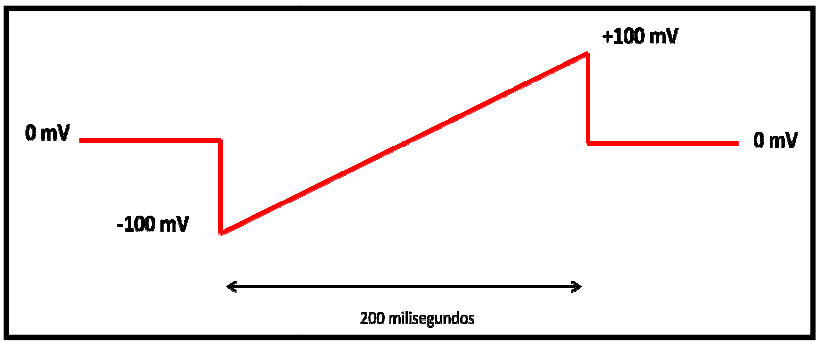

Figura 3: Protocolo de voltaje aplicado.

Los registros obtenidos se analizaron con el programa Clampfit 10.2. Los datos obtenidos de cada registro se representan como la intensidad corriente en pA por el protocolo de voltaje aplicado, mostrando desde los -80 mV hasta los +80 mV. Otra forma de representar los datos y que nos permite ver de una manera más general los efectos de los diferentes inhibidores o estímulos aplicados, es mediante la selección de los

valores máximos y mínimos de intesidad de corriente de cada registro. Se hará seleccionando los valores de pA a -80 mV y a +80 mV de cada uno de los registros.

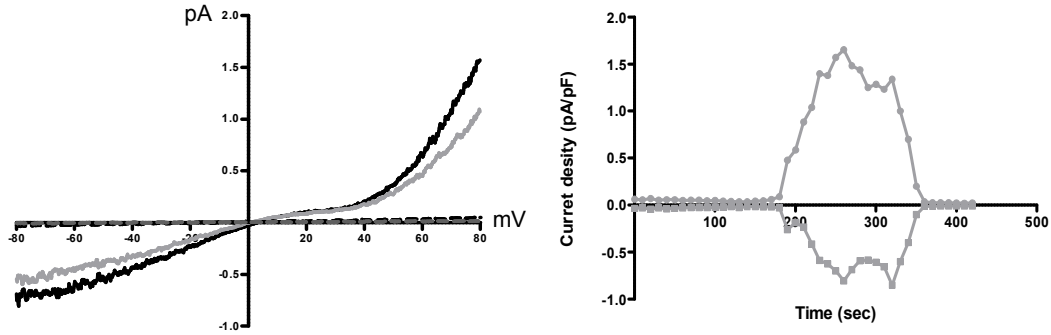

Figura 4: Representación registros patch clamp

13. Aislamiento y cuantificación de proteínas.

13.1. Inmunoprecipitación.

La inmunoprecipitación es una técnica que nos va a permitir aislar la proteína en estudio de un lisado celular para poder analizarla de forma más específica, como investigar sus asociaciones con otras proteínas o su papel en una ruta de señalización determinada.

Las células resuspendidas en HBS (con apirasa en el caso de las plaquetas) se llevaron a una concentración de 10^8-10^9 células/mL en el caso de plaquetas y 10^7 células/mL para las MEG-01. Las suspensiones celulares se repartieron en viales con 500 µL y se trataron a 37ºC, con agonistas tanto fisiológicos como farmacológicos y con activadores o inhibidores de los distintos procesos a estudiar.

Una vez tratadas, las plaquetas se lisaron con el detergente RIPA + Na_3VO_4 + Inhibidor de proteasas, en una proporción 1:1 (500 µL células: 500 µL RIPA 2*x*, para que la concentración final de RIPA sea 1*x*) y se mantuvieron 10 minutos en hielo para evitar proteólisis, desfosforilación y desnaturalización de las proteínas (procesos que comienzan inmediatamente después del lisado, y que se detienen o al menos ralentizan al mantener las muestras a 4ºC a la vez que se añaden los correspondientes inhibidores).

REACTIVOS	Concentración (mM)	(g/L)
Tris	20	2,42
NaCl	316	18,50
Deoxicolato sódico	2 %	20,00
EGTA	2	0,76
SDS	0,2 %	2,00
Tritón X-100	2 %	20 mL

Tabla 6: Ripa 2x (pH=7,2)

Las MEG-01 se lisaron con la solución NP40 (2*x*) (Tabla 7) + Na_3VO_4 + Inhibidor de proteasas. Se dejaron en hielo 30 min y se les dio un pulso de sonicación.

REACTIVOS	Concentración (mM)	(g/L)
Tris-HCl	40	6,30
NaCl	277	16,19
Glicerol	20 %	200 mL
Nonidet	2 %	20 mL
EDTA	4	1,17

Tabla 7: NP40 (2x)

Los lisados celulares, previamente centrifugados a 16000 *g* y retirados los restos no solubilizados por el RIPA o por el NP40, se transfieren a otros eppendorf donde son incubados con anticuerpos que reconocen específicamente la proteína que queremos detectar y se une a ella. Este anticuerpo primario, a su vez, se adhiere a unas bolitas de agarosa (resina insoluble) mediante el empleo de proteína A/G que posee afinidad por la región constante del anticuerpo primario. La incubación de las muestras con el anticuerpo y las bolitas de agarosa se realiza durante toda la noche a 4ºC con agitación constante.

Tras la formación de los complejos de anticuerpo primario y bolitas de agarosa, realizamos 5 lavados de las muestras centrifugándolas a 1600 *g* durante 1 minuto. El

pellet, que contiene las proteínas objeto de estudio, se lava para poder eliminar el resto de proteínas celulares añadiéndole PBS (1*x*).

Después de la última centrifugación, se fijan las muestras con LB (1*x*) + 5 % DTT y se calientan a 70ºC durante 10 minutos para desnaturalizar las proteínas a su estructura lineal, lo que permitirá su posterior separación y análisis mediante western blotting.

REACTIVOS	Concentración (mM)	(g/100 mL)
Tris	280	3,40
Glicerol	40 %	40 mL
Azul de bromofenol	0,008 %	0,008
SDS	8 %	8

Tabla 8: Solución de Laemmli 4x (pH=7,4)

13.2. Cuantificación de proteínas.

El contenido en proteína total se determina empleando el método de Bradford (1976). Este método consiste en una técnica colorimétrica basada en la existencia de dos formas diferentes del Coomasie Brilliant Blue G-250 (una roja y otra azul), que en contacto con las proteínas el colorante pasa de color rojo a azul, pudiendo ser detectado ese cambio mediante el empleo de un espectofotómetro.

Para la determinación del contenido de proteínas en nuestras muestras y su posterior homogenización, empleamos un lector de placas ELISA (TECAM), en el que añadiremos a cada pocillo 190 µL de colorante y 10 µL de muestra, siempre que la muestra no esté muy concentrada, en tal caso se diluirá. Después de 10 minutos de incubación en oscuridad, procedemos a la lectura empleando una longitud de onda de 595 nm. Las lecturas de densidad óptica correspondientes a las muestras se compararon con una curva estándar realizada con albúmina bovina a diferentes concentraciones.

Obtenemos resultados de concentración de proteína en mg/mL y calculamos la cantidad de muestra que tenemos que añadir en cada pocillo.

13.3. Western blotting.

13.3.1. Electroforesis.

Esta técnica se desarrolló para la separación y análisis de una mezcla de proteínas que se encuentran en un material biológico, para ello, las proteínas celulares se separaron mediante electroforesis. El principio del Western blotting se fundamenta en el hecho de que las velocidades de arrastre de las proteínas (V) en un campo eléctrico dependen de la fuerza del campo eléctrico a la que se ven sometidas (E), la carga eléctrica neta de la proteína (Z) y la resistencia de fricción (F) que depende de la forma y el tamaño de la proteína. La velocidad de migración viene relacionada con estas variables por la fórmula:

$$V = \frac{E \cdot Z}{F}$$

La adición de solución de Laemmli a las muestras más la acción del calor, permiten que las proteínas se desnaturalicen en presencia de agentes como beta-mercaptoetanol que destruye los puentes disulfuro, haciéndoles perder su conformación ternaria y pasando a la conformación primaria lineal. Las proteínas se recubren con cargas netas negativas gracias al poder reductor del dodecilsulfato sódico (SDS), permitiendo que se muevan solamente en función de su tamaño, siendo las más pequeñas las que se desplacen más en el gel. Además, el Laemmli´s buffer es un medio reductor que hará que los anticuerpos se separen de las bolas de agarosa cuando se ha realizado la técnica de inmunoprecipitación. Para asegurar la completa desnaturalización de la proteína, se calentaron las muestras durante 10 minutos a 70ºC en un termobloque y se centrifugaron.

Una vez obtenidas las muestras se procedió a separar las proteínas en función de su tamaño, usando para ello un gel separador de diferente porcentaje de acrilamida dependiendo de la proteína de estudio.

Las muestras de proteínas se depositan en los pocillos creados en el gel de carga (añadiendo un volumen apropiado para que exista la misma cantidad de proteína en cada pocillo, calculado a partir de los resultados obtenidos con la técnica de

Bradford). Este gel contiene una concentración de acrilamida y bisacrilamida muy pequeña (4 %) que asegura la migración de todas las proteínas compactadas en el frente de migración. Luego el frente de proteínas migrará por el gel separador, quedándose retenidas a diferentes alturas del gel en función del peso molecular de cada proteína. Por ello la concentración de acrilamida y bisacrilamida variará en función del tamaño de la proteína buscada. Así, a mayor concentración de acrilamida/bisacrilamida el poro formado disminuye, por lo que se separarán proteínas de pequeño peso molecular, mientras que para separar proteínas de gran tamaño se usarán bajas concentraciones de acrilamida y bisacrilamida.

REACTIVOS	10 mL (Gel 10 %)	10 mL (Gel 4 %)
Solución A	3,33 mL	1,32 mL
Solución B	2,5 mL	
Solución C	0,10 mL	0,1 mL
Solución D		2,5 mL
AMPS	5 mg	10 mg
H_20	4,067 mL	6,07 mL
Temed	5 µL	10 µL

Tabla 9: Gel acrilamida

Solución A: 30 % de acrilamida/bis-acrilamida (Ratio 37,5:1).
Solución B: Tris 1,5 M (72,6 g en 400 mL de H_2O) pH= 8,8.
Solución C: SDS 10 % (10 g en 100 mL de H_2O).
Solución D: Tris 0,5 M (24,24 g en 400 mL de H_2O) pH= 6,8.
AMPS: Persulfato de Amonio
Temed: Catalizador que comienza la reacción de polimerización.

Para su separación, las proteínas se sometieron a un campo eléctrico de 30 mA por gel mediante el empleo del tampón de electroforesis o *running buffer* (Tabla 10). La duración de la electroforesis dependió de la velocidad de avance del frente de carga indicado por el azul de bromofenol.

REACTIVOS	Concentración (mM)	(g/L) 5*x*
Tris	124	15
Glicina	1260	77
SDS	0,5 %	5

Tabla 10: Tampón de electroforesis (pH=8,3)

13.3.2. Transferencia.

Una vez separadas las proteínas por electroforesis, éstas se transfirieron desde el gel de acrilamida hasta una membrana de nitrocelulosa, que es un soporte más estable para realizar los estudios posteriores. Para la transferencia, se sometió a las proteínas a un campo eléctrico, empleando un amperaje de 0,8 mA/cm^2 durante 2 horas, en un equipo de transferencia de proteínas semiseco que necesita una solución de transferencia o blotting buffer (Tabla 11).

REACTIVOS	Concentración (mM)	(g/L) 5*x*
Tris	25	3,03
Glicina	150	8,5
MeOH	20 %	200 mL

Tabla 11: Solución de transferencia (pH=8,3)

El contenido de MeOH de la solución de transferencia disminuye al 15 % cuando lo que nos interesa es transferir proteína de muy elevado peso molecular como por ejemplo mTOR.

13.3.3. Bloqueo.

Una vez que las proteínas han sido transferidas a la membrana, es necesario evitar las interacciones inespecíficas que pueda ocasionar la incubación con anticuerpos. Para ello, la membrana de nitrocelulosa se bloqueó con una solución de bloqueo (Tabla 12), encargada de cubrir las zonas conocidas como sitios de unión

inespecíficos. Esta solución de bloqueo contiene alta cantidad de BSA, una proteína frente a la que no reaccionarán los anticuerpos que usaremos a continuación. El bloqueo se puede realizar de dos formas: bien se deja la solución 1 hora en agitación a temperatura ambiente o bien podemos dejarla una noche completa (un mínimo de 8 horas) a 4ºC y sin agitación. El fabricante de ciertos anticuerpos recomienda resuspenderlos en leche desnatada (5 %), con lo que en la solución de bloqueo se sustituye la BSA por leche desnatada.

REACTIVOS	Concentración (mM)	g/L
Tris	25	15
NaCl	150	5
BSA	10 %	100
Tween-20	0,1 %	1mL
Azida sódica	0,02 %	0,2

Tabla 12: Solución de bloqueo (pH=8)

13.3.4. Incubación con anticuerpos.

Tras el bloqueo, la membrana se incubó con un anticuerpo primario, que reconoce de forma específica una secuencia de la proteína diana a detectar. Dependiendo del anticuerpo, los tiempos de incubación pueden variar desde 1 h a temperatura ambiente hasta toda la noche a 4ºC. Asimismo, la concentración del mismo también varía en función de la especificidad del anticuerpo (según la casa comercial). A menudo han de hacerse pruebas complementarias para optimizar dichos parámetros.

Una vez terminada la incubación con el anticuerpo primario, éste se retiró y la membrana se lavó para retirar el exceso de anticuerpo primario que no hubiera quedado unido específicamente a la proteína. Para ello, se lavó la membrana 6 veces durante 5 minutos cada vez, con una solución salina con detergente (Tween), (TBST).

REACTIVOS	Concentración (mM)	g/L
Tris	25	25
NaCl	150	5
Tween-20	0,1 %	1 mL

Tabla 13: Solución de lavado (TBST) (pH=7,6)

A continuación, la membrana se incubó con una inmunoglobulina G (IgG) específica del tipo animal del que se obtiene el anticuerpo primario, puesto que reconocerá la fracción constante del anticuerpo primario y se unirá a ella. El tiempo de incubación de todos los anticuerpos secundarios fue de 1 h a temperatura ambiente. La concentración de anticuerpo secundario que se empleó varió entre 1:5000 y 1:10000.

Una vez finalizada la incubación del secundario, la membrana se lavó para retirar el exceso de anticuerpo secundario que no se ha unido, siguiendo el protocolo de lavado descrito anteriormente.

13.3.5. Revelado.

El anticuerpo secundario está conjugado con peroxidasa de rábano picante (HRP), que al combinarse con un reactivo comercializado por Pearce (Solución ECL), generará una reacción quimioluminiscente que será detectada por una película fotográfica con una alta sensibilidad. Al exponer la película fotográfica a esta reacción lumínica, se obtuvieron unas manchas (blot) que correspondían a la proteína buscada. También se dispuso de un escáner de membranas CDigits (Licor®) que detecta la quimioluminiscencia emitida por la membrana y obtiene una imagen digital de la misma

Estas manchas fueron analizadas con el programa informático Image J (N.I.H), expresando los resultados como diferencias en los porcentajes de densidad óptica entre las manchas de los diferentes tratamientos.

14. Tratamiento estadístico.

Los datos obtenidos en estos estudios se expresan como media ± el error estándar de la media (E.S.M.). Las curvas representativas de la movilidad de Ca^{2+}

intracelular se presentan como cambios en concentración intracelular expresados en nM·s.

Para el análisis estadístico de los resultados obtenidos se utiliza el análisis de la varianza o ANOVA de una vía, cuando los grupos experimentales a comparar son más de dos, o el test t-Student para comparar sólo dos grupos. En los experimentos en los que el análisis de la varianza resultaba significativo, se realizaron además comparaciones entre los grupos empleando según convenía el test de Dunnett, el de Bonferroni o el de Turkey.

Los resultados obtenidos se consideraron estadísticamente significativos cuando la $p < 0.05$.

RESULTADOS

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FKBP52 is involved in the regulation of SOCE channels in the human platelets and MEG 01 cells

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ABSTRACT

Immunophilins are FK506-binding proteins that have been involved in the regulation of calcium homeostasis, either by modulating Ca^{2+} channels located in the plasma membrane or in the rough endoplasmic reticulum (RE). We have investigated whether immunophilins would participate in the regulation of stored-operated Ca^{2+} entry (SOCE) in human platelets and MEG 01. Both cell types were loaded with fura-2 for determining cytosolic calcium concentration changes ($[Ca^{2+}]_c$), or stimulated and fixed to evaluate the protein interaction profile by performing immunoprecipitation and western blotting. We have found that incubation of platelets with FK506 increases Ca^{2+} mobilization. Thapsigargin (TG)-evoked, Thr-evoked SOCE and TG-evoked Mn^{2+} entry resulted in significant reduction by treatment of platelets with immunophilin antagonists. We confirmed by immunoprecipitation that immunophilins interact with transient receptor potential channel 1 (TRPC1) and Orai1 in human platelets. FK506 and rapamycin reduced the association between TRPC1 and Orai1 with FK506 binding protein (52) (FKBP52) in human platelets, and between TRPC1 and the type II IP_3R, which association is known to be crucial for the maintenance of SOCE in human platelets. FKBP52 role in SOCE activation was confirmed by silencing FKBP52 using SiRNA FKBP52 in MEG 01 as demonstrated by single cell configuration imaging technique. TRPC1 silencing and depletion of cell of TRPC1 and FKBP52 simultaneously, impair activation of SOCE evoked by TG in MEG 01. Finally, in MEG 01 incubated with FK506 we observed a reduction in TRPC1/FKBP52 coupling, and similarly, FKBP52 silencing reduced the association between IP_3R type II and TRPC1 during SOCE. All together, these results demonstrate that immunophilins participate in the regulation of SOCE in human platelets.

1. Introduction

Stored-operated Ca^{2+} entry (SOCE) is a major mechanism for conducting Ca^{2+} influx in human platelets, as well as in other non-excitable cell types [1–4]. In platelets, association of STIM1 and IP_3RII located in the membranes of internal stores, with Ca^{2+} channels expressed in the plasma membrane, like Orai1 and TRPC1, promotes SOCE [4–8]. In human platelets, several members of the canonical transient receptor potential channels, such as TRPC1, TRPC4 [6,8,9], TRPC3 and TRPC6 [6,10] have been reported to associate to Orai1; thus conforming heteromultimeric channel complexes, which have been shown to present different sensitivity to store depletion, which relies on STIM1 function [11–13].

Immunophilin family groups proteins with chaperone activity (PPIase-activity) have been classified into three main subfamilies: cyclophilins (Cyps) [14,15], that bind cyclosporin A (CsA) [14,16,17]; immunophilins (FK506-binding proteins or FKBPs) inhibited by FK506 (tacrolimus) and rapamycin (sirolimus), both structurally unrelated to cyclosporin A (CsA); and FCBPs (FK506- and cyclosporin-binding proteins) that bind both macrolides [16]. In human lymphocytes and other cell types, including human platelets, impairment of immunophilin activity evokes deregulation of intracellular Ca^{2+} homeostasis. In this sense, calstabin 12 (FKBP12.6) silencing in the mdx mice model evoked alteration of Ca^{2+} leak from the endoplasmic reticulum (ER), which resulted in a weak myocyte contraction that is characteristic of Duchene's disease [18,19]. In addition, several Ca^{2+}-handling proteins, which activity is crucial for cellular processes, have been shown to be compromised in the presence of antagonists of the immunophilin subfamily, such as cyclophilins. Treatment of cells with these antagonists impaired, for instance, the activity of the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) in human platelets

Abbreviations: Cyps, cyclophilins; FKBPs, immunophilin; FKBP52, FK506 binding protein (52); ER, rough endoplasmic reticulum; SERCA2b, sarco-endoplasmic Ca^{2+}-ATPase isotype 2b; IP_3R II, inositol 1,4,5-trisphosphate receptor; TRPC, transient receptor potential channel; CsA, cyclosporin A; FK506, tacrolimus; PPIase, peptidyl-prolyl isomerase activity; CN, calcineurin; CaM, calmodulin; Rapa, rapamycin; Cyp, cypermethrin; $[Ca^{2+}]_c$, cytosolic calcium concentration; TG, thapsigargin; PBS, phosphate-buffered saline; HBS, HEPES-buffered saline; BSA, bovine serum albumin; Thr, thrombin; SiRNA FKBP52, small interfering RNA of FKBP52; ShRNA TRPC1, short hairpin RNA of TRPC1

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[20] and other cell types [21], and PMCA4C or calcineurin (CN) [22,23]. Finally, there is scarce information regarding the participation of immunophilins in SOCE, or whether they associate with SOC channels [24,25]. Hence, taking into account that some FKBP subfamily members regulate the permeability of intracellular Ca^{2+} channels, such as ryanodine receptors and IP_3 receptors [19,23], here we address the role of FKBPs in SOCE in human platelets and MEG01.

2. Material and methods

2.1. Materials

Apyrase (grade VII), aspirin, thrombin (Thr), dithiothreitol (DTT), thapsigargin (TG), sodium dodecyl sulphate (SDS), ionic detergent tween 20 (Tween-20), cypermethrin, ECL reagents and bovine serum albumin (BSA), rabbit anti-Orai1 antibody (c-terminal) were from Sigma (Madrid, Spain). FK506, cyclosporin A and rapamycin were from Selleck Chemical® (Huston, Texas, U. S. A). Protein A-agarose was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-hTRPC1 antibody, Anti-CN (PP2B) antibody, anti-FKBP52 antibody, anti-IP_3R type II antibody and SiRNA against FKBP52 were from (SantaCruz biotechnology®). ShRNA against TRPC1 was kindly provided by Dr. Ambudkar. MEG 01 culture medium RPMI, streptomycin/penicillin and other elements required for MEG 01 cell culture and protein silencing (KIT-C Amaxa) were purchased from LONZA®. Horseradish peroxide-conjugated anti-rabbit or anti-mouse IgG antibodies, hyperfilm ECL and molecular weight markers were from GE Healthcare UK Ltd (Chalfont St. Giles, UK). All other reagents were of analytical grade.

2.2. Platelet preparation

Fura-2-loaded platelets were prepared as described previously [2–4]. Briefly, blood was obtained from healthy drug-free volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700×*g* and then aspirin (100 μM) and apyrase (40 μg/mL) were added. For intracellular Ca^{2+} concentration measurement, the platelet-rich plasma was incubated at 37°C with 2 μM of fura-2/AM for 45 min. Platelets were collected by centrifugation at 350×*g* for 20 min, then resuspended in HEPES-buffered saline (HBS), and finally subjected to the appropriate stimulation protocol as required. HBS contains (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45 and supplemented with 0.1% w/v bovine serum albumin and 40 μg/mL apyrase.

2.3. Cell culture and protein silencing procedure

The human megakaryoblastic cell line, MEG 01 cells, was obtained from ATCC (Manassas, VA, USA) and cultured at 37 °C with a 5% CO_2 in RPMI media, supplemented with 10% fetal calf serum, 2 mM L-glutamine and a cocktail of penicillin and streptomycin as recommended by the supplier. Cells were transiently transfected with SiRNA against FKBP52, or with ShRNA against TRPC1, and with both ShTRPC1 and SiFKBP52 simultaneously, using the kit-C and Amaxa Nucleofection system®, and following the manufacturer's instructions which in our hand have been previously shown to obtain efficient results [26]. Transfected MEG 01 cells were used 72 h after transfection upon ensuring by western blotting that targeted protein expression was efficiently reduced.

2.4. Measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$)

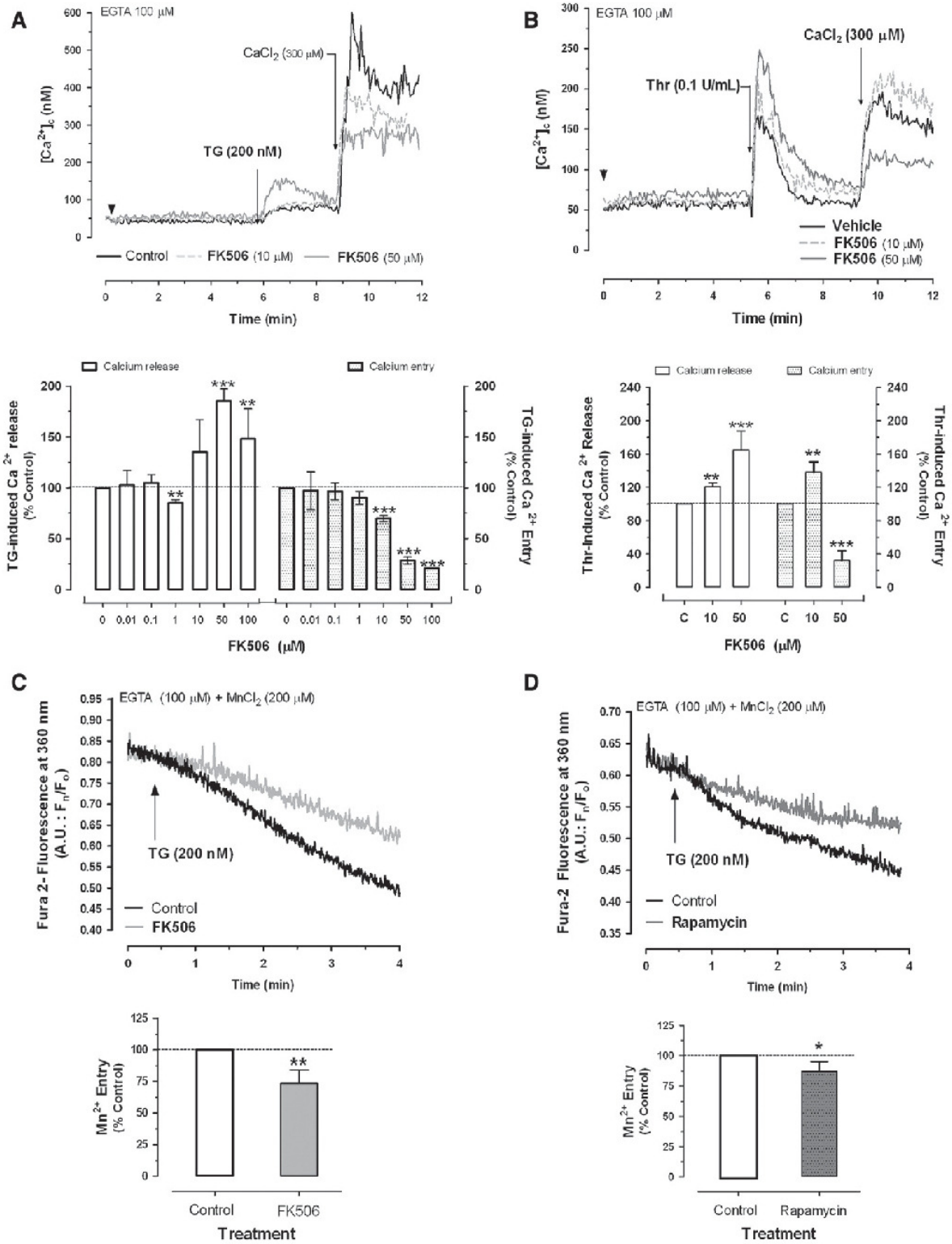
Fluorescence was recorded from 2 mL aliquots of magnetically stirred platelet suspension (10^8 cells/mL) at 37 °C using a spectrophotometer (Cary Eclipse, Varian, Madrid) with excitation wavelengths of 340 and 380 nm and emission at 510 nm. Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz [27]. Alternatively, MEG 01 cells were incubated for 30 min with 2 μM of fura-2/AM at room temperature and then centrifuged for 2 min at 100×*g*, and resuspended in fresh HBS medium supplemented with 50 μM of $CaCl_2$. MEG 01 cells were then transferred to a perfusion chamber that was placed under an inverted microscope. Changes in fura-2 fluorescence as result of changes in the $[Ca^{2+}]_c$ in MEG 01 cells were monitored using a single-cell configuration equipment and were processed using Aquacosmos software (Hamamatsu ®). Data are expressed as change in fluorescence after the addition of different stimulus (F_n) divided by fluorescence emitted by the cells under resting conditions (F_0). Additionally, Mn^{2+}-induced quenching of fura-2 fluorescence excited at 360 nm was used as a surrogate for monitoring Ca^{2+} entry, since both cations share the same channels and mechanisms for entering platelets, as previously described [28]. Data in the histogram are expressed as percentage of the changes in the decay rate at 360 nm evoked by fura-2-fluorescence quenching of Mn^{2+}, after addition of the stimulus (F_n) and compared to control non-stimulated cells (F_0).

2.5. Immunoprecipitation and western blotting

The immunoprecipitation and western blotting were performed as described previously [2–4]. Briefly, 250 μL aliquots of platelet suspension (10^9 cells/mL), MEG 01 cells wt (10×10^6 cells/mL) and MEG 01 cells transfected with the FKBP52 Si RNA or TRPC1 Sh RNA, or both RNAs, were then stimulated and lysed by mixing with an equal volume of lysis buffer, RIPA at pH 7.2, that contains; 316 mM NaCl, 20 mM Tris, 2 mM EGTA, 0.2% SDS, 2% sodium deoxycholate, 2% triton X-100, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 100 μg/mL leupeptin and 10 mM benzamidine. Aliquots of platelet and MEG 01 cell lysates (500 μL) were immunoprecipitated by incubating cells with 2 μg/mL of anti-IP_3R type II, anti-FKBP52, anti-CN and 25 μL of protein A-agarose, overnight at 4 °C and placed in a rocking platform. The immunoprecipitates were resolved by 10 % SDS-PAGE and separated proteins were electrophoretically transferred onto nitrocellulose membranes for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA or skimmed milk in tris-buffered saline with 0.1 % Tween 20 (TBST) to block residual protein binding sites. Immunodetection was achieved using the anti-TRPC1 polyclonal antibody diluted 1:200 in TBST (BSA), anti-FKBP52 diluted 1:200 in TBST (skimmed milk) and incubated for 2 h, or using an anti-Orai1 antibody incubated overnight at 4 °C and diluted 1:1000 in TBST (BSA). The primary antibody was removed and blots were washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted from 1:2500 to 1:10000 in TBST (BSA or skimmed milk), depending of the primary antibody used, and then exposed to enhanced chemiluminescence reagents for 4 min. Blots were then exposed to a photographic film. The density of bands on the film was measured using the Image J free software from NIH. Stripping of the membranes and reprobing using anti-IP_3RII, anti-CN, and anti-FKBP52, was done to corroborate that similar amount of protein was loaded in each gel lane.

2.6. Statistical analysis

Analysis of statistical significance was performed using Student's unpaired *t*-test and only values with $p<0.05$ were accepted as significant.


A
EGTA 100 μM
CaCl2 (300 μM)
TG (200 nM)
[Ca2+]c (nM)
Control
FK506 (10 μM)
FK506 (50 μM)
Time (min)
B
EGTA 100 μM
CaCl2 (300 μM)
Thr (0.1 U/mL)
[Ca2+]c (nM)
Vehicle
FK506 (10 μM)
FK506 (50 μM)
Time (min)
Calcium release
Calcium entry
TG-induced Ca 2+ release (% Control)
TG-induced Ca 2+ Entry (% Control)
FK506 (μM)
Calcium release
Calcium entry
Thr-induced Ca 2+ Release (% Control)
Thr-induced Ca 2+ Entry (% Control)
FK506 (μM)
C
EGTA (100 μM) + MnCl2 (200 μM)
Fura 2-Fluorescence at 360 nm (A.U.: Fn/Fo)
TG (200 nM)
Control
FK506
Time (min)
Mn2+ Entry (% Control)
Control
FK506
Treatment
D
EGTA (100 μM) + MnCl2 (200 μM)
Fura-2 Fluorescence at 360 nm (A.U.: Fn/Fo)
TG (200 nM)
Control
Rapamycin
Time (min)
Mn2+ Entry (% Control)
Control
Rapamycin
Treatment

3. Results

3.1. FK506 alters Ca^{2+} leakage and active Ca^{2+} release from intracellular stores induced by agonists in human platelets

Treatment of human platelets in a Ca^{2+}-free medium with the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) blocker, thapsigargin (TG) or with the physiological agonist thrombin (Thr), resulted in sustained or transient increase in $[Ca^{2+}]_c$, respectively (Fig. 1A–B). As shown in Fig. 1A, incubation of platelets with the immunophilin antagonist, FK506, increased TG- and Thr-evoked Ca^{2+} release from the intracellular pools. The effect of FK506 pretreatment for 5 min was dose-dependent. Thus, we found that TG-evoked Ca^{2+} release was increased by 36.6 ± 17.8% using 10 μM of FK506 (Fig. 1A, dark-gray dotted line; $p<0.05$; n = 6) and 85.4 ± 11.6% in cells incubated with 50 μM (Fig. 1A, dark-gray solid trace; $p<0.001$; n = 6). We have also found that in human platelets stimulated with TG, EC_{50} of FK506 was 9.75 μM. Similarly, active Ca^{2+} release from the stores evoked by thrombin (Thr, 0.1 U/mL) was enhanced in the presence of FK506 (20.63 ± 4.9% using 10 μM; Fig. 1B, dark-gray dotted line; $p<0.05$; n = 6; and 65.02 ± 22.4% in the presence of 50 μM; Fig. 1B, dark-gray solid line; $p<0.01$; n = 6).

3.2. FK506 alters TG- and Thr-induced Ca^{2+} entry in human platelets

As shown in Fig. 1A–B, Ca^{2+} entry induced by incubating human platelets either with TG or Thr, was altered in platelets previously incubated for 5 min with FK506. Incubation of human platelets with increased concentration of FK506 (0.01–100 μM) evoked a dose-dependent reduction in TG-evoked SOCE. IC_{50} of FK506 in SOCE observed was 18.5 μM. Furthermore, FK506 also altered Thr-evoked SOCE. In this case, different results were obtained depending of the FK506 dose used, thus platelets incubation with 10 μM of FK506 enhanced by 38.2 ± 12.5 % in Thr-evoked SOCE ($p<0.01$; n = 6), while 50 μM FK506 induced a significant attenuation of 67.8 ± 11.2% in Thr-evoked Ca^{2+}-entry ($p<0.001$; n = 8).

In order to further explore whether FK506 alters SOCE, experiments in the absence of extracellular Ca^{2+} (EGTA 100 μM was added), but in the presence of extracellular $MnCl_2$ (200 μM) were performed. Mn^{2+} extrusion from the cellular cytosol through PMCA and Na^+/Ca^{2+} is very low, as previously reported, therefore by using this cation we excluded possible interferences with Ca^{2+} extrusion mechanisms during cation entry monitoring [29,30]. As observed in Fig. 1C, incubation of platelets with 50 μM of FK506 significantly reduced TG-evoked Mn^{2+} entry by 26.7 ± 10.7% ($p<0.01$; n = 6).

3.3. Immunophilins regulate SOCE by a calcineurin (CN)-independent signaling pathway

Immunophilin inhibitors have been commonly referred in the literature as specific inhibitors of calcineurin, moreover their actual inhibitory effect that is based on a previous binding and subsequently inhibition of FKBPs, like FKBP12 or FKBP52, has remained unconsidered [22,23] [31]. However, it is widely accepted in the literature that FK506, upon binding to its target immunophilins, acquire a three-dimensional conformation that adapts inside the functional domain of CN inhibiting its activity [32]. Rapamycin is another immunophilin subfamily antagonist that upon complexing to FBKPs, reduces mTOR activity, without affecting CN activity; therefore, we have used rapamycin to ascertain whether CN is involved in SOCE. As shown in Fig. 1D, rapamycin treatment reduced Mn^{2+} entry by 13.1 ± 8.2 % ($p<0.05$; n = 6). Impairment of SOCE by rapamycin was then corroborated by performing experiments in the presence of 300 μM of $CaCl_2$. Under these experimental conditions, rapamycin administration significantly reduced TG-evoked Ca^{2+}-entry by 19.5 ± 9.4% (Fig. 2A; $p<0.05$; n = 6), and contrary to FK506 the Ca^{2+} leak from stores resulted in inhibition by 11.1 ± 4.8% ($p<0.05$; n = 6).

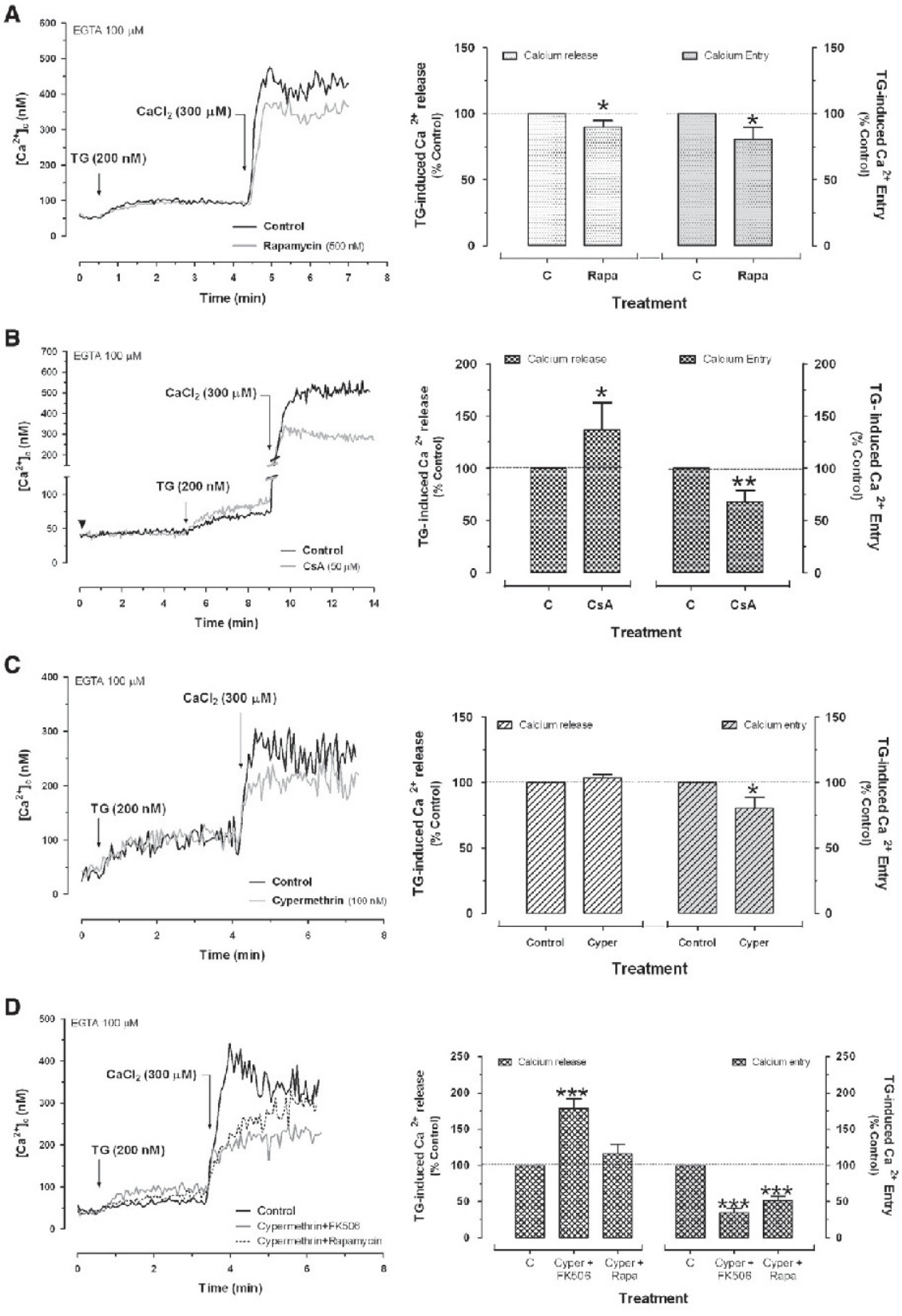
Inhibition of the other main immunophilin subfamily, cyclophilins, by incubating platelets with CsA (50 μM) for 5 min at 37 °C, also reduced TG-activated SOCE by 32.4 ± 11.0% (Fig. 2B; $p<0.01$; n = 6), while an increase on TG-evoked Ca^{2+} release of 36.6 ± 25.9% was observed ($p<0.05$; n = 6), perhaps due to more rapid inhibition of SERCA2b activity, as previously described [20].

Alternatively, incubation of human platelets with the specific CN inhibitor, cypermethrin (100 nM) for 30 min, whose action mechanism is different from that reported for immunophilin antagonist, reduced TG-induced SOCE by 14.0 ± 7.8% (Fig. 2C; $p<0.05$; n = 6), but without altering Ca^{2+} release from the stores. Finally, demonstration of a CN-independent role of immunophilins in SOCE activation is presented in Fig. 2D, where human platelets were incubated for 30 min with cypermethrin (100 nM) + rapamycin (500 nM), or with cypermethrin (100 nM) + FK506 (50 μM; for the latest 5 min previous to $[Ca^{2+}]_c$ determination). As demonstrated by comparing Fig. 2C and D, combined treatment with CN antagonist and immunophilin antagonists evoked bigger SOCE inhibition than cypermethrin alone (51.7 ± 5.8 % and 35.0 ± 5.9%, respectively; $p<0.001$; n = 4). As shown in Fig. 2D, only in the presence of FK506 a significant increase in TG Ca^{2+} release was observed (212.5 ± 20.8%, light-gray traces; $p<0.001$; n = 4). Hence, our results indicate that despite CN is regulating SOCE, an alternative regulatory pathway that relies on immunophilin activity is also present in human platelets.

3.4. Immunophilins are required for complexing Ca^{2+} channels during SOCE activation in platelets

Studies on the role of TRPC1 in SOCE are controversial. While studies in platelets from KO mice where TRPC1 reported no differences in SOCE activation [33], several others independent groups have demonstrated that TRPC1, alone or coupled to Orai1 and IP_3R type II, is an important elements for the activation and maintenance of SOCE in platelets and other cell types [34–36]. Here, we have evaluated the role of FKBPs in the formation of these proteins complexes by performing immunoprecipitations. Briefly, human platelets were treated at 37 °C for 5 min with FK506 (50 μM) or left untreated, and then stimulated in a Ca^{2+}-free medium for 1 min with Thr (0.1 U/mL). As shown in Fig. 3A, stimulation of human platelets with Thr (0.1 U/mL) evokes the formation of TRPC1/type II IP_3R complexes. The generation of this complex was attenuated by 40.2 ± 21.7% ($p<0.05$; n = 4) in platelets previously incubated for 5 min with FK506 (50 μM; Fig. 3A and histogram below). Additionally, in platelets treated for 30 min with the vehicle or rapamycin (500 nM) and then stimulated with Thr (Fig. 3B), the coupling between both elements was reduced by 27.9 ± 2.5% ($p<0.01$; n = 4). Furthermore, incubation of human platelets with FK506 (50 μM) equally impaired the association between TRPC1 and CN

Fig. 1. Effect of FK506 on cytosolic calcium homeostasis induced by TG and thrombin in human platelets. (A–B) Human platelets were suspended in a Ca^{2+}-free medium (100 μM of EGTA was added as indicated by arrowhead), and preincubated for 5 min at 37 °C in the absence (solid black traces) or presence of increased concentrations (0.01–100 μM) of FK506 (light-doted and dark solid-gray traces, respectively). Cells were then stimulated with TG (200 nM; A) or Thr (0.1 U/mL; B) and 3 min later 300 μM of $CaCl_2$ was added to extracellular medium to visualize calcium entry. (C–D) Platelets were suspended in calcium free-HBS and incubated for 5 min with FK506 (50 μM; C, gray trace) or for 30 min with rapamycin (500 nM; D, gray trace) or their respective vehicles (black traces) and upon addition of $MnCl_2$ (200 μM) platelets were stimulated with TG to enhance the opening of SOCE channels. Changes in fura-2 fluorescence were monitored using the 340/380 nm ratio and calibrated in terms of $[Ca^{2+}]_c$. Alternatively, Mn^{2+} quenching properties over 360 nm wavelength of fura-2 is used as surrogated of Ca^{2+} to monitor SOCE activation which is represented as fluorescence initial (F_0) divided by fluorescence upon TG stimulation. Histograms represent mean ± SEM of six to eight separate experiments and they are presented as percentage of control. *, **, and *** represent $p<0.05$, $p<0.01$ and $p<0.001$, with respect to control platelets.


A
EGTA 100 μM
CaCl2 (300 μM)
TG (200 nM)
Control
Rapamycin (500 nM)
[Ca2+]c (nM)
Time (min)
Calcium release
Calcium Entry
TG-induced Ca 2+ release (% Control)
TG-induced Ca 2+ Entry (% Control)
C
Rapa
Treatment
B
EGTA 100 μM
CaCl2 (300 μM)
TG (200 nM)
Control
CsA (50 μM)
Time (min)
Calcium release
Calcium Entry
C
CsA
Treatment
C
EGTA 100 μM
CaCl2 (300 μM)
TG (200 nM)
Control
Cypermethrin (100 nM)
Time (min)
Calcium release
Calcium entry
Control
Cyper
Treatment
D
EGTA 100 μM
CaCl2 (300 μM)
TG (200 nM)
Control
Cypermethrin+FK506
Cypermethrin+Rapamycin
Time (min)
Calcium release
Calcium entry
C
Cyper + FK506
Cyper + Rapa
Treatment

induced by Thr in a 53.9 ± 7.7%, respect to platelets non-incubated with FK506 but stimulated with Thr (Fig. 3C and histogram below; $p<0.05$; n = 4). As expected, treatment with rapamycin (500 nM) for 30 min was without effect (Fig. 3D and histogram below; $p<0.05$; n = 4). Furthermore, we have found that treatment of platelets with FK506 and rapamycin prevented the increase in the association between FKBP52 and TRPC1 in platelets stimulated with Thr (0.1 U/mL), which in cells non-treated with FK506 was enhanced by 22.8 ± 16.9% (Fig. 3E;*, $p<0.05$; n = 4).

On the other hand, Orai1 have recently been described in human platelets and megakaryocytes as a key element during the activation of SOCE [11,37–39]. Hence, we have evaluated whether immunophilins might be associated to Orai1 during calcium entry in human platelets. As shown in Fig. 4A, we have found that in resting conditions FKBP52 complexed to Orai1, and upon platelet stimulation with Thr, FKBP52/Orai1 interaction was enhanced by 57.2 ± 34.7% ($p<0.05$; n = 4). As reported above for the TRPC1/FKBP52 complex, platelets preincubation for 5 min with FK506 (50 μM) evoked a significant reduction in FKBP52/Orai1 complex, both in resting conditions (25.5 ± 10.1%; ***, $p<0.001$; n = 4), and upon stimulation with Thr (98.3 ± 17.6%; $$$, $p<0.001$; n = 4). Similarly, incubation with rapamycin (500 nM) for 30 min reduced by 86.3 ± 9.5% the coupling between FKBP52 and Orai1 induced by Thr in these cells ($$$, $p<0.001$; n = 4).

3.5. FKBP52 is required for SOCE activation in human platelets and MEG 01 cells

In order to corroborate the involvement of FKBP52 in SOCE, we have silenced FKBP52 in MEG 01 cells, which is a megakaryoblastic cell line that in presence of thrombopoietin and under the adequate experimental conditions have been derived into platelets [40]. It has been reported that MEG 01 cells express most of the elements that participates in calcium homeostasis and in particular that have been previously described participating in SOCE activation mechanism in human platelets [41–45]. Hence, by using Amaxa® nucleofector and Si RNA FKBP52 (2 μg/mL), we were able to reduce efficiently the expression of FKBP52 upon 72 h of transfection procedure, as revealed Western blotting (see Fig. 5A left-hand side image). Furthermore, we have also silenced TRPC1 alone using a Sh RNA TRPC1 (see Fig. 5A right-hand side image). As shown in Fig. 5B, single-cell imaging experiments using MEG 01 cells with fura-2 revealed that silencing of FKBP52, TRPC1 or both reduced significantly TG-evoked SOCE by 25.4 ± 10.1%; $p<0.05$; n = 4), 33.0 ± 4.0% ($p<0.01$; n = 4) and 46.2 ± 18.3% ($p<0.01$; n = 4), respectively. Transfection itself was without effect on Ca^{2+} accumulation inside the intracellular stores, as demonstrated by the fact that irrelevant alteration in calcium leak in response to TG is found upon comparing the areas under the curves as described in the Material and methods section (Fig. 5B and C).

Additionally, MEG 01 cells transfected with Si RNA FKBP52 and incubated for 5 min with FK506 showed reduced TG-evoked Ca^{2+}-release (61.6 ± 14.1%; $p<0,001$; n = 4; Fig. 5C) and SOCE (32,3 ± 21.3%; $p<0,01$; n = 4; Fig. 5C).

Finally, we have observed that in MEG 01 cells, FKBP52/Orai1 complex is detected under resting conditions and it is significantly enhanced by 35.1 ± 15.1% upon stimulation with TG for 1 min (Fig. 4B; $p<0.01$; n = 6), and as occurred in platelets, preincubation with FK506 (50 uM for 5 min) significantly reduces FKBP52/Orai 1 complex generated by stimulating with TG (200 nM) (61.9 ± 28.4%; $p<0.01$; n = 6). Additionally, we have observed lack or very low association between TRPC1-FKBP52 in MEG 01 cells under resting condition, but a very significant TRPC1/FKBP52 coupling was found upon SOCE stimulation with TG (51.5 ± 20.2%; $p<0.05$; n = 4; Fig. 5D). Treatment of MEG 01 cells with 50 μM FK506 for 5 min reduced by 72.8 ± 17.1% the TRPC1/FKBP52 association ($p<0.001$; n = 4). We further explore the role of FKBP52 in generation of SOCE-associated complexes, by silencing FKBP52, which reduced by 127.2 ± 40.7% TG-evoked type II IP_3R/TRPC1 coupling as compared to controls (Fig. 5E; $p<0.001$; n = 4), thus revealing a direct role in the regulation of TRPC1 function in MEG 01, as occurred in platelets.

4. Discussion

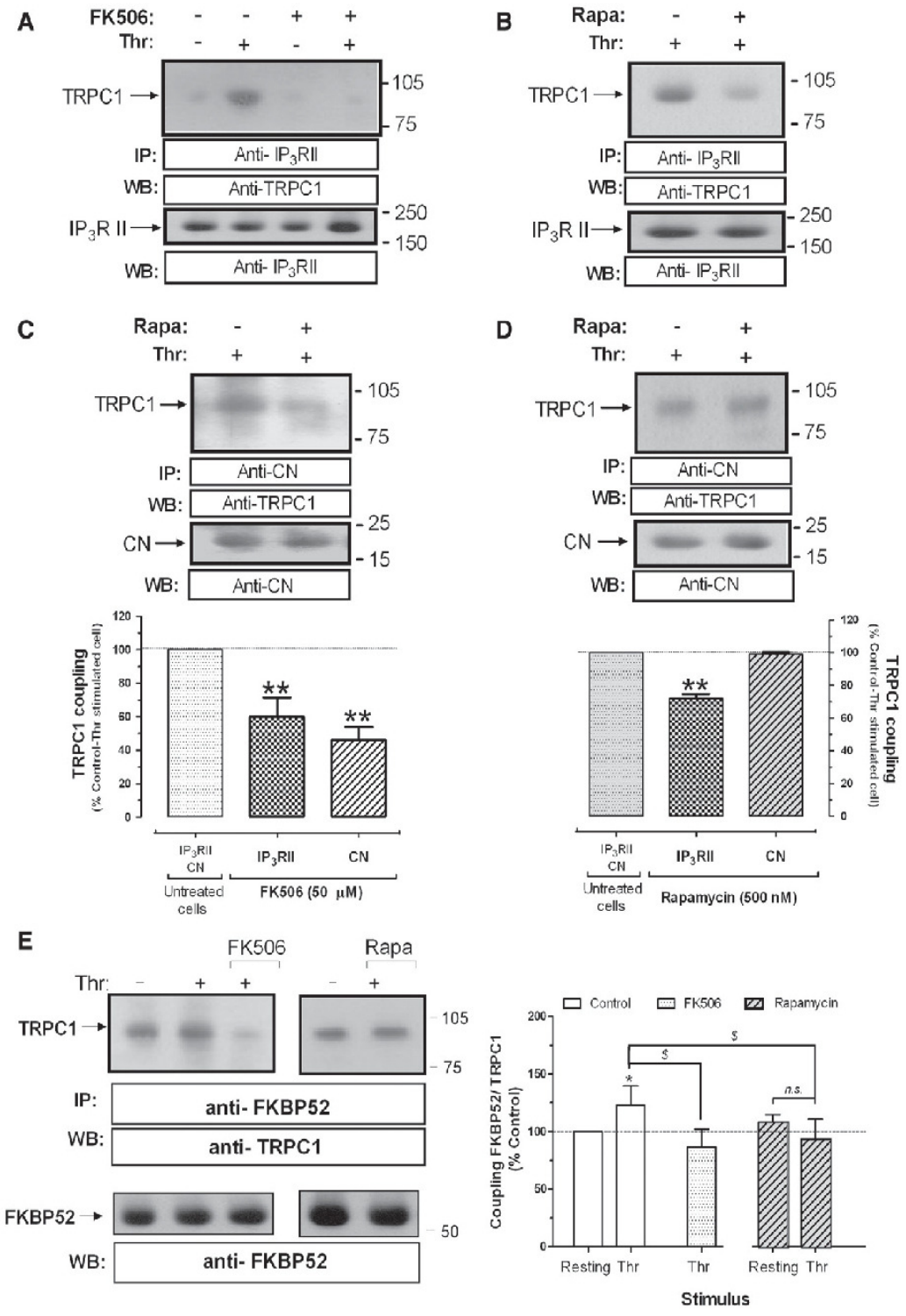
Immunophilin inhibitors have been often considered as specific CN activity antagonists [22,23,31], since immunophilins-FK506 and CsA-immunophilins complexes inhibits CN [46], despite the fact that FK506 or CsA specifically target the peptidyl-prolyl isomerase active domain of immunophilins. Hence, in the present study we have evaluated the possible participation of immunophilins in intracellular Ca^{2+} homeostasis in human platelets independently of CN.

The increasing effect induced by FK506 on Ca^{2+} release may be attributed to the activity of CaM- and CN-dependent phosphorylation/dephosphorylation mechanisms of ER-resident Ca^{2+} channels as previously reported in other cells types [47–50]. However in human platelets, as observed in Fig. 3D, despite CN might participate in the regulation of Ca^{2+} leak from the stores, a CN independent Ca^{2+} leak evoked by FK506 is demonstrated, by using cypermethrin, a CN antagonist, which was reported without effect in Ca^{2+} leak. Under these experimental conditions, FK506 was still able to further increase Ca^{2+} leakage in platelets stimulated with TG. Nevertheless, a regulatory role for CN on Ca^{2+} leakage is probably present in human platelets, due to the significant differences observed in Ca^{2+} leakage between cell treated with cypermethrin and rapamycin and compared with platelets treated with rapamycin alone (see Fig. 3A vs D). In this sense, CN-dependent mechanisms might be also present in MEG 01 cells, where treatments of Si RNA FKBP52 transfected cells with FK506 showed a significant reduction in the Ca^{2+} leak upon stimulation with TG, indicative of a role for FKBP52 Ca^{2+} leak. Furthermore, participation of other immunophilins cannot be rule out.

Our results concerning SOCE regulation by immunophilins are consistent with previous observations in other cell models [31,51]. Nevertheless, other authors have considered a unique retrograde CN-dependent mechanism for regulating Ca^{2+} entry activation. The mechanisms currently proposed suggest that only in conditions where $[Ca^{2+}]_c$ reaches a critical value, CN may evoke the activation of non-capacitative arachidonic acid-dependent Ca^{2+} channels (ARC). Furthermore, these authors contemplate the possibility of an alternative participation of immunophilins, different from the CN pathway in HEK293 cells [31]. In human platelets, as shown in Fig. 2, an alternative CN-independent pathway might also coexist, since as we have shown, FK506 has a dual effect in SOCE depending on the concentration used (see Fig. 1), while rapamycin incubation also inhibits SOCE, and this effect has been linked to inhibition of the mTOR (AKT/PKB) pathway instead of the classically proposed CN mechanism [17,52]. The latest observations in MEG 01 and platelets suggest that FKBP52, or other immunophilin activities, like FKBP12, are impaired by complexing with their target drugs, such as FK506 or rapamycin, being enough to alter SOCE in our cellular lineage.

Fig. 2. Immunophilins participate in SOCE activation by CN-dependent but also CN-independent signaling pathways. Fura-2 loaded platelets were suspended in HBS and subsequently incubated at 37 °C for 30 min with either rapamycin (500 nM; A) or cypermethin (100 nM; C); or a combination of cypermethrin + rapamycin (D, dark-doted trace) and cypermethrin + FK506 (50 μM; D, light-solid gray trace). Alternatively, platelets are incubated for 5 min with cyclosporin A (CsA, 50 μM; B). Once incubation times with the different conditions was over, platelets were stimulated with TG (200 nM) in a calcium free-HBS (EGTA 100 μM was added as indicated the by arrowhead) and 4 min later $CaCl_2$ (300 μM) was added to visualized calcium entry. Changes in fura-2 fluorescence were monitored using the 340/380 nm ratio and calibrated in terms of $[Ca^{2+}]_c$. Traces are representative of four to six independent experiments. *, **, and *** represents $p<0.05$, $p<0.01$ and $p<0.001$, with respect to control platelets.

Despite that other groups have presented evidences against the participation of TRPCs and in particular TRPC1 in SOCE, using protocols that include TRPC1-knockout mice model [33], here we show that in MEG 01 cells TRPC1 silencing has a clear negative effect on SOCE, thus demonstrating that, as previously reported in human platelets and in several other cell types, TRPC1 has an important



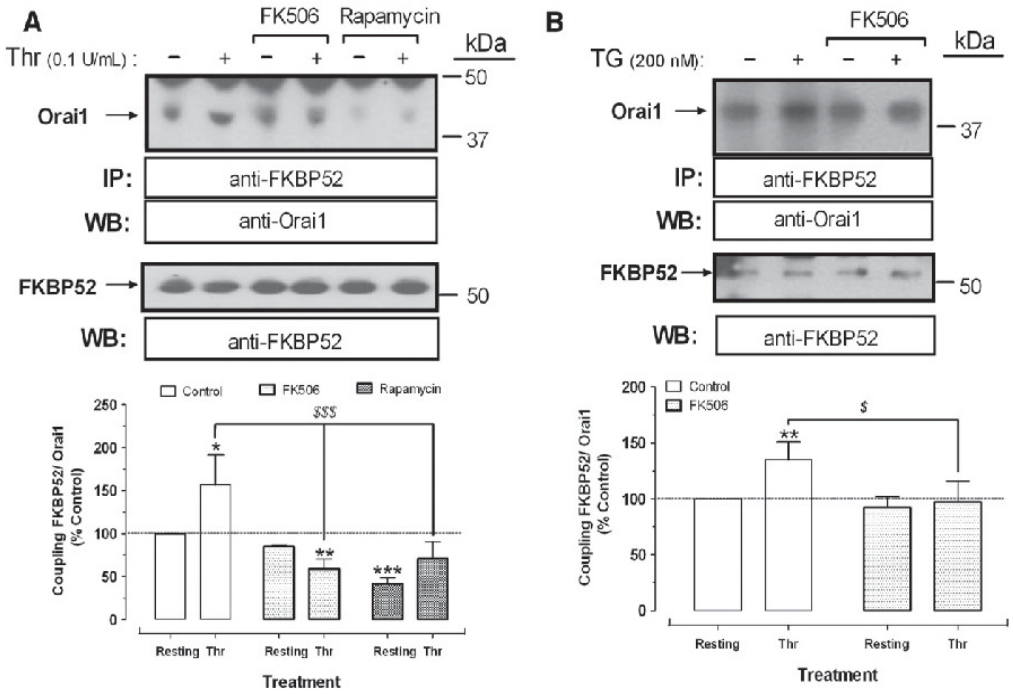


Fig. 4. Platelets treatment with FK506 and rapamycin impairs FKBP52 association to Orai1 in human platelets and MEG 01 cells. Human platelets (A) and MEG 01 (B) suspended in free-Ca^{2+} medium (EGTA, 100 μM) were incubated for 5 min at 37 °C with FK506 (50 μM) or for 30 min with rapamycin (500 nM) as indicated. Cells were then stimulated for 1 min with Thr (0.1 U/mL, A) or TG (200 nM; B) to induce Ca^{2+} entry, and then they were fixed by mixing for 10 min with equal volume of ice-cold RIPA. FKBP52 was immunoprecipitated from platelet lysates by incubating overnight at 4 °C with 2 μgr/mL of specific anti-FKBP52 antibody as described under Material and methods. Western blotting was done by incubation with a specific anti-Orai1 antibody diluted 1:1000 in blocking buffer overnight. Reproving of the membranes was done using the specific anti-FKBP52 antibody for 2 h diluted 1:200 in blocking buffer containing skimmed milk. Images shown are representative of four independent experiment and histograms represents the percentage of coupling as compared to control and expressed as means and standard errors. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$, as compared to untreated resting cells.

role in SOCE [34–36]. Experimental data, obtained by our group and others, suggest that TRPC1 is a relevant element of the macromolecular complex newly generated during SOCE [6,11,53].

Recently studies have described by using nuclear magnetic resonance (NMR) exchange spectroscopy, a region within TRPC1 structure, which has been designated as TRP box 2. This region has been described as a suitable target region for the immunophilin-PPIase activity. It has been also suggested that after conformational changes induced by FKBP52 or FKBP12, consisting on isomerization of the proline residues found within this target region, TRPC1 would be unrecognized by Homer. Homer has recently been described as a structural element that would support the complexes of TRPC1 during SOCE activation. On the other hand, it has also being involved in SOCE inhibition due to its complexing to an extra homer-binding domain identified in TRPC1 structure [54]. Hence, we suggest that removal of immunophilins from TRPC1 heteromultimeric complex disturbs the fragile stoichiometry of the macromolecular complex generated during activation of SOCE, being this role independently of calcineurin activity. Evidences of this role of immunophilin in the generation of the macromolecular complexes are presented in Fig. 4, where both, FK506 and rapamycin significantly block the coupling between TRPC1 and type II IP_3R. Similarly in MEG 01 cells transfected with Si RNA FKBP52, a reduction in TRPC1/type II IP_3R was also observed. Furthermore, as mentioned above, TRP regulation by physical interaction of immunophilin members has already been reported mainly linked to non-capacitative Ca^{2+} channels, such as TRPL, TRPV, and TRPC6. Here, we have shown for the very first time that FKBP52 and TRPC1 interaction occurs in human platelets and in MEG 01 cells, and how these complexes are impaired by treating human platelets with the FKBP inhibitors, FK506 and rapamycin. In other cell models, FKBP12 associates with TRPC3 and TRPC6, and FKBP52 binds to TRPC1, TRPC4 and TRPC5, hence participation of additional FKBPs during SOCE activation in human platelets cannot be excluded, since in MEG 01 where FKBP52 was silenced FK506 was still able to evoke a significant reduction of SOCE.

To our knowledge this is the first description that Orai1 interacts with immunophilin members, and this mechanism is found not only in the platelet but also in megakaryoblastic cell line, MEG 01 hence, indicating that this mechanism is not exclusive of differentiated cells like platelets.

Finally, alternative explanation for FKBP52-dependent regulatory mechanism of SOCE would involve a competitive association with the scaffolding protein homer, that has been recently shown to

Fig. 3. Immunophilins regulate the generation of the macromolecular TRPC1-complex during Thr-evoked calcium entry in human platelets. Human platelets suspended in calcium-free medium (EGTA, 100 μM) were incubated for 5 min at 37 °C with FK506 (50 μM; A, C and E) or for 30 min with rapamycin (500 nM; B, D and E), as indicated. Platelets were then stimulated for 1 min with Thr (0.1 U/mL) and lysed by mixing for 10 min with equal volume of ice-cold RIPA. Immunoprecipitation of either the type II IP_3R, CN and FKBP52 was achieved using 2 μgr/mL of specific antibodies as described under Material and methods. Western blotting using an anti-TRPC1 antibody diluted in TBST containing BSA (1:200) for 2 h was done to analyze coupling formation. Membrane reproving with the antibody used for immunoprecipitation confirmed that similar amount of protein was loaded in all gel lanes. Histograms below show the percentage of association of the different proteins with TRPC1 in Thr-stimulated platelets incubated under the different experimental conditions, and compared to platelets left untreated but stimulated with Thr. *, $p<0.05$ and **, $p<0.01$ with respect to resting untreated platelets and $, $p<0.05$ with respect to untreated but Thr-stimulated platelets. Images are representative of additional four independent experiments.

interact with TRPC1 and Orai1 and regulates SOCE in several cell types including platelets [55–58]. In this sense, in neurons it has recently been shown that homer shares a binding region with FKBP52 that is localized within the TRPC1 C-terminal domain, however the existence of such a competence has not been confirmed in human platelets yet and to our knowledge would deserved future investigation according to the relevance of the result concerning FKBP52 presented here [59].

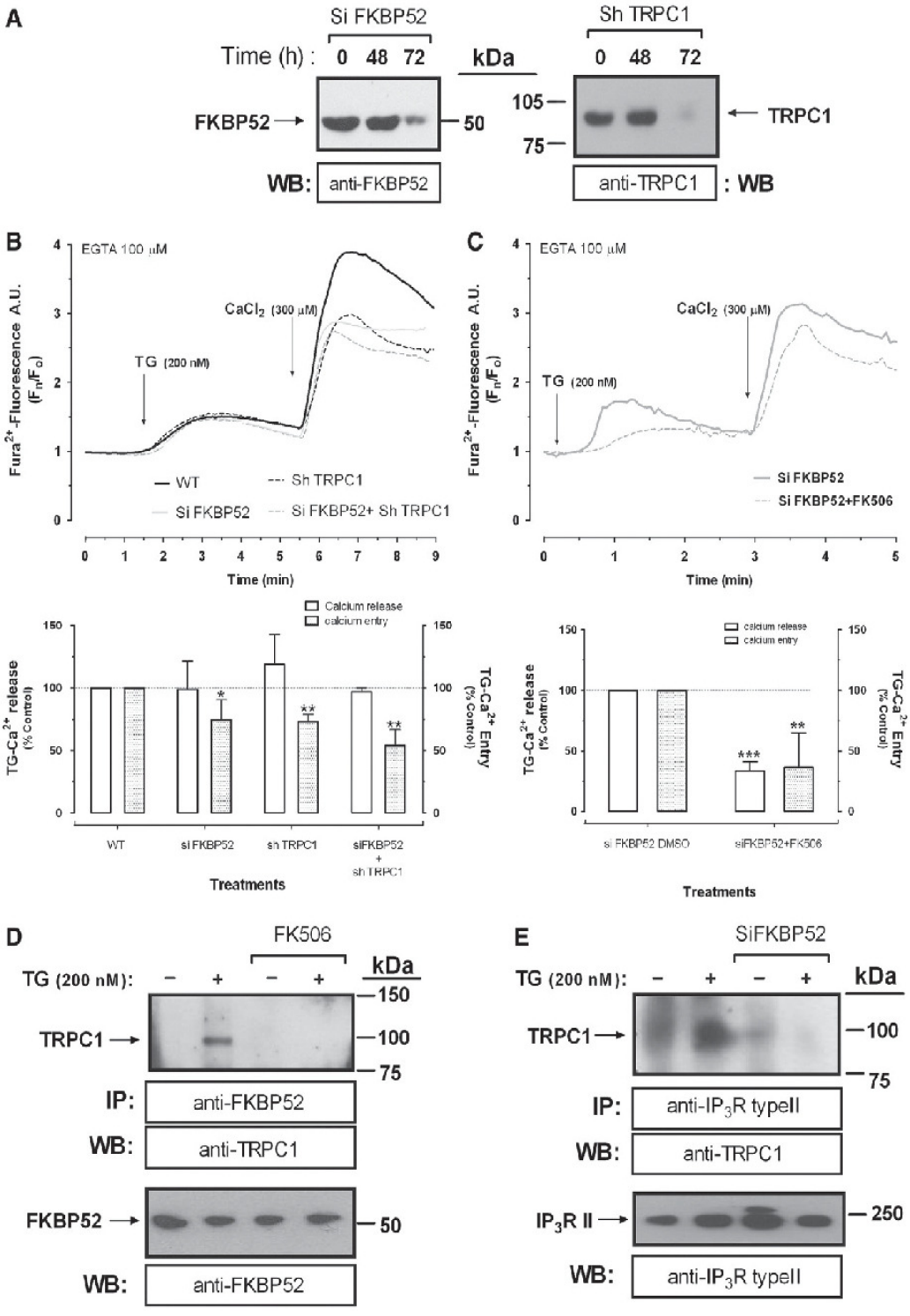


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Fig. 5. Effect of FKBP52 and TRPC1 silencing in SOCE in MEG 01 cells. (A–C, E) MEG 01 cells were transfected either with empty plasmid (WT), or with Si RNA FKBP52, Sh RNA TRPC1 or both for 72 h, or left untreated (D). (A) Evaluation of the silencing efficiency was corroborated after 72 h by Western blotting using anti-FKBP52 and anti-TRPC1 antibodies as described under Material and methods (B–C). MEG 01 cells transfected as indicated were loaded with fura-2 and additionally incubated for 5 min in absence (B) or presence of FK506 (50 μM; C). TG-evoked Ca^{2+} release and entry were monitored by using a flow chamber and single-cell imaging configuration in cells alternatively excited at 340/380. Additionally, MEG 01 cells non transfected (D) or transfected with siRNA FKBP52 (E), were left under resting condition or incubated for 5 min with FK506 (50 μM) as indicated. Cells were then stimulated with TG (200 nM) for 1 min and then lysed. Immunoprecipitation of either FKBP52 (D) or type II IP_3R (E) was achieved using the appropriate antibody, and subsequent Western blotting was performed using an anti-TRPC1 antibody as described under Material and methods section. Histograms represent means and standard errors of four to six independent experiments, while images are representatives of four independent coimmunoprecipitations. *, $p<0.05$, **, $p<0.01$. and ***, $p<0.001$ compared to MEG 01 WT, or siRNA-FKBP52 transfected MEG 01 cells treated with vehicle.

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Long-term mTOR inhibitors administration evokes altered calcium homeostasis and platelet dysfunction in kidney transplant patients

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Abstract

The use of the mammal target of rapamycin (mTOR) inhibitors has been consolidated as the therapy of election for preventing graft rejection in kidney transplant patients, despite their immunosuppressive activity is less strong than anti-calcineurin agents like tacrolimus and cyclosporine A. Furthermore, as mTOR is widely expressed, rapamycin (a macrolide antibiotic produced by *Streptomyces hygroscopicus*) is recommended in patients presenting neoplasia due to its antiproliferative actions. Hence, we have investigated whether rapamycin presents side effects in the physiology of other cell types different from leucocytes, such as platelets. Blood samples were drawn from healthy volunteers and kidney transplant patients long-term medicated with rapamycin: sirolimus and everolimus. Platelets were either loaded with fura-2 or directly stimulated, and immunoassayed or fixed with Laemmli's buffer to perform the subsequent analysis of platelet physiology. Our results indicate that rapamycin evokes a biphasic time-dependent alteration in calcium homeostasis and function in platelets from kidney transplant patients under rapamycin regime, as demonstrated by the reduction in granule secretion observed and subsequent impairment of platelet aggregation in these patients compared with healthy volunteers. Platelet count was also reduced in these patients, thus 41% of patients presented thrombocytopenia. All together our results show that long-term administration of rapamycin to kidney transplant patients evokes alteration in platelet function.

Keywords: Platelets • rapamycin • calcium • mTOR • thrombosis

Introduction

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase downstream of Akt/PKB that is activated either by intracellular second messengers or receptor-associated kinases like insulin receptors [1–4]. Two mTOR complexes have been identified and, they are designated as mTOR complex 1 (mTOR1) or mTOR complex 2 (mTOR2) [1, 5, 6], involving the proteins raptor and rictor respectively. mTOR1/2 resulting complexes regulate different downstream pathways by phosphorylation. For instance, mTOR1 impairs protein phosphatase 2A activity [7] and, contrary, it activates by phosphorylation the transcription factor activators 4EBP, HIF1α [8] and S6K [9]. In addition, mTOR2, among other functions, regulates actin cytoskeleton reorganization by up-regulating PKC, Rho and Rac activities. Furthermore, mTOR2 has been described upstream of Akt/PKB. Hence, several key intracellular pathways require mTOR activity, being mTOR particularly relevant in the cellular cycle through the control of cell growing, proliferation and apoptosis; therefore, it often represents a good target to prevent neoplasia and other illnesses [10].

Some investigations have revealed that rapamycin is neither so good nor specific mTOR inhibitor, as its administration would inhibit mTOR1 upon complexing with several members of the immunophilin family, like FKBP12 or FKBP52 [11–13]. By contrast, mTOR2 complex activity would remain unaltered in the presence of the drug, unless

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that high concentrations or chronic administration are used [1, 14]. Furthermore, rapamycin complexing to immunophilins might be involved in the activation of calcium-ATPases, like the sarcoendoplasmic Ca^{2+}-ATPase (SERCA) [15, 16], and plasma-membrane Ca^{2+}-ATPase 4C, as well as the inositol 1,4,5-trisphosphate receptor type I in neurons [17, 18].

Several new potent drugs have been designed nowadays and some mTOR inhibitors showed satisfactory immunosuppressor activity, like everolimus [19]. Nevertheless, sirolimus (rapamycin) is the therapy of election to prevent graph rejection in kidney transplant patients, where renal function has been compromised owing to previously administration of other immunosuppressors that target calcineurin, such as CsA or tacrolimus [20, 21].

Hence, we have explored here the possible side effects of two mTOR inhibitors, sirolimus and everolimus, in platelets from kidney transplant patients long-term medicated with mTOR inhibitors.

Materials and methods

Materials

Fura-2 acetoxymethyl ester (Fura-2/AM) was from Molecular Probes (Leiden, The Netherlands). Apyrase (grade VII), aspirin, bovine serum albumin (BSA), dithiothreitol (DTT), quinacrine, adenosine 5′-diphosphate (ADP) and thrombin (Thr) were from Sigma-Aldrich (Madrid, Spain). Tert-Butyl hydroquinone (TBHQ) was from Alexis (Nottingham, UK). Anti-CD62P-PE antibody, anti-CD41-a PerCP (clone HIP8) and anti-PE isotype were from Becton Dickinson Transduction Laboratories (Madrid, Spain). Anti-phospho-mTOR (Ser 2481) and anti-phospho-raptor (Ser 722) antibodies were from Millipore (Hayward, CA, USA). Anti-phospho-Akt (Thr 308) antibody was from Cell Signalling technology (Beverly, MA, USA). Horseradish peroxidase-conjugated antimouse IgG antibody was from Amersham (Buckinghamshire, UK). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, UK). All other reagents were of analytical grade.

Selection of patients, blood processing and platelet samples preparation

Kidney transplant patients and healthy volunteers were selected by the Department of Renal Transplantation of Infanta Cristina Hospital (Badajoz, Spain). Twenty nine kidney transplant patients ranging from 35 to 72 years old under sirolimus treatment (Rapamune®) administered at 1.88 ± 0.5 mg/12 hr, $n = 21$ patients) or everolimus (Certican® administered at 1.81 ± 0.3 mg/24 hr, $n = 8$ patients), and administration of mTOR inhibitor was combined with daily administration of prednisone (up to 10 mg) and healthy volunteers of similar age range were selected ($n = 6$). A similar number of men (17 patients and 3 healthy volunteers) and women (12 patients and 3 healthy volunteers) have been considered in both patients and control groups included in the present investigation. Vascular or thrombotic problems were not diagnostized either before or after transplantation proceeds. Selected patients presented at the time of the study creatinin concentration and clearance rate of 1.64 ± 0.63 (mg/dl) and 61.93 ± 25.68 (ml/min.) respectively. The blood glucose values observed in the selected patients were 95.52 ± 15.82 (mg/dl). Two patients were excluded from the results during the study mainly as they required hospitalization and further surgical intervention, hence rapamycin treatment had to be removed previous to rehospitalization. Finally, at the time of blood extraction, trough level monitored of sirolimus and everolimus was 8.59 ± 2.34 and 6.75 ± 1.27 ng/ml respectively.

Upon informative consents were given according to Helsinki's declaration, early morning blood samples were drawn by venipuncture during common patients controls (performed by qualified staff) using vacutainer tubes with 6.3 mg EDTA-K3 to prevent coagulation. The tubes and sampling procedure have been demonstrated to keep platelet size and other platelet parameters within the 180 min. after blood drawn [22]. One of the tubes extracted was used for evaluating general wellness parameters, like trough levels of sirolimus and everolimus, creatinine clearance rate, plasma creatinine concentration, platelets count and volume and blood glucose concentration. The second tube was supplemented with apyrase alone (40 μg/ml) or in combination with aspirin (100 μM), and used for platelet calcium homeostasis and granule secretion determinations. All determinations were done during the following 3–4 hr from blood extraction.

Measurement of cytosolic-free calcium concentration ($[Ca^{2+}]_c$)

Fura-2-loaded platelets were prepared as described previously [23–25]. Platelet-rich plasma obtained upon sequential centrifugation was incubated at 37°C with 2 μM fura-2/AM for 45 min. Cells were then collected by centrifugation at $350 \times g$ for 20 min. and resuspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.40 and supplemented with 0.01% w/v bovine serum albumin and 40 μg/ml apyrase.

Fluorescence was recorded from 1.0 ml of platelet suspension aliquots (2×10^8 cells/ml) using a fluorimeter (Cary Eclipse, Varian, Madrid, Spain). Monitored fluorescence records were transformed into cytosolic-free calcium concentrations ($[Ca^{2+}]_c$) using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz [26].

Determination of platelet granule content and secretion

Platelets were first gated by size (FSC) and complexity (SSC) and 8000 events were counted. α- and δ-granule secretion was monitored in CD41-gated platelets by monitoring fluorescence change in platelet samples using a flow cytometer (FASCcan cytometer; Becton-Dickinson, San Jose, CA, USA). Samples of 50 μl of plasma rich platelets (PRP) were suspended in 450 μl of tempered HBS and platelet δ-granules were stained by incubating at 37°C for 30 min. with 10 μM of the quinacrine fluorescence probe. The attenuation in quinacrine fluorescence of platelets is indicative of δ-granule secretion and it is expressed as mean fluorescence intensity (MFI = quinacrine fluorescence − endogenous fluorescence) [27–29]. Meanwhile, α-granules secretion was monitored using a specific anti-P-selectin antibody (anti-CD62P-PE) [30]. Incubation with anti-CD62P antibody was done for 10 min. upon cell stimulation

with the physiological agonist thrombin (Thr), and incubation time was finished by mixing with ice-cold phosphate buffer saline. Fluorescence emitted by anti-CD62P-PE antibody and quinacrine was gated in cell positively stained with anti-CD41-a PerCP (clone HIP8) antibody that is indicative of positive platelet identification.

Aggregometry

The percentage and delay time of aggregation was monitored from aliquots of 400 μl of washed platelets isolated from kidney transplant patients treated with either sirolimus and everolimus, using a Chronolog aggregometer (Havertown®, Havertown, PA, USA) at 37°C under stirring at 1200 r.p.m. [31]. Percentage of aggregation was estimated as the percentage of the difference in light transmission between the platelet suspended in HBS and HBS alone, and it is shown as the percentage of platelet aggregated in response to Thr (0.1 U/ml) or ADP (10 μM), compared to resting platelets. HBS-free platelet medium is considered to be 100% of aggregation and resting platelets is arbitrarily 0%. The delay time is considered as the time required for reaching the maximum aggregation percentage in each platelet suspension.

Western blotting

Western blotting was performed as described previously [32, 33]. Briefly, 250 μl aliquots of platelet suspension (1×10^8 cell/ml) were stimulated with Thr (0.1 U/ml) for 1 min. and fixed by mixing with equal volume of Laemmli's buffer (2×) using reducing conditions (5% final concentration of dithiotheitrol, DTT). Proteins were isolated in a 6% acrilamyde-bisacrilamide SDS-PAGE and separated proteins were electrophoretically transferred onto nitrocellulose membranes for subsequent analysis by Western blotting (WB). Blots were incubated overnight with blocking buffer, containing 5% (w/v) skimmed milk, to block residual protein-binding sites. Immunodetection of mTOR and evaluation of the phosphorylation state of mTOR and ractor activation were achieved using an anti-phospho-mTOR (Ser 2481, autophosphorylation residue) and phospho-raptor (Ser 722) antibodies [34, 35], overnight at 4°C and diluted 1:1000 in blocking buffer. The primary antibody was removed and blots were washed with Tris-buffered saline supplemented with tween 20 (TBST) six times for 5 min. To detect the primary antibodies, blots were incubated for 1 hr with the appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:7500 in TBST [containing 5% (w/v)]. Membranes were then incubated with enhanced chemiluminescence reagent for 4 min. and they were subsequently exposed to photographic films. The density of bands on the film was measured using the Image J free software from national health institute of USA (NIH). Reprobing of the membranes with anti-actin antibody was done to assess that a similar amount of proteins was loaded in all gel lanes.

Statistical analysis

Patients were included in four groups according to the time of administration of either sirolimus (six patients within each group) or everolimus (four patients within each group); hence, patients medicated less than 24 months were considered as group I. Group II were medicated during 24–36 months, Group III were medicated during 36–60 months and group IV were medicated over 60 months. Analysis of statistical significance was performed using Student's unpaired *t*-test. In addition, one-way ANOVA was performed, and to evaluate differences between groups we used the Dunnett's test. Only values with $P < 0.05$ were accepted as significant.

Results

Altered calcium homeostasis in platelets from kidney transplant patients treated with sirolimus and everolimus

Correlation analysis performed in kidney transplant patients, revealed that sirolimus administration for long periods might alter calcium entry, being particularly affected the group II of patients (medicated for 24–36 months; Table 1), although trough levels of sirolimus are unlikely the key factors. As shown in Figure 1, fura-2-loaded platelets from patients and healthy individuals were suspended in a Ca^{2+}-free HBS medium (100 μM EGTA was added), and were stimulated for 3 min. with thrombin (Thr; 0.1 U/ml; Fig. 1A) or ADP (10 μM; Fig. 1B) and then 300 μM $CaCl_2$ was added to the extracellular medium to initiate calcium entry. Our results indicate that both Ca^{2+} release and entry in response to Thr were altered in most of the groups analysed, being most evident in group II of patients compared with healthy individuals (see Fig. 1A and 1B, where sirolimus reduced in Ca^{2+} entry evoked by Thr in a $59.8 \pm 14.1\%$ ($P < 0.01$; $n = 6$)). The effect of sirolimus on ADP-evoked Ca^{2+} mobilization was not so evident as presented for Thr, but it resulted in a small and time-dependent increase in Ca^{2+} release among the different patient groups as compared with healthy individuals. Meanwhile, reduced Ca^{2+} entry in these groups was observed, and despite this difference was not statistically significant a clear tendency was found [$32.9 \pm 28.0\%$ ($P > 0.05$; $n = 4$) in group II]. The different effect on Thr- and ADP-evoked Ca^{2+} signals might be explained because of the fact that ADP releases Ca^{2+} from the dense tubular system (DTS; similar to the endoplasmic reticulum in other cells) and Thr mobilizes calcium from the DTS and the acidic stores [36].

On the other hand, tert-butyl hydroquinone (TBHQ) releases Ca^{2+} from the acidic stores in platelets [36–38]. As shown in Figure 1C, in group II of patients, sirolimus induced a reduction of $34.9 \pm 14.9\%$ in TBHQ-evoked Ca^{2+} entry ($P > 0.05$; $n = 6$). Hence, considering that ADP-evoked Ca^{2+} entry resulted unaltered, we suggest that sirolimus mostly affects SOCE controlled by acidic granules.

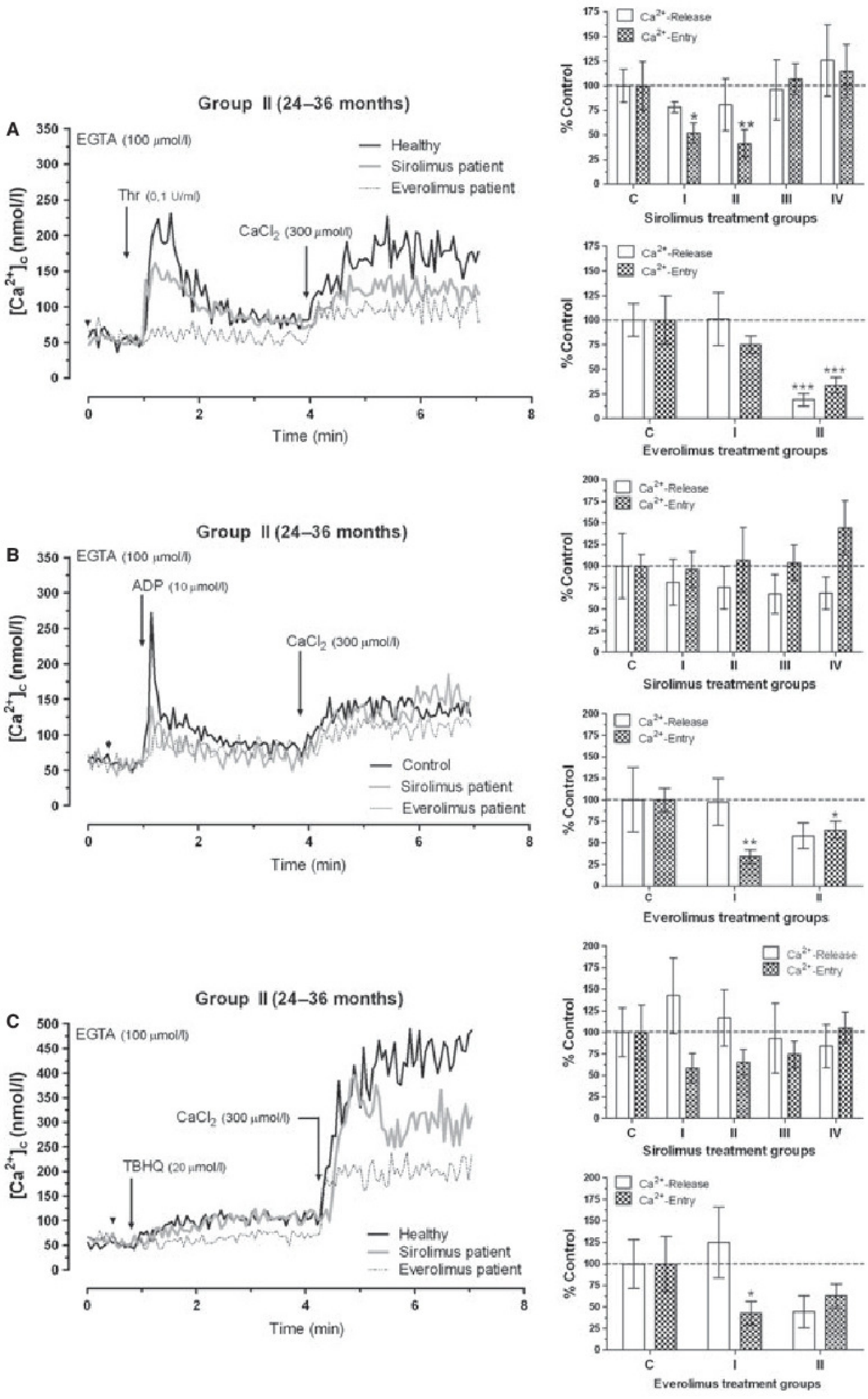
Treatment with everolimus altered Ca^{2+} homeostasis evoked by Thr, ADP and TBHQ (Fig. 1, see graphs and right hand side histograms). We have found that in the group II of patients (which received everolimus for more than 24 months), Ca^{2+} release was reduced by $80.8 \pm 6.3\%$ ($P < 0.001$; $n = 4$), $41.7 \pm 14.7\%$ ($P > 0.05$; $n = 4$) and $55.6 \pm 18.97\%$ ($P > 0.05$; $n = 4$) in platelets stimulated with Thr, ADP and TBHQ respectively. Similarly, Ca^{2+} entry was reduced by $66.1 \pm 8.0\%$ ($P < 0.001$; $n = 4$), $35.2 \pm 10.1\%$

Table 1 Correlation analysis of demographic and physiological variables of kidney transplanted patients administered sirolimus and everolimus. Grey background boxes highlights variables that are significantly correlated

	Age		Dose administered		Time transplanted		Time medicated		Platelet count		Platelet volume		Glucose levels		Trough levels		Calcium entry		Calcium release		Aggregation		CD62 positive		Quinacrine stain	
	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square
Platelet count	-0.296	0.087	-0.188	0.035	0.340	0.115	0.183	0.034																		
Platelet volume	-0.278	0.077	-0.012	0.000	-0.058	0.003	-0.298	0.089	0.106	0.040																
Glucose levels	0.217	0.047	-0.184	0.034	-0.085	0.007	-0.085	0.050	0.200	0.011	-0.387	0.150														
Trough levels	-0.054	0.003	0.099	0.010	-0.092	0.009	0.223	0.050	* -0.466	0.217	-0.162	0.026	-0.787	0.006												
Calcium entry (Thr)	-0.318	0.101	0.110	0.012	0.252	0.063	** 0.563	0.317	0.352	0.124	-0.067	0.005	-0.343	0.118	0.160	0.006										
Calcium release (Thr)	-0.188	0.035	0.173	0.030	0.012	0.000	0.259	0.067	0.418	0.175	-0.004	0.000	-0.298	0.089	-0.087	0.007	** 0.715	0.511								
Aggregation	-0.136	0.019	0.16	0.026	** 0.555	0.307	0.152	0.023	0.268	0.072	0.177	0.031	-0.031	0.001	0.143	0.020	0.411	0.169	0.014	0.000						
CD62 positive	-0.123	0.015	0.146	0.021	-0.029	0.001	* -0.532	0.283	* 0.518	0.268	0.210	0.044	-0.042	0.018	-0.482	0.232	-0.030	0.001	0.203	0.041	0.101	0.010				
Quinacrine stain	-0.008	0.000	0.389	0.151	0.344	0.119	0.108	0.116	-0.036	0.010	0.099	0.010	-0.106	0.011	-0.188	0.035	-0.036	0.001	-0.036	0.001	0.132	0.018	-0.111	0.012		

	Age		Dose administered		Time transplanted		Time medicated		Platelet count		Platelet volume		Glucose levels		Trough levels		Calcium entry		Calcium release		Aggregation	
	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square	Pearson r	R-Square
Platelet count	-0.155	0.024	-0.199	0.040	0.326	0.106	0.684	0.468														
Platelet volume	-0.392	0.154	-0.021	0.000	-0.662	0.438	-0.336	0.113	-0.510	0.260												
Glucose levels	0.614	0.377	0.626	0.392	0.125	0.016	-0.037	0.000	-0.067	0.004	-0.421	0.178										
Trough levels	-0.293	0.086	-0.137	0.019	* -0.712	0.507	-0.142	0.020	-0.294	0.087	0.526	0.276	-0.366	0.134								
Calcium entry (Thr)	0.187	0.035	-0.163	0.026	-0.190	0.036	-0.494	0.244	-0.198	0.039	0.268	0.072	-0.458	0.209	-0.279	0.006						
Calcium release (Thr)	0.207	0.043	-0.050	0.002	0.012	0.000	-0.550	0.302	-0.415	0.172	0.387	0.150	-0.266	0.071	-0.313	0.982	** 0.866	0.750				
Aggregation	0.088	0.078	-0.028	0.001	0.651	0.424	0.427	0.182	0.195	0.038	-0.670	0.449	0.432	0.186	* -0.795	0.632	-0.218	0.048	-0.0300.	0.001		

*$P < 0.05$.
**$P < 0.01$.


Group II (24–36 months)
A
EGTA (100 µmol/l)
Thr (0,1 U/ml)
CaCl2 (300 µmol/l)
Healthy
Sirolimus patient
Everolimus patient
[Ca2+]c (nmol/l)
Time (min)
Ca2+-Release
Ca2+-Entry
% Control
C I II III IV
Sirolimus treatment groups
C I II
Everolimus treatment groups
Group II (24–36 months)
B
EGTA (100 µmol/l)
ADP (10 µmol/l)
CaCl2 (300 µmol/l)
Control
Sirolimus patient
Everolimus patient
Time (min)
Sirolimus treatment groups
Everolimus treatment groups
Group II (24–36 months)
C
EGTA (100 µmol/l)
CaCl2 (300 µmol/l)
TBHQ (20 µmol/l)
Healthy
Sirolimus patient
Everolimus patient
Time (min)
Sirolimus treatment groups
Everolimus treatment groups

($P < 0.05$; $n = 4$) and 37.0 ± 14.0% ($P < 0.05$; $n = 4$) in platelets stimulated with Thr, ADP and TBHQ respectively.

Sirolimus evokes reduction in platelet granule secretion from kidney transplant patients

Ca^{2+} homeostasis regulates several intracellular mechanisms in human platelets like actin cytoskeleton reorganization, shape change or granule secretion. Hence, using flow cytometry, we gated CD41+ cells (platelet positive staining), and fluorescence of anti-P-selectin (CD62P) antibody and quinacrine was monitored. Fluorescence protocols have been widely used to evaluate alpha (α-) and dense (δ-) granule secretion [39]. As shown in Figure 2A, platelets present low levels of surface-exposed P-selectin under resting conditions (Fig. 2A; C: white bars representing resting platelets from healthy individuals), which is drastically enhanced upon α-granule secretion stimulated by Thr. Furthermore, we found that sirolimus-treated patients presented enhanced P-selecting membrane exposure under resting conditions, and subsequently, Thr-evoked P-selectin exposure was significantly lower ($P < 0.001$; $n = 6$); thus, the reduction in the fold increase observed between platelets from the group II of patients treated with sirolimus compared with control was of 0.18 ± 0.06 (Fig. 2A, right-hand side histogram; $P < 0.05$; $n = 6$). P-selecting exposition reached a 4.6 ± 0.1 fold increase ($P < 0.001$; $n = 6$) in Thr-stimulated platelets from healthy individuals. Hence, α-granule secretion was altered by sirolimus in a time-dependent manner (Fig. 2A, right-hand side histogram). Regarding everolimus patients, the most samples in resting conditions presented a very high elevated P-selectin exposure under resting condition, which makes subsequent evaluation of granule secretion difficult.

In addition, in healthy individuals, Thr (0.1 U/ml) reduced quinacrine staining by 1.6 ± 0.04 fold decrease respect to the fluorescence found in platelets under resting conditions ($P < 0.05$; $n = 4$). Thr-evoked δ-granules secretion, and subsequently, lost of quinacrine fluorescence. Upon platelets stimulation with Thr quinacrine stain remaining inside the platelets was higher in patients treated with sirolimus than in control, owing to the inhibition of granule secretion. Thus, a 1.3 ± 0.10 fold increase ($P < 0.01$; $n = 4$), 1.4 ± 0.04 ($P < 0.001$; $n = 4$), 1.3 ± 0.05 ($P < 0.001$; $n = 4$), 1.4 ± 0.10 ($P < 0.001$; $n = 4$) was observed in groups I, II, III and IV patients treated with sirolimus respectively (Fig. 2B). As it has been shown for α-granule secretion, δ-granule secretion resulted higher in platelets from patients under resting condition compared with platelets from healthy individuals.

Long-term administration of sirolimus and everolimus significantly alter platelet aggregation in response to physiological agonists

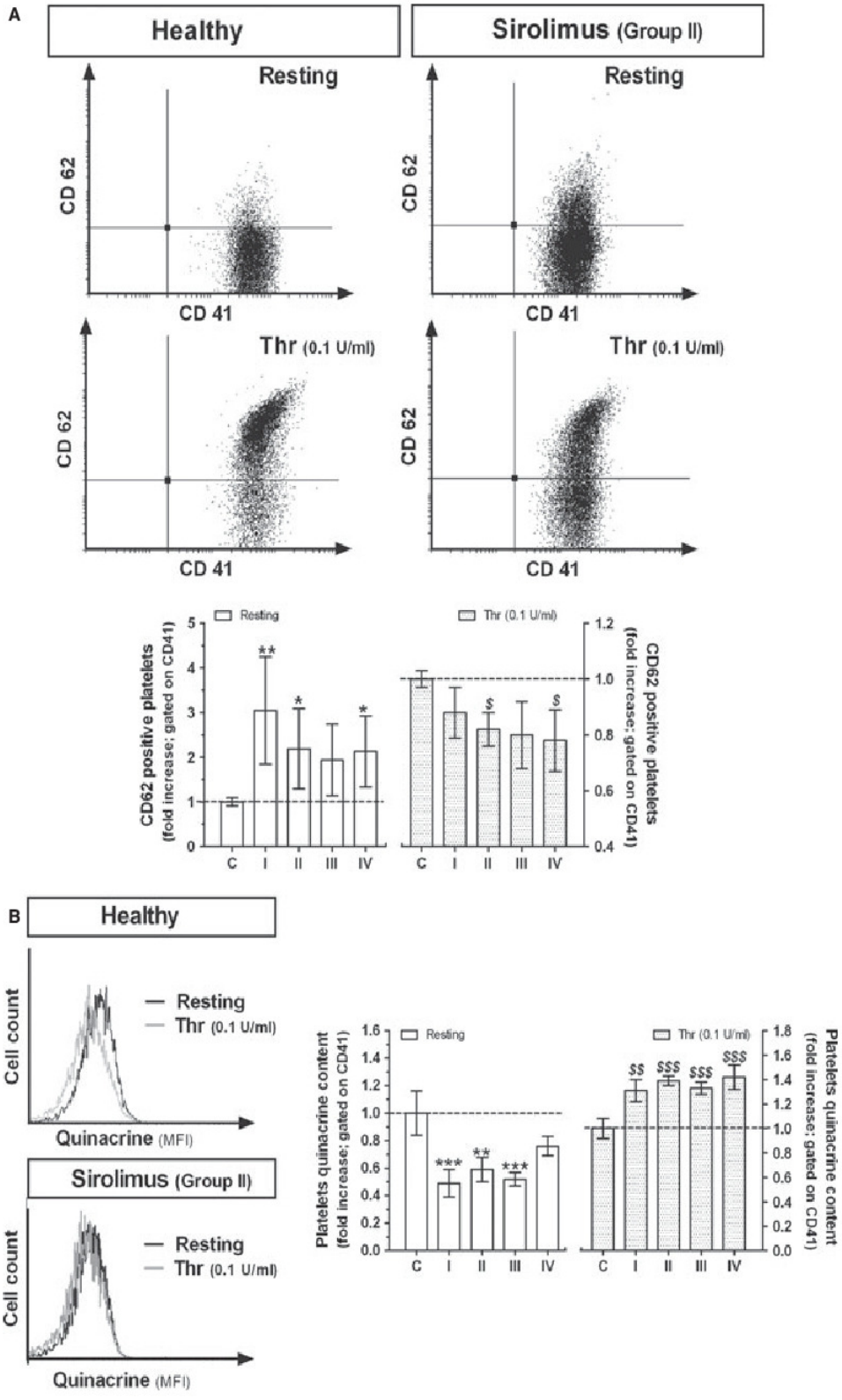
Aggregation is a process finely regulated by, among others, Ca^{2+} homeostasis, protein phosphorylation, and other events like surface exposure of molecules, such as P-selectin (CD62P) or tetraspanin (CD63), which favour platelet–platelet and platelet–endothelium interaction [40]. As shown in Figure 3, sirolimus and everolimus administration perturbed platelet aggregation in response to Thr and ADP, as demonstrated by observing the percentage of aggregation and, even more evidently, by evaluating the delay time, which is considered as the time required to reach the maximum percentage of aggregation in each platelet sample. Percentage of aggregation was significantly reduced in group II of patients treated with sirolimus (by 26.1 ± 8.8% compared with healthy individuals; Fig. 3A; $P < 0.01$; $n = 6$). The decrease in percentage of aggregation was accompanied of an increase of 227.2 ± 52.0% ($P < 0.01$; $n = 6$) in the delay time compared with healthy individuals where it never exceeded 6.8 ± 2.3 min. ($P < 0.01$; $n = 6$). In the case of everolimus, Thr-evoked aggregation was also found reduced by 57.2 ± 2.3% ($P < 0.01$; $n = 6$) in patients treated for less than 24 months (group I), while the delay time was enhanced by 317.6 ± 55.6% ($P < 0.001$; $n = 6$) as compared with platelets from healthy individuals. Furthermore, sirolimus caused greater alterations in ADP-evoked aggregation in the group II of patients (Fig. 3B, 94.8 ± 3.0%; $P < 0.001$; $n = 6$); meanwhile everolimus mostly affected the group I patients (93.4 ± 2.7%; $P < 0.001$; $n = 6$).

Sirolimus reduces phosphorylation by altering mTOR activation in human platelets

As shown in Figure 4, Thr stimulation evokes an increase in mTOR phosphorylation in platelets from healthy individuals, and subsequently, as a result of an enhanced mTOR activation an increased phosphoserine levels of raptor was observed. As expected, and it is shown in Figure 4A and B (representative experiment of patients belonging to group II of patients is shown) the phosphorylation levels of both mTOR and raptor were attenuated in patients that were long-term treated with sirolimus.

To ascertain whether these changes in proteins belonging to mTOR complex might affect to the activity of the mTOR complex, we

Fig. 1 Calcium homeostasis in patients under sirolimus and everolimus medication. Fura-2-loaded platelets isolated from healthy (black solid lines) and patients treated with sirolimus (grey lines) or everolimus (black-doted lines), were suspended in Ca^{2+}-free HBS medium (100 μM EGTA was added; arrowheads) and subsequently stimulated either with Thr (**A**), ADP (**B**) and THBQ (**C**) for 3 min., followed by addition of 300 μM of $CaCl_2$ to the extracellular medium to initiate calcium entry. Representative calcium signals of patients belonging to group II are plotted and histograms on the right hand side, represent calcium release and entry as percentage of control of patients treated with sirolimus ($n = 6$ each group) and everolimus ($n = 4$ each group) and medicated during less than 24 (I), 24–36 (II), 36–60 (III) and over 60 months (IV). *, ** and ***, represents $P < 0.05$, <0.01 and <0.01 compared with healthy individuals respectively.


A
Healthy
Sirolimus (Group II)
Resting
Thr (0.1 U/ml)
CD 62
CD 41
Resting
Thr (0.1 U/ml)
CD62 positive platelets (fold increase; gated on CD41)
C I II III IV
B
Healthy
Sirolimus (Group II)
Cell count
Quinacrine (MFI)
Resting
Thr (0.1 U/ml)
Platelets quinacrine content (fold increase; gated on CD41)

have investigated the phosphorylation state of Akt, which has been described belonging to the same mTOR signalling pathway, and it has been reported to be crucial during platelet activation. As presented in Figure 4C, Akt resulted dephosphorylated during the initial steps of the platelet activation with Thr (0.1 U/ml), which agrees with previous observation in other cells type upon G protein-coupled receptor activation, like thrombin receptor or colecystokinin receptor [41]. Akt phosphorylation pattern was not significantly altered in presence of rapamycin upon stimulation with Thr as previously reported in platelets [42]. Membranes were reprobed using anti-α-actin antibody to asses that similar amount of proteins have been loaded in all lanes.

Correlation analysis of different variables in kidney transplant patients receiving long-term administration of sirolimus and everolimus

To further explore the possible impairment of platelet function by administration of mTOR inhibitors, several variables were analysed in the different patient groups. As shown in Table 1, we found positive correlation between the *time transplanted* and *platelet aggregation* in patients treated with sirolimus ($P < 0.05$; $n = 19$), indicating that platelets aggregation from transplanted patients recovered functionality 5 years after the transplant. Interestingly, aggregation values were found similar to those in healthy individuals. We have also observed correlation between the *time medicated* and *Ca^{2+} entry* ($P < 0.01$; $n = 21$). Interestingly, patients with smaller *platelet count* presented also reduced platelet *α-granule secretion* in response to Thr ($P < 0.05$; $n = 16$), which might be indicative of a greater clearance rate of pre-stimulated platelets in these patients. Furthermore, we observed negative correlation between *time medicated* and platelet *α-granule secretion* ($P < 0.05$; $n = 16$), and between *trough levels* of sirolimus and *platelet count* (R squared: 0.2196; $P < 0.05$; $n = 21$). Regarding the rest of correlations analysed, none of them presented R squared values high enough to result statistically significant (see Table 1). Furthermore, we have observed that patients presenting higher trough levels of mTOR inhibitors, also showed significant reduction in platelet count, which might be indicative of a lower platelet generation from bone marrow or higher rate of platelet clearance.

On the other hand, patients treated with everolimus presented lower rate of correlation among the variables considered in this study. We found negative correlation between time transplanted and trough levels ($P < 0.05$; $n = 8$), and also between trough levels and aggregation percentage ($P < 0.05$; $n = 8$). These results suggest that patients accumulate more levels of everolimus within the first months after transplantation, and elevated circulating everolimus have a negative effect in platelet function.

Discussion

Rapamycin-based therapies represent a good alternative in cardiac-transplanted patients, in patients presenting cardiovascular complications and particularly, in those patients where immunosupression-related neoplasias have been diagnostized. Despite the information concerning the effects of mTOR inhibitors in platelets function is scarce, the reports available in the literature regarding sirolimus effects in platelets function are controversial.

Our results indicate that long-term mTOR inhibitors administration alters Ca^{2+} release and impairs Ca^{2+} entry in response to Thr and also dependent of acidic store depletion using TBHQ. Contrary, in kidney-transplanted patients under sirolimus medication no significant alteration has been observed in Ca^{2+} homeostasis induced by ADP in platelets. Furthermore, everolimus modified the Ca^{2+} homeostasis pattern induced by all stimuli used.

Interestingly, the alteration observed in Ca^{2+} homeostasis resulted most evident in patients treated with sirolimus during 24–26 months; after that, most patients included in this study recovered Ca^{2+} mobilization patterns in response to Thr. This finding explains the high correlation coefficient found between Ca^{2+} entry levels and time medicated. In this sense, rapamycin-dependent inhibition of SOCE evoked by the SERCA inhibitor, cyclopiazonic acid, has been recently reported in pulmonary vascular cells [43], and in human pulmonary arterial smooth muscle cells, suggesting that mTOR is downstream to PDGF receptor participating in the association between STIM1/Orai and subsequently regulating SOCE [44].

Recent studies have shown that platelet aggregation is enhanced in response to ADP [45]. Furthermore, other studies have proposed that sirolimus might enhance cyclooxygenase activity, as they presented evidence for a reduced aspirin effect by increasing rapamycin concentration in '*in vitro*' assay using shorter incubation time, which is different to a cronical exposition during years presented here [45]. More specific mTOR inhibitors, like PP242 and torin1, enhanced platelet aggregation in response to SFLLRN, a PAR-1 receptor agonist. Moreover, platelets incubated for 15 min. with rapamycin (200 nM) does not reported significant alteration in aggregation activated by SFLLRN, suggesting that under these experimental conditions mTOR2 regulates Akt function and subsequently platelet activity, through this pathway that would be non-sensitive to rapamycin

Fig. 2 Granule secretion in patients medicated with sirolimus. Platelets isolated from kidney-transplanted patients treated with sirolimus were incubated in plasma rich platelets (PRP) for 30 min. at 37 °C with anti-CD41 and quinacrine (10 μM). Platelets were then left under resting condition or stimulated for 10 min. with Thr (0.1 U/ml), and simultaneously, anti-CD62 antibody (P-selectin; diluted 1:50) was added to medium. Fluorescence of CD41 was used for selecting platelet positive cells. Fluorescence was analysed using flow cytometry, from patients under sirolimus medication for different periods of time (<24: I), (24–36: II),(36–60: III) and (>60 months: IV). The fluorescence results of recording CD41 and CD62-positive platelets (**A**; α-granules) and simultaneously, CD41 and quinacrine (**B**; δ-granules) in the same platelets sample. Histograms show either the increase in P-selectin membrane exposition or quinacrine fluorescence remaining in the platelets in fold increase. *, ** and ***, represent $P < 0.05$, <0.01 and <0.01 compared with values found in resting platelets from healthy individuals ($n = 6$). While, $, $$, $$$, represent $P < 0.05$, <0.01 and <0.01 compared with Thr-stimulated values found in healthy individuals.

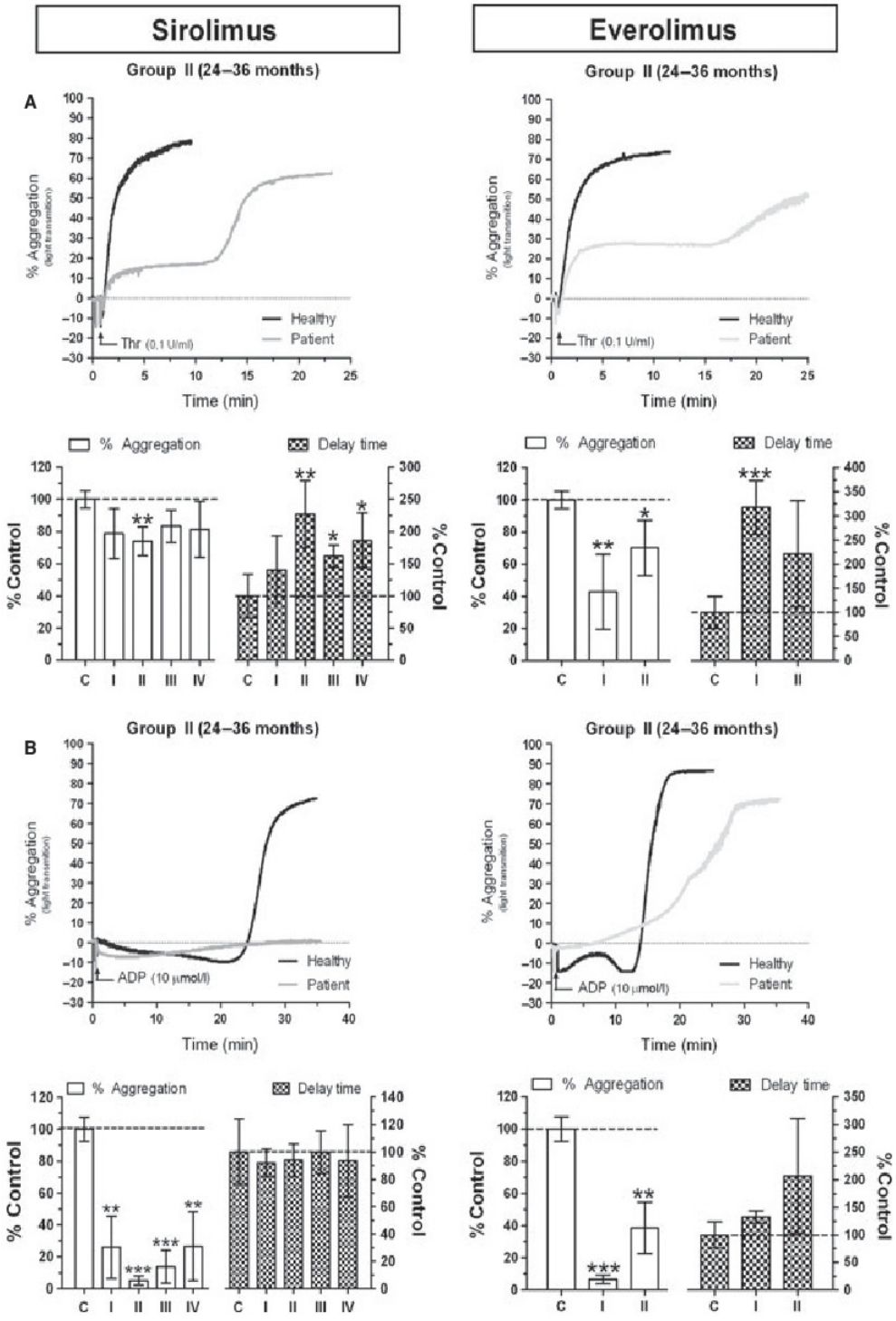


Fig. 3 Sirolimus and everolimus alter aggregation in long-term medicated patients. Aliquots of platelets (400 µl) were suspended in fresh rich Ca^{2+}-free HBS medium (1 mM), and then, they were stimulated with 0.1 U/ml of Thr (**A**) or 10 µM of ADP (**B**) to determine platelet aggregation as it is described in Materials and Methods. Histograms represent the percentage of aggregation and delay time, considering the latest as the time required to reach maximum percentage of aggregation, compared with healthy individuals. *, ** and ***, represent $P < 0.05$, <0.01 and <0.01 compared with healthy individuals, respectively.

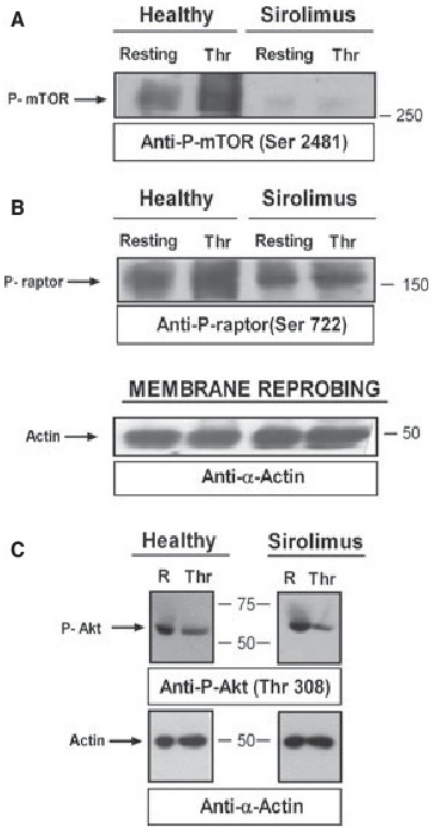


Fig. 4 Long-term administration of sirolimus evokes reduction in phosphorylation of proteins belonging to mTOR signalling cascade. Platelets from healthy individual or sirolimus-treated patients (during 24–36 months) were stimulated in Ca^{2+}-free HBS medium for 1 min. with Thr and then fixed in 2xLB (5% final DTT concentration). Western blotting was performed using anti-phospho-mTOR (Ser 2481; **A**), anti-phospho-raptor (Ser 722; **B**), anti-phospho-Akt (Thr 308; **C**) antibodies, all of them diluted (1:1000) in blocking buffer containing skimmed milk. Specific secondary antibody was used to develop the membranes as described under Material and methods. Membranes were reprobed using an anti-α-actin antibody to corroborate that similar amount of proteins were loaded in each lane. Panels are representative of four independent experiments using samples belonging to group II of patients and healthy individuals.

[1, 14]. In our hands, Akt resulted dephosphorylated during the initial steps of platelets activation, which would allow cytoskeleton reorganization. After the initial steps of platelet activation, Akt would probably become highly phosphorylated as reported in a very recent publication, in which the author evaluated phospho-Akt upon 15 min. of stimulation with Thr [42]. We have not found changes in Akt phosphorylation pattern between healthy individual and patients belonging to group II, which would indicate that mTOR1 would not be involved in the modification of Akt activity, as previously reported [42].

However, a recent study has reported that mTOR2 is sensitive to rapamycin depending on the time of exposure to the drug [46]. In this sense, our findings are unlikely explained by the exclusive participation of mTOR2 but also to the participation of mTOR1 instead, since mTOR1 downstream protein kinase, p70S6K1 has been presented previously regulating Bcl-3 secretion of glycoprotein αIIb/β3 evoked by Thr, which is required for platelets response to fibrinogen, von Willebrand factor, vitronectin and fibronectin [47–49]. We suggest that this controversy relies in the agonist used for stimulating platelets, since despite stimulating platelets with Thr, after granules secretion other autocrine stimuli, like ADP or serotonin, might modify the initial stimulation evoked by Thr, or by contacting with disrupted vessel exposing collagen, vWF, etc.

Time-dependent membrane exposure of P-selectin, as well as a reduced δ-granule secretion containing autocrine and paracrine substances, is presented here. Using cytometry we have evaluated resting platelets (CD41+ cells) and Thr-stimulated platelets from sirolimus-treated kidney transplant patients. In our patients, an elevated degranulation is observed in resting platelets, which might explain the reduced response and impaired aggregation found upon application of external stimulus presented above.

Finally, as first clinical trials were set up, thrombocytopenia is the most evident side-effect reports in patients under rapamycin medication, or rapamycin analogues administration [50, 51]. We have observed that almost half of our patients presented very low platelet count, and even some of them presented severe thrombocytopenia. Enhanced clearance of pre-activated platelets or reduced platelets generation from bone marrow alteration, leads to thrombocytopenia. These hypotheses are under an intense investigation nowadays, revealing that mTOR might play an important role in platelet production [52–54].

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Disclosures

No disclosure of interest.

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Immunophilins are Involved in the Altered Platelet Aggregation Observed in Patients with Type 2 Diabetes Mellitus

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Abstract: Platelet hyperaggregability might contribute to vascular complications associated with type 2 diabetes mellitus (DM2). Experimental evidence supports a direct link between altered Ca^{2+} entry and hyperaggregability in DM2 patients. *Objectives:* We aimed to investigate whether altered immunophilin expression and function are involved in the abnormal Ca^{2+} entry observed in platelets from DM2 patients. *Results:* Inhibition of immunophilins by tacrolimus (FK506) and sirolimus (rapamycin) reduced Ca^{2+} entry in platelets from healthy donors and DM2 patients. Similarly, immunophilin inhibitors reduced platelet degranulation in both healthy and DM2 subjects. Nevertheless, α-granule secretion reduction was greater than that observed for dense granules in platelets from DM2 patients. However, no difference was observed in the inhibition of secretion in platelets from healthy subjects. Additionally, altered expression of FK506 binding protein-52 (FKBP52) and coupling to Ca^{2+} channels were found in platelets from DM2 patients compared to healthy subjects. Finally, reduction in platelet function from healthy subjects and DM2 patients in the presence of immunophilin antagonists was observed, being this dysfunction more evident in platelets from DM2 patients. *Conclusions:* We suggest that, among others, FKBP52 expression and function are altered in platelets from DM2 patients, contributing to the altered Ca^{2+} entry and hyperaggregability in these cells.

Keywords: Aggregation, DM2, FKBP52, immunophilin, secretion, SOCE

INTRODUCTION

Type 2 diabetes mellitus (DM2) is associated with thrombopathy, retinopathy and nephropathy [1, 2]. Platelet hyperactivity and hyperaggregability have been suggested to promote the appearance of these vascular complications in DM2 patients. In this sense, a direct link between impairment of Ca^{2+} homeostatic mechanisms and platelet hyperactivity has been reported in human platelets from DM2 subjects [3-6]. Platelets from DM2 patients show enhanced Ca^{2+} leakage [6], reduced Ca^{2+} extrusion through plasma membrane Ca^{2+}-ATPase [7] and increased Ca^{2+} entry from the extracellular medium [4]. Furthermore, distinct intracellular Ca^{2+}-dependent and -independent mechanisms are also altered, thus leading to platelet hyperaggregability in DM2 patients [5, 8]. Among them, resting cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) is enhanced in DM2 platelets, thus reflecting the increased Ca^{2+} leak from the intracellular stores [3, 6, 8]. In addition, the metabolic disorder induced by diabetes leads to oxidative stress, with increased levels of H_2O_2 and $ONOO^-$, which alters Ca^{2+} influx through store-operated Ca^{2+} entry (SOCE) in these cells. Reduction of glucose levels in the medium or platelet incubation with oxidant scavengers reduces SOCE to a level comparable to healthy donors [4, 7, 9-11].

Immunophilins are proteins with peptidyl-prolyl isomerase activity that have been recently identified to regulate Ca^{2+} homeostasis in human platelets and other cell types. Immunophilins are widely expressed and can be grouped into cyclophilins and FK506-binding proteins (FKBPs)[12]. In human platelets, cyclophilin A controls SERCA2b activity and cyclophilin D regulates mitochondrial integrity, and subsequently cell survival [13, 14]. Furthermore, FKBP members, like FKBP12 and FKBP52, regulate Ca^{2+} channels involved in Ca^{2+} release from intracellular stores and Ca^{2+} entry across the plasma membrane. FKBP12 and FKBP52 co-immunoprecipitate with TRPC channels [15]. In particular, FKBP52 activity controls Ca^{2+} permeability of TRPC1 in response to physiological agonists by altering its structure; meanwhile, interaction of FKBP12 with TRPC1 facilitates uncontrolled Ca^{2+} permeability of the channel [16].

There is a growing body of evidence [17, 18] suggesting that immunophilin expression and activity might be altered in DM2. In fact, immunophilin antagonists, like FK1706, reduce DM2 associated symptoms like diabetic neuropathy. Interestingly, FKBP12.6 expression was reported to be downregulated in rats where diabetes was induced using streptozotocin [17]. Hence, we aimed to explore whether altered immunophilin expression and function are involved in the enhanced Ca^{2+} entry observed in platelets from DM2 patients.

MATERIALS AND METHODS

Materials

Fura-2 acetoxymethyl ester (Fura-2/AM) was from Molecular Probes (Leiden, The Netherlands). Apyrase (grade

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VII), aspirin, thrombin (Thr), dithiothreitol (DTT), thapsigargin (Tg), sodium dodecyl sulphate (SDS), ionic detergent tween 20, Na_3VO_4, quinacrine, ECL reagents, anti-α-actin antibody, anti-Orai1 antibody and bovine serum albumin (BSA) were from Sigma (Poole, Dorset, U.K.). FK506 and rapamycin were from Selleck Chemical (Huston, Texas, U.S.A). Anti-FKBP52 and anti-FKBP12 antibody were from Santa Cruz Biotechnology (Dallas, Texas, USA). Anti-CD62P-PE antibody, anti-CD41-a PerCP (clone HIP8) and anti-PE isotype were from Becton Dickinson Transduction Laboratories (Madrid, Spain). Anti-horseradish peroxide-conjugated anti-rabbit or anti-mouse IgG antibodies, hyperfilm ECL and molecular weight markers were from GE Healthcare (Chalfont St. Giles, UK). All other reagents were of analytical grade.

Selection of Patients, Blood Processing and Platelet Sample Preparation

Diabetes mellitus type 2 patients and healthy volunteers were selected from the Hematology Clinic Hernandez-Cruz (Cáceres, Spain). Blood was drawn upon informative consent of the patients , in accordance with the Declaration of Helsinki. Type 2 diabetic patients not suffering other disorders were selected, and confirmation of high blood glucose concentration was done, which resulted in the range of 180–240 mg/dL. The glycosylated hemoglobin level (HbA1c) was used as an index of metabolic control. Only blood from diabetic patients with a level of HbA1c > 6% was selected for experiments. Meanwhile, the control subjects were age- and gender-matched healthy people with HbA1c levels in the normal range (3.5–5%). Blood was drawn during analyses routinely performed to the patients in order to avoid further alteration of the patients. Platelets are obtained by sequential centrifugation, as previously described elsewhere, and suspended in HBS medium containing high glucose [4].

Measurement of Cytosolic free Ca^{2+} Concentration ($[Ca^{2+}]_c$)

Platelets were incubated with 2 μM of fura-2-AM for 45 min at 37 °C, as previously described [18-20]. Cells were collected by centrifugation and suspended in HEPES-buffered saline (HBS, pH 7.45) and supplemented with 0.01% w/v BSA and 40 μg/mL apyrase. Fluorescence was recorded from 2.0 mL aliquots of platelet suspensions ($2x10^8$ cells/mL) at 37 °C using a fluorimeter (Cary Eclipse, Varian, Madrid). Samples were alternatively excited at 340 and 380 nm and fluorescence emission was recorded at 515 nm. Data were calibrated in terms of $[Ca^{2+}]_c$ as described by Grynkiewicz *et al.* [21].

Cytometry Analysis of Platelet Granule Content and Secretion

Platelet α- and δ-granule secretion was determined by monitoring fluorescence changes in platelet samples using a flow cytometer (FASCcan cytometer, Becton-Dickinson, CA, USA). Briefly, approximately $1x10^6$ platelets/mL were suspended in temperate HBS and the platelet δ-granules were stained by incubation at 37 ºC for 30 min with 10 μM quinacrine fluorescence probe. Decreased quinacrine fluorescence is indicative of δ-granule secretion, which is expressed as mean fluorescence intensity (MFI = quinacrine fluorescence minus endogenous fluorescence) [22-24]. α-granule secretion was monitored by using a specific anti-P-selectin antibody (anti-CD62P-PE) [25], which was added to the platelet extracellular medium during 10 min upon cell stimulation with thrombin (Thr). Antibody incubation was finished by mixing with phosphate buffer saline (PBS). Fluorescence derived from anti-CD62P-PE antibody and quinacrine was gated in cell positively stained with anti-CD41-a PerCP (clone HIP8) antibody that is indicative of positive platelet identification.

Aggregometry

Platelet aggregation was monitored in 400 μL-samples of washed platelets using a Chronolog aggregometer (Havertown, Pa, USA). Platelets were kept during the experiments at 37 ºC under and stirring at 1200 rpm [26].

Immunoprecipitation and Western Blotting

Immunoprecipitation and Western blotting were performed as described previously [27, 28]. Briefly, 250 μL aliquots of platelet suspension ($1 x 10^8$ cell/mL) were stimulated with Thr (0.1 U/mL) for 1 min and fixed by mixing with equal volume of Laemmli's buffer (2x) using reducing conditions (5% final concentration of dithiotheitrol, DTT) or with equal volume of ice-cold RIPA (2x; for immunoprecipitation). Immunoprecipitation was achieved by incubation with specific anti-FKBP52 and anti-FKBP12 antibodies and 25 μg agarose beads. Proteins were separated by 6% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes for subsequent analysis. TRPC1, Orai1, FKBP52 and FKBP12 were detected using specific antibodies diluted either 1:200 or 1:1000 in blocking buffer. To detect the primary antibodies, blots were incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:5000 in TBST. Membranes were then incubated with enhanced chemiluminicence reagent for 4 min and exposed to photographic films. The band density of the film was measured using the Image J free software (NIH, USA). For protein loading control, reprobing of the membranes was done using anti-α-actin antibody diluted 1:1000 in blocking buffer (BSA) in order to assess the similar amount of proteins loaded in all lanes.

Statistical Analysis

Analysis of statistical significance was performed using Student's unpaired *t*-test. Only values with $p<0.05$ were considered as significant.

RESULTS

Immunophilins Control Intracellular Ca^{2+} Homeostasis in Platelets from DM2 Patients and Healthy Subjects

Fura 2-loaded platelets from healthy subjects and DM2 patients were suspended in a Ca^{2+}-free HBS medium, and subsequently, incubated for 5 min with the vehicle or increasing concentrations (1-50 μM) of FK506, a general immunophilin antagonist [12]. Platelets were then stimulated with Thr for 3 min, and finally, 300 μM of $CaCl_2$ was added to the extracellular medium to initiate Ca^{2+} entry. As shown in (Fig. **1A**), FK506 (50 μM) reduced Ca^{2+} entry both in DM2 patients and healthy donors as compared to their respective controls (Ca^{2+} entry in the presence of FK506 was

53.2 ± 18.1% and 49.6 ± 26.3% of control in DM2 and healthy donors, respectively; $p<0.01$, n=6). However, no significant differences were detected in the effect of FK506 in healthy and DM2 subjects. FK506 also attenuated SOCE evoked by Tg. As shown in (Fig. **1B**), administration of 50 μM FK506 for 5 min reduced Tg-evoked SOCE by 53.7 ± 13.8% in platelets from healthy subjects and by 62.8 ± 22.6% in platelets from DM2 patients ($p<0.001$ as compared to their respective controls; n=6). Hence immunophilins are involved in the regulation of Ca^{2+} entry in platelets from healthy and DM2 subjects, being this effect slightly more relevant in platelets from DM2 patients.

To investigate whether the effect of FK506 on Ca^{2+} entry is independent on calcineurin, as reported by others [29, 30], similar experiments were performed in the absence or presence of rapamycin, which has no effect on calcineurin [31, 32]. As shown in (Fig. **1C**), incubation for 30 min with ra-

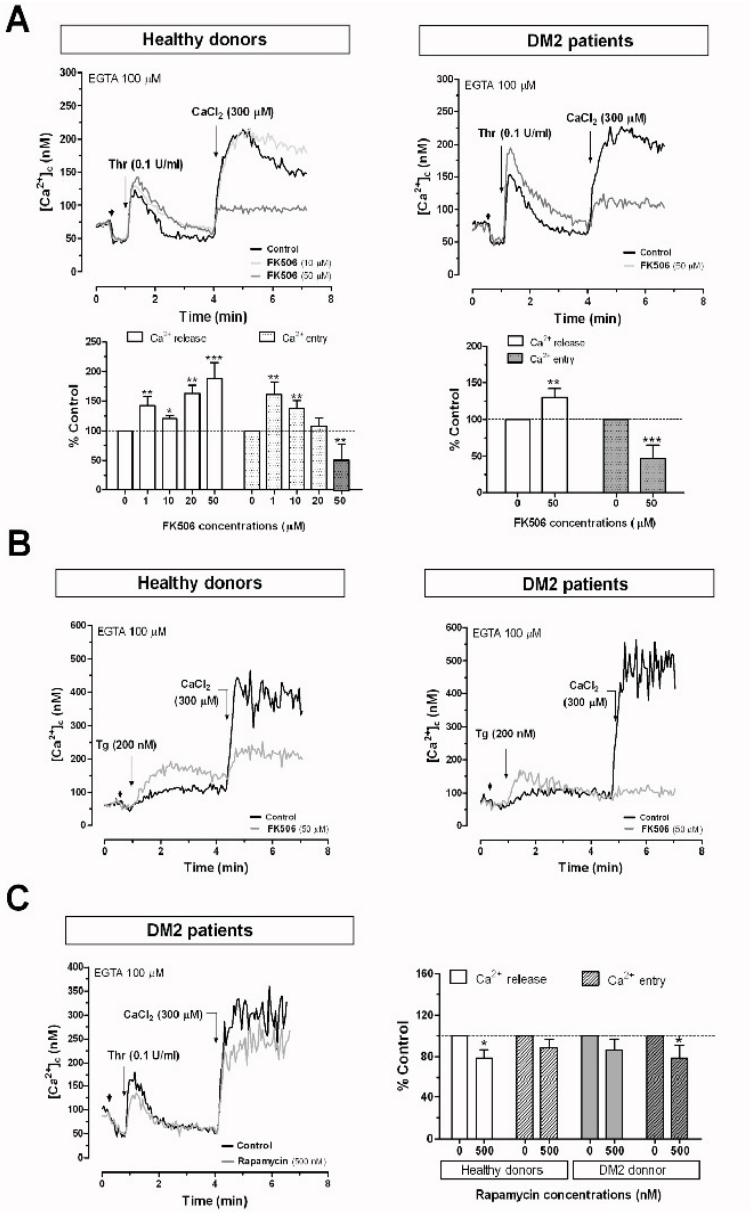

Fig. (1). Role of immunophilins in $[Ca^{2+}]_c$ homeostasis in platelets from DM2 patients and healthy subjects. Fura-2-loaded platelets from DM2 patients and healthy subjects, suspended in Ca^{2+}-free HBS, were preincubated either for 5 min with 50 μM FK506 (A & B) or for 30 min with 500 nM rapamycin (C). Platelets were stimulated either with Thr (0.1 U/ml; A & C) or Tg (200 nm; C) (B) for 3 min and 300 μM $CaCl_2$ was added to the extracellular medium to initiate Ca^{2+} entry. Traces are representative of four to six independent experiments and histograms represent mean ± ESM. *,**,*** represent P < 0.05, 0.01, 0.001.

pamycin (500 nM) reduced Ca^{2+} entry evoked by Thr in platelets from both, healthy (11.3 ± 7.0%; p>0.05; n=4) and DM2 patients (21.5 ± 12.5%; p>0.05; n=4). Contrary to FK506, rapamycin administration resulted in a reduction of Thr-evoked Ca^{2+} release which was much more evident in platelets from healthy subjects, thus suggesting that mostly immunophilins, and not calcineurin, are involved in the alteration showed above. The greater inhibition of SOCE stimulated by Thr observed in platelets from DM2 patients treated with rapamycin might be indicative of a more relevant role of immunophilins in SOCE in these patients.

Inhibition of Immunophilins Results in a Greater Attenuation of α- and δ-Granule Secretion in Platelets from DM2 Patients than in Controls

Changes in Ca^{2+} homeostasis are required for platelet secretion. Thus, we have further investigated whether the alteration evoked by immunophilin antagonists in Ca^{2+} homeostasis might be reflected in an altered platelet secretion. As shown in (Fig. **2**), incubation of platelets from healthy subjects in a Ca^{2+}-free medium with FK506 (50 μM; 5 min) and rapamycin (500 nM; 30 min) slightly, reduced CD62P exposure under resting condition; nevertheless a significant reduction of CD62P exposure induced by stimulation with Thr during 10 min in presence of the immunophilin antagonist. We observe a reduction of 31.1 ± 5.0% (p<0.001; n=8) and 32.6 ± 6.2% (p<0.001; n=8) in presence of FK506 and rapamycin compared to control non-treated platelets, respectively. Additionally, in platelets from DM2 patients a greater inhibition of α-granule secretion in the presence of immunophilin antagonists was observed, where CD62P exposure was reduced by 45.4 ± 4.2% (p<0.001; n=6) and 46.7 ± 6.0%; p<0.001; n=6) in the presence of FK506 and rapamycin, respectively.

On the other hand, in platelets from healthy subjects FK506 reduced Thr-evoked δ-granules secretion as observed by the fluorescence of quinacrine remaining within the cells upon Thr stimulation in the presence of FK506, compared to Thr-stimulated platelets non treated with FK506 (Fig. **3**; 25.4 ± 15.8%; p<0.05; n=6). In contrast, non-significant differences were observed in Thr-evoked δ-granules secretion in the presence of rapamycin under resting condition, but secretion of δ-granules in the presence of rapamycin was also reduced to a similar extend that FK506. Although, contrary to the differences induced by immunophilin antagonists over

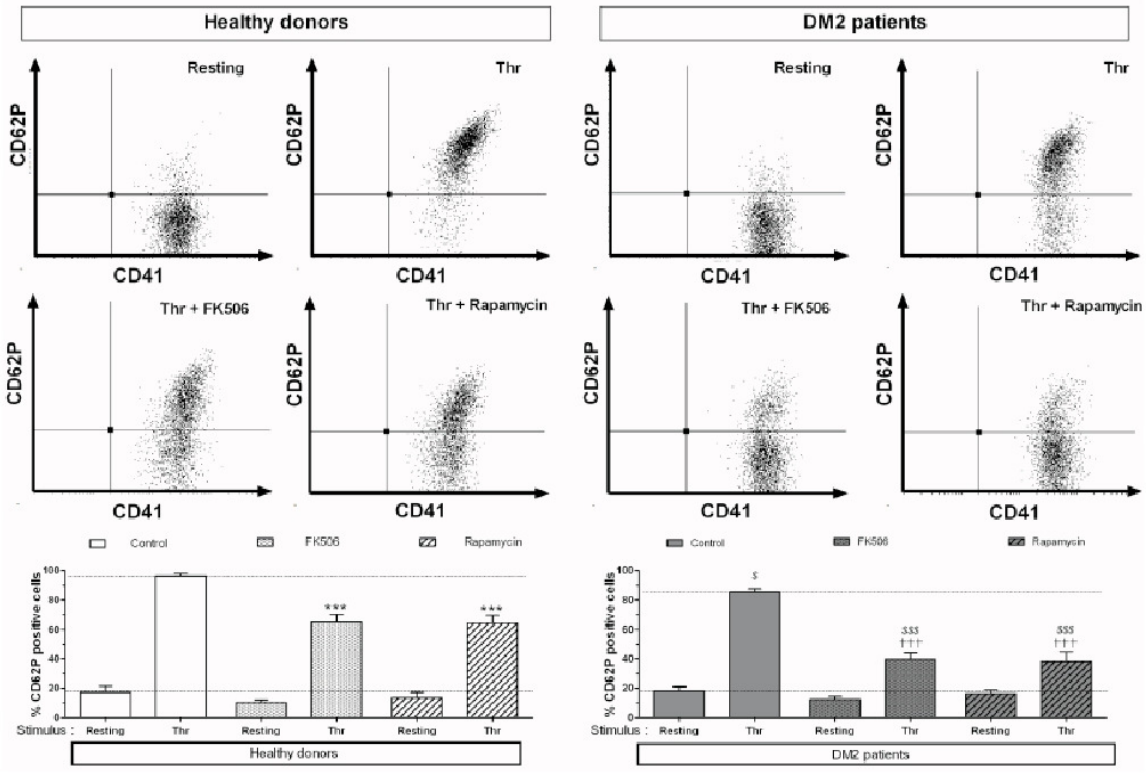

Fig. (2). Immunophilins are involved in platelet α-granules secretion in DM2 patients and healthy subjects. Platelets from DM2 patients and healthy subject were stained with an anti-CD41 antibody in the absence or presence of either rapamycin or FK506 (only for the latest 5 min), and stimulated for 10 min with Thr (0.1 U/ml) in presence of anti-CD62P antibody. Fluorescence of anti-CD62P antibody (α-granules) was monitored in platelets positive for anti-CD41 antibody by flow cytometry. Data shown are representative of 6 to 8 independent experiments. Histograms represent percentage of anti-CD62P positive cells. **, ***: P < 0.01, P < 0.001 compared to Thr-stimulated healthy platelets but non-treated with immunophilin antagonists; ††, †††: P < 0.01, P < 0.001 compared to Thr-stimulated DM2 platelets but non-treated with immunophilin antagonists; $, $$$, P < 0.001, compared to platelets from healthy subjects.

the secretion of α-granules when comparing DM2 patients versus healthy subjects, no differences were observed between the quinacrine fluorescence remaining in Thr-stimulated platelets from DM2 patients and healthy subject in presence of immunophilin antagonists (Fig. **3**, upper histogram compared to lower histogram where δ-granule secretion of platelets from healthy and DM2 patients is represented, respectively; n=6).

According to these results, the alteration in Ca^{2+} homeostasis evoked by incubating platelets with the FKBP antagonist, FK506, is large enough to alter Ca^{2+}-dependent mechanisms such as platelet granules secretion. Interestingly, evaluation of size and complexity of platelets positive particles (CD41 positive cells) by flow cytometry revealed that platelets from DM2 patients were smaller and also presented lower intracellular complexity.

Role of Immunophilins in the Aggregation of Platelets from DM2 Patients and Healthy Subjects

Platelet hyperaggregability in DM2 patients has been reported in the literature [6, 33, 34]. In order to evaluate whether immunophilins are involved in the exacerbated platelet aggregation found in DM2 patients, we have tested the effect of immunophilin antagonists on Thr-evoked aggregation. As shown in (Fig. **4A**), treatment of platelets from healthy subject with FK506 (50 μM; 5 min) resulted in a reduced percentage of aggregation and slope compared to untreated platelets (71.1 ± 9.8% and 81.4 ± 3.2%, respectively; $p<0.001$; n=8). Nevertheless, we observed a lower reduction in the percentage of aggregation and almost no alteration in the slope in platelets from healthy subject preincubated during 30 min with 500 nM rapamycin (80.5 ± 17.1% and 94.0 ± 22.8%, respectively; $p>0.05$; n=8). In contrast, incubation of platelets from DM2 patients with both immunophilin antagonists evoked a significant alteration in all the aggregation parameters evaluated. Thus, the percentage of aggregation was reduced by 88.6 ± 3.1% ($p<0.001$; n=8) and 27.3 ± 5.1% (Fig. **4B**; $p<0.001$; n=8) after treatment with FK506 and rapamycin, respectively. Meanwhile, the slope of aggregation was reduced by 80.5 ± 4.9% ($p<0.001$; n=8) and 16.1 ± 5.1% ($p<0.01$; n=8), in the presence of FK506 and rapamycin, respectively.

These results indicate that immunophilins participate in the regulation of platelet aggregation in human platelets. Since the immunophilins inhibitors induced a greater reduction in platelets from DM2 patients, our results indicate that immunophilins might be involved in the platelet hyperaggregability previously described in the diabetic patients.

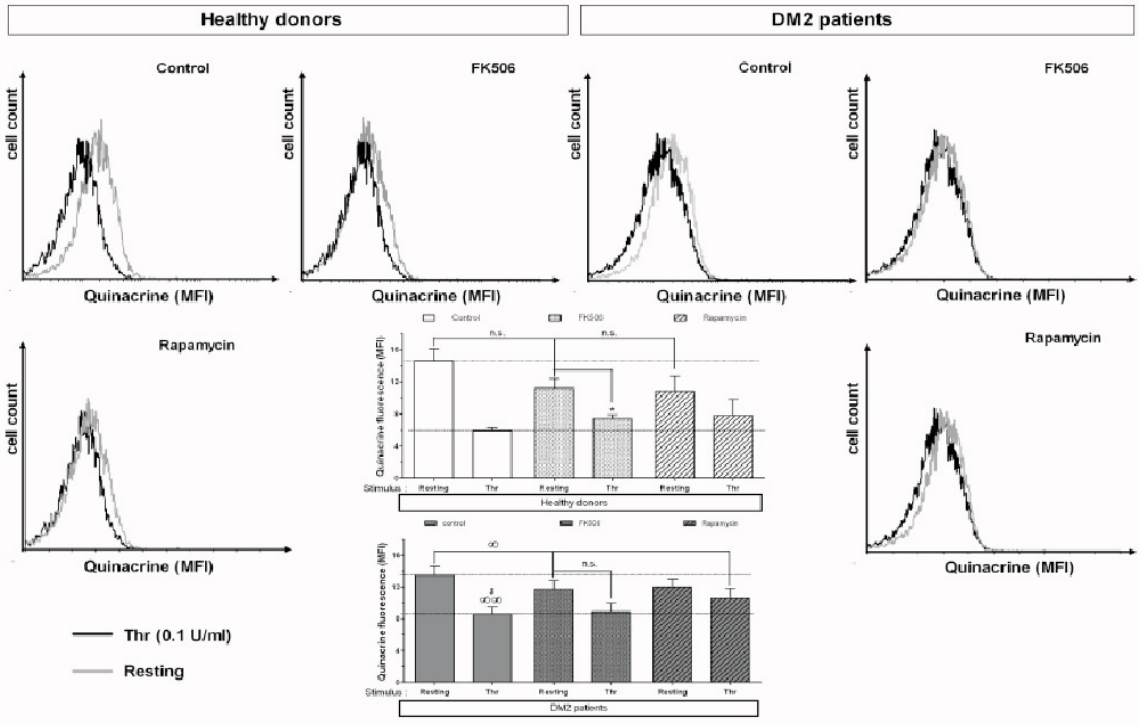

Fig. (3). Immunophilins are involved in platelet α-granules secretion in DM2 patients and healthy subjects. Platelets from DM2 patients and healthy subjects were stained with an anti-CD41 antibody and 10 μM quinacrine in the absence or presence of either rapamycin or FK506 (only for the latest 5 min), and stimulated for 10 min with Thr (0.1 U/ml) in the presence of anti-CD62P antibody. Fluorescence of anti-CD62P antibody (α-granules) was monitored in CD41 positive platelets by flow cytometry. Data shown are representative of 6 to 8 independent experiments. Histograms represent percentage mean fluorescence intensity against cell count. *, **: $P < 0.05$, 0.01 compared to healthy platelets non-treated with immunophilin antagonists; $^{\$}$:$P < 0.05$, platelets from DM2 patients compared to platelets from healthy subjects; $^{\infty}$, $^{\infty\infty\infty}$: $P < 0.01$, $P < 0.001$ compared to DM2 platelets but non-treated with immunophilin antagonists.

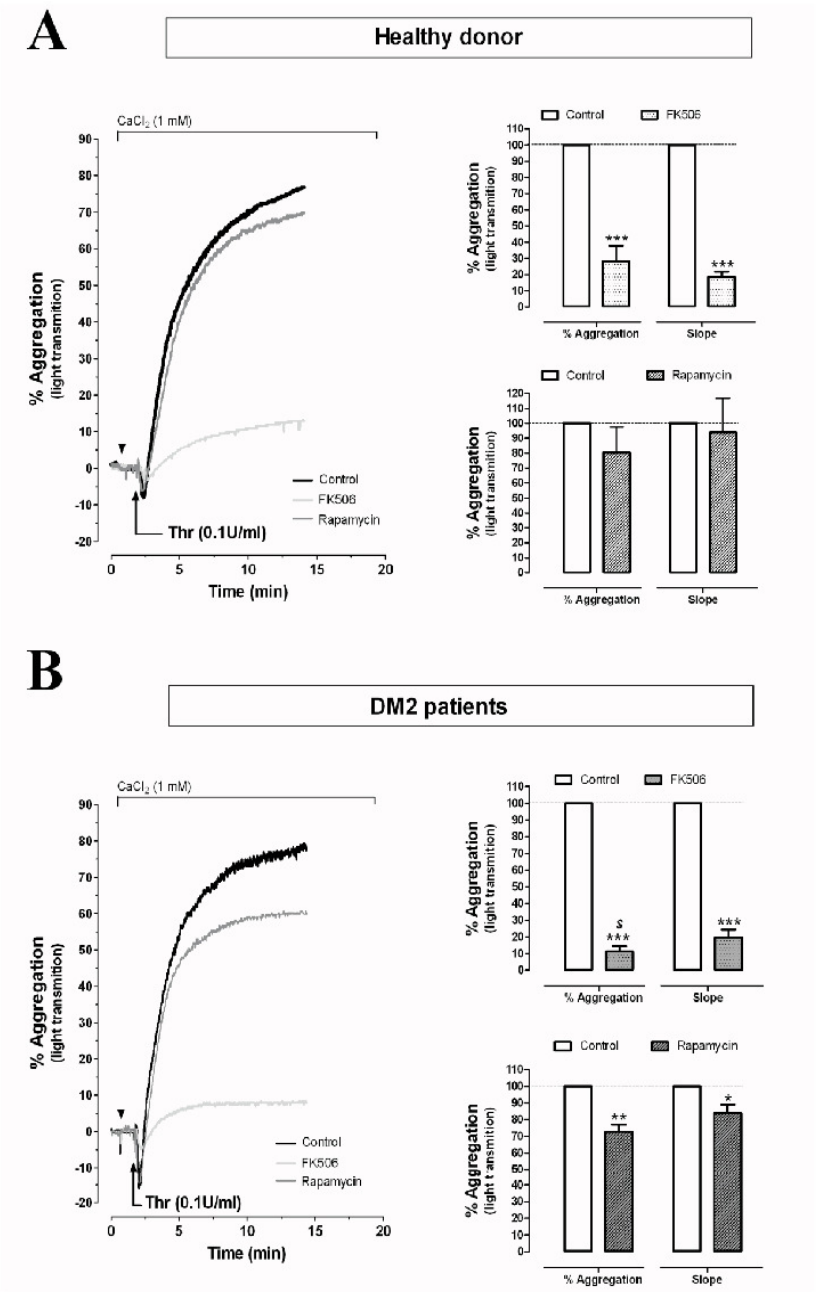

Fig. (4). Role of immunophilins in platelet aggregation in DM2 patients and healthy subjects. Platelets from healthy subjects (A) and DM2 patients (B) were suspended in Ca^{2+}-free HBS and incubated for 5 min with FK506 (50 μM) or for 30 min with rapamycin (500 nM). Platelets were then stimulated with Thr (0.1 U/ml) in the presence of 1 mM of $CaCl_2$ and aggregation was analyzed using an aggregometer. Traces are representative of 8 independent experiments. Histograms represent the percentage of aggregation expressed as mean ± ESM. *, **, ***, $P < 0.05, 0.01, 0.001$; $^{\$}$, $P < 0.05$, platelets from DM2 patients compared to platelets from healthy subject.

Elevated Expression of Immunophilin Members and Complexing to Ca^{2+} Channels in Platelets from DM2 Patients Compared to Platelets from Healthy Subjects

Expression of FKBP12 and FKBP52, which are involved in the regulation of intracellular Ca^{2+} homeostasis, was investigated by Western blotting. Briefly, a similar amount of platelets from DM2 patients and healthy subjects were lysed, and the cell lysates were subjected to Western blot analysis with anti-FKBP12 and FKBP52 specific antibodies. As shown in (Fig. **5**), despite both immunophilins are overexpressed in patients suffering DM2, only the expression of

FKBP52 was found significantly greater in DM2 patients (Fig. **5**). Additionally, coupling between FKBP12 and FKBP52 and the Ca^{2+} channels TRPC1 and Orai1 was investigated. As shown in (Fig. **6**), FKBP12 association with TRPC1 and Orai1 was enhanced by Thr, being this coupling greater, although not significantly, in healthy subjects than in DM2 patients. In contrast, FKBP52 was found to co-immunoprecipite with Orai1 in a larger extent than TRPC1 and significant differences were observed between healthy subject and DM2 patients. FKBP52/TRPC1 and FKBP52/Orai1 were not enhanced upon Thr stimulation of platelets from DM2 patients. Hence FKBP52 in DM2 patients could generate a complex with other Ca^{2+} channels like TRPC3, favoring a larger and deregulated Ca^{2+} entry known as non-capacitative calcium entry. Finally, as shown in (Fig. **6**), treatment of both healthy and DM2 patients platelets with immunophilin antagonist modified the coupling pattern previously described, subsequently altering the permeability of the channel to Ca^{2+}, thus evoking impaired Ca^{2+} homeostasis.

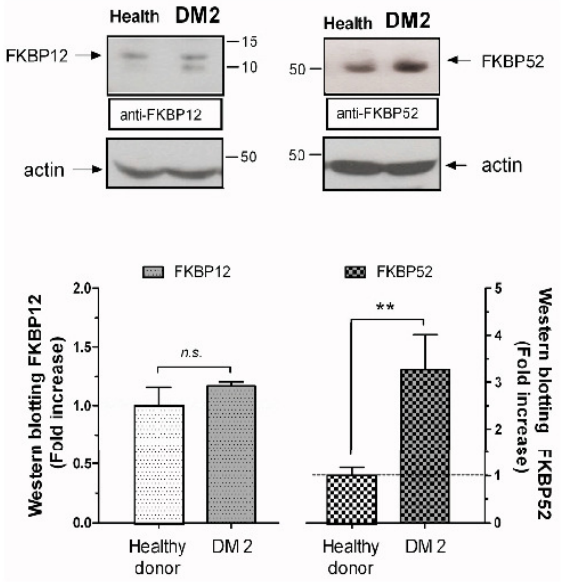

Fig. (5). Expression of immunophilins in platelets from DM2 patients. Platelets from healthy subjects and DM2 patients were suspended in Ca^{2+}-free HBS medium (2 x 10^8) and then lysed by mixing with LB for subsequent Western blotting. Images are representative of 4 independent experiments. Histograms represent fold increase of protein expression in platelets from DM2 patients and healthy subjects. ***, $P < 0.001$ expression in platelets DM2 compared to healthy subjects. Positions of molecular-mass markers are shown by the images.

DISCUSSION

Immunophilin antagonists have been widely used as immunosuppressors and the effects of tacrolimus (FK506) or sirolimus (rapamycin) on the intracellular Ca^{2+} homeostasis have been already reported in leukocytes and other cell types. In this sense, ryanodine receptors have been identified as the main target of FK506 in a pathway that involves calcineurin phosphatase activity. Furthermore, several Ca^{2+}-handling proteins, such as IP_3R and SERCA, have been shown to be altered in the presence of immunophilin antagonists. Here, we have shown evidences for the role of immunophilins on intracellular Ca^{2+} homeostasis in platelets from healthy and DM2 patients. As presented in (Fig. **1**), and as previously reported in other cell types, administration of immunophilin antagonists evoked alteration in Ca^{2+} release and Ca^{2+} entry. Interestingly, the effect of tacrolimus on Thr-evoked Ca^{2+} entry was concentration dependent; while low concentrations enhanced Ca^{2+} release and Ca^{2+} entry high concentrations inhibited Ca^{2+} entry. Therefore, we suggest being cautious with the dose administered to patients and the evaluation of aggregation parameters in patients treated with immunophilin antagonists.

In this sense, an early study reported enhanced FK506 effect on platelet function as determined by evaluation of platelet serotonin secretion and aggregation in response to ADP [35]. Similarly, rapamycin was later reported to enhance dose and time-dependently platelet aggregation and ATP secretion in response to ADP and TRAP-6, which mimics Thr stimulation [36]. In our hands, the immunophilin antagonists, FK506 and rapamycin, reduced platelet α-granule and δ-granule secretion in both platelets from DM2 patients and healthy subjects. Interestingly, evaluation of size and complexity of CD41 positive cells by flow cytometry revealed that platelets from DM2 patients were smaller and also presented lower intracellular complexity, which is indicative of lower granule content. This reduced granule content may be the cause of the reduced α- and δ-granule content, and might result in the reduced secretion observed in platelets from DM2 patients stimulated by Thr.

Furthermore, contrary to the previously described effect for ADP-induced aggregation, we have observed that both immunophilin antagonists reduce platelet aggregation stimulated by Thr in both healthy and DM2 patients. The inhibitory effect of FK506 on platelet aggregation was greater than that of rapamycin, which might be attributed to the broader spectrum of FK506 as immunophilin inhibitor. The effect of rapamycin on aggregation *in vitro* was promising, since it has a negligible effect on aggregation in healthy subjects but significantly reduced hyperaggregability in platelets from DM2 patients. Thus, considering that rapamycin is already used in clinic, we would recommend it use as a therapeutic strategy in DM2 patients to minimize the vascular complications associated to platelets. Although, we also suggest that it is need to be cautious with the dose administered to patients and the evaluation of the immunological system as well as the aggregation parameters in patients treated with immunophilin antagonists.

Finally, we report elevated FKBP12 expression in platelets from DM2 patients, but values of expression were not significant compared with those found in healthy subjects, which is opposite to previous observation in rats where diabetes mellitus have been artificially induced by streptozotocin administration [17]. Similarly, an exacerbated expression of FKBP52 was found in platelets from DM2 patients compared to healthy subjects. In this sense, we have found impaired association of FKBP52 with Ca^{2+} channels in DM2 patients, which might explain the enhanced expression of this protein in DM2 patients, perhaps as a mechanism of compensation. The impaired association between FKBP52

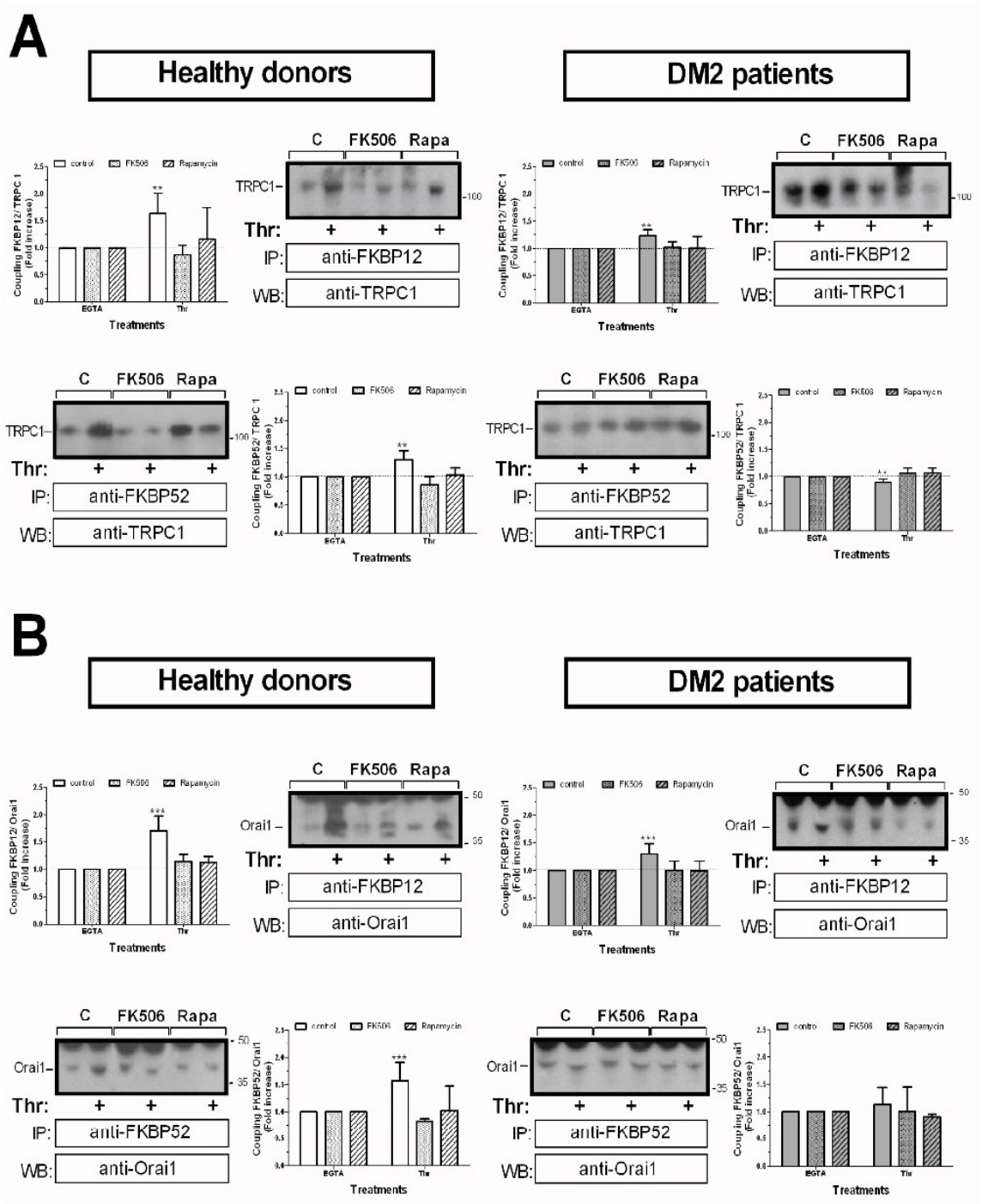

Fig. (6). Differences in FKBP12 and FKBP52 complexing to TRPC1 and Orai1 in diabetic and healthy subjects. Resting and Thr-stimulated platelets from DM2 patients and healthy subjects were immunoprecipitated with anti-FKBP52 or anti-FKBP12 antibodies and Western blotting was performed using anti-TRPC1 and anti-Orai1 antibodies as indicated. Panels are representative of 4 to 6 independent experiments **, ***: $P < 0.05, 0.01$, respectively.

and both Orai1 and TRPC1, together with previous findings demonstrating the effect of FKBP52 in SOCE in platelets [37], might explain the attenuated SOCE observed in platelets from diabetics, where we have reported decreased SOCE and enhanced non-capacitative Ca^{2+} influx, the latter leading to a greater global Ca^{2+} entry signal [38]. Meanwhile,

FKBP12 association with Ca^{2+} channels resulted enhanced by Thr in both DM2 patients and healthy subjects. Further analysis will allow us to better understand the role of immunophilins in Ca^{2+} entry and platelet function, which might lead to the design of new pharmacological tools to regulate the impaired platelet function characteristic of certain diseases, such as DM2.

Summarizing, we have found that immunophilins participate in intracellular Ca^{2+} homeostasis, secretion and aggregation in platelets from healthy subjects and DM2 patients. Inhibition of immunophilins affects in a larger extent to the function of platelets from DM2 patients, which agree with the fact that FKBP12 and FKBP52 are overexpressed in platelets from DM2 patients, and that an impaired coupling of FKBP52 to Ca^{2+} channels is present in these patients, Thus we strongly suggest that immunophilins are involved in the alterations presented in platelets from type 2 diabetic patients.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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RESEARCH ARTICLE

FKBP25 and FKBP38 regulate non-capacitative calcium entry through TRPC6.

López E., Berna-Erro A., Salido G.M., Rosado, J.A., Redondo P.C.*

ABSTRACT

Non-capacitative calcium entry (NCCE) contributes to cell activation in response to the occupation of G protein-coupled membrane receptors. The platelet physiological agonist, thrombin evokes the synthesis of diacylglycerol downstream of PAR receptors activation. Diacylglycerol has been described as a major agonist of the transient receptor potential channels (TRPCs) involved in NCCE, including TRPC3 and TRPC6 in human platelets. Although, it is known that immunophilins interact with TRPCs and regulate their activity in the activation of store-operated calcium entry (SOCE), the role of immunophilins in the regulation of NCCE remain unknown. Platelet incubation with the immunophilin antagonist, FK506 reduced dose-dependently OAG-evoked NCCE, an effect that was independent on the inactivation of calcineurin (CaN). FK506 was unable to reduce NCCE evoked by OAG in platelets from TRPC6 knockout mice. Ca^{2+} entry evoked by Hyp 9, a synthetic agonist of TRPC6, was also found to be sensitive to inhibition of immunophilins. Further evicende was obtained by analyzing the Ba^{2+} current in HEK-293 cells overexpressing TRPC6 in the presence of the immunophilin inhibitor. FK506 modified the pattern of association between FKBP25 and FKBP38 with TRPCs as well as impaired OAG-evoked TRPC3 and TRPC6 coupling in both human and mouse platelets. Finally, FKBP25 and FKBP38 expression silencing resulted in a significant reduction of OAG-evoked NCCE in MEG01 and HEK293. These findings provide strong evidence for a role of immunophilins, especially FKBP25 and FKBP38, in NCCE mediated by TRPC6.

KEY WORDS: TPRC6, platelets, NCCE, FKBP25 and FKBP38

INTRODUCTION

In human platelets Ca^{2+} entry from the extracellular medium is a key signaling event for both secretion and aggregation upon plasma membrane receptor occupation. Two major receptor-operated Ca^{2+} influx mechanisms have been described in these cells: store-operated or capacitative Ca^{2+} entry (SOCE) and non-capacitative Ca^{2+} entry (NCCE) (Berna-Erro et al., 2012b; Harper and Poole, 2011; Hassock et al., 2002; Jardin et al., 2009). In human platelets, and other cells types, both mechanisms would occur simultaneously upon the activation of G-protein coupled membrane receptors that activate downstream proteins like phospholipase C, which, in turn, generates diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) (Szumilo and Rahden-Staron, 2008). IP_3 mobilizes Ca^{2+} from the internal stores that subsequently evokes the activation of the stromal interaction molecule 1 (STIM1) (Putney, 2013; Shaw et al., 2013; Soboloff et al., 2012), which regulates the activation of the store-operated Ca^{2+} (SOC) channels in the plasma membrane. On the other hand, diacylglycerol has been presented as a second messenger that directly or indirectly gates non-capacitative Ca^{2+} channels (Rosado and Sage, 2000; Szumilo and Rahden-Staron, 2008).

The precise configuration of the Ca^{2+} channels involved in both mechanisms is still discussed nowadays; nevertheless, the hypothesis that SOCE occurs through Orai1, as well as transient receptor potential channels, such as TRPC1, has received support during the last decade (Cheng et al., 2013). Certain TRPC and Orai subtypes are claimed to constitute the channels involved in NCCE (Berna-Erro et al., 2012a; Hassock et al., 2002; Lee et al., 2014; Zhang et al., 2014). In human platelets, it was initially demonstrated the generation of the TRPC1/4/5 and TRPC3/6 heteromultimeric complexes in response to physiological agonists (Brownlow and Sage, 2005). SOC channels have been found to take part of a functional signalplex that includes other proteins and channels, including the scaffolding protein homer, IP_3 receptors (IP_3Rs), calmodulin (CaM), or α-actinin (Dionisio et al., 2011; Jardin et al., 2013; Redondo et al., 2007; Redondo et al., 2008). Contrary, the NCCE mechanism has received less attention and it has been generally accepted that it is conducted by heteromultimeric channels resulting from the association between TRPC3 and TRPC6 (Carrillo et al., 2012; Harper and Sage, 2010; Imai et al., 2012). Recently, Orai3 has been also reported to conduct NCCE in neurons and platelets (Berna-Erro et al., 2012a; Tang et al., 2001; Zhang et al., 2001).

The immunophilin family comprises immunophilins (FKBPs), cyclophilins (CyPs) and junglone. Some members of this family have been reported to be involved in the regulation of the intracellular Ca^{2+} homeostasis (MacMillan, 2013), trough interaction with different Ca^{2+} channels (Scaramello et al., 2009; Wagenknecht et al., 1996); (Cameron et al., 1995; Rosado et al., 2010), and even some members of this family belong to the permeable transition pore of mitochondria, like cyclophilin D (Elrod and Molkentin, 2013). FKBPs are characterized by binding to FK506, a chemical that impairs their chaperone activity, being FKBP12 and FKBP52 the most frecuently studied until now. These FKBPs are involved in the regulation of intracellular Ca^{2+} homeostasis via interaction with RYRs and even IP_3Rs (Cameron et al., 1995; Scaramello et al., 2009; Wagenknecht et al., 1996). FKBP12 and FKBP52 have also been reported to interact with TRPCs (Lopez et al., 2013b; Lopez et al., 2013c; Shim et al., 2009; Sinkins et al., 2004) and in human platelets, SOC channels like Orai1 or TRPC1 interact with both FKBP12 and FKBP52 in response to strong agonists, such as thrombin (Thr), or the SERCA inhibitor thapsigarsin (Lopez et al., 2013b). Furthermore,

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studies in cells from the rat cerebral cortex and HEK cells have revealed that FKB12 interacts with TRPC3, TRPC6 and TRPC7, while FKBP52 co-immunoprecipitates with TRPC1, TRPC4 and TRPC5, which might denote a different role of these proteins in the SOCE or NCCE (Sinkins et al., 2004). In HEK293 overexpressing TRPC6 it has been described that this channel also interacts with the native FKBP13 (Sinkins et al., 2004). In the present study we have investigated the role of immunophilins in the mechanism underlying DAG-induced NCCE in non-excitable cells.

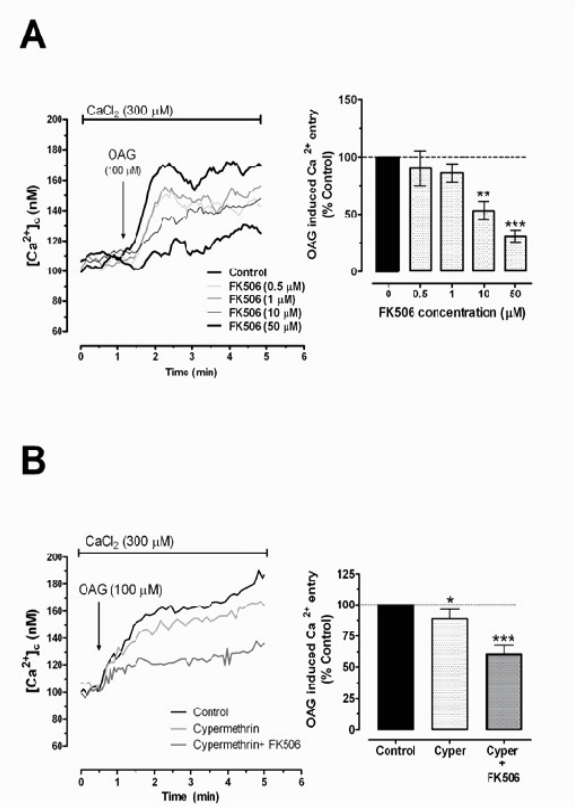

Fig. 1. FK506 reduces NCCE independently of its inhibitory effect in CaN activity. A) Fura-2-loaded human platelets were suspended in HBS containing 300 µM Ca^{2+} and preincubated for 5 min with increasing concentration of FK506 (0.5-50 µM). OAG (100 µM) was then added to initiate NCCE. B) NCCE was stimulated by adding OAG (100 µM) to platelets suspended in HBS containing 300 µM Ca^{2+} and previously incubated for 30 min in the absence or presence of the CaN inhibitor cypermethrin (100 nM) alone or in combination with FK506 (50 µM) that was added to the cuvette 5 min before starting the Ca^{2+} records. Graphs are representative of six independent experiments using different blood donors. Histograms represent OAG-evoked Ca^{2+} entry expressed as percentage of control (cells not treated with FK506). Data are presented as mean ± S.E.M. *,**, ***: represent P<0.05, <0.01 and < 0.001, respectively, as compared to control.

RESULTS

Immunophilins participates in NCCE in human platelets

Association and regulation of SOCE channels by FKBPs have been previously demonstrated, but their role in NCCE remains poorly described (Lopez et al., 2013c; Shim et al., 2009; Sinkins et al., 2004). As shown in Figure 1, incubation of human platelets with increasing FK506 concentrations (5 min. at 37ºC) resulted in a dose-dependent inhibition of NCCE stimulated by OAG (100 µM). Submicromolar concentrations of the inhibitor did not significantly alter NCCE but a significant reduction of 47.0 ± 8.0% ($P < 0.01$; n=6) was observed using 10 µM of FK506, as previously shown in neurons (Gibon et al., 2010; Shim et al., 2009), therefore, we have used 50 µM of FK506 throughout the study, since using this concentration we have observed a very significant inhibition of 69.0 ± 5.0% ($P < 0.001$; n=6) in OAG-evoked NCCE, and it was the concentration used to explore the role of immunophilins in SOCE previously published by our group (Lopez et al., 2013c). Additionally, FK506 (50 µM) was still able to reduce OAG-evoked NCCE in platelets previously incubated for 30 min with 100 nM of cypermethrin, a specific CaN antagonist (Figure 1B); thus suggesting that the effect of FK506 cannot be attributed to impairment of CaN activity.

Immunophilins regulate NCCE through TRPC6 in human platelets.

Previously, it was described NCCE is conducted by activating TRPC6 and TRPC3 in human platelets (Berna-Erro et al., 2012a; Brownlow and Sage, 2005). We have used the specific TRPC6 synthetic agonist derivated from hyperforin, Hyp 9, in order to establish whether TRPC6 might be regulated by the immunophilin activity (Gibon et al., 2010). As shown in Figure 2, FK506 (50 µM) evoked a very significant reduction of 57.2 ± 6.5% ($P < 0.001$; n=6) in the Hyp 9-evoked Ca^{2+} entry through TRPC6 in human platelets where mitochondria were previously disrupted using rotenone (10 µM), to impair complex IV of the electron transport chain, combined with oligomycin (10 µM), in order to impair ATP consumption due to the mitochondrial H^{+}-ATPase working in reverse mode (Tu et al., 2010). To further demonstrate that TRPC6 was the Ca^{2+}-channel targeted by immunophilin activity, we reproduced the same protocol in platelets from TRPC6 KO mice (TPRC6 -/-) and the results were compared with those obtained in WT mouse platelets (Albarran et al., 2014; Berna-Erro et al., 2014). FK506 reduced in 33.6 ± 4.9% ($P < 0.001$; n=8; Figure 3A) the OAG-evoked Ca^{2+}-entry in platelets from WT mice. Contrary, FK506 (50 µM) was unable to reduce the Ca^{2+} entry evoked by OAG in platelets from TRPC6 KO mice, which exhibited a significantly smaller Ca^{2+} entry upon OAG addition as expected due to the lack of TRPC6 expression. Additionally, we have performed patch-clamp experiments in HEK293 cells constitutively overexpressing TRPC6, and as shown in Figure 4A, inward and outward TRPC6 current resulted modified by preincubating cells FK506 for 5 min. Ga^{3+} was added to the cells at the end of each experimental protocol to corroborate that the currents recorded were due to OAG through TRPC6. We observed a reduction of 75.2 ± 4.4% ($P < 0.001$; n=8, Figure 4A) in the density current through TRPC6 channel in presence of FK506 as compared with control untreated cells. Similarly, we have found that NCCE induced by stimulating HEK293 (overexpressing TRPC6) with 100 µM OAG was reduced by 59.5 ± 22.4% ($P < 0.001$; n= 4; Figure 4B) in the presence of FK506, when analyzed with a Flexstation device and using Fluo-4 for monitoring $[Ca^{2+}]_c$ changes.

FKBP25 and FKBP38 interact with TRPCs

FKBPs were shown to interact with TRPCs in several cells models. In non-excitable cells, we and others have recently identified FKBP52 and FKBP12 as possible regulators of the SOC channels Orai1 and TRPC1 (Beech, 2005; Lopez et al., 2013c; Shim et al., 2009; Sinkins et al., 2004). Here, we have looked for immunophilins such as FKBP25 and FKBP38 that are receiving more attention from the scientific community due to their role in cells survival and subsequently cancer progression. In

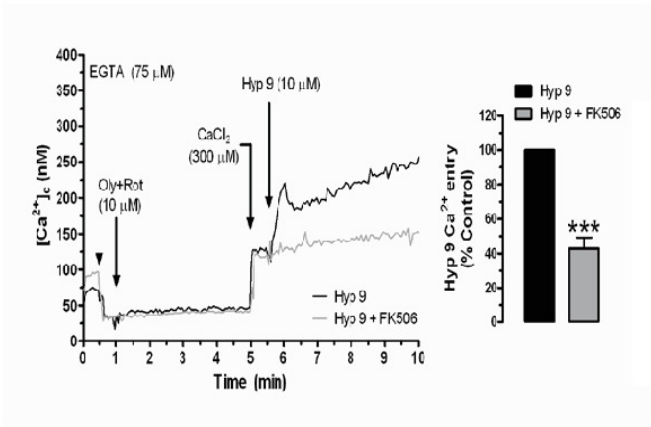

Fig. 2 . FK506 inhibits Hyp-9 activation of TRPC6. Fura-2-loaded human platelets were suspended in HBS-Ca^{2+} free medium (75 μM of EGTA was added, arrow head) and they were incubated for 4 min with olygomicin (10 mM, Oly) and rotenone (10 mM, Rot) alone, or in presence of FK506 (50 μM), and then, $CaCl_2$ (300 μM) was added to visualized mitochondrial-dependent Ca^{2+} entry, upon which TRPC6 opening was evoked by adding the synthetic hyperforin analogue, Hyp 9 (10 μM). Graph is representative of six independent experiments using different blood donors. Data are presented as mean ± S.E.M. ***: represents $P < 0.001$, as compared to control.

Figure 5, the interaction between FKBP38 and FKBP25 with TRPCs is shown. In human platelets, FKBP25 was found associated with TRPC6 and TRPC3 under resting conditions. FKBP25 and TRPC6 coupling was enhanced by 24.7 ± 10.9% ($P < 0.01$; n= 4; Figure 5A) upon platelet stimulation with OAG (100 μM) for 1 min, which was unaffected by treatment with FK506 (50 μM) for 5 min. Stimulation with OAG had not effect on the association between FKBP25 and TRPC3, which was detectable under resting conditions ($P > 0.05$; n=4). Interestingly, FKBP25 was also found to interact with TRPC1 under resting conditions and a very significant increase of 39.1 ± 15.3% ($P < 0.001$; n= 4; Figure 5C) was observed in platelets stimulated with OAG. Treatment for 5 min with FK506 significantly reduced OAG-evoked FKBP25/TRPC1 coupling (79.5 ± 13.2%; $P < 0.001$; n= 4; Figure 5C).

In human platelets under resting conditions, FKBP38 was found to associate with TRPC6, TRPC3, and, to a smaller extent, with TRPC1, as observed in Figure 5C. Treatment with OAG reduced the association between TRPC6 and FKBP38 and slightly (non-significantly) enhanced TRPC3 and FKBP38 interaction. FK506 significantly increased the association between FKBP38 and TRPC6 by 11.1 ± 4.0% ($P < 0.05$; n= 4; Figure 5A). In contrast TRPC3/FKBP38 association resulted significantly attenuated in presence of FK506 by 15.1 ± 8.7% ($P < 0.05$; n= 4; Figure 5B). Finally, association between TRPC1 and FKBP38 remains unaltered upon OAG stimulation in both resting or FK506 treated platelets.

By evaluating these patterns of interaction between the immunophilins and the different TRPCs, we concluded that TRPC6 seems to have much more relevance than TRPC3 during

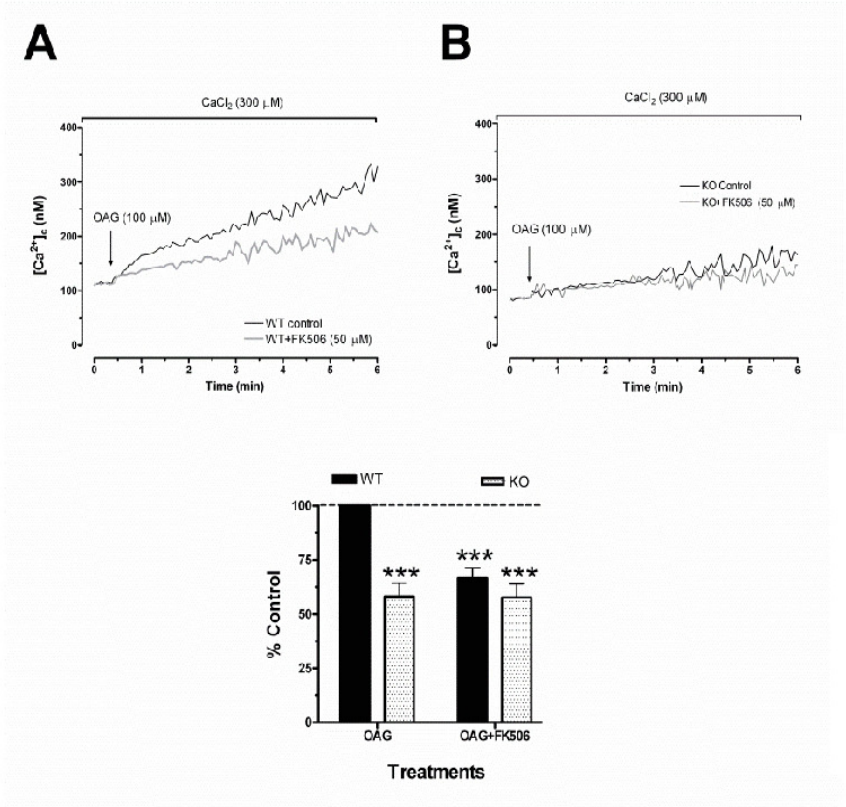

Fig. 3. NCCE entry is not affected by FK506 in murine TRPC6 -/- platelets. Fura-2-loaded platelets drawn from WT (A) and TRPC6 -/- mice (B), were suspended in HBS-rich Ca^{2+} medium (300 μM) and tyrode's buffer-Ca^{2+} rich medium, respectively, and then incubated for 5 min with FK506 (50 μM), upon which NCCE was stimulated with 100 μM of OAG. Graphs are representative of six independent experiments using different mice of 8 to 10 week old, while histograms show the average of the percentage and S.E.M. respect to the controls. ***: $P < 0.001$.

the coupling process evoked by OAG. Hence, we reproduced the same experimental protocols in the murine model lacking of TRPC6 (TRPC6 -/-), and the results are shown in Figure 6. In platelets drawn from WT mice, both immunophilins were found associated with TRPC6 (Figure 6A; n=3). Nevertheless, as shown in the Figure 6B, the interaction between the immunophilins and TRPCs was impaired in the murine TRPC6 KO platelets. Hence, these observations reinforce the idea of the existence of different configurations of the NCCE channels, and it would be TRPC6, the channels that immunophilins interact with during this process.

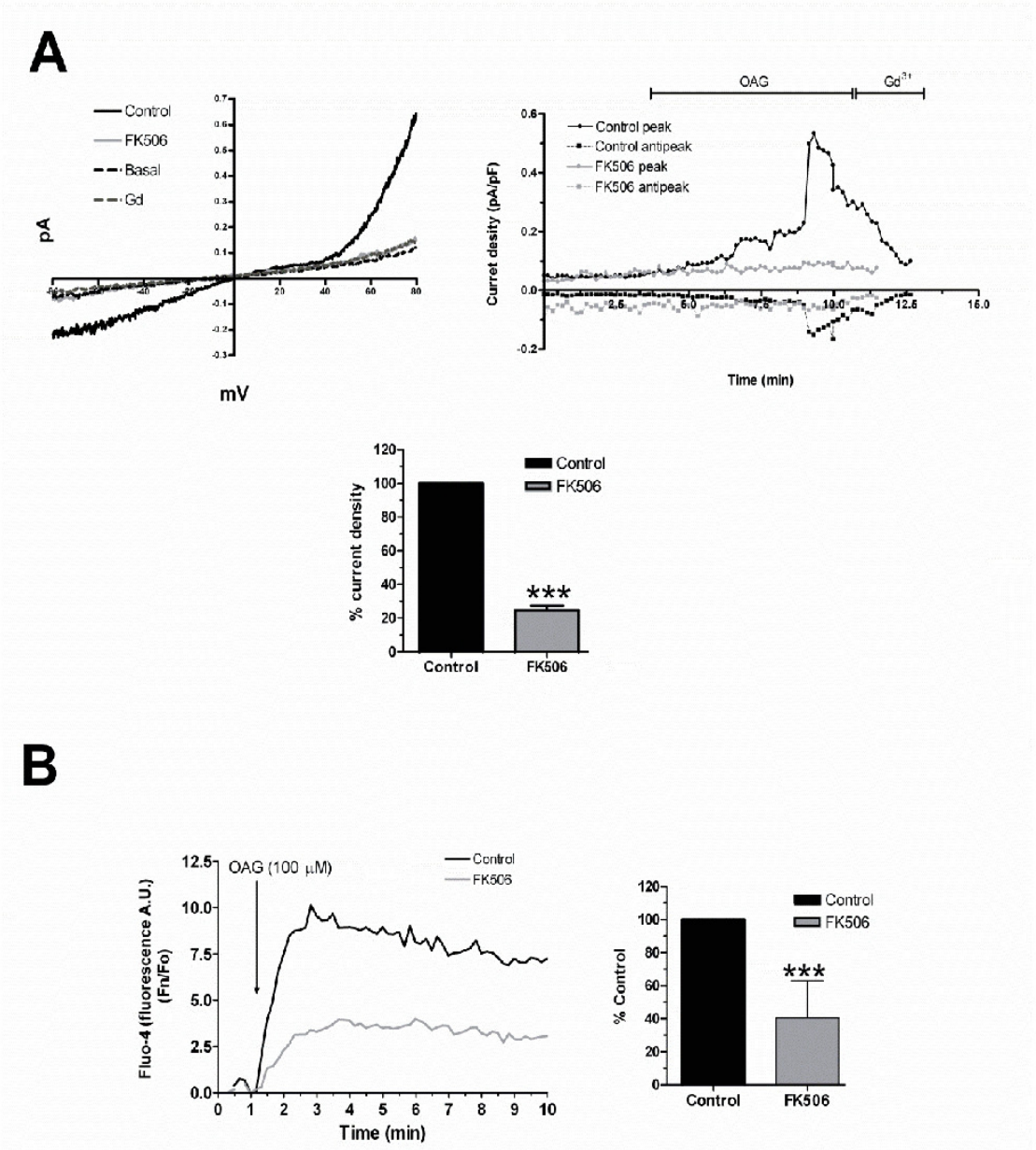

Fig. 4. FK506 almost completely abolishes the TRPC6 current and Ca^{2+} permeability in HEK293 cells overexpressing TRPC6. HEK293 constitutively expressing TRPC6 cell line, were culture in DMEN over coverslips. A) Patch-clamp experiments were performed to monitor TRPC6-dependent current in presence or absence of FK506 (50 µM). Time course of inhibition of OAG (100 µM) activated hTRPC6 current by FK506 is represented on the left hand side; current amplitudes were measured at -80 mV and +80 mV. Inward and outward current densities of OAG-activated hTRPC6 are represented in the right hand side. Gd^{3+} was added to the chamber at the end of each cuvette to corroborate that current monitored relay on TRPC6 permeability. B) HEK293 constitutively expressing TRPC6 were culture in DMEN over coverslips and they were detached 24 h before the beginning of the experiments and added to the 98-hole plate. Fluo-4 loaded cells with were incubated in the presence or absence of FK506 (50 µM) for 5 min, they where automatically stimulated with OAG (100 µM) and $[Ca^{2+}]_c$ changes was analyzed using a Flexstation system®. Patch-clamp results are reprensentative of nine independent experiments, while Flexstation experiments were reproduced six times. ***: P< 0.001, as compared to control.

Interestingly, we have not observed compensation of TRPC6 with TRPC3 in TRPC6 -/- mice (see Figure 6B middle panels where TRPC3 expression in WT and TRPC6-/- is analyzed by Western lotting).

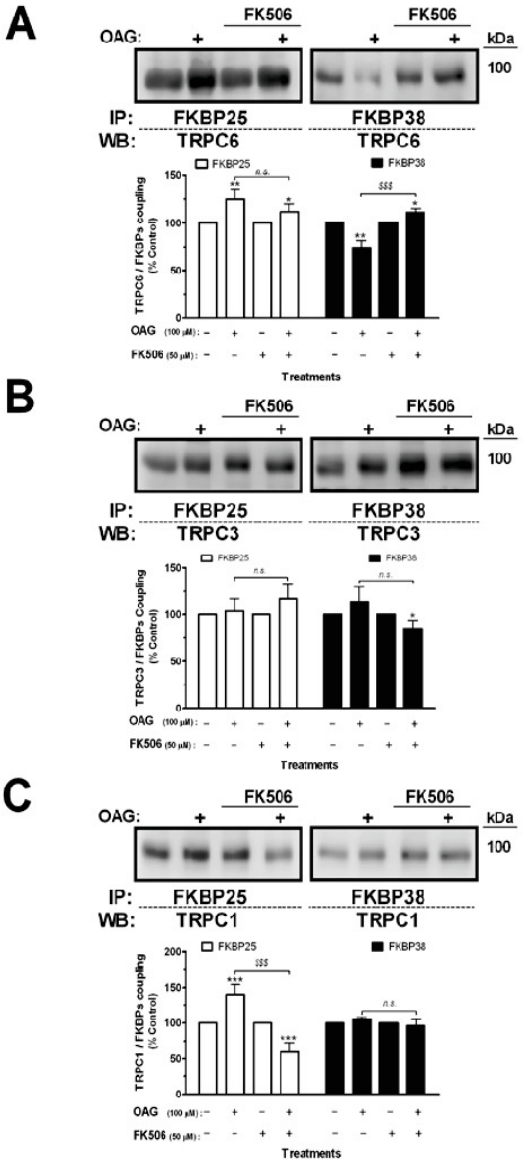

Fig. 5. FKBPs interact with TRPCs in human platelets. Human platelets were incubated for 5 min in absence or presence of FK506 (50 µM) and subsequently stimulated with OAG for 1 min (100 µM) in HBS-rich Ca^{2+} medium or kept under resting conditions, after which platelets were fixed by mixing with RIPA. FKBP25 and FKBP38 were immunoprecipitated from the cell fragments solution by incubating overnight with 1 µg/ml of anti-FKBP25 and anti-FKBP38 and agarose bead conjugated with protein A. Upon washing, immunoprecipitated complexes were analyzed by performing Western blotting using anti-TRPC3, anti-TRPC6 and anti-TRPC1 antibodies. Reprobing of the membranes was done with either anti-FKBP25 or anti-FKBP38 to corroborate the homogeneity of the samples loaded in the gel (data not shown). Images in the panels are representative of four independent experiments, while histograms represent the changes in the protein complexes expressed as the percentage and the S.E.M respect to control resting platelets. *,**, ***: represent $P < 0.05$, < 0.01 , < 0.001 respect to control resting platelets; $$$: $P < 0.001$ respect OAG stimulated platelets but non-treated with FK506.

FKBPs modify the structural composition of NCCE channels.

The previous disparity observed regarding the association of the different immunophilins with both NCCE channels in our cells model might be explained by the generation of different NCCE heteromultimeric channels; thus, we have evaluated the association between TRPC6 and TRPC3 in both murine and human platelets during NCCE activation. As shown in Figure 7A, during OAG-evoked NCCE (100 µM) a significant increase of TRPC3/6 coupling was observed (109.9 ± 3.0%; $P < 0.05$; n= 4), and this interaction was significantly reversed by incubating platelets for 5 min with 50 µM of FK506 (85.5 ± 4.8%; $P < 0.05$; n= 4). OAG-evoked association between TRPC6 and TRPC3 resulted also impaired in WT mice platelets incubated with FK506 (50 µM for 5 min.; Figure 7B; n=4). Thus, all together these results provide evidence that immunophilins participate in the assembly of TRPC6/TRPC3 heteromultimeric complex.

FKBP25 and FKBP38 silencing inhibit the activation of NCCE.

In Figure 8, we explored the extension of the mechanism described here by silencing the expression of FKBP25 and FKBP38 in two different cell types, MEG-01 and HEK293. As expected the reduction in the expression of FKBP25 or FKBP38 significantly attenuated the OAG-evoked NCCE by 52.1 ± 14.4% ($P < 0.001$; n= 4) and 37.59 ± 15.5% ($P < 0.001$; n= 4) in MEG-01 cells, and by 38.9 ± 11.9% ($P < 0.001$; n= 4) and 48.5 ± 14.4% ($P < 0.001$; n= 4) in HEK293, respectively. Silencing the expression of FKBP25 and FKBP38 in HEK293 cells did not alter the expression of TRPC3 or TRPC6 but slightly but non-significantly attenuated the coupling between TRPC6 and TRPC3. Hence, these particular inmunophilins would not be involved in the structure of the NCCE channels but regulate their function.

DISCUSSION

TRPC channels are subjected to different postraductional structural modifications that affect to their permeability (Bousquet et al., 2011; Hassock et al., 2002; Redondo et al., 2007; Shen et al., 2011; Shi et al., 2014). TRPC6 is one of the two classic TRPCs involved in NCCE operated by DAG. The relevance of NCCE in human platelets is evidenced in patients suffering type 2 diabetes mellitus, where an enhanced permeability through TRPC6 seems to be in the background of the hyperaggregability observed in these patients (Alexandru et al., 2008; Bouaziz et al., 2007; Mita et al., 2010). Here, we have explored the possible role of immunophilins in the regulation of NCCE stimulated by OAG, a cell-permeant DAG synthetic derivative.

Our results provide clear evidence for a role of immunophilins in NCCE that can be attributed to the activity of the FKBP members and not to the indirect inhibitory effect of FK506 on CaN, as previously suggested by others (Gibon et al., 2010; Liu and Ji, 2012). Inhibition of immunophilins did not alter NCCE in platelets from mice lacking TRPC6, which provides strong evidence that TRPC6 might be the target of the immunophilin activity (Kim and Saffen, 2005). Previous studies have reported direct interaction of immunophilins with TRPC channels. In neurons, FKBP52 presents two possible binding region in both extremes of the TRPC1 structure (19LPSSP23 and 644LPPPF648), being the latest region overlapped with the homer binding region and close to the STIM1 interaction region (consisting in two aspartate residues immediately after TRP box 2) (Shim et al., 2009). Interaction between immunophilins and TRPC6 might occur through a unique residue like FKBP12 that recognizes the Ser768 and Ser714 of TRPC6 upon phosphorylation by PKC (Kim and Saffen, 2005).

Here we have found interaction between TRPC6 and FKBP25 and FKBP38 under resting conditions that is modulated upon stimulation with OAG. FKBP25 has been described as a nuclear factor that have the ability to interact with pre-rRNA thus affecting to ribosomal protein synthesis (Gudavicius et al., 2014), and subsequently, general protein expression in the cells. FKBP25 is downregulated by rapamycin and FK506, having more affinity for rapamycin than FK506, hence it represents a good candidate for the alteration observed in patients suffering cancer or neurodegenerative disorders and are medicated with this macrolide (Jin et al., 1992; Klettner et al., 2001; Lopez et al., 2013a). Meanwhile, FKBP38 is activated by CaM/Ca^{2+} and it is inhibited by HSP90 (Edlich et al., 2007). It has been described that FKBP38 is an inhibitor of the pro-survival Bcl-2, as well as a constitutive inhibitor of mTOR, which also favored cell survival, thus its role in cancer progression is of a great interest nowadays (Romano et al., 2010; Yoon et al., 2011). Our results indicate for the first time that both immunophilins associate with TRPC6 to regulate NCCE, and according to the coupling pattern observed, FKBP25 could be acting as a positive activator, while FKBP38 could be acting as negative regulator. This statement is based on the results obtained under resting conditions, where FKBP38 appears associated with TRPC6 and stimulation of platelets with OAG evoked its dissociation from the channel, while inhibition of FKBPs activity with FK506 reverted the dissociation from TRPC6. Hence, FKBP38 might be impairing TRPC3 and TRPC6 interaction under resting condition, while FKBP25 and other immunophilins might be favoring the generation of the full active NCCE channels resulting of the complex between both channels. The fact that there is specific antagonist of FKBP38, like the N-(N',N'-dimethylcarboxamidomethyl)cycloheximide that reported promising results for prevent acute and/or chronic neurodegenerative diseases in rats (Edlich et al., 2006), lead us to think that this drug can be also used to evoke Ca^{2+} entry in platelets in patients where platelet aggregation is reduced, and subsequently, leading to the appearance of the spontaneous bleeding.

Summarizing, here we describe for the first time the interaction between FKBP25 and FKBP38 with TRPC6, which might be necessary for the regulation of NCCE. Both proteins seem to interact with the NCCE channel through TRPC6 as demonstrated in the murine TRPC6 KO model and both, expression silencing and pharmacological inhibition, using FK506, affect OAG-evoked Ca^{2+} entry and current through the NCCE channel.

MATERIALS AND METHODS

Materials

Apyrase (grade VII), aspirin, dithiothreitol (DTT), sodium dodecyl sulphate (SDS), ionic detergent tween 20 (Tween-20), Na_3VO_4, cypermethrin, bovine serum albumin (BSA), TRPC6 specific synthetic agonist (Hyp 9), geneticin G418 disulfate salt solution, and anti-actin antibody were from Sigma® (Madrid, Spain). FK506 were from Selleck Chemical® (Huston, Texas, U.S.A). OAG, oligomycin and rotenone were from Calbiochem® (Merck© Millipore, Darmstadt, Germany, UK). Protein A-agarose was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Kit-C of MEG-01 transfection was from Amaxa, and other medium for cell growing are supplied by LONZA® (CULTEK, Madrid, Spain). Anti-FKBP38 antibody and SiRNAs against FKBP25 and FKBP38 were purchase by Santa Cruz biotechnology Inc. (Texas, U.S.A.). Transfecting Lipid Reagent (Bio-Rad®, Madrid, Spain) Anti-TRPC1, anti-TRPC3 and anti-TRPC1 antibodies were from Alomone (Israel). Horseradish peroxide-conjugated anti-rabbit or anti-mouse IgG antibodies, hyperfilm ECL and molecular weight markers were from GE Healthcare Ltd (Chalfont St. Giles, UK). All other reagents were of analytical grade.

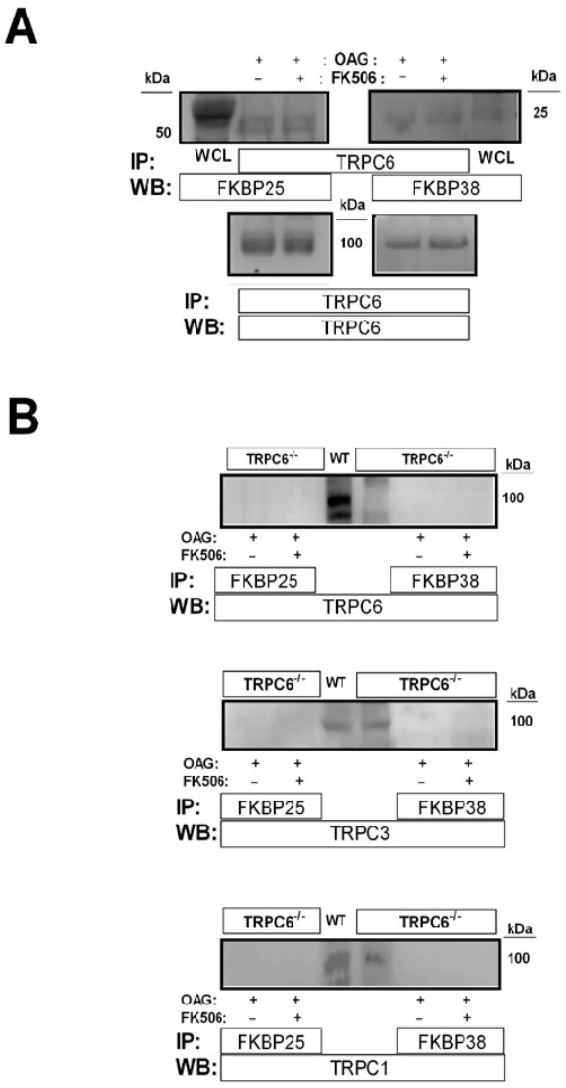

Fig. 6. FKBP25 and FKBP38 interact with TRPC6 but not with other TRPCs. Platelets drawn from WT (A) or TRPC6 -/- mice were suspended in a HBS- Ca^{2+} rich medium or tyrode's Ca^{2+}-rich medium (300 μM was added), respectively, and then were incubated for 5 min in absence or presence of FK506 (50 μM), after which they were stimulated for 1 min with OAG (100 μM) or kept under resting conditions, and finally fixed using RIPA. Immunoprecipitation of the proteins of interest was achieved by incubating the cell sample solutions with either anti-TRPC6 (A) or anti-FKBP25 (B) or FKBP38 (B) and beads of agarose conjugated with protein A. Immunoprecipitated protein complexes were analyzed by Western blotting using anti-FKBP25 (A) and anti-FKBP38 (A), or using anti-TRPC1(B), anti-TRPC3 (B) and anti-TRPC6 (B). Reprobing of the membranes with the appropriate antibody confirmed that same amount of proteins were loaded in each gel lane.

Animals

TRPC6-deficient (TRPC6 KO) and wild type (WT) mice were purchased from Deltagen, Inc. (San Mateo, CA) through the European Mouse Mutant Archive (EMMA) network (B6;129P2-Trpc6tm1Dgen/H; http://www.emmanet.org/deltagen/DELTAGEN _T881/). Briefly, murine TRPC6 allele expression was disrupted by substitution of 13 nucleotides located into the exon four (E4) with a selection cassette containing an internal ribosome entry site, β-galactosidase and the neomycin resistant gene. Chimeric mice were backcrossed with C56Bl6 mice to obtain the first generation of heterozygous mice, which were subsequently intercrossed to obtain homozygous mice for the Trpc6-null allele. TRPC6 silencing using this crossing has been previously reported by our research group (Albarran et al., 2014; Berna-Erro et al., 2014). All experiments were conducted on mice between 8 and 12 weeks of age. Experiments involving animals were conducted in accordance with the regulations of the local authorities and were approved by the Local Ethical Committee.

Human and mouse platelet preparations.

Human platelets were prepared as described previously (Lopez et al., 2013b; Redondo et al., 2007) . Briefly, blood was obtained from healthy drug-free volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma (PRP) was then prepared by centrifugation for 5 min at 700 × g and then aspirin (100 µM) and apyrase (40 µg/ml) was added. Platelets were then centrifuged at 350 × g for 20 min, then resuspended in HEPES-buffered saline (HBS), and finally subjected to the appropriate stimulation protocol as required. HBS contains (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45 and supplemented with 0.1% w/v bovine serum albumin and 40 µg/ml apyrase.

Mice were bled from the retroorbital plexus under isoflurane anesthesia (Albarran et al., 2014; Berna-Erro et al., 2014) and blood was collected into an Eppendorf tube containing 300 µl acid citrate dextrose buffer (ACD, 85 mM sodium citrate, 78 mM citric acid, 111 mM glucose, pH 7.3). Mouse blood was centrifuged in a Galaxy 7D centrifuge (VWR International, PA, U.S.A.) at 300 × g for 5 min. The supernatant was centrifuged again at 100 × g for 5 min to obtain platelet rich plasma (PRP). PRP was centrifuged at 600 × *g* for 5 min in the presence of acetylsalicylic acid (0.1 µg/ml) and the pellet was resuspended in Ca^{2+}-free Tyrode's buffer (NaCl 137 mM, KCl 2.7 mM, $NaHCO_3$ 12 mM, NaH_2PO_4 0.43 mM, glucose 0.1%, HEPES 5 mM, BSA 0.35%, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, pH 7.13) containing apyrase (40 µg/ml).

Both platelet samples (mice and human platelets) were subjected to Ca^{2+} monitorization or immunoprecipitation experiments according to the protocols described below.

Culture cell lines and protein silencing reactives

HEK293 stably expressing TRPC6 were grown using DMEN supplemented with antibiotic and 10% FBS at 37˚C with 5% CO_2. HEK 293 cells were detached from the culture dish using trypsin solution (0.25% trypsin+0.1% EDTA) and stored in the culture medium at room temperature for patch-clamp experiments within 5 hours (Seo et al., 2014; Xu et al., 2005). Additionally, for Ca^{2+} experiments, cells were passed to a 98-holes plate 24 h before the beginning of the experiments.

The human megakaryoblastic cell line, MEG-01 cells were obtained from ATCC (Manassas, VA, USA) and cultured at 37 °C and 5% CO_2 in RPMI media, supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 µg/ml gentamycin. Cells were transiently transfected with SiRNA FKBP25 and SiRNA FKBP38, using the kit-C, Amaxa Nucleofection system®, and following manufacturer's instructions, which in our hand have been previously shown to obtain satisfactory percentage of transfected cells (Lopez et al., 2013c; Lopez et al., 2012). 2 µg of siRNA was added to the Amaxa cubette. Upon transfection, cells were transferred to a fresh medium and kept for 72 h. Positive silencing of the protein was verified by performing Western blotting using the specific antibodies as described below.

HEK293 at 60-90% confluence were transfected with 7 µg SiRNA FKBP25 and SiRNA FKBP38 using Transfecting Lipid Reagent (Bio-Rad®). Upon 5 hours of incubation at 37ºC with the mix, cells were supplemented with fresh medium and they were analyzed 72 h after transfection.

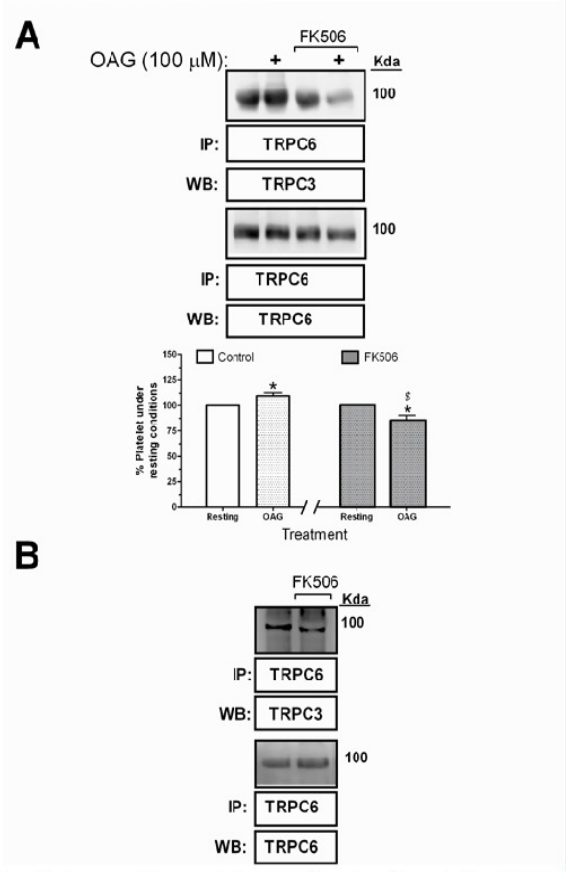

Fig. 7. Immunophilins participate in the formation of the NCCE heteromultimeric complexes. Human (A) or WT mice (B) platelets were suspended in a HBS- Ca^{2+} rich medium or tyrode's Ca^{2+}-rich medium, respectively, and incubated for 5 min in presence or absence of FK506 (50 µM), and then were subsequently stimulated with OAG (100 µM) for 1 min or left non-stimulated. Upon cell fixation by mixing with equal volume of RIPA, protein of interest were immunoprecipitated using anti-TRPC6 and beads of agarose conjugated with protein A. Immunoprecipitated proteins were solved by Western blotting using anti-TRPC3 antibody. Images in the panel are representative of four independent experiments. As shown, reprobing of the membranes was done with anti-TRPC6 antibody to corroborate the homogeneity of the sample loaded in each gel lane. *: $P < 0.05$ respect control platelet under resting conditions, and $: $P < 0.05$ respect to control platelets stimulated with OAG.

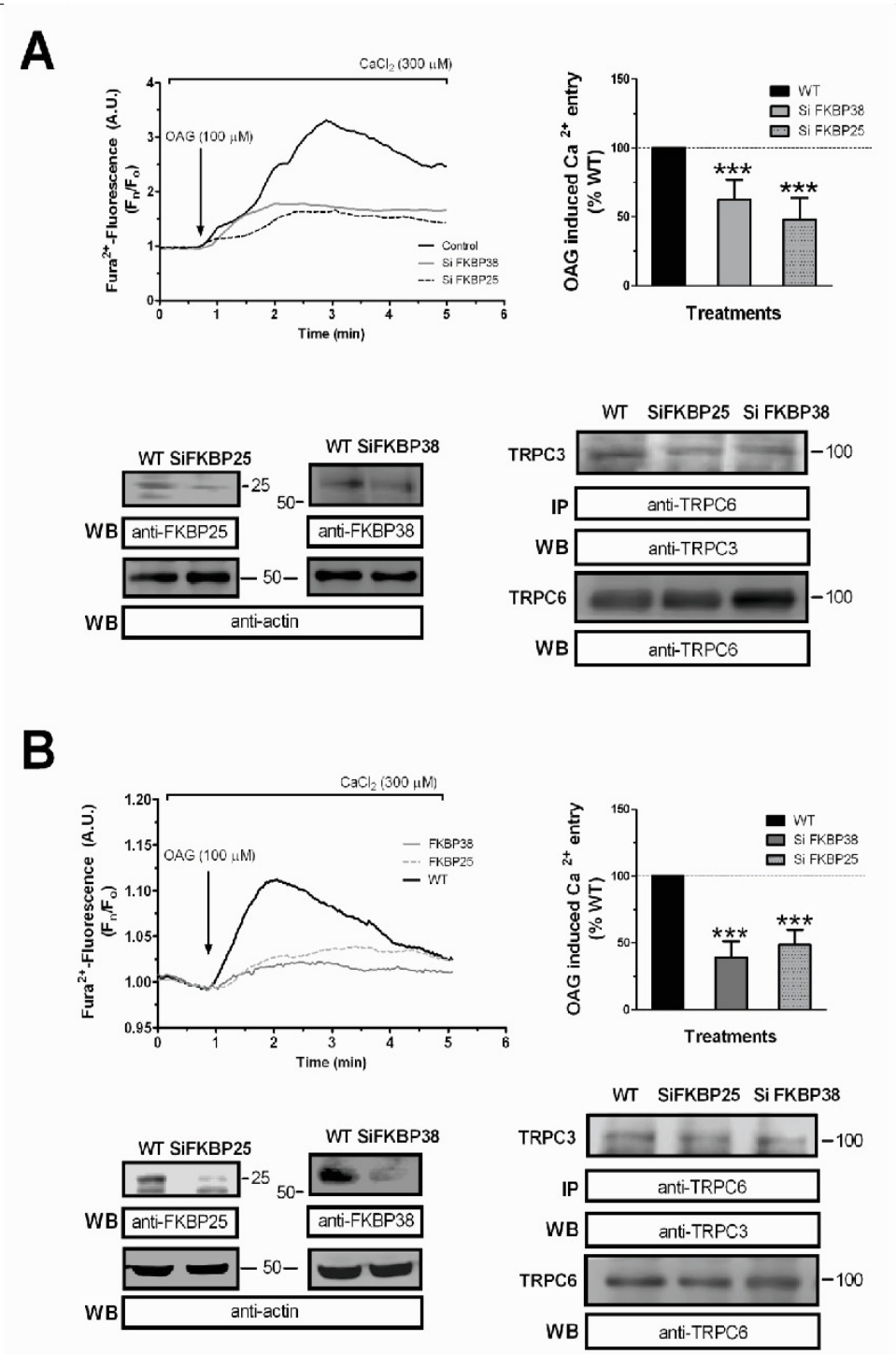

Fig. 8. Silencing of FKBP25 and FKBP38 reduces NCCE in MEG-01 and HEK293 cells. MEG-01 (A) and HEK293 (B) cells were transfected with 2 µg/ml of SiRNA against FKBP25 and FKBP38. Upon confirming the reduction in the expression of the FKBPs which occurred 72 h after transfection, cell were loaded with fura-2 and mounted in a perfusion chamber of a single cells imaging system. Cell were then perfused with HBS-rich Ca^{2+} (50 µM) containing or lacking FK506 (50 µM), and after 5 min cells were stimulated with OAG solution (containing both Ca^{2+} (300 µM) and FK506, or containing only Ca^{2+}). Additionally, FKBP25 and FKBP38 expression was observed in both MEG-01 and HEK293 transfected with SiFKBP25 or SiFKBP38 by Western blotting using the specific antibodies. Finally, coupling between TRPC6 and TRPC3 was explored in both cell types and compared to control non-transfected cells. Reprobing of the membranes with anti-TRPC6 antibodies or anti-actin antibodies was done following the appropriate protocols in order to corroborate that similar amount of proteins was loaded in each gel lane. Graphs are representative of 4 independent cells transfections, meanwhile histograms represent the mean of the percentage of control ± S.E.M. Images in the panels are representative of 3 to 4 independent experiments. ***: P < 0.001.

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Measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$)

The platelet-rich plasma isolated from both human and mouse blood was incubated at 37ºC with 2 µM of fura-2/AM for 40 min in order to evaluate the changes in the $[Ca^{2+}]_c$ evoked during platelets activation. Fluorescence was recorded from 2 ml aliquots of magnetically stirred human platelet suspensions (10^8 cells/ml in HBS) at 37°C using a spectrophotometer (Cary Eclipse, Varian, Madrid). Mouse platelets were suspended in 600 µl of tyrode's buffer containing 50 µM of $CaCl_2$, and the $[Ca^{2+}]_c$ was monitored using a RF-5301PC spectrofluorophotometer (Shimadzu®, Japan). Samples were alternatively excited at the wavelengths of 340 and 380 nm and emission was recorded at 510 nm. Changes in $[Ca^{2+}]_c$ were determined using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz (Grynkiewicz et al., 1985). Alternatively, MEG-01 and HEK293 cells were incubated for 30 min with 2 µM of fura-2/AM at room temperature and then centrifuged for 2 min at 100 × *g* and resuspended in fresh HBS medium supplemented with 50 µM of $CaCl_2$. Culture cells were then transferred to a perfusion chamber that was placed under an inverted microscope. Emission of fura-2 fluorescence as result of changes in the $[Ca^{2+}]_c$ in culture cells were monitored using a single-cell configuration equipment and were processed using Aquacosmos software (Hamamatsu ®, Japan). Data are expressed as change in fluorescence after the addition of different stimulus (Fn) divided by fluorescence emitted by the cells under resting conditions (Fo). Finally, HEK293 overexpressing TRPC6 were loaded with Fluo-4 by incubating the cells for 60 min at 37ºC. $[Ca^{2+}]_c$ in HEK293 cells were monitored using a Flexstation 3 system (Molecular Devices®, USA), in which cell growing in a 98-holes culture plate are stimulated by adding 100 µM of OAG, 300 µM of $CaCl_2$, and then the calibration buffers using a robotic arm, and following the protocol setting established by the main handling control unit.

Patch-clamp experiments

Whole-cell patch-clamp recordings were performed as described previously (Seo et al., 2014; Xu et al., 2005). Patch pipette solution contained (in mM): 40 EGTA, 10 HEPES, 48 L-glutamic acid, 8 NaCl, 2 $MgCl_2$, 10 Na^{2+}-ATP, 10 Hepes, 17 $CaCl_2$ (osmolarity was adjusted to ~300 mOsm with mannitol and pH was titrated to 7.2 using CsOH). The extracellular (bath) solution contained (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 1 $BaCl_2$, 10 HEPES, 10 Glucosa (osmolarity ~290 mOsm and pH 7). A salt–agar bridge was used to connect the Ag–AgCl ground wire to the bath. Signals were amplified with an Axopatch 200B patch-clamp amplifier and controlled with Signal software 3.05 (CED). A 200 ms ramp protocol from −100 mV to +100 mV was applied at a frequency of 0.1 Hz from a holding potential of −60 mV. Current signals were analogue filtered (Bessel) at 1 kHz and digitally sampled at 3 kHz. TRPC6 current was measured at -80 and +80 mV, and the differences in the current between control and FK506- treated cells was estimated and represented as % respect to control.

Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed as described previously [2-4]. Briefly, 500 µl aliquots of human platelet and mouse platelet suspensions (10^9 cells/ml) were stimulated with OAG in the absence or presence of FK506, and then, cells were lysed by mixing with an equal volume of lysis buffer, RIPA (2x) (pH 7.2) containing; 316 mM NaCl, 20 mM Tris, 2 mM EGTA, 0.2% SDS, 2% sodium deoxycholate, 2% triton X-100, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 100 µg/ml leupeptin and 10 mM benzamidine. MEG-01 and HEK293 cells lysed in buffer NP-40 (2x) containing: 40 Tris-HCl, 277 NaCl, 20% Glycerol, 2% Nonidet, 4 EDTA. Aliquots of platelet, MEG-01 and HEK293 cell lysates (1 ml) were immunoprecipitated by incubating cells with 1 µg/ml of anti-TRPC6, anti-FKBP25, anti-FKBP38 antibodies and 25 µl of protein A-agarose overnight at 4°C on a rocking platform. The immunoprecipitates were solved by 10% SDS-PAGE and separated proteins were electrophoretically transferred onto nitrocellulose membranes for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Immunodetection was achieved using the anti-TRPC6 antibody diluted 1:1000 in TBST (+BSA), anti-TRPC3 antibody diluted 1:1000 in TBST (+BSA), anti-TRPC1 antibody diluted 1:200 in TBST (+BSA), anti-FKBP25 diluted 1:500 in TBST (+BSA), anti-FKBP38 diluted 1:500 in TBST (+BSA), and incubated for 2 h, or using an anti-actin antibody incubated for 1 hour at 4ºC and diluted 1:1000 in TBST (+BSA). The primary antibody was removed and blots were washed six times for 5 min each with TBST (+BSA). To detect the primary antibody, blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:10000 in TBST (BSA), depending of the primary antibody used, and then exposed to enhanced chemiluminescence reagents for 5 min. Blots were then exposed to photographic film. The density of bands on the film was measured using the Image J free software from NIH. Stripping of the membranes and reprobing using antibody against the immunoprecipitated proteins or with anti-actin antibody was done to corroborate that a similar amount of protein was loaded in each lane.

STATISTICAL ANALISIS

Analysis of statistical significance was performed using Student's unpaired t-test and only values with $p < 0.05$ were accepted as significant.

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Competing interests

The authors declare no competing interests.

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DISCUSIÓN

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La homeostasis del Ca^{2+} está en parte regulada por las inmunofilinas ya que se ha descrito que estas proteínas controlan los canales de Ca^{2+} como el RyR y el IP_3R. En esta Tesis Doctoral hemos querido demostrar la importancia que tienen las FKBPs en la regulación del Ca^{2+} en plaquetas y otros modelos celulares

En el primer artículo publicado de la Tesis Doctoral demostramos cómo las inmunofilinas, en concreto las FKBPs, participan en la regulación de la ECC y cuáles de estas proteínas se encuentran involucradas en este proceso. Una de las vías evaluadas ha sido la de la CaN ya que se conoce que los complejos inmunofilinas/FK506 y CsA/inmunofilinas inhiben la CaN, a pesar del hecho de que el FK506 o la CsA se unen específicamente al dominio activo de las inmunofilinas.

El incremento inducido por el FK506 en la salida de Ca^{2+} desde los depósitos intracelulares podría estar atribuido a la actividad de fosforilación/defosforilación de las proteínas CaM y CaN sobre los canales IP_3R y RyR, como se ha visto en otros tipos celulares (Kasri et al., 2004; MacMillan et al., 2005; Vanderheyden et al., 2009; Yamaguchi et al., 2005). Sin embargo, en plaquetas humanas, hemos observado que la inhibición de la CaN con cipermetrina, no produce alteración en la salida de Ca^{2+} desde los depósitos, de tal manera que se podría atribuir todo el aumento provocado por el FK506 a su efecto inhibitorio sobre las inmunofilinas. Sin embargo, no se descarta el papel regulador de la CaN en la salida de Ca^{2+} en plaquetas humanas, debido a las diferencias significativas observadas entre las células tratadas con rapamicina + cipermetrina, en las que la combinación de ambas drogas no parece tener ningún efecto, en comparación con las tratadas solas con rapamicina, que sí presentaron una disminución en la liberación. Este mecanismo dependiente de CaN podría también estar presente en células MEG-01, ya que al silenciar la proteína FKBP52 se redujo la salida de Ca^{2+} estimulada con tapsigargina, pero ésta se inhibió aún más al tratar estas células con FK506. Esto es indicativo del papel de la FKBP52 en este mecanismo, además de que la participación de otras inmunofilinas en este proceso no puede ser descartado. Por lo tanto podemos afirmar que la salida de Ca^{2+} estimulada por tapsigargina en plaquetas está regulada por la CaN y la FKBP52 sin descartar otras FKBPs.

La participación de las inmunofilinas sobre la regulación de la ECC, ha sido poco estudiada hasta el momento. Sin embargo, algunos autores han considerado que existe un mecanismo dependiente de CaN que regula la activación de la entrada de Ca^{2+}. El

mecanismo propuesto sugiere que sólo en condiciones donde las $[Ca^{2+}]_c$ alcanzan un valor crítico, la CaN puede activar los canales no capacitativos de Ca^{2+} (Mignen et al., 2003). En plaquetas humanas hemos observado que, una vía independiente de CaN podría también existir ya que la incubación solo con rapamicina también produce una inhibición de la entrada de Ca^{2+}, en la que los efectos observados están relacionados con la inhibición de la ruta de mTOR, vía independiente de CaN. Las últimas observaciones vistas en MEG-01 y en plaquetas, sugieren que la FKBP52 y las actividades de otras inmunofilinas como la FKBP12, se inhiben al unirse con drogas como el FK506 o la rapamicina, siendo suficiente para alterar la ECC en nuestra línea celular.

A pesar de que otros grupos de investigación han presentado evidencias en contra de que los TRPCs, y en partícular TRPC1, participen en la ECC en plaquetas, usando protocolos que incluyen ratones KO (Varga-Szabo et al., 2008), nosotros hemos mostrado que el silenciamiento de TRPC1 en MEG-01 tiene un claro efecto negativo en la ECC, por lo tanto se demuestra que TRPC1 tiene un papel relevante en este mecanismo (Cheng et al., 2011b; Kim et al., 2009; Olah et al., 2011). Estos datos concluyentes avalan otros resultados previos obtenidos por nuestro grupo de investigación, en los que se sugiere que TRPC1 es un elemento relevante del complejo macromolecular generado durante la ECC (Redondo et al., 2008a; Rosado and Sage, 2000a).

Recientes estudios han descubierto una región dentro de la estructura de TRPC1, que ha sido descrita como una región diana para la actividad PPIase de las inmunofilinas. Esto sugiere que FKBP12 o FKBP52 se pueden unir a TRPC1 y provocarle una isomerización de los residuos de prolina presentes en esta región. Este cambio conformacional de TRPC1 le haría irreconocible para proteínas como homer. Homer ha sido descrito como un elemento estructural que estabiliza la unión entre los complejo TRPC1 durante la activación de la ECC. Por ello, sugerimos que la disociación de las inmunofilinas del complejo heteromultimérico de TRPC1 interrumpe la frágil estequiometria del complejo macromolecular generado durante la activación de la ECC, siendo este papel independiente de la actividad de la CaN. La presencia de las inmunofilinas en estos complejos se observa al ver que tanto el FK506 como la rapamicina bloquean significativamente la asociación entre TRPC1 y el IP_3R tipo II. De la misma manera, en células MEG-01 en las que está silenciada la proteína FKBP52, también se observa esta reducción. La interacción entre los TRPs y las inmunofilinas ya ha sido demostrada anteriormente, principalmente en canales no capacitativos como

TRPL, TRPV y TRPC6. En este trabajo hemos mostrado por primera vez que hay asociación entre la FKBP52 y TRPC1 tanto en plaquetas como en MEG-01, y que estos complejos se alteran en presencia de los inhibidores de las inmunofilinas como el FK506 y la rapamicina. En otros modelos celulares, FKBP12 se asocia con TRPC3 y TRPC6, mientras que FKBP52 con TRPC1, TRPC4 y TRPC5, por lo que no podemos excluir la participación de otras inmunofilinas en la activación de la ECC en plaquetas humanas, ya que en MEG-01, donde fue silenciada la FKBP52, el FK506 todavía produjo una reducción mayor de la ECC. La participación de las inmunofilinas en la ECC también queda confirmada por esta vía.

También hemos descrito por vez primera que Orai1 interactúa con miembros de las inmunofilinas tanto en plaquetas como en MEG-01, confirmando la participación de estas proteínas en la ECC, ya que Orai1 es un elemento clave del canal capacitativo.

Una explicación alternativa para la regulación de la ECC mediada por la FKBP52, podría ser la competencia con homer, que se ha demostrado que interactúa con TRPC1 y Orai1 regulando la ECC en varios tipos celulares incluyendo plaquetas (Jardin et al., 2012). En neuronas se ha demostrado que homer comparte una región con FKBP52 que se encuentra localizada dentro del dominio C-terminal de TRPC1, sin embargo, la existencia de tal competencia no ha sido todavía confirmada en plaquetas (Shim et al., 2009).

Una vez confirmada que las inmunofilinas participan en la homeostasis del Ca^{2+}, quisimos ver el efecto que tenía su inhibición sobre la funcionalidad plaquetaria en pacientes trasplantados, ya que los artículos disponibles en la literatura acerca de los efectos de sirolimus en la función plaquetaria son controvertidos. Se conoce que terapias basadas en la administración de rapamicina representan una buena alternativa en pacientes trasplantados de corazón, en pacientes que presentan complicaciones vasculares y particularmente, en aquellos en los que la inmunosupresión es debida al diagnóstico de neoplasias. Pero poco se conoce de los efectos adversos en plaquetas como consecuencia de la terapia.

Nuestros resultados indican que la administración prolongada de un análogo de la rapamicina, el everolimus, modifica el patrón de homeostasis del Ca^{2+} en plaquetas humanas con todos los estímulos utilizados. Donde realmente no se observaron diferencias significativas fue en los pacientes tratados con sirolimus al estimular las plaquetas con ADP.

Curiosamente, las alteraciones observadas en la homeostasis del Ca^{2+} resultaron ser más evidentes en pacientes tratados con sirolimus durante los 24-36 meses de tratamiento; después de este tiempo, la mayoría de los pacientes incluidos en este estudio recuperaron el patrón normal de movilización de Ca^{2+} en respuesta a Thr. Este hecho explica el elevado coeficiente de correlación encontrado entre los niveles de entrada de Ca^{2+} y el tiempo de administración. Además, una correlación similar ha sido demostrada en células vasculares pulmonares donde el tratamiento con rapamicina inhibe la ECC en presencia del inhibidor de SERCA, el ácido ciclopiazónico (Ogawa et al., 2009). También se ha visto en células de músculo liso de la arteria pulmonar humana, en las que activando el receptor PDGF se activa la vía Akt/mTOR produciéndose un aumento de la ECC. La activación de esta vía provoca un aumento en la expresión de las proteínas STIM1 y Orai1 lo que da lugar a ese aumento visto en la ECC y por lo tanto en la proliferación de estas células (Ogawa et al., 2012).

Se conoce que elevadas concentraciones de rapamicina pueden aumentar la agregación plaquetaria en repuesta a ADP y disminuir la capacidad de la aspirina para inhibir la agregación plaquetaria. En este último caso la rapamicina estaría favoreciendo la actividad de la ciclooxigenasa (Wu et al., 2009). Estos estudios han sido realizados *in vitro* usando tiempos cortos de incubación, lo cual difiere de la exposición crónica durante años, que ha sido objeto de parte de los estudios recopilados en la presente Tesis Doctoral.

Estudios previos muestran que inhibidores específicos de mTOR, como PP242 y torin1, no produjeron un aumento en la fosforilación de Akt en el residuo treonina 308 (Moore et al., 2011). Nosotros tampoco hemos observado cambios en el patrón de fosforilación de Akt entre individuos sanos y pacientes pertenecientes al grupo II (24-36 meses), lo cual podría indicar que mTOR podría no estar participando en la modificación de la actividad de Akt. En ese mismo estudio se observó que en plaquetas incubadas durante 15 min con rapamicina (200 nM) no se producía una alteración significativa en la agregación activada por SFLLR, indicando que bajo estas condiciones experimentales mTOR2 regula la función de Akt y en consecuencia, la actividad plaquetaria (Moore et al., 2011).

Sin embargo, un estudio ha demostrado que mTOR2 es sensible a rapamicina en períodos de exposición de larga duración (Sarbassov et al., 2006). De tal manera que tanto mTOR1 como mTOR2 podría estar participando en la agregación plaquetaria.

Las diferencias observadas en la agregación plaquetaria entre individuos sanos y pacientes tratados puede deberse al hecho de que mTOR está regulando la síntesis de proteínas en respuesta a mecanismos de adhesión a la superficie plaquetaria. La integrina αIIb/β3 regula la síntesis de Bcl-3 en respuesta a Thr, es decir, en plaquetas humanas activadas esta integrina es capaz de unirse al colágeno y al fibrinógeno y activar mTOR para que module la síntesis de Bcl-3. Bcl-3 es una proteína que se ha visto que participa en la agregación plaquetaria, la retracción del coágulo formado y en la remodelación de las plaquetas activadas (Aslan et al., 2011; Law et al., 1996; Weyrich et al., 2007).

En este estudio hemos analizado mediante citometría la exposición de P-selectina en la membrana plaquetaria en función del tiempo de tratamiento de los pacientes. Se observa una elevada degranulación en plaquetas en reposo, en comparación con los individuos sanos, lo cual explica la reducida respuesta a Thr y la alteración en la agregación encontrada tras la aplicación del estímulo. Estos gránulos contienen sustancias autocrinas y paracrinas que favorecen la activación de mecanismos auxiliares que inducen una mayor agregación plaquetaria, lo que explicaría, la baja respuesta a Thr observada en estos pacientes.

En los primeros ensayos clínicos se estableció que en pacientes tratados con rapamicina o análogos de ésta, dichos pacientes presentaban trombocitopenia (Hong and Kahan, 2000; Johnson et al., 1996). Nosotros hemos observado que al menos la mitad de nuestros pacientes presentaban un recuento bajo de plaquetas, y que alguno de ellos presentaba una trombocitopenia severa. El aumento del aclaramiento de plaquetas preactivadas o la reducción de la producción en la médula ósea, podrían explicar dicha trombocitopenia observada. Estas hipótesis están siendo investigadas, revelando que mTOR podría jugar un papel importante en la producción de plaquetas (Chanprasert et al., 2006; Liu et al., 2011; Raslova et al., 2006).

Por otro lado, estudios previos relacionan las inmunofilinas con alteraciones en la homeostasis del Ca^{2+} observadas en síntomas causadas por la diabetes (Qi et al., 2006), además las alteraciones vistas en la agregación plaquetaria de pacientes diagnosticados de diabetes mellitus tipo 2 nos indujeron a iniciar el estudio acerca del papel de las inmunofilinas en estas alteraciones.

En la presente Tesis Doctoral, hemos mostrado evidencias del papel de las inmunofilinas en la homeostasis del Ca^{2+} intracelular en plaquetas de individuos sanos y pacientes diagnosticados de diabetes mellitus tipo 2. Como ya hemos visto

anteriormente la administración de inhibidores de las inmunofilinas alteran tanto la salida como la entrada de Ca^{2+}. Por lo tanto, esto sugiere que hay que ser cuidadoso con las dosis administradas a pacientes y evaluar los parámetros de agregabilidad plaquetaria.

Estudios previos demuestran que al incubar las plaquetas con FK506 existe un aumento de la función plaquetaria como se determina al evaluar la secreción de serotonina y la agregación en respuesta a ADP (Fernandes et al., 1993). De manera similar, se demostró después, que la rapamicina de manera dosis dependiente también aumentaba la agregación plaquetaria y la secreción de ATP en respuesta a ADP y TRAP-6, que simula una estimulación con Thr (Babinska et al., 1998). Nosotros hemos visto que el FK506 y la rapamicina reducen la secreción plaquetaria de gránulos α y δ tanto en individuos sanos como en pacientes diabético tipo 2. Interesantemente, la evaluación mediante citometría del tamaño y la complejidad de células CD41 positivas nos mostró que las plaquetas de los pacientes diabéticos tipo 2 eran más pequeñas y presentaban menor complejidad que las de los individuos sanos, lo cual es indicativo de un menor contenido de gránulos. Esta disminución del contenido granular podría ser la causa de la reducción en la secreción de gránulos α y δ estimulada por Thr en pacientes diabéticos tipo 2.

Además, contrario al efecto descrito anteriormente en la agregación inducida por ADP, hemos observado que ambos inhibidores de las inmunofilinas reducen la agregación plaquetaria inducida por Thr tanto en individuos sanos como pacientes diabéticos tipo 2. El efecto inhibitorio del FK506 en la agregación plaquetaria fue mayor que el observado con rapamicina, lo cual se podría atribuir al amplio espectro del FK506 como inhibidor. El efecto de la rapamicina en la agregación *in vitro* fue prometedora, ya que tiene un efecto insignificante sobre la agregación en individuos sanos, pero redujo significativamente la hiperagregabilidad en pacientes diabéticos tipo 2. Por lo tanto, considerando que la rapamicina se usa actualmente en clínica, y en consecuencia ha pasado todos los estudios previos necesarios para su aplicación en humanos, se podría recomendar el uso como una estrategia terapéutica basada en la administración de rapamicina a los pacientes diabéticos tipo 2 para minimizar las complicaciones vasculares asociadas a plaquetas. A pesar de ello, sugerimos ser cautelosos con las dosis administradas a pacientes y realizar evaluaciones periódicas del sistema inmunológico, así como los parámetros de agregabilidad en pacientes tratados con inhibidores de las inmunofilinas.

Finalmente, hemos encontrado un aumento en la expresión de FKBP12 en plaquetas de pacientes diabéticos tipo 2, pero los valores de expresión no fueron significativos comparados con los encontrados en individuos sanos, lo cual es opuesto a lo encontrado en ratas donde la diabetes fue inducida artificialmente con estreptozotocina (Qi et al., 2006). De manera similar, hemos visto un aumento exacerbado en la expresión de FKBP52 en las plaquetas de estos pacientes en comparación con individuos sanos. Además, se observa una alteración en la asociación de FKBP52 con canales de Ca^{2+}, lo cual podría explicar el aumento de la expresión de esta proteína, quizás como mecanismo de compensación. La alteración en la asociación entre FKBP52 con Orai1 y con TRPC1, junto con los resultados previos demostrando el efecto de la FKBP52 en la ECC en plaquetas, podrían explicar la inhibición de la ECC observada en pacientes diabéticos tipo 2, donde hemos demostrado una disminución de la ECC y un aumento del componente no capacitativo de la entrada de Ca^{2+}, el cual provoca una señal global de mayor entrada de Ca^{2+} (Jardin et al., 2011).

Por lo tanto, hemos visto que las inmunofilinas participan en la homeostasis intracelular de Ca^{2+}, en la secreción y agregación plaquetaria de individuos sanos y pacientes diabéticos tipo 2. La inhibición de las inmunofilinas afecta en mayor medida a la función de las plaquetas en los pacientes diabéticos tipo 2, lo cual concuerda con el hecho de que las proteínas FKBP12 y FKBP52 estén sobreexpresadas en estos pacientes y que esté alterada la asociación entre FKBP52 y los canales de Ca^{2+}. Según esto, se puede concluir que las inmunofilinas están involucradas en las alteraciones plaquetarias vistas en pacientes diabéticos tipo 2.

En estos pacientes se ha demostrado que la alteración en la agregación es debida en mayor parte al componente no capacitativo de la entrada de Ca^{2+}, debido a un aumento de la permeabilidad del TRPC6 (Alexandru et al., 2008; Bouaziz et al., 2007; Mita et al., 2010). Por esta razón, investigamos si además las inmunofilinas participaban en este mecanismo no capacitativo.

Nuestros resultados muestran que el papel de las inmunofilinas en el mecanismo de NCCE es debido a la actividad de las FKBPs y no al efecto indirecto del FK506 de inhibir la CaN, como sugieren otros autores (Gibon et al., 2010; Liu and Ji, 2012). La inhibición de las inmunofilinas no altera la NCCE en plaquetas de ratones carentes de TRPC6, lo cual nos indica que el TRPC6 podría ser una proteína diana para la actividad de las inmunofilinas (Kim and Saffen, 2005). Existen estudios en los que se ha demostrado la interacción de las inmunofilinas con canales TRPCs. De hecho, en

neuronas FKBP52 puede unirse a dos posibles regiones que presenta TRPC1 en su estructura (^{19}LPSSP^{23} and ^{644}LPPPF^{648}), siendo la última región una región que se superpone con sitio de unión de homer y cerca del lugar de interacción con STIM1 (Shim et al., 2009). En el TRPC6 existe además un dominio en el que es fosforilado por PKC (Ser(768/714)) y que sirve de unión para FKBP12, CaM y CaN (Kim and Saffen, 2005) donde podría ocurrir dicha interacción.

Nuestros resultados muestran la existencia de interacciónes entre el TRPC6 y FKBPs, como la FKBP25 y FKBP38 en condiciones de reposo. Además, tras la estimulación plaquetaria con OAG dichas interacciones se ven modificadas, lo cual indica un papel relevante de las mismas durante la activación plaquetaria. FKBP25 ha sido descrita como un factor nuclear que regula la expresión proteica. Además se conoce que tiene mayor afinidad por la rapamicina que por el FK506, con lo que podría estar participando en las alteraciones observadas en pacientes tratados con este macrólido (Jin et al., 1992; Klettner et al., 2001). En cambio, FKBP38 se activa mediante CaM/Ca^{2+} y se inhibe por HSP90 (Edlich et al., 2007). Se trata de un inhibidor de la proteína antiapoptótica Bcl-2 además de ser inhibidor constitutivo de mTOR muy importante en la proliferación celular (Romano et al., 2010; Yoon et al., 2011).

En el último trabajo recopilado en esta Tesis Doctoral, hemos mostramos que ambas FKBPs se asocian con TRPC6 regulando la NCCE, pero FKBP25 actuaría como un regulador positivo mientras que FKBP38 podría estar actuando como un regulador negativo. Estos indicios están avalados por el hecho de que FKBP38 aparece asociado a TRPC6 en condiciones basales, mientras que cuando se estimulan las plaquetas con OAG, ésta se disocia del complejo. Tras su inhibición con FK506 dicha disociación se revierte. Por lo tanto, FKBP38 podría estar impidiendo la asociación entre TRPC3 y TRPC6 en condiciones basales mientras que FKBP25 y otras inmunofilinas estarían favoreciendo la activación de la NCCE gracias a la asociación de ambos canales.

El hecho de que se hayan mostrado resultados prometedores en ratas con enfermedades neurodegenerativas al ser tratadas con drogas específicas contra el FKBP38, como el N-(N',N'-dimethylcarboxamidomethyl)cycloheximide (Edlich et al., 2006), nos permite pensar que este compuesto puede ser usado para promover la entrada de Ca^{2+} en pacientes que tienen reducida la agregación plaquetaria, impidiendo con ello la aparición de sangrados espontáneos.

Por lo que durante el desarrollo del último objetivo de la Tesis Doctoral, hemos descrito por primera vez que dos nuevas proteínas como FKBP25 y FKBP38,

interaccionan con TRPC6 y podrían ser necesarias para la regulación de la NCCE, como se demuestra en los experimentos realizados en ratones deficientes de TRPC6. Además, el silenciamiento de estas proteínas y su inhibición usando FK506, afectan tanto a la entrada de Ca^{2+} promovida por OAG como a las corrientes a través de los canales no capacitativos en varios modelos celulares.

Resumiendo, las inmunofilinas desempeñan un papel fundamental en la homeostasis del Ca^{2+}, y principalmente sobre la entrada de Ca^{2+} (tanto capacitativa como no capacitativa) desde el exterior de la célula. Aunque la mayoría de resultados presentados han sido obtenidos en plaquetas humanas, el empleo de líneas celulares en cultivo, como MEG-01 y HEK293, implica que dicho papel regulador puede ser universal, aunque somos conscientes de que cada modelo celular puede presentar alteraciones con respecto al tipo de inmunofilina que participe o en cuanto al tipo de canal de Ca^{2+} implicado, de ahí que sugerimos que se realice un estudio más amplio empleando más modelos celulares para validar dicha universalidad. La importancia del papel de las inmunofilinas en la biología celular también se refleja en las alteraciones encontradas en las plaquetas de pacientes tan dispares como son los pacientes afectados de diabetes mellitus tipo 2 o en los pacientes trasplantados de riñón, ya que ambos pacientes tan solo tienen en común el efecto inhibitorio observado sobre la entrada de Ca^{2+} una vez se les administra los antagonistas de las inmunofilinas. Del mismo modo, hemos identificado algunas inmunofilinas que hasta ahora no se consideraban como reguladores del Ca^{2+} intracelular, y por tanto, pueden ser diana de nuevos fármacos diseñados con el fin de regular las concentraciones intracelulares de Ca^{2+} y con ello determinadas funciones celulares.

DISCUSSION.

Calcium homeostasis is controlled by immunophilins through regulating the permeability of intracellular calcium channels, such a RyR and IP_3R. During the present Thesis we aimed to demonstrate the relevance of immunophilins in platelet calcium homeostasis.

The role of FKBPs in capacitative calcium entry (CCE) and the proteins involved in this mechanism is shown in the first article. We have evaluated the CaN pathway because, despite FK506 or CsA specifically target the peptidyl-prolyl isomerase active domain of immunophilins, both have been often considered as specific CaN antagonist.

The dose-dependent effect induced by FK506 on Ca^{2+} release may be attributed to the activity of CaM and CaN-dependent phosphorylation/dephosphorylation mechanisms of ER-resident Ca^{2+} channels as previously reported in other cell types (Kasri et al., 2004; MacMillan et al., 2005; Vanderheyden et al., 2009; Yamaguchi et al., 2005). However in human platelets, cypermethrin, a CaN inhibitor, did not alter Ca^{2+} leak, therefore Ca^{2+} leak evoked by FK506 is CaN independent. A regulatory role for CaN on Ca^{2+} leakage is likely to be present in human platelets, due to the significant differences observed in Ca^{2+} leakage in cells treated with cypermethrin and rapamycin and comparing them with platelets treated with rapamycin alone. In this sense, CaN-dependent mechanisms might be also present in MEG-01 cells, where treatment of siRNA FKBP52 transfected cells with FK506 reported a significant reduction in Ca^{2+} leak upon stimulation with TG, hence evidencing a role for FKBP52 in Ca^{2+} leak. Furthermore, participation of other immunophilins cannot be rule out.

Currently, the role of immunophilins in CCE remains unclear. Nevertheless, some authors have considered a unique retrograde CaN-dependent mechanism for regulating Ca^{2+} entry activation. The mechanism proposed suggests that only in conditions where $[Ca^{2+}]_c$ reaches a critical value, CaN may evoke the activation of non capacitative Ca^{2+} channels (Mignen et al., 2003). In human platelets, an alternative CaN-independent pathway might also coexist because rapamycin incubation also inhibits Ca^{2+} entry, and this effect has been linked to inhibition of the mTOR pathway, which is independent of CaN. The latest observations in MEG-01 and platelets, suggest that FKBP52, or other immunophilins like FKBP12, are impaired by complexing with their targets drugs, therefore being enough to alter CCE in our cellular lineage.

Despite other groups have presented evidences against the participation of TRPCs and in particular TRPC1 in CCE, using protocols that include TRPC1 KO mice model (Varga-Szabo et al., 2008), we have shown that in MEG-01 cells TRPC1 silencing has a clear negative effect on CCE, thus demonstrating that TRPC1 has an important role in this mechanism (Cheng et al., 2011b; Kim et al., 2009; Olah et al., 2011). Experimental data, obtained by our group and others, suggest that TRPC1 is a relevant element of the macromolecular complex newly generated during CCE (Redondo et al., 2008a; Rosado and Sage, 2000a).

Recent studies have reported a region within the TRPC1 structure, which has been described as a suitable target region for the immunophilins-PPIase activity. It has also been suggested that after conformational changes induced by FKBP12 or FKBP52, consisting on isomerization of the proline residues localized within these regions, TRPC1 would be recognized by homer. Homer has been described as a structural element that would support the complexes of TRPC1 during ECC activation. Hence, we suggest that removal of immunophilins from TRPC1 heteromultimeric complex disturbs the fragile stoichiometry of the macromolecular complex generated during activation of ECC, being this role independently of CaN activity. The presence of immunophilins in these complexes is evidenced by the fact that both immunophilin antagonists, FK506 and rapamycin, block the coupling between TRPC1 and type II IP_3R. Similarly in MEG-01 cell transfected with siRNA FKBP52, the reduction in the generation of the complex mentioned above was also observed. Furthermore, TRP regulation by physical interaction of immunophilins members has already been reported mainly linked to non capacitative Ca^{2+} channels, such as TRPL, TRPV and TRPC6. Here, we have shown for the first time that FKBP52 and TRPC1 interaction occurs in human platelets and MEG-01 cells, and these complexes are impaired by treating human platelets with FKBP inhibitors, like FK506 and rapamycin. In other cell models, FKBP12 associated with TRPC3 and TRPC6, and FKBP52 binds to TRPC1, TRPC4 and TRPC5, hence participation of additional FKBPs during ECC activation in human platelets cannot be excluded, since in MEG-01 where FKBP52 was silenced, FK506 was still able to evoke a significant reduction of CCE.

We report for the very first time that Orai1 interacts with immunophilin members in human platelets and MEG-01, confirming their involvement in CCE.

An alternative explanation for the FKBP52-dependent regulatory mechanism of CCE would involve a competitive association with homer, which has been shown to

interact with TRPC1 and Orai1, and regulates CCE in several cell types including platelets (Jardin et al., 2012). In neurons it has been shown that homer shares a binding region with FKBP52 that is localized within TRPC1 C-terminal domain; however the existence of such a competence has not been confirmed in human platelets yet (Shim et al., 2009).

After confirmation that immunophilins are involved in Ca^{2+} homeostasis, we wanted to show the effect of immunophilins inhibition in platelet function of transplant patient, due to the controversy regarding this issue that exist in the literature. It is known that in cardiac transplanted patients, rapamycin based therapies represent a good alternative to classic treatment. In patients presenting cardiovascular complications and particularly, in patients medicated with immunosuppressants, neoplasias have been diagnostized. However, little is known about adverse effects of these therapies in platelet activity.

Our results indicate that long-term everolimus (a rapamycin analogue) administration modified the Ca^{2+} homeostasis pattern in human platelets induced by all stimuli used, except ADP.

Interestingly, the alterations observed in Ca^{2+} homeostasis resulted most evident in patients treated with sirolimus during 24-36 months; after that, most patients included in this study recovered the Ca^{2+} mobilization patterns in response to Thr. This finding explains the high correlation coefficient found between Ca^{2+} entry levels and the extent of the administration period. In this sense, rapamycin dependent inhibition of CCE evoked by SERCA inhibitor, cyclopiazonic acid, has been recently reported in both pulmonary vascular cells (Ogawa et al., 2009), and in human pulmonary arterial smooth muscle cells, suggesting that mTOR is downstream to PDGF receptor participating in the association between STIM1/Orai and subsequently regulating CCE (Ogawa et al., 2012).

Other authors have shown that specific mTOR inhibitors, like PP242 or torin1, do not enhance Akt phosphorylation (threonine 308) (Moore et al., 2011). We have not found changes in Akt phosphorylation pattern between healthy individual and patients belonging to group II (24-36 months), which would indicate that mTOR would not be involved in the modification of Akt activity. In this study it was observed that platelets incubated for 15 min with rapamycin (200 nM) does not reported significant alteration in aggregation activated by SFLLRN, suggesting that under those experimental conditions mTOR2 regulates Akt function and subsequently platelet activity (Moore et al., 2011). However, a study reported that mTOR2 is sensitive to rapamycin depending on the time

of exposure to the drug (Sarbassov et al., 2006). Hence, our findings are unlikely explained by the exclusive participation of mTOR2, but would suggest the participation of mTOR1 instead.

The differences observed in platelet aggregation between healthy individuals and medicated patients might be due to the role of mTOR in the regulation of protein synthesis in response to adhesion molecules of platelets surface. Glycoprotein αIIb/β3 regulates the Bcl-3 synthesis evoked by Thr, so in human platelets this glycoprotein would be able to link the collagen or fibrinogen receptors and activate mTOR, which would induce Bcl-3 synthesis. Bcl-3 is a protein that participates in platelet aggregation, retraction of fibrin clots and wound remodeling (Aslan et al., 2011; Law et al., 1996; Weyrich et al., 2007).

Using flow-cytometry we have evaluated time-dependent membrane exposure of P-selectin in human platelets. In medicated patients, an elevated degranulation is observed in resting platelets, which might explain the reduced response and impaired aggregation found upon application of external stimuli. The secretion granules contain autocrine and paracrine substances that stimulate the activation of other mechanisms that enhanced platelet aggregation. These findings would explain the low response of platelets to Thr in these patients.

Since the first clinical trials were set up, thrombocytopenia has been the most evident side-effect reported in patients receiving rapamycin or treatments with the rapamycin analogues (Hong and Kahan, 2000; Johnson et al., 1996). We have observed that almost half of our patients presented very low platelet count, and even some of them presented severe thrombocytopenia. Enhanced clearance of pre-activated platelets or reduced platelets generation from bone marrow alteration, leads to thrombocytopenia. These hypotheses are under intense investigation nowadays, revealing that mTOR might play an important role in platelet production (Chanprasert et al., 2006; Liu et al., 2011; Raslova et al., 2006).

On the other hand, previous reports associated the immunophilins with Ca^{2+} homeostasis alterations in patients with diabetes mellitus type 2 (DM2) (Qi et al., 2006). Moreover, the enhanced platelet aggregation in these patients was the key in order to begin our study regarding the role of immunophilins in these alterations.

In the present Thesis, we have shown evidence for the role of immunophilins on intracellular Ca^{2+} homeostasis in platelets from DM2 patients. As previously reported in

other cell types, administration of immunophilin antagonists evoked an altered Ca^{2+} release and Ca^{2+} entry. Therefore, we suggest being cautious with the dose administrated to patients and the evaluation of aggregation parameters in patients treated with immunophilin antagonists.

An early study reported enhanced FK506 effect on platelet function as determined by evaluating the platelet serotonin secretion and aggregation in response to ADP (Fernandes et al., 1993). Similarly, rapamycin was later reported to enhanced dose and time-dependent platelet aggregation and ATP secretion in response to ADP and TRAP-6, which mimics Thr stimulation (Babinska et al., 1998). In our hands, FK506 and rapamycin reduced platelet α-granule and δ-granule secretion in both platelets from DM2 patients and healthy subjects. Interestingly, evaluation of size and complexity of CD41 positive cells by flow cytometry revealed that platelets from DM2 patients were smaller and also presented lower intracellular complexity, which is indicative of less granule content. This reduced granule content may be the cause of the reduced α- and δ-granule content, and might lead to the reduced secretion observed in platelets from DM2 patients stimulated by Thr.

Furthermore, contrary to previously described effects on ADP-induced aggregation, we have observed that both immunophilin antagonists reduce platelet aggregation stimulated by Thr in both healthy and DM2 patients. The inhibitory effect of FK506 on platelet aggregation was greater than the found in presence of rapamycin, which might be attributed to the broader spectrum of FK506 as immunophilin inhibitor. The effect of rapamycin on aggregation *in vitro* was promising, since it has a negligible effect on aggregation in healthy subjects but significantly reduced hyperaggregability in platelets from DM2 patients. Thus, considering that rapamycin is already been used in the clinical practice, we would recommend its use as a therapeutic strategy in DM2 patients to minimize the vascular complications associated to platelets. Although, we also suggest that it is need to be cautious with the dose administered to patients and the evaluation of the immunological system as well as the aggregation parameters in patients treated with the immunophilin antagonists.

We reported an elevated FKBP12 expression in platelets from DM2 patients, but the expression values were not significantly different when compared with those found in healthy subjects, which disagrees to previous observation in rats where diabetes mellitus have been artificially induced by streptozotocin administration (Qi et al., 2006). Similarly, an exacerbated expression of FKBP52 was found in platelets from DM2 patients

compared to healthy subjects. In this sense, we have found impaired association of FKBP52 with Ca^{2+} channels in DM2 patients, which might explain the enhanced expression of this protein in DM2 patients, perhaps as a mechanism of compensation. The impaired association between FKBP52 and both Orai1 and TRPC1, together with previous findings demonstrating the effect of FKBP52 in CCE observed in platelets from diabetics, agree with our previous studies where we have reported decreased CCE and enhanced non capacitative Ca^{2+} influx, the latter leading to a greater global Ca^{2+} entry signal (Jardin et al., 2011).

Therefore, we have found that immunophilins participate in intracellular Ca^{2+} homeostasis, secretion and aggregation in platelets from healthy subjects and DM2 patients. Inhibition of immunophilins affects in a larger extent to the function of platelets from DM2 patients, which agree with the fact that FKBP12 and FKBP52 are overexpressed in platelets from DM2 patients, and that an impaired coupling of FKBP52 to Ca^{2+} channels is present in these patients. Thus we strongly suggest that immunophilins are involved in the alterations presented in platelets from type 2 diabetic patients.

In DM2 patients we have demonstrated that the alterations in platelet aggregation is mainly caused by non capacitative Ca^{2+} entry trough the enhancement of TPRC6 permeability (Alexandru et al., 2008; Bouaziz et al., 2007; Mita et al., 2010); hence, we have also investigated the role of immunophilins in the Non Capacitative Ca^{2+} Entry (NCCE).

Our results provide clear evidence for a role of immunophilins in NCCE that can be attributed to the activity of the FKBP subfamily members and not to the indirect effect of FK506 on CaN, as previously suggested by other authors (Gibon et al., 2010; Liu and Ji, 2012). Inhibition of immunophilins did not alter NCCE induced by the diacyl glycerol analogue, OAG, in platelets from mice lacking TRPC6, which provides strong evidences in favor of TRPC6 as the molecular target of the immunophilin activity (Kim and Saffen, 2005). Previous studies have reported direct interaction of immunophilins with TRPC channels, as mentioned above. This is, FKBP52 presents two possible binding regions in both extremes of the TRPC1 structure (^{19}LPSSP^{23} and ^{644}LPPPF^{648}), being the latest region overlapped with the homer binding region and close to the STIM1 interaction region (Shim et al., 2009). In the TRPC6 structure exists a domain that can be phosphorilated by PKC (Ser(768/714)) and that serves of binding region to FKBP12,

CaM y CaN (Kim and Saffen, 2005), thus allowing the interaction between immunophilins and TRPC6.

Here we have found interaction between TRPC6 and FKBP25 and FKBP38 under resting conditions that is altered upon stimulation with OAG. FKBP25 has been described as a nuclear factor that affects to the protein expression in the cells. FKBP25 presented more affinity for rapamycin than FK506, hence it represents a good candidate for the alteration observed in medicated patients with this macrolide (Jin et al., 1992; Klettner et al., 2001). Meanwhile, FKBP38 is activated by CaM/Ca^{2+} and it is inhibited by HSP90 (Edlich et al., 2007). It has been described that FKBP38 is an inhibitor of the pro-survival Bcl-2, as well as a constitutive inhibitor of mTOR which also favores cell survival (Romano et al., 2010; Yoon et al., 2011).

In the last study of this Thesis, we have shown that these two FKBPs associate with TRPC6 to modulate NCCE, and according to the coupling pattern observed, FKBP25 could be acting as a positive activator, while FKBP38 could be a possible negative regulator. This statement is based on the results obtained under resting conditions, where FKBP38 appears associated with TRPC6 and stimulation of platelets with OAG evoked its dissociation from the channel, while inhibition of FKBPs activity with FK506 reverses the dissociation from TRPC6. Hence, FKBP38 might be impairing TRPC3 and TRPC6 interaction under resting condition, while FKBP25 and other immunophilins might be favoring the generation of the full active NCCE channels resulting of the complex between both channels.

The fact that a specific antagonist of FKBP38, the N-(N',N'-dimethylcarboxamidomethyl)cycloheximide, reported promising results to prevent acute and/or chronic neurodegenerative diseases in rats (Edlich et al., 2006), lead us to think that this drug can be also used to evoke Ca^{2+} entry in platelets in patients where platelet aggregation is reduced, and subsequently, leading to the appearance of the spontaneous bleeding.

Here we describe for the first time the interaction between FKBP25 and FKBP38 with TRPC6, which might be necessary for the regulation of NCCE. Both proteins seem to interact with the NCCE channel through TRPC6 as demonstrated in the murine TRPC6 KO model and both, expression silencing and pharmacological inhibition, using FK506, which affected OAG-evoked Ca^{2+} entry and also impaired the current through the NCCE channel.

Summarizing, immunophilins play a key role in Ca^{2+} homeostasis, mainly in Ca^{2+} entry (capacitative and non capacitative). Although, the majority of the results were obtained using human platelets as the cell model, the alternative use of culture cell lines, such as MEG-01 and HEK293, indicates that the regulatory role of immunophilins in Ca^{2+} homeostasis may be universal; however, we always have to consider that each cell line has different Ca^{2+} channels and immunophilins participating in the same mechanism, and for this reason, we suggest that it is needed to extend our study to other cell lines in order to confirm the universality of our statement. The importance of immunophilins in the cell biology is also presented here, due to the alterations observed in platelets from DM2 patients and kidney transplant patients, who have in common the inhibitory effect in Ca^{2+} entry when they are treated with the antagonists of the immunophilins. Similarly, we have identified some immunophilins which has not been considered intracellular Ca^{2+} regulators yet; hence, they might represent new pharmacological targets for drugs designing with the purpose of regulating intracellular Ca^{2+} concentrations and thereby, cellular functions.

CONCLUSIONES

CONCLUSIONES

- Las inmunofilinas participan en la regulación de la ECC, esta regulación se basa en la asociación de FKBP52 con TRPC1 y Orai1, aunque no descartamos que otras inmunofilinas puedan participar en dicho proceso.
- La inhibición de la vía mTOR provoca alteraciones en la homeostasis del Ca^{2+} y en la funcionalidad plaquetaria en pacientes tratados con inhibidores de las inmunofilinas.
- La sobreexpresión de las inmunofilinas en pacientes diabéticos tipo2 contribuyen la hiperagregabilidad y en el aumento de secreción plaquetaria.
- La disminución de la ECC en plaquetas de pacientes diabéticos tipo 2 es debida a la sobreexpresión de FKBP52 y su alteración en la asociación con Ora1 y TRPC1.
- FKBP25 y FKBP38 interactúan con TRPC6 regulando el mecanismo de NCCE.

CONCLUSIONS

- Immunophilins are involved in the capacitative calcium entry through the association of FKBP52 with TRPC1 and Orai1.
- mTOR inhibition evokes alterations in calcium homeostasis and platelet function in patients treated with immunophilins inhibitors.
- Immunophilin overexpresion in patients with type 2 diabetes mellitus promotes hyperagregability and enhanced platelet secretion.
- Capacitative calcium entry decrease is due to FKBP52 overexpresion and impaired association between Orai1 and TRPC1.
- FKBP25 and FKBP38 interact with TRPC6 for the regulation of non capacitative calcium entry.

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