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PAPEL DE LAS PROTEÍNAS STIM1 Y ORAI1 EN LA RESPUESTA DE LOS ASTROCITOS A LA TROMBINA

TESIS

QUE PARA OBTENER EL GRADO ACADÉMICO DE

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PRESENTA

CLAUDIA MARCELA MORENO MORENO

DIRECTOR DE TESIS:

DR. LUIS ALFONSO VACA DOMÍNGUEZ

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"Nuestra recompensa se encuentra en el esfuerzo y no en el resultado. Un esfuerzo total es una victoria completa"

Mahatma Gandhi

"Todos somos muy ignorantes. Lo que ocurre es que no todos ignoramos las mismas cosas"

Albert Einstein

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ABREVIATURAS

DAG	Diacilglicerol
GFP	Proteína verde fluorescente
GPCRs	Receptores acoplados a proteínas G
IP_3	Inositol 1, 4, 5-trifosfato
IP_3R	Receptor de IP_3
PCR	Reacción en cadena de la polimerasa
PIP_2	Fosfatidil inositol 4, 5- bifosfato
PLC	Fosfolipasa C
RE	Retículo Endoplásmico
RNAi	RNA de interferencia
ROC	Canales operados por receptor
ROCE	Entrada de calcio operada por receptores
RyR	Receptor de rianodina
SERCA	ATPasa de calcio del retículo sarcoplásmico
siRNA	RNA pequeño interferente
STIM1	Stromal interacting molecule 1
SOC	Canales operados por las reservas
SOCE	Entrada de calcio operada por las reservas
SOCIC	Complejo de entrada de calcio operado por las reservas
TRPC	Transient receptor potential canonical channel

RESÚMEN

La trombina es una proteasa que participa en la cascada de coagulación y que también desencadena una respuesta celular mediada por su unión a receptores presentes en la membrana de diferentes tipos celulares. En el cerebro la trombina puede ser liberada durante un accidente cerebrovascular. En este órgano, los astrocitos son las células más susceptibles a la acción de la trombina, la cual induce una variedad de respuestas que van desde el aumento en la proliferación hasta la muerte celular. Los astrocitos responden a la trombina con una elevación citoplasmática de calcio que es generada por la liberación de calcio desde el retículo endoplásmico y por la entrada de calcio a través de canales en la membrana plasmática. La identidad de los canales responsables de esta entrada de calcio en los astrocitos permanece aún desconocida. En muchos tipos celulares la entrada de calcio activada por el vaciamiento del retículo está mediada dos proteínas: STIM1 que censa la disminución de calcio y Orai1 que es el canal responsable de la entrada de calcio. En los astrocitos no hay estudios sobre el papel de estas dos proteínas y no se sabe si STIM1 y Orai1 son activadas como parte de la respuesta a la trombina. En este estudio nosotros empleamos ensayos de inmunocitoquímica, sobreexpresión de proteínas y RNA de interferencia para investigar la participación de STIM1 y Orai1 en la respuesta de los astrocitos corticales de rata a la trombina. Nosotros encontramos que los astrocitos expresan endógenamente las proteínas STIM1 y Orai1 y que STIM1 es relocalizada durante la estimulación con trombina. Además la sobreexpresión de estas dos proteínas duplicó la entrada de calcio inducida por la trombina en los astrocitos, mientras que la inhibición de su expresión redujo significativamente la entrada de calcio. Estos resultados en conjunto sugieren que STIM1 y Orai1 participan en la respuesta de los astrocitos a la trombina y que la entrada de calcio a través del canal Orai1 es responsable de más del 70% de esta respuesta.

1. INTRODUCCIÓN

El calcio es uno de los segundos mensajeros más importantes en la célula, este ión regula a nivel del organismo procesos fisiológicos tan importantes como la contracción muscular, la regulación endocrina, el aprendizaje, algunos ciclos circadianos y la memoria [1-5]. Debido a sus múltiples funciones, la célula controla finamente la concentración de calcio libre en el citoplasma mediante la extrusión y el almacenaje, de esta forma se pueden generar elevaciones de calcio que funcionan como un código que permite activar diferentes vías de señalización. En general, la célula tiene dos mecanismos para que el calcio aumente, el primero de ellos es la liberación de calcio desde las reservas de almacenamiento, principalmente desde el retículo endoplásmico (RE), y el segundo es la entrada de calcio a través de la membrana plasmática [6]. El vaciamiento del RE es inducido principalmente por una vía de señalización que inicia con la activación de receptores metabotrópicos acoplados a proteínas G (GPCRs). Como consecuencia del vaciamiento del RE se activa una entrada de calcio que se conoce como entrada de calcio operada por las reservas o SOCE por sus siglas en inglés (*Store-Operated Calcium Entry*).

SOCE es un mecanismo ubicuo que se basa en la detección de la salida de calcio desde el RE y la posterior activación de canales en la membrana plasmática [7]. Debido a que ésta entrada de calcio ocurre en todos los tipos celulares y a que puede ser activada por cualquier agonista que induzca la liberación de calcio desde el RE, es de imaginarse la amplia gama de procesos fisiológicos y patológicos en los cuales está involucrada. Sin embargo, el hecho de que la identidad de las proteínas responsables de SOCE permaneciera desconocida hasta el año 2005 había limitado el estudio de su papel fisiológico. El descubrimiento de STIM1, un sensor de calcio en el RE, y de Orai1, el canal operado por las reservas, ha contribuido enormemente al entendimiento de SOCE [8]. En los últimos 7 años se ha empezado a dilucidar la importancia de la entrada de calcio mediada por estas dos proteínas y hoy se sabe, por ejemplo, que SOCE es esencial para la función del sistema inmune adaptativo e innato, controlando la activación de los linfocitos T, la producción de anticuerpos por parte de los linfocitos B y el funcionamiento de las células NK, neutrófilos, macrófagos y células dendríticas [9]. En cuanto al sistema circulatorio se

ha visto que SOCE está implicada en muchas patologías como la trombosis, en donde la acción de agonistas como la trombina sobre las plaquetas activa esta entrada de calcio, causando su agregación y la formación del trombo [10, 11]. SOCE también participa en el desarrollo de arteriosclerosis, restenosis e hipertensión [10]. Aunque inicialmente se planteó que SOCE era una entrada exclusiva de células no excitables, ahora se sabe que también está presente en miocitos y neuronas. Su importancia en el músculo se ha revelado por su participación en el desarrollo y remodelación del músculo esquelético [12], el funcionamiento del endotelio [13] y del marcapasos del corazón [14], esto ha develado su participación en patologías cardíacas, vasculares y en diversas miopatías. Como se puede notar, la entrada de calcio mediada por STIM1 y Orai1 tiene un sin número de funciones en diversos tejidos, sin embargo en lo que respecta al sistema nervioso no hay muchos estudios. Por esta razón aún hay muchas incógnitas sobre el papel de estas dos proteínas en la fisiología y patología del cerebro.

El sistema nervioso central está compuesto por neuronas y células gliales, estas últimas incluyen los astrocitos, los oligodendrocitos y la microglia. Los astrocitos son el tipo celular más abundante en el cerebro, ocupando casi el 50% del volumen cerebral en algunos organismos [15]. Debido a que los astrocitos carecen de propiedades eléctricas que les permitan disparar potenciales de acción, se creía que su única función era servir como soporte estructural y metabólico para las neuronas. Si bien los astrocitos sí desempeñan estas dos funciones, ahora empieza a entenderse que estas células participan en procesos mucho más complejos del funcionamiento cerebral. La participación de los astrocitos en estas funciones cerebrales depende de una importante característica: su capacidad para producir y propagar señales de calcio [16, 17]. Los aumentos de calcio en el astrocito llevan a la liberación de diferentes moléculas que les permiten comunicarse con las neuronas y los vasos sanguíneos, modulando la actividad de estas células en un proceso que ha sido denominado gliotransmisión [18, 19]. Dentro de los procesos fisiológicos modulados por los astrocitos se encuentran la neurogénesis, el desarrollo de circuitos cerebrales, la transmisión y la plasticidad sináptica, la presión sanguínea, los patrones de sueño y la memoria [15, 20-22]. En cuanto a las patologías cerebrales, los astrocitos están involucrados en epilepsia, enfermedad de Parkinson, Alzheimer, isquemia y accidentes cerebrovasculares[23].

Debido a que SOCE es la principal entrada de calcio en células no excitables, es de esperarse que en los astrocitos esta entrada de calcio y sus proteínas efectoras, STIM1 y Orai1, jueguen un papel crucial en la señalización de calcio. Sin embargo, este es un campo que aún no ha sido explorado. El entendimiento de las bases moleculares que subyacen a los aumentos de calcio que experimentan los astrocitos en respuesta a diferentes agonistas, contribuirá a comprender cómo estas células desempeñan funciones tan importantes en la fisiología del cerebro, y más aún, cómo una alteración en su señalización podría contribuir al desarrollo de diferentes patologías.

2. ANTECEDENTES

2.1. LOS ASTROCITOS Y SU PAPEL EN LA FISIOLOGÍA Y PATOLOGÍA DEL CEREBRO

Los astrocitos (del griego *astron* que significa estrella y *cyte* que significa célula), son un tipo de célula glial propio del sistema nervioso central. Identificados en 1858 por Rudolf Virchow, quien los describió como “una substancia que reside entre las partes nerviosas manteniéndolas unidas” [24], son el tipo celular más abundante en el cerebro. Estas células fueron posteriormente teñidas y observadas por Camilo Golgi lo que permitió establecer su morfología [25, 26]. Su nombre les fue otorgado por Michael von Lenhossek en 1891 [27]. Los astrocitos son células de morfología estrellada, organizadas en dominios celulares independientes no superpuestos que crean una red continua que ocupa todo el cerebro y que contacta a las neuronas y a los vasos sanguíneos. Según su morfología y ubicación los astrocitos se clasifican en fibrosos y protoplásmicos, los astrocitos fibrosos son propios de la materia blanca y poseen largas proyecciones sin ramificaciones (Figura 1A). Por su parte, los astrocitos protoplásmicos, dentro de los cuales encontramos a los astrocitos corticales que son el objeto de este estudio, se encuentran en la materia gris y se caracterizan por poseer múltiples proyecciones altamente ramificadas (Figura 1B). Las finas ramificaciones de los astrocitos envuelven a las sinápsis mientras que proyecciones especializadas conocidas como pedículos entran en contacto con los vasos sanguíneos (Figura 1B y 1C). Los astrocitos también establecen contactos con los somas neuronales y la barrera hematoencefálica. Todas estas conexiones les permiten establecer una comunicación bidireccional con las neuronas y la vasculatura siendo esto fundamental para que los astrocitos lleven a cabo sus funciones.

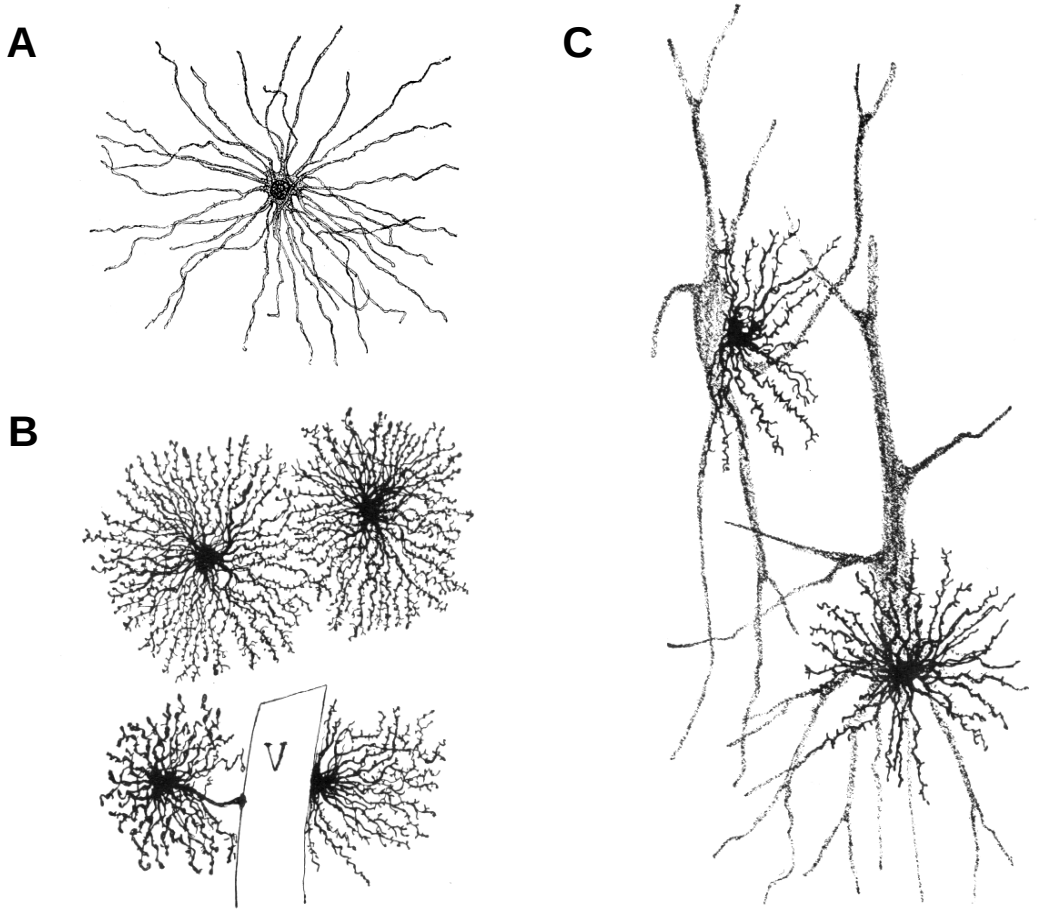

Figura 1. Diferencias morfológicas de los dos tipos de astrocitos **A.** Astrocito fibroso de la materia blanca de la médula espinal. **B.** Astrocito protoplásmico de la materia gris del cerebro, se ilustra el pedículo contactando un vaso sanguíneo (V). **C.** Astrocitos protoplásmicos pericelulares contactando neuronas de la corteza cerebral. Tinciones e ilustraciones hechas por Ramón y Cajal. (Modificado de Ramón y Cajal, 1899 [28])

Antiguamente se creía que los astrocitos solo cumplían una función de soporte estructural para las neuronas, sin embargo los últimos 25 años de estudios han revelado que los astrocitos son elementos activos de la fisiología y patología del cerebro. La Tabla 1 resume las principales funciones de los astrocitos en el cerebro y los mecanismos que emplean para llevar a cabo dichas funciones.

Tabla 1. Principales funciones de los astrocitos en el cerebro

Función	Mecanismo	Referencias
Desarrollo		
Guía para el crecimiento neurítico	Formación de puentes de Tenascina-C y proteoglicanos, expresión de moléculas de adhesión celular (N-Cadherina)	*[29, 30]*
Sinaptogénesis	Liberación de trombospondina, remodelación de la matriz extracelular	*[31, 32]*
Homeostasis		
Iónica	Acuaporinas, Transportadores de K^+	*[33, 34]*
Ph	Intercambiador Na+/H+, transportadores de $NaHCO_3$, transportadores de ácido monocarboxílico, ATPasa de protones	*[35]*
Recaptura de neurotransmisores	Transportadores de Glutamato, GABA y Glicina	*[36-38]*
Modulación sináptica		
Precursores de neurotransmisores	Producción de Glicina	
Liberación de gliotransmisores	Glutamato, GABA, purinas y D-serina	*[22, 39]*
Regulación de la liberación sináptica	Liberación de purinas	*[21]*
Memoria a largo plazo	Producción de lactato	*[40]*
Metabolismo	Almacenamiento de glicógeno, producción de lactato	*[41]*
Regulación del flujo sanguíneo	Liberación de prostaglandinas, óxido nítrico y ácido araquidónico	*[20, 42]*

Dentro de las funciones principales que desempeñan los astrocitos se encuentran la homeostasis iónica, la recaptura de neurotransmisores, el soporte metabólico neuronal y la modulación sináptica [15, 22]. Los astrocitos liberan diferentes moléculas que se han denominado gliotransmisores y que incluyen a las purinas, la D-serina y el glutamato, los cuales actúan directamente sobre receptores neuronales permitiendo la comunicación entre estos dos tipos

celulares, así como la regulación de la actividad neuronal por parte de los astrocitos, por esta razón los astrocitos han sido considerados como un componente más de la sinápsis en el modelo de “sinápsis tripartita” [39]. Recientemente ha sido demostrado que los astrocitos potencian la liberación sináptica basal mediante la liberación de purinas [21] y que participan en la formación de la memoria a largo plazo por medio de la liberación de lactato, él cual es esencial para la actividad neuronal [40]. Adicionalmente los astrocitos regulan el flujo de sangre local en respuesta a la actividad neuronal, el glutamato liberado por las neuronas se une a receptores en la membrana del astrocito desencadenando una serie de eventos que llevan a la liberación de moléculas que regulan la dilatación y la contracción arterial, como es el caso de las prostaglandinas [42], esta función es de suma importancia ya que a mayor actividad neuronal se requiere una mayor irrigación sanguínea para suplir los requerimientos de oxígeno y glucosa (Figura 2).

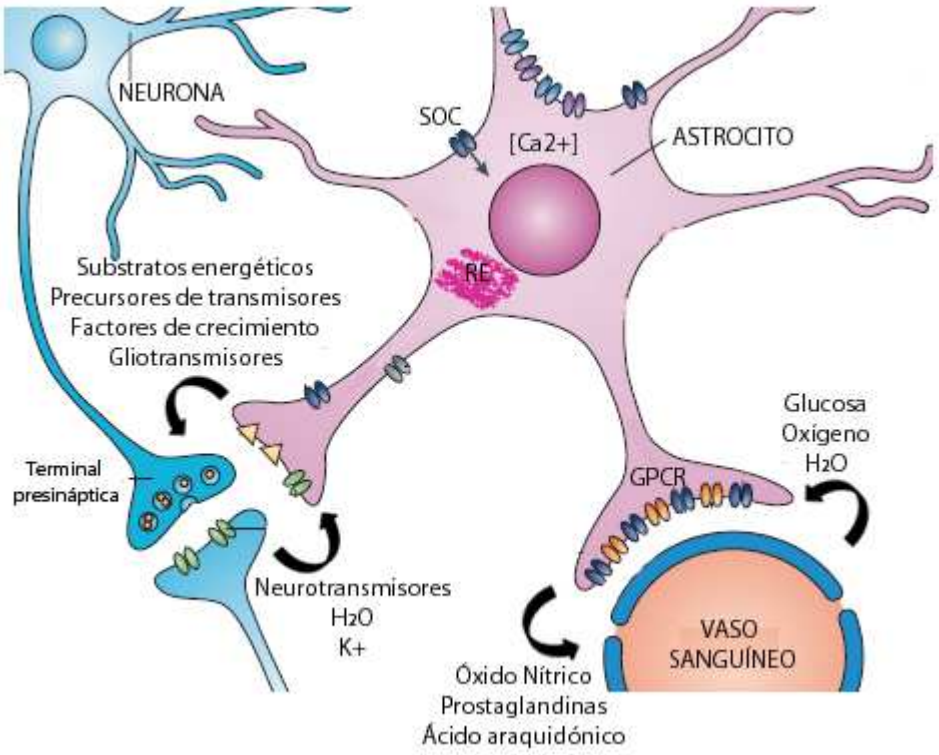

Figura 2. Moléculas importantes para la comunicación de los astrocitos con las neuronas y los vasos sanguíneos. (Modificado de Seifert et al. 2006 [43])

Aunque los astrocitos expresan canales dependientes del voltaje incluyendo algunos canales de potasio, de sodio e incluso de calcio, estas células no tienen la capacidad de disparar potenciales de acción como lo hacen las neuronas [44]. Sin embargo, los astrocitos son células altamente activas que se comunican constantemente con su entorno, para esto han desarrollado una forma de "excitabilidad" basada en variaciones de la concentración intracelular de calcio, la cual les permite comunicarse con otros astrocitos mediante ondas de calcio intercelulares y con las neuronas mediante la liberación dependiente de calcio de gliotransmisores [45, 46]. La excitabilidad de los astrocitos depende principalmente de la liberación de calcio desde el RE [47, 48], esta liberación está mediada por la activación de receptores metabotrópicos en la membrana plasmática del astrocito, dentro de los cuales los acoplados a proteínas G (GPCRs) son los más importantes [17]. La vía de señalización de los GPCRs que conlleva a la activación de los receptores de IP_3 (IP_3R) y a la liberación de calcio desde el RE se explicará en la siguiente sección. Los astrocitos expresan una gran variedad de GPCRs que causan elevaciones citoplasmáticas de calcio, incluyendo receptores a glutamato, purinas, serotonina, acetilcolina, bradicinina, dopamina y trombina [4, 16, 47]. La liberación de calcio a través del IP_3R es indispensable para el inicio y el mantenimiento de las ondas de calcio en los astrocitos [46, 49]. Aunque hay evidencia de que los astrocitos también expresan receptores de rianodina (RyR), existe controversia acerca de su papel, algunos estudios muestran que los RyR participan junto con los IP_3R en el fenómeno de liberación de calcio inducida por calcio [4, 50], mientras que otros reportes descartan que los astrocitos presenten este tipo de liberación de calcio [51]. En los astrocitos, la liberación de calcio desde el RE conlleva a la activación de canales en la membrana plasmática responsables de la entrada de calcio operada por las reservas. Sin embargo hasta la fecha hay muy pocos estudios acerca de la identidad de los canales operados por las reservas que participan en la respuesta de los astrocitos a diferentes agonistas.

2.2. EL RETÍCULO ENDOPLÁSMICO COMO PRINCIPAL RESERVA DE CALCIO EN LA CÉLULA

El calcio es uno de los segundos mensajeros más importantes en la célula, regula múltiples procesos celulares entre los cuales se destacan la división celular, la diferenciación, la apoptosis y el cáncer [41, 52]. Debido a sus múltiples funciones, las células han diseñado diversos mecanismos para mantener una concentración baja de calcio libre en el citoplasma, siendo ésta de alrededor de 100 nM, 10.000 veces menor que la concentración afuera de la célula. Uno de estos mecanismos es el almacenamiento de calcio en el RE, la principal reserva de calcio en la célula. El RE es un organelo altamente dinámico que cumple dos funciones fundamentales, la primera de ellas es la síntesis de proteínas y la segunda es la señalización celular a través de la liberación de calcio [46]. El RE recibe dos señales principales que inducen la liberación de calcio, el IP_3 y el propio ion calcio.

En cuanto a su estructura el RE es una red tubular de forma variable y altamente dinámica. El RE se divide en dos tipos, rugoso (RER) y liso (REL), mientras que la síntesis de proteínas está restringida al RER parece que la señalización de calcio se da principalmente en el REL [53]. En ciertas regiones, el RE se encuentra estrechamente cercano a la membrana plasmática (MP), estas zonas son conocidas como uniones RE-MP, y en ellas existe un acople estructural entre proteínas de ambas membranas que es fundamental para que se dé la entrada de calcio operada por las reservas de una manera localizada (Figura 3).

La principal vía de señalización que activa la liberación de calcio desde el RE inicia con la unión de un agonista a su receptor del tipo GPCR acoplado a la proteína Gαq, la subunidad α de la proteína G se libera y activa a la fosfolipasa C (PLC), esta enzima hidroliza el fosfatidilinositol 4,5-bifosfato (PIP_2) produciendo diacilglicerol (DAG) e inositol trifosfato (IP_3), este último se une a los IP_3R en la membrana del RE que funcionan como canales a través de los cuales se libera el calcio [54]. El calcio a su vez puede activar a los RyR también localizados en la membrana del RE permitiendo que se amplifique la señal de calcio.

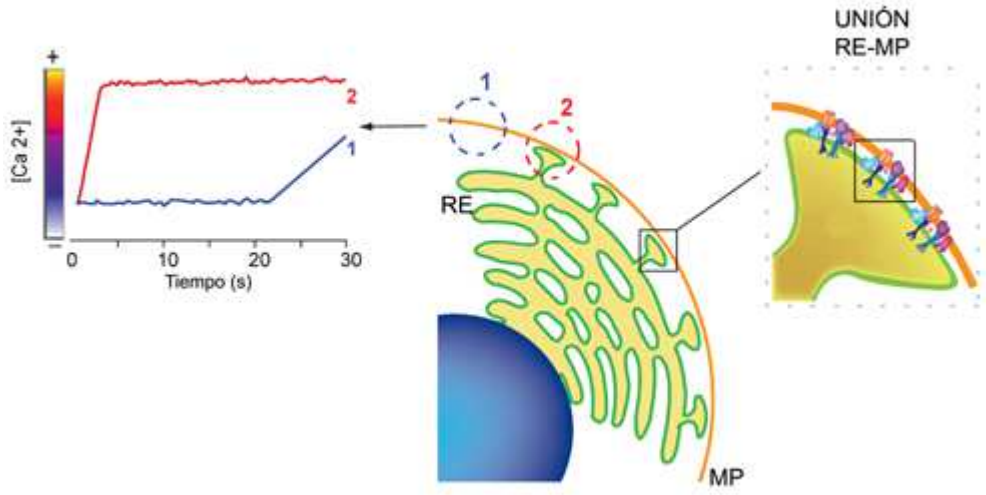

Figura 3. Uniones entre el retículo endoplásmico y la membrana plasmática (RE-MP). Estas asociaciones estructurales son esenciales para que SOCE ocurra y para que las elevaciones de calcio se den de manera localizada. (Modificado de Moreno y Vaca 2012 [55])

El vaciamiento del RE lleva a la activación de STIM1, un sensor de calcio que se localiza en la membrana del RE y que se encarga de detectar la salida de calcio y de activar la entrada de calcio operada por las reservas (SOCE). SOCE es la entrada de calcio más importante en células no excitables, ya que en células excitables el calcio entra a través de otras dos importantes vías, los canales de calcio dependientes de voltaje (VGCC) [56] y la acción reversa del intercambiador Na^{2+}/ Ca^{2+} (NCX) [57]. Otra proteína importante en la señalización de calcio del RE es la ATPasa de calcio (SERCA), la cual es responsable del transporte activo de calcio hacia el interior del RE, de rellenar el RE después del vaciamiento y de facilitar el proceso de liberación de calcio por los IP_3R y los RyR [58, 59]. El RE tiene una fuga constitutiva de calcio la cual es importante en el establecimiento de la concentración basal de calcio, aunque aún no se ha establecido el mecanismo responsable de dicha fuga, hay varias hipótesis que platean que la SERCA operando en forma reversa o un poro acuoso formado por el complejo ribosoma-translocón, podrían ser los responsables de dicha fuga [39, 59]. Adicionalmente, la dinámica de calcio del RE está íntimamente relacionada con la de la mitocondria, el segundo organelo más importante en el almacenamiento de calcio [60]; la mitocondria captura rápidamente el calcio liberado por RE a través de un uniporter modulando así la elevación de calcio en el citoplasma y luego lo libera a

través de un cotransportador Na^{2+}/ Ca^{2+} para que el RE se rellene [47]. La Figura 4 resume las principales proteínas implicadas en el manejo del calcio en las células.

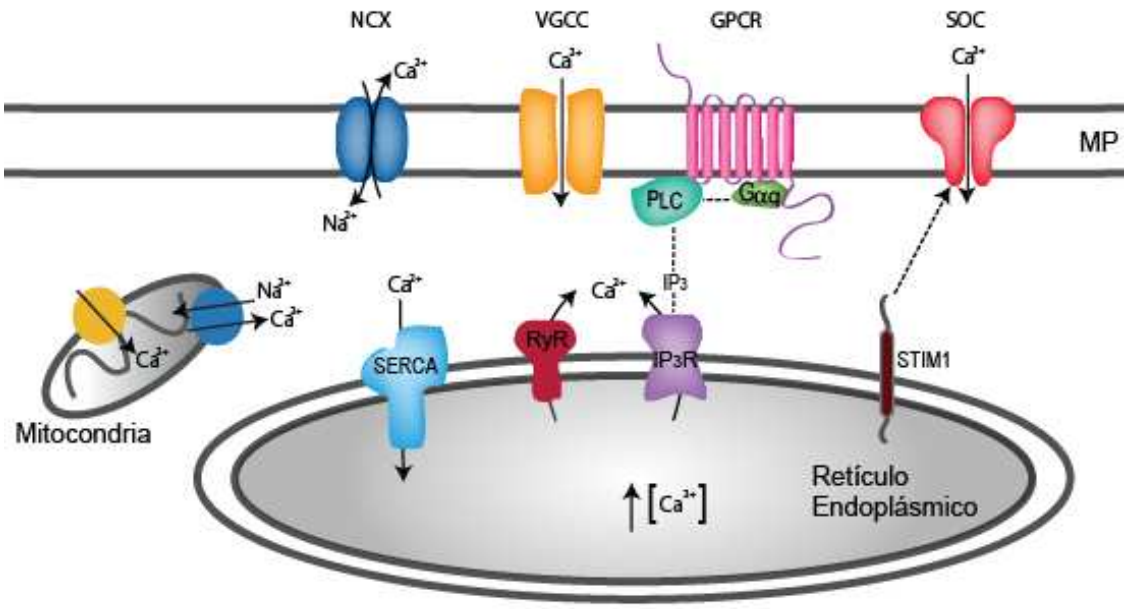

Figura 4. Principales proteínas involucradas en la señalización de calcio en la célula.

2.3. ENTRADA DE CALCIO OPERADA POR LAS RESERVAS INTRACELULARES (SOCE).

La entrada de calcio operada por las reservas intracelulares fue descrita por primera vez en 1986 por Putney [61]. Sus estudios le permitieron establecer que tras la estimulación de células de la glándula salival con diferentes agonistas que se unían a los GPCRs, activando la vía de la PLC, se producía un aumento intracelular de calcio con dos componentes característicos, un pico inicial que dependía de la salida de calcio desde el RE y una fase sostenida que podía ser inhibida al quitar el calcio de la solución extracelular, mostrando que este último componente dependía de la entrada de calcio a través de la membrana plasmática (Figura 5). Sin embargo, el mecanismo responsable de esta entrada de calcio fue develado hasta dos décadas después con el descubrimiento de dos proteínas claves: STIM1 y Orai1.

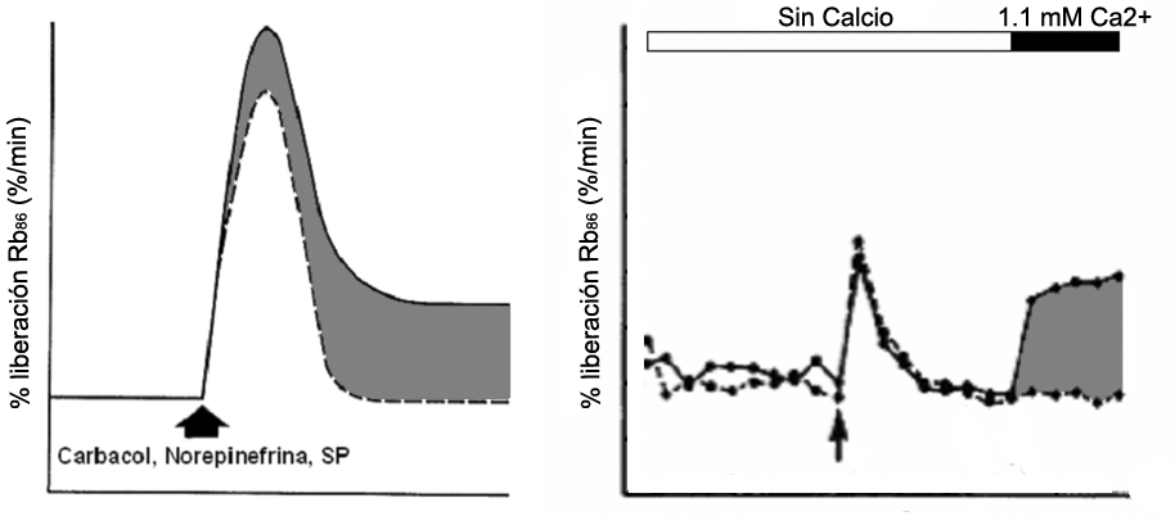

Figura 5. Respuesta de las células de la glándula parótida a la estimulación con agonistas de los GPCRs. La cuantificación de la liberación de Rb^{86} como medida del aumento de calcio intracelular muestra la dependencia de esta respuesta a la concentración de calcio extracelular. En ambos trazos se pueden apreciar los dos componentes de la respuesta, la región en banco corresponde a la liberación de calcio desde el RE y la región en gris a SOCE. (Modificado de Putney 1977 [62] y Putney 1986 [61])

2.3.1. STIM1, comunicando el vaciamiento del retículo a la membrana plasmática.

Por sus múltiples funciones, la proteína STIM1 (stromal interacting molecule 1) puede ser considerada como la proteína más importante para el funcionamiento de SOCE. STIM1 fue la primera proteína de la vía de SOCE identificada, y su hallazgo tuvo lugar casi 20 años después del descubrimiento de esta entrada de calcio. Su participación en SOCE fue determinada por experimentos de RNA de interferencia en células S2 de Drosophila [63-65]. STIM1 tiene dos funciones fundamentales en SOCE, la primera es censar la disminución de calcio en el interior del RE y la segunda es activar los canales operados por las reservas (SOC, *store-operated channels*) [65]. STIM1 es una proteína de un solo cruce transmembranal que se localiza principalmente en la membrana del RE. Se han identificado varios dominios importantes para las diversas funciones de STIM1 [66, 67]. En la Figura 6 se muestra un esquema de la estructura de STIM1 para ilustrar los diferentes dominios que se explican a continuación.

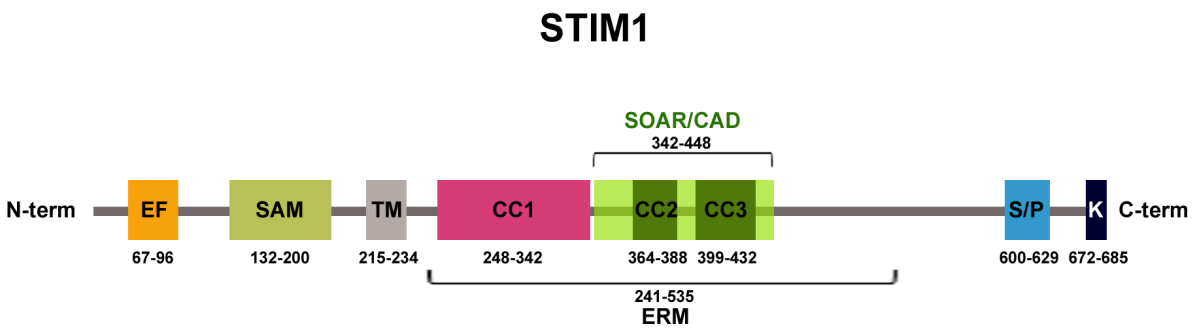

Figura 6. Esquema de la estructura de la proteína STIM1. El N-terminal se encuentra dirigido hacia el lumen del RE. Se ilustran sus dominios más importantes, EF: dominio de unión a calcio, SAM: motivo α-estéril, TM: dominio transmembranal, CC: coiled-coil, ERM: ezrin, radixin, moesin, SOAR: STIM1-Orai1 activating region, CAD: CRAC-activating domain, S/P: región rica en serina y prolina, K: región rica en lisina. Los números indican las posiciones de los aminoácidos.

El N-terminal de STIM1 se encuentra dirigido hacia el lumen del RE y contiene dos dominios de unión a calcio tipo EF (uno clásico y otro denominado "*hidden-EF*") y un motivo α-estéril (SAM). El dominio EF clásico se encarga de censar la concentración de calcio al interior del RE con una baja afinidad de entre 200 y 600 µM [68], lo cual le permite a esta proteína detectar disminuciones en la concentración de calcio al interior del RE. Cuando el RE está lleno (~1 mM) el calcio está unido al dominio EF de STIM1 y la proteína se encuentra en estado inactivo, una disminución en el calcio del RE causa el desprendimiento de los iones calcio del dominio EF lo cual conlleva a un cambio conformacional de STIM1 que permite su oligomerización, formando cúmulos en la uniones RE-MP e iniciando el proceso que lleva a la activación de SOCE [64, 69]. La oligomerización de STIM1 está mediada por una interacción entre los dominios SAM y entre los dominios *hidden-EF* de cada proteína [70].

Por otro lado el C-terminal de STIM1 está constituido por un dominio rico en serina/prolina, el cual está involucrado en la interacción de STIM1 con el citoesqueleto [71] y un dominio rico en lisina que media tanto el anclaje de STIM1 a la membrana plasmática [72], como la activación del TRPC1, uno de los canales operados por las reservas [73]. Estos dos dominios polibásicos también son importantes para la translocación de STIM1 hacia las uniones RE-MP. Además en esta región también se encuentra un dominio ERM (*ezrin, radixin, moesin*) dentro del cual se

localizan tres dominios *coiled coil* y una región conocida como SOAR (*STIM1-Orai1 activating region*, 344-442) o CAD (*CRAC-activating domain*, 342-448), la cual es responsable de la activación del Orai, el principal canal operado por las reservas [74, 75].

2.3.2. Orai, el canal activado por el vaciamiento del retículo

En 1992 Hoth y Penner identificaron una corriente iónica activada en respuesta al vaciamiento del RE, la cual denominaron I_{crac} (*calcium release activated current*), que se caracterizaba por ser altamente selectiva a calcio (Ca^{2+}/Na^{+}= 1.000), con una fuerte rectificación de entrada en su relación I/V, una amplitud de corriente de 0.6 pA/pF y una conductancia unitaria muy pequeña (<1pS) [76]. La identificación del canal responsable de esta corriente no ocurrió sino hasta el año 2006, mediante el mapeo genético de linfocitos que carecían de SOCE, provenientes de un paciente con un síndrome de inmunodeficiencia combinado severo [77]. En estos estudios se descubrió una nueva familia de proteínas que se denominó Orai, una mutación puntual (R91W) en esta proteína era la causante del síndrome de inmunodeficiencia, mostrando la relevancia fisiológica de Orai. En vertebrados se han identificado tres miembros de la familia Orai (Orai1, Orai2 y Orai3) [78], siendo Orai1 el responsable de la mayor parte de SOCE en las diferentes células estudiadas. Estudios posteriores mostraron que las corrientes del Orai1 cumplen todas la características de la I_{crac} y que esta proteína es esencial para el funcionamiento de SOCE [77-81].

Orai1 es una proteína con cuatro cruces transmembranales que se localiza en la membrana plasmática, tanto su C-terminal como su N-terminal se encuentran dirigidos hacia el citoplasma y parece formar tetrámeros después de su interacción con STIM1, lo cual lleva a su estado funcional [82]. Las Figuras 7A y 7B muestran la estructura de Orai1 y los aminoácidos que han sido identificados como importantes para su funcionamiento, entre ellos podemos encontrar a dos residuos de glutamato (E106 y E190), que están localizados en la región del poro y están involucrados en la selectividad del canal al calcio [80, 83], mientras que el C-terminal posee las regiones que permiten el reclutamiento de Orai1 y su unión con STIM1 para que se dé su

activación [75, 84]. Tres glutamatos conservados en el C-terminal parecen participar en el mecanismo de activación del canal al unirse a una región polibásica (4 lisinas) del SOAR [85, 86].

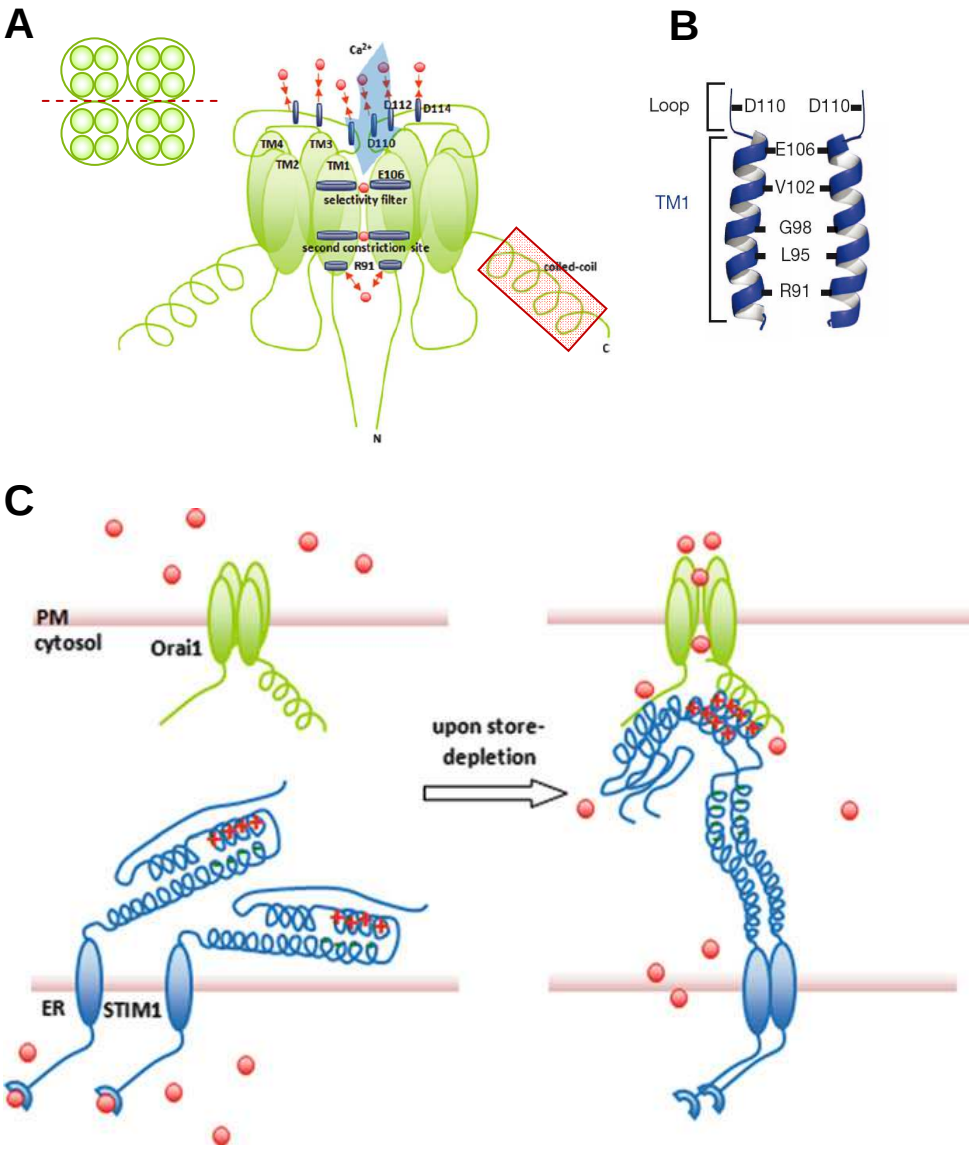

Figura 7. A. Estructura del canal Orai1, un tetrámero formado por 4 subunidades independientes, cada una con 4 cruces transmembranales (TM1-4). Dentro de los aminoácidos importantes se han identificado varios aspartatos (D110, D112, D114) ubicados en el primer loop extracelular, los cuales forman un vestíbulo que estabiliza los iones calcio y facilitan su entrada. La activación del Orai1 por parte de STIM1 se da mediante un dominio coiled-coil ubicado en su C-terminal. **B.** Estructura del poro del canal formado por aminoácidos presentes en el TM1 de cada subunidad, el filtro de selectividad está formado por el glutamato E106. **C.** Esquema del proceso de activación de Orai1 por STIM1 tras el vaciamiento del RE (Modificado de Derler et al. 2012 [87] y McNally et al. 2012 [88])

Por otro lado, en el N-terminal tiene una región rica en prolina que está involucrada en una regulación negativa mediada por STIM1 [75] y en la unión a la calmodulina (CaM) [89]. La Figura 7C ilustra el proceso de activación de Orai1, como se mencionó anteriormente, cuando el RE se encuentra lleno STIM1 tiene el calcio unido a su dominio EF y se distribuye homogéneamente en la membrana del RE a manera de monómero, mientras que el Orai1 se encuentra cerrado. Una vez se libera el calcio del RE, los iones calcio unidos a STIM1 se desprenden causando un cambio conformacional que lleva a STIM1 a formar oligómeros que se ubican en las uniones RE-MP permitiendo la interacción del SOAR con el C-terminal de Orai1, esta interacción causa la apertura del canal permitiendo el flujo de calcio[90, 91].

2.3.3. TRPCs y otras proteínas involucradas en SOCE

Por muchos años la búsqueda del canal operado por las reservas se centró en la familia de los TRPC (*Transient receptor potential canonical*), debido a que la identificación de la familia de los TRP se dio precisamente mediante experimentos que mostraban la activación de una corriente en respuesta al vaciamiento del RE en células de Drosophila [92]. Aunque hay suficiente evidencia que apoya que varios de estos canales pueden ser activados en respuesta al vaciamiento del RE [93-95], existe controversia sobre su papel en SOCE ya que estos canales también pueden ser activados por otros mecanismos como por ejemplo la PKC, la unión del DAG o la hidrólisis del PIP_2 [93]. Por esta razón los canales TRPC podrían ser considerados como SOC condicionales, cuya operación por las reservas podría depender tanto del agonista como del tipo celular, mientras que el Orai1, el cual es activado exclusivamente por el vaciamiento del RE, estaría funcionando como un SOC incondicional (Figura 8).

Ha sido visto que el TRPC1 puede ser activado por STIM1 mediante una interacción electrostática entre dos aspartatos en el C-terminal del canal (D639 y D640) y la región rica en lisina del STIM1, siendo esta la evidencia más fuerte de que el TRPC1 funciona como un SOC [73].

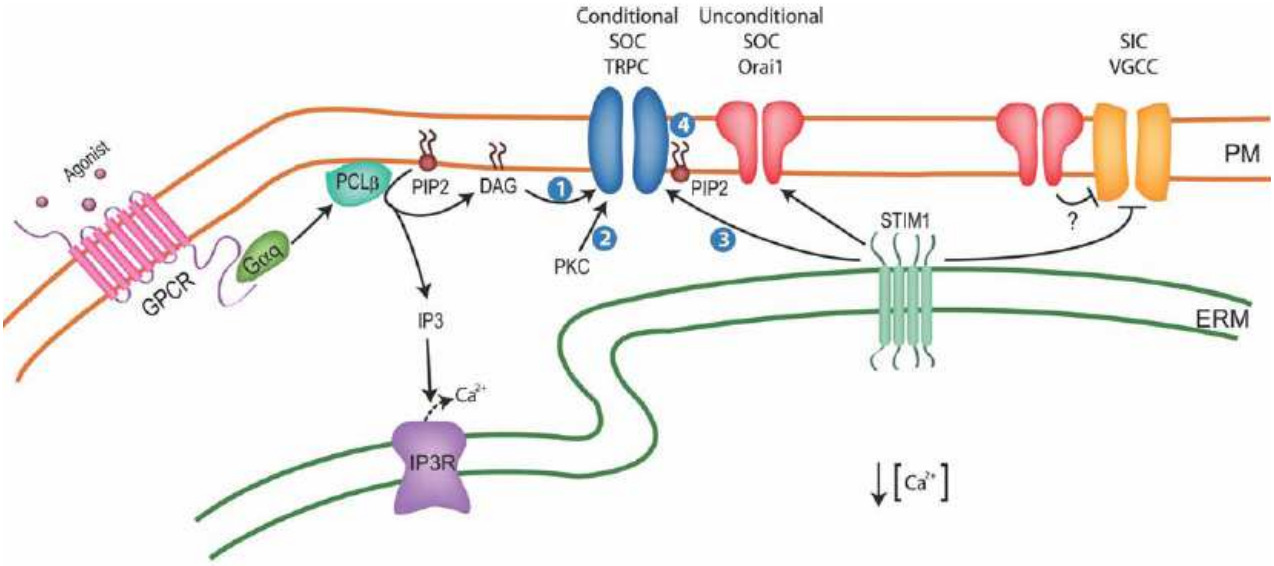

Figura 8. Canales operados por las reservas (SOC). Mientras el Orai1 es exclusivamente activado por STIM1 tras el vaciamiento del RE, el TRPC puede ser activado por STIM1 y por otras moléculas producidas tras la activación de los GPCR acoplados a proteínas Gαq (1-4). Hay evidencia de que STIM1 también puede inhibir a los canales de calcio dependientes de voltaje (VGCC). (Tomado de Moreno y Vaca 2011[96]).

Aunque el STIM1 y el Orai1 son suficientes para que SOCE ocurra, existen otras proteínas involucradas en esta entrada de calcio. Este conjunto de proteínas forma un complejo que se ha denominado SOCIC (*store-operated calcium influx complex*) [97]. Dentro de los miembros de SOCIC identificados hasta ahora podemos encontrar a STIM1, a los canales Orai1 y TRPC1, a algunas proteínas reguladoras como la CaM, la CRACR2A (*calcium-release activated channel regulator 2A*) y la proteína Junctate; y a proteínas efectoras como la SERCA y algunas adenilato ciclasas (AC) (Figura 9).

La CaM interactúa con el dominio rico en lisina de STIM1 y se cree que participa en la relocalización de STIM1 hacia las uniones RE-MP y en el desensamblaje de STIM1 y Orai1 [98]. Además la CaM regula la inactivación dependiente de calcio tanto del Orai1 como del TRPC1 por una interacción directa con estos canales [89, 99, 100]. De esta manera, la CaM actúa como un regulador negativo de SOCE al participar en la inactivación de los SOCs. Otras dos proteínas reguladoras de SOCE que han sido recientemente descubiertas mediante ensayos de inmunopurificación son CRACR2A y Junctate [52, 101]. Ambas proteínas tienen dominios EF de

unión a calcio. CRACR2A se encuentra en el citoplasma y parece estabilizar la interacción STIM1-Orai1 al unirse al N-terminal de Orai1 y al dominio polibásico de STIM1, formando un complejo a concentraciones altas de calcio. Por otro lado, Junctate reside en la membrana del RE y parece participar en la relocalización de STIM1 hacia las uniones del RE-MP al interactuar con el N-terminal de STIM1, siendo esencial para que se dé la formación de los oligómeros y la activación de Orai1.

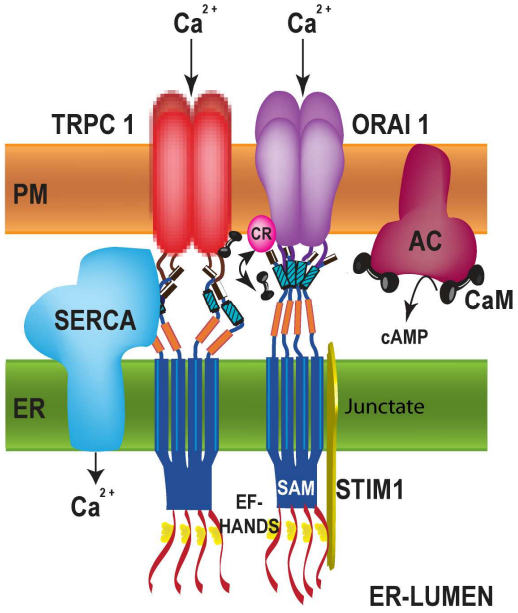

Figura 9. SOCIC. El complejo de entrada de calcio activado por el vaciamiento del RE. PM: Membrana plasmática, ER: Retículo endoplásmico, CR: CRACR2A. (Modificado de Moreno y Vaca 2012 [55]).

Otro componente importante de SOCIC es la SERCA la cual se encarga de rellenar el RE. Ha sido visto que la activación de SOCE ocurre en microdominios en los cuales la concentración de calcio aumenta transitoriamente sin causar una elevación citoplasmática global [102]. Esto se logra por la eficiencia de la SERCA para bombear el calcio hacia el interior del RE [103]. La SERCA es reclutada a las uniones RE-MP después del vaciamiento del RE [104] y su función es importante para mantener las concentraciones basales de calcio al interior del RE y así permitir que las células puedan responder a una estimulación repetitiva. Aunque siempre se ha creído que la función más importante de SOCE es rellenar el RE, la elevación del calcio citoplasmático en los microdominios en los que ocurre SOCE, activa proteínas dependientes de calcio y participa

así en diferentes vías de señalización. Este es el caso de la AC8, la cual es reclutada a las uniones RE-MP y se activa exclusivamente por la entrada de calcio a través del Orai1, produciendo AMPc, otro de los segundos mensajeros importantes en la célula [105-108].

2.4. LA RESPUESTA DE LOS ASTROCITOS A LA TROMBINA

La trombina es una proteasa de la familia de las tripsinas, generada a partir del clivaje de la protrombina mediado por el complejo conocido como protrombinasa [109]. Su estructura consiste en una cadena ligera de 49 aminoácidos y una cadena pesada catalítica de 259 aminoácidos conectadas por un puente disulfuro. Si bien la trombina es conocida principalmente por su función en la cascada de coagulación, esta proteasa también es capaz de unirse a receptores en la membrana plasmática desencadenando diferentes respuestas en muchos tejidos incluyendo el cerebro [110]. La trombina puede ser sintetizada en el cerebro durante episodios de isquemia o puede entrar al cerebro tras una hemorragia o una ruptura de la barrera hemato-encefálica, causando activación de las células endoteliales, infiltración de linfocitos y edema [111, 112]. Adicionalmente activa a los astrocitos, siendo estos las células cerebrales más susceptibles a la acción de la trombina, su activación causa una potenciación de la inflamación local y diversos efectos neurotóxicos [113, 114].

La cascada de señalización de la trombina inicia con su unión a receptores PAR (*Protease-activated receptors)*, los cuales pertenecen a la familia de los GPCRs. Existen 4 miembros llamados PAR-1, -2, -3 y -4, a excepción del PAR-2 todos los demás son activados por trombina. La trombina corta el extremo N-terminal del receptor dando origen a un nuevo extremo que sirve de ligando y que se une al segundo loop extracelular del receptor auto-activándolo (Figura 10A) [110]. Los astrocitos expresan los tres receptores PAR activados por trombina, siendo el PAR-1 el más relevante en estas células [113, 115]. Este receptor PAR tiene la propiedad de acoplarse a diferentes proteínas G incluyendo la Gαq, la Gαi/0 y la Gα11/12, lo que resulta en la activación de diferentes vías de señalización (Figura 10B) [116]. La proteína Gαq se une al segundo loop

intracelular del receptor y es la responsable de inducir los aumentos de calcio en el astrocito [117].

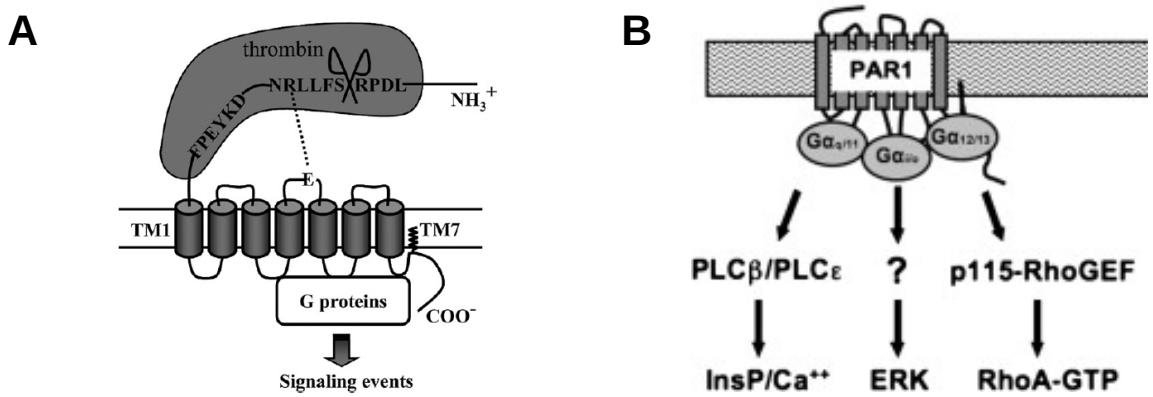

Figura 10. **A.** Mecanismo de activación del receptor PAR. **B.** Proteínas G acopladas al receptor PAR-1 y las vías de señalización activadas. (Tomado de Luo et al. 2007 [118] y McCoy et al. 2102 [117]).

La respuesta de los astrocitos a la trombina se caracteriza por un aumento bifásico en la concentración citoplasmática de calcio, una primera fase que corresponde a la liberación de calcio desde el RE y una segunda fase que depende de la entrada de calcio a través de canales en la membrana plasmática (Figura 11) [119].

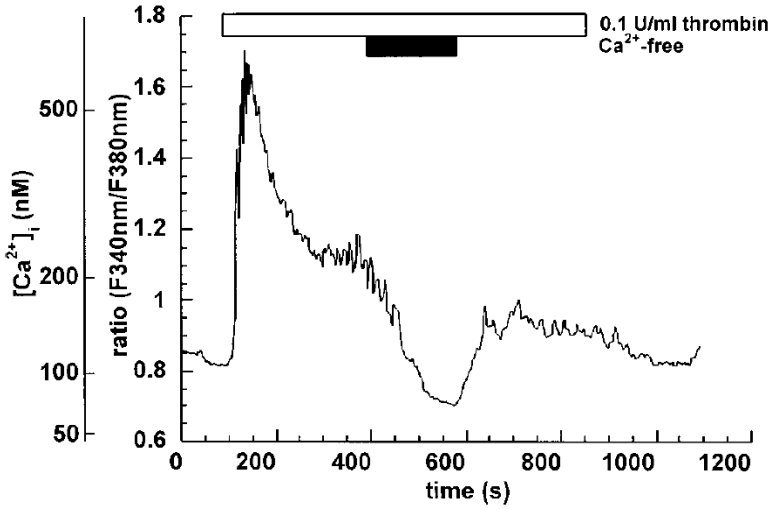

Figura 11. Respuesta de los astrocitos a la trombina. La trombina induce un aumento bifásico en la concentración citoplasmática de calcio de los astrocitos que depende del calcio extracelular. (Tomado de Ubl y Reiser 1997 [119])

En los astrocitos la trombina, dependiendo de su concentración, induce una variedad de respuestas mediadas por el calcio que van desde respuestas citoprotectoras hasta citotóxicas [118]. A concentraciones bajas (0.1 – 1 U/ml), la trombina es neuroprotectora, mientras que a altas concentraciones (≥10 U/ml) causa daño cerebral, llevando a los astrocitos hacia una serie de cambios morfológicos y moleculares conocidos como un estado de activación o astrogliosis [18, 120]. Dentro de las respuestas desencadenadas por la trombina encontramos un aumento en la proliferación [115, 121], cambios en la morfología, siendo la retracción de las proyecciones la más común [122], cambios en la movilidad celular [114] y muerte celular [123]. La activación de los astrocitos causa además la liberación de muchas moléculas que inducen la inflamación cerebral, como el AA, las interleucinas, moléculas quimioatrayentes y el óxido nítrico [109, 124]. Adicionalmente, se ha visto que la trombina causa la potenciación de la liberación dependiente de calcio del glutamato, uno de los procesos que causa más daño en el cerebro [125, 126].

3. PLANTEAMIENTO DEL PROBLEMA

SOCE es una entrada de calcio que se activa en respuesta a la liberación de calcio desde el RE. Esta entrada de calcio es crítica para el funcionamiento de muchos tipos celulares [127-129], entre los cuales los astrocitos parecen no ser la excepción [130, 131]. Se ha visto que en astrocitos y astrocitomas SOCE se activa en respuesta a la estimulación con glutamato [132], histamina [133] y trombina [119], sugiriendo que estas células expresan la maquinaria necesaria para que SOCE ocurra. Como se explicó anteriormente hay dos componentes centrales de SOCE, el primero de ellos es STIM1 el cual es el sensor de calcio que comunica el vaciamiento del RE y activa los SOC en la membrana plasmática [63, 65], el segundo es el canal Orai1. Los canales TRPC también participan en SOCE, sin embargo estos canales, a diferencia del Orai1, funcionan como SOCs condicionales que pueden operar de manera independiente al vaciamiento del RE [96].

En los astrocitos los estudios de la maquinaria molecular que da lugar a SOCE se han enfocado en los canales TRPC. Hay evidencia de que el TRPC1 participa en SOCE en astrocitos corticales de ratón tratados con bloqueadores de la SERCA [102] y en astrocitos corticales de rata estimulados con glutamato [134]. Además se ha visto que el TRPC3 participa en SOCE cuando los astrocitos se estimulan con substancia P [135]. En cuanto a la trombina, el TRPC3 es el único canal que ha sido demostrado que participa en la respuesta de los astrocitos a este agonista, aunque por sí solo no explica toda la entrada de calcio observada ante este estímulo [136, 137].

Aunque es sabido que en plaquetas y células endoteliales STIM1 y Orai1 son esenciales para que se dé la entrada de calcio inducida por trombina [138-142], el papel de STIM1 y Orai1 en la entrada de calcio inducida por este agonista en los astrocitos permanece aún sin explorar. ¿Causa la trombina la activación de STIM1 en los astrocitos?, ¿Participa el canal Orai1 en la entrada de calcio inducida por trombina en los astrocitos?, ¿Qué porcentaje de la entrada de calcio activada por la trombina en los astrocitos es debido a la activación del canal Orai1 por STIM1?, ¿Cuál es el papel de STIM1 y Orai1 en la fisiología y patología de los astrocitos?, son algunas de las

preguntas que aún faltan por contestar. La Figura 12 ilustra la posible participación de STIM1 y Orai1 en la entrada de calcio activada por la trombina en los astrocitos, la cual es la pregunta central de este trabajo.

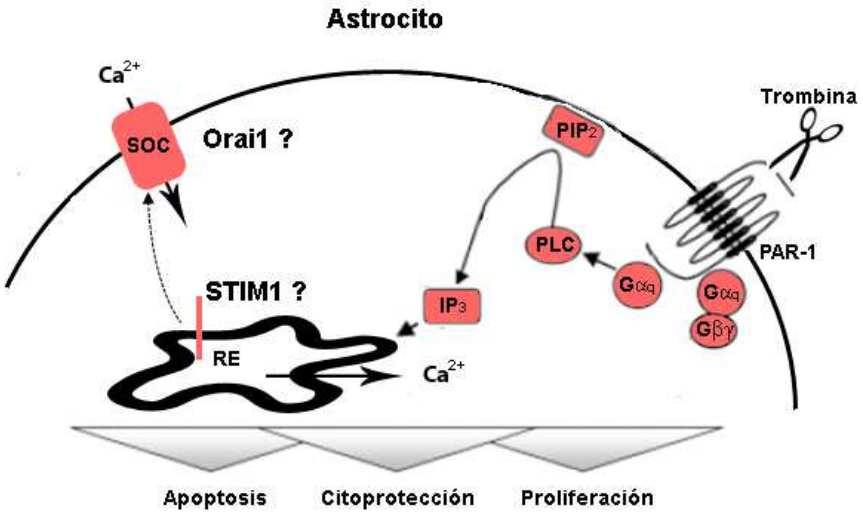

Figura 12. Hipótesis de la participación de STIM1 y Orai1 en la entrada de calcio inducida por la trombina en astrocitos.

4. HIPÓTESIS

Ya que STIM1 y Orai1 son los principales efectores de la entrada de calcio operada por el vaciamiento del RE y que ha sido demostrado que la trombina causa la liberación de calcio desde el RE. Nosotros creemos que STIM1 y Orai1 participan en el aumento de la concentración de calcio intracelular inducido por la trombina en los astrocitos. La estimulación con trombina causará la salida de calcio desde el RE, llevando a la oligomerización y reorganización de STIM1 para finalmente activar el canal Orai1. Este canal funcionaría en los astrocitos como la principal vía de entrada de calcio durante la respuesta a la trombina.

5. OBJETIVOS

OBJETIVO GENERAL

Determinar la participación de STIM1 y Orai1 en la entrada de calcio activada tras la liberación de calcio desde el retículo endoplásmico en astrocitos corticales de rata estimulados con trombina.

OBJETIVOS ESPECÍFICOS

1. Determinar la expresión endógena de STIM1 Y Orai1 en astrocitos corticales de rata en cultivo.

2. Estudiar la reorganización de STIM1 durante la estimulación de los astrocitos con trombina.

3. Evaluar el efecto de la sobreexpresión de STIM1 y Orai1 sobre la entrada de calcio operada por las reservas intracelulares y sobre la respuesta de los astrocitos a la trombina.

4. Analizar el efecto de la inhibición de la expresión de STIM1 y Orai1 endógenos, mediante RNA de interferencia, sobre la entrada de calcio operada por las reservas intracelulares y sobre la respuesta de los astrocitos a la trombina.

5. Evaluar el efecto de la expresión de una mutante dominante negativa del canal Orai1 sobre la entrada de calcio inducida por la trombina en los astrocitos.

6. MÉTODOS

6.1. CULTIVO DE ASTROCITOS CORTICALES DE RATA

Todos los procedimientos para el mantenimiento de los animales, su manejo y la extracción de tejido para el cultivo de astrocitos fueron aprobados por el Comité del Cuidado de Animales del Instituto de Fisiología Celular, UNAM, y cumplieron con todos los requisitos establecidos por el "*Council for International Organizations of Medical Sciences, 2010"*. Para el cultivo de astrocitos corticales se utilizaron ratas de la cepa Wistar de 1 día de nacidas. Se utilizaron 6 animales por cultivo, éstos fueron decapitados y el cerebro colocado en una solución Krebs en donde se retiraron las meninges con ayuda de pinzas. Las cortezas fueron extraídas y cortadas en pequeñas secciones para facilitar la digestión enzimática. El tejido fue disociado por 10 minutos en una solución Krebs que contenía 4800U/ml de tripsina (Sigma). Luego se adicionó una solución Krebs que contenía 15% de Suero Fetal Bovino (SFB, Gibco) para detener la reacción de la tripsina y el tejido fue recuperado por centrifugación a 1000 rpm. Posteriormente el tejido fue disociado mecánicamente por pipeteo en una solución Krebs que contenía 110 U/ml de DNAasa (Sigma) hasta obtener una suspensión homogénea de células. El tejido disociado fue pasado a través de una malla de nylon con poro de 80 µm de diámetro. La suspensión celular resultante de la filtración fue centrifugada a 1000 rpm por 5 minutos y resuspendida en medio BME (Basal Medium Eagle, Gibco), suplementado con 10% de SFB, 2 mM de glutamina (Sigma), 50 U/ml de penicilina y 50 µg/ml de estreptomicina. Las células fueron sembradas en cajas de plástico de 10 cm de diámetro e incubadas a 37°C y 5% CO_2. Después de 2-3 semanas, al alcanzar el 90% de confluencia, las células fueron tripsinizadas y sembradas a una densidad de 0.5×10^6 células/caja de 60mm sobre vidrios cubiertos con poli-L-lisina. Las células fueron usadas para los experimentos al alcanzar la confluencia deseada (aproximadamente 1 semana después de la resiembra).

6.2. TRANSFECCIÓN DE PLÁSMIDOS

En este trabajo se utilizaron los siguientes plásmidos: STIM1-YFP (Addgene, plásmido #18857 [64]), Orai1-YFP (Addgene, plásmido #19756 [83]) y Orai1 E106A el cual fue una generosa donación del Dr. Shenyuan Zhang (Texas A&M University). Para las transfecciones se utilizaron cultivos de astrocitos con una confluencia de entre 80% y 90%. La transfección se realizó con Lipofectamina PLUS™ (Invitrogen). El protocolo utilizado se resume a continuación: para cajas de 35 mm se usó 1 µg del plásmido correspondiente, se mezcló con 6 µl de PLUS en 100 µl de Opti-MEM (Gibco) y se incubó a temperatura ambiente por 15 min. Posteriormente, se adicionaron 4 µl de Lipofectamina diluidos en 100 µl de Opti-MEM y se incubó por otros 15 min. El medio de cultivo de la caja fue reemplazado con 800 µl de Opti-MEM y el complejo DNA-Lipofectamina preparado anteriormente fue adicionado. Las células se incubaron a 37°C y 5% CO_2 durante 6 horas, después de este tiempo el medio fue reemplazado por medio BME suplementado con suero. Las pruebas de expresión de las proteínas y las mediciones de calcio se realizaron entre las 24 y las 36 horas post-transfección. Para las células sembradas en cajas de 60 mm las cantidades de DNA y Lipofectamina fueron escaladas según la superficie del plato de cultivo.

6.3. INMUNOCITOQUÍMICA Y MICROSCOPÍA CONFOCAL.

Para la detección de proteínas por inmunocitoquímica, los astrocitos fueron cultivados sobre vidrios de 9 x 9 mm cubiertos con poli-L-lisina. Las células fueron lavadas con un buffer salino de fosfatos (PBS) e inmediatamente fijadas con metanol puro a -20°C por 10 minutos. Después de la fijación se incubaron en PBS con 50 mM de glicina por 10 minutos a 4°C y posteriormente en solución de bloqueo (5% de BSA en PBS) por 1 hora a temperatura ambiente. Los anticuerpos primarios anti-STIM1 (BD Biosciences) y anti-Orai1 (Santa Cruz Biotech.), se utilizaron a una dilución 1:40 en PBS-1%BSA y fueron incubados durante toda la noche a 4°C. Al día siguiente las células fueron lavadas 3 veces con PBS durante 10 minutos, posteriormente se incubaron con

el anticuerpo secundario fluorescente respectivo, DyLight-549 donkey anti-mouse IgG o DyLight-649 donkey anti-goat IgG (Jackson ImmunoResearch Labs Inc), a una dilución 1:200 en PBS-1%BSA durante 1 hora a temperatura ambiente y en condiciones de oscuridad. Las células fueron nuevamente lavadas 3 veces con PBS e incubadas con DAPI (Molecular Probes) a una dilución 1:1000 por 5 minutos. Las laminillas fueron montadas en láminas portaobjetos con el medio de montaje DAKO (Invitrogen).

Las imágenes de inmunofluorescencia fueron tomadas usando un microscopio confocal Olympus FV10i equipado con un objetivo de 60x y fueron analizadas usando el programa Fluoview 10-ASW2.1. Para los experimentos de visualización de las proteínas fluorescentes STIM1-YFP y Orai1-YFP en células vivas se utilizó el microscopio confocal Olympus FV1000 y un objetivo de 60x.

6.4. MEDICIONES DE CALCIO

Para la medición del calcio intracelular los astrocitos fueron cultivados sobre láminas de vidrio de 10 x 40 mm cubiertas con poli-L-lisina. Las células fueron incubadas por 30 minutos a temperatura ambiente con el indicador fluorescente Fura-2 AM (Molecular Probes) a una concentración de 5 µM en solución fisiológica (Tabla 1) con 1% de ácido plurónico. Las células fueron lavadas con solución fisiológica y la lámina fue posicionada en una cubeta a un ángulo de 50° respecto a la vía de luz de excitación de un espectrómetro de luminiscencia Aminco-Bowman (Thermo Electron). La longitud de onda de excitación fue alternada entre 340 nm y 380 nm y la emisión fue colectada a 510 nm. La cubeta contenía solución fisiológica con o sin calcio según las condiciones requeridas para cada experimento. La composición de ambas soluciones se describe en la Tabla 2.

Tabla 2. Composición de las soluciones fisiológicas usadas.

	Solución con Ca^{2+}	***Solución sin Ca^{2+}***
NaCl	120 mM	120 mM
KCl	4.75 mM	4.75 mM
$MgCl_2$	1.2 mM	1.2 mM
HEPES	20 mM	20 mM
$CaCl_2$	1.8 mM	-
Glucosa	10mM	10mM
KH_2PO_4	1.17	1.17
BSA	0.05%	0.05%
EGTA	-	200 µM

Ajustadas a 296 mOsm, pH 7.4

Para inducir el vaciamiento del RE se utilizó Tapsigargina (Tg, 200 nM, Calbiochem), un bloqueador irreversible de la SERCA. La trombina (BioPharm Labs) se utilizó a una concentración de 5 U/ml. El protocolo usado para los experimentos de medición de calcio se detalla en la Figura 13. La frecuencia de muestro fue de 2 segundos y el registro tuvo una duración total de 1300 segundos. Los 100 primeros segundos corresponden a la señal basal, a los 100 segundos las células fueron estimuladas con Tg o con Trombina en ausencia de calcio intracelular para inducir la salida de calcio desde el RE. A los 700 segundos se adicionó 1.8 mM de $CaCl_2$ a la solución extracelular para inducir la entrada de calcio (SOCE). Los resultados son presentados como la relación de la fluorescencia emitida para las dos longitudes de excitación (F340/F380). Los cambios en la concentración de calcio fueron estimados calculando el área total bajo la curva del primer pico para la liberación de calcio desde el RE y del segundo pico para la entrada de calcio (SOCE).

Como en estos experimentos se colectó toda la luz emitida en la vía del laser, estas mediciones corresponden al cambio poblacional de la concentración de calcio intracelular (en un mínimo de 50 células). Para cada condición se realizaron mínimo 3 experimentos independientes. Los datos obtenidos se presentan como el promedio ± S.E.M. La significancia estadística de las diferencias

encontradas en cada tratamiento se determinó por una prueba t-student no pareada ($p < 0.05$). Todos los datos fueron graficados usando el programa Igor Pro 6 (WaveMetrics).

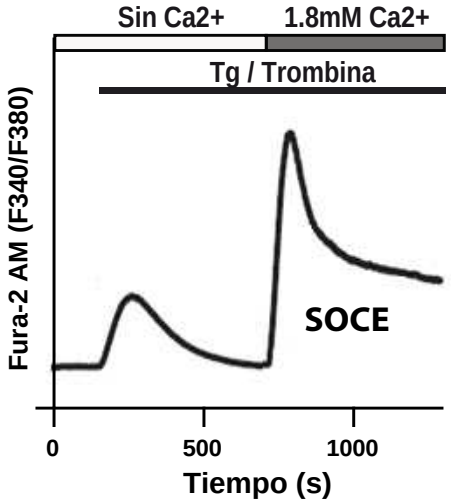

Figura 13. Protocolo de estimulación usado para la medición de cambios en la concentración de calcio intracelular en los astrocitos. El primer pico corresponde a la salida de calcio desde el RE inducida por la aplicación de Tg o trombina en la ausencia de calcio extracelular, mientras que el segundo pico corresponde a la entrada de calcio operada por las reservas (SOCE) activada cuando se adiciona 1.8 mM de calcio a la solución extracelular.

6.5. RNA DE INTERFERENCIA

Para la inhibición de la expresión de STIM1 y Orai1 en los astrocitos de rata se utilizaron siRNAs (small interference RNAs). Se utilizó un set de tres oligonucleótidos *Stealth-siRNAs* (Invitrogen) dirigidos contra cada gen (rStim1:RSS324019, RSS360174, RSS324020 y rOrai1: RSS357633, RSS357634, RSS357635). Como control negativo se usó el *Stealth-RNAi scramble negative control duplex* (Invitrogen), el cual no reconoce ninguna secuencia del genoma de la rata. La transfección de los siRNA se llevo a cabo utilizando Lipofectamina 2000 (Invitrogen). El protocolo de transfección se detalla a continuación: en un primer tubo se mezclaron 200 pmoles de la tripleta de siRNAs o del siRNA-scramble con 500 µl de Opti-MEM, en un segundo tubo se mezclaron 15 µL de Lipofectamina con 500 µL de Opti-MEM, ambos tubos fueron incubados

durante 5 minutos a temperatura ambiente. Posteriormente se mezclaron las dos diluciones y se incubaron por 25 minutos. El medio de cultivo de una caja de 60 mm con una confluencia entre el 70% y el 80% fue reemplazado por 1 ml de Opti-MEM y la mezcla preparada anteriormente fue adicionada a la caja. La concentración final de siRNA fue de 100 nM. Las células se incubaron durante toda la noche a 37°C y 5% CO_2. Al siguiente día el medio fue reemplazado con BME suplementado con SFB sin antibióticos para evitar la citotoxicidad. Los experimentos para determinar los niveles de expresión del RNAm por PCR en tiempo real así como las mediciones de calcio se realizaron después de las 36 horas post-transfección. Para la transfección de cajas de 35 mm las cantidades fueron escaladas según el área de la superficie.

La eficiencia de transfección de este protocolo se evaluó mediante la transfección del oligonucleótido fluorescente BLOCK-iT (Alexa-Fluor Red, Invitrogen). Por otro lado la eficiencia de inhibición mediante siRNAs en los astrocitos se verificó expresando simultáneamente un plásmido de la proteína verde fluorescente (GFP) y un siRNA dirigido contra esta proteína (GFP-22 siRNA,1022064,Qiagen), para estos experimentos se utilizó el siRNA 1022076 (Qiagen) como control negativo.

6.6. EXTRACCIÓN DE RNA Y SÍNTESIS DE cDNA

Para la extracción de RNA se utilizaron astrocitos cultivados en cajas de 100 mm a una confluencia de 90%, tratados con el siRNA correspondiente (siRNA scramble, siRNA STIM1 o siRNA Orai1). El RNA fue aislado y purificado mediante el tratamiento con Trizol (Invitrogen). El protocolo usado se detalla a continuación, el medio de cultivo fue removido y se adicionaron 4 ml de Trizol, mediante pipeteo se despegó la monocapa celular hasta obtener un lisado homogéneo, el cual se incubó por 20 minutos a temperatura ambiente en agitación constante. Posteriormente se adicionaron 4 ml de cloroformo, se mezcló por inversión durante 15 segundos y se incubó por 3 minutos a temperatura ambiente. Luego se centrifugó a 12.000 g por 15 minutos a 4°C. Después de la centrifugación, el RNA permanece en la fase acuosa (fase

superior), esta fase fue separada y el RNA fue precipitado adicionando 2 ml de isopropanol e incubando por 10 minutos a temperatura ambiente (o toda la noche a -20°C). Se centrifugó a 12.000 g durante 10 minutos a 4°C. El sobrenadante fue descartado y se adicionaron 4 ml de etanol al 75% en agua con 0.05% DEPC. Se centrifugó nuevamente a 7.500 g por 5 minutos a 4°C. Finalmente se descartó el sobrenadante y se dejó secar el pellet a temperatura ambiente por 5 minutos. El pellet fue resuspendido en 100 µl de agua estéril e incubado por 15 minutos a 55°C. La integridad de los RNAs extraídos se verificó por medio de electroforesis en geles de agarosa al 1% en condiciones desnaturalizantes.

Para la síntesis del DNA complementario (cDNA) se realizó la transcripción reversa de 2.5 µg de RNA utilizando la retrotranscriptasa SuperScript III RT (Invitrogen). En un tubo se adicionó 1 µl del *primer* Oligo dT (50µM, Invitrogen), 2.5 µg de RNA, 1 µl de dNTPs (10 mM, Invitrogen) y se llevó a un volumen final de 13 µl con agua inyectable. La mezcla se calentó a 65°C por 5 minutos y luego se incubó por 1 minuto en hielo. Se adicionó 1 µl de DTT (0.1M, Invitrogen), 4 µl de buffer first strand 5x (Invitrogen), 1 µl de RNAseOUT (40U/µl, Invitrogen) y 1 µl de Superscript III RT (200U/µl). Se incubó a 50°C por 1 hora, luego a 70°C por 15 minutos y finalmente en hielo por 1 minuto.

6.7. PCR EN TIEMPO REAL

Para cuantificar la expresión del RNAm de STIM1 y Orai1, en condiciones control y bajo la acción de los siRNAs, se realizaron ensayos de PCR en tiempo real. Utilizando el programa Primer Express se diseñaron *primers* para STIM1, Orai1, HPRT y GAPDH, estos dos últimos como genes de referencia internos. Los *primers* se diseñaron siguiendo los siguientes criterios: longitud entre 18 y 22 nucleótidos, complementariedad a una región de unión exón-exón para asegurar la amplificación del RNAm, tamaño del amplicón entre 70 y 110 pb y Tm de 60°C. Todos los primers fueron analizados por BLAST para asegurar que no fueran complementarios a

ninguna otra secuencia dentro del genoma de la rata. Las secuencias de los primers se muestran en la tabla 3.

Tabla 3. Secuencias de los oligonucleótidos usados como *primers* en los ensayos de PCR en tiempo real.

Gen	No. de Acceso	Tamaño pb	Primers		Posición
rOrai1	NM_001013982.1	75	FW: 5-CATGGTAGCGATGGTGGAAGT-3	RV: 5-GGCGCTGAAGACGATGAGTAAC-3	303-378
rStim1	NM_001108496.2	104	FW: 5-AGCTGAAGGCTCTGGACACAGT-3	RV: 5-CACCCACACCAATCACGATAGA-3	572-676
rGapdh	NM_017008.3	91	FW: 5-ACTCTACCCACGGCAAGTTCAA-3	RV: 5-CGCTCCTGGAAGATGGTGAT-3	143-233
rHprt1	NM_012583.2	93	FW: 5-TTGCTCGAGATGTCATGAAGGA-3	RV: 5-CCAGCAGGTCAGCAAAGAACT-3	146-238

Los experimentos de PCR en tiempo real se realizaron en un termociclador ABI PRISM7000 (Applied Biosystems), utilizando SYBRgreen (PCR MasterMix, Applied Biosystems) como reportero. Para cada reacción se utilizaron 250 ng de cDNA (2 µl del cDNA obtenido a partir de 2.5 ug de RNA), 200nM de cada *primer* (Fw y Rv), 10 µl de MasterMix y se llevó a un volumen final de reacción de 20 µl con agua. El protocolo utilizado fue: una desnaturalización inicial de 10 minutos a 95°C, seguida de 40 ciclos que comprendían desnaturalización de 15 segundos a 95°C y extensión de 30 segundos a 60°C. Al final de cada experimento se realizó la curva de disociación para verificar la obtención de un solo producto de amplificación. Adicionalmente se realizaron electroforesis de los productos de PCR en geles de poliacrilamida en condiciones no desnaturalizantes. El cambio en la expresión del RNAm de rStim1 y rOrai1 se calculó mediante el método de $2^{-\Delta\Delta CT}$ tomando a rGapdh y rHprt como genes control de referencia. Siguiendo la fórmula:

$$\Delta\Delta Ct = (Ct_{\text{gen problema}} - Ct_{\text{gen referencia}})_{\mathbf{A}} - (Ct_{\text{gen problema}} - Ct_{\text{gen referencia}})_{\mathbf{B}}$$

En donde **A** es la condición experimental (siRNA STIM1 o siRNA Orai1) y **B** es la condición control (siRNA scramble). El gen problema corresponde a STIM1 u Orai1 dependiendo del siRNA usado y el gen de referencia al Gapdh o Hprt.

7. RESULTADOS

STIM1 y Orai1 son dos componentes esenciales de SOCE, los cuales se activan en respuesta de la liberación de calcio desde el RE. La trombina induce en los astrocitos un aumento de calcio que tiene dos componentes, la salida de calcio desde el RE y SOCE [119]. Sin embargo el papel de STIM1 y Orai1 en los astrocitos, tanto en SOCE en general, como en la respuesta de estas células a la trombina no había sido estudiado. En este trabajo nosotros aportamos evidencia que soporta la hipótesis de que en los astrocitos la trombina activa la entrada de calcio a través del canal Orai1, y que esta proteína junto con el STIM1, es esencial para que estas células respondan ante esta estimulación.

7.1. LA RESPUESTA DE LOS ASTROCITOS A LA TROMBINA DEPENDE PRINCIPALMENTE DEL CALCIO EXTRACELULAR

El primer paso en nuestro estudio fue realizar experimentos de mediciones de calcio usando Fura-2 AM en astrocitos en cultivo para verificar que la respuesta de los astrocitos a la trombina tenía un componente dependiente del calcio extracelular. En todos nuestros experimentos además de evaluar la entrada de calcio inducida por trombina, se utilizó la Tg como una estrategia para vaciar el RE sin activar la vía de los GPCRs. De esta forma se evaluó simultáneamente el papel de STIM1 y Orai1 en SOCE y en la respuesta de los astrocitos a la trombina. La aplicación de Tg (200nM) causó una elevación citoplasmática de calcio cuya cinética y magnitud fue evidentemente modificada al retirar el calcio de la solución extracelular. Como se observa en la Figura 14A, la remoción del calcio causó una disminución de cerca del 80% en SOCE, apoyando la idea de que los astrocitos presentan una prominente entrada de calcio activada en respuesta al vaciamiento del RE. En la Figura 14B se muestra la respuesta de los astrocitos ante la estimulación con trombina (5 U/ml), en este caso también se observa que la respuesta inducida por este agonista tiene un componente importante (cercano al 80%) que depende del calcio extracelular, similar a lo observado con la Tg. En presencia de calcio extracelular la respuesta

observada para los dos estímulos tiene dos componentes, la salida de calcio desde el RE y SOCE, para separar estos dos componentes se utilizó el protocolo ilustrado en la Figura 13. La separación de los componentes mostró tanto para la Tg como para la trombina un componente prominente de SOCE (Figura 14C y D). Estos resultados confirman que tras la estimulación con trombina se activa una entrada de calcio y que esta entrada es la responsable de la mayor parte de la elevación citoplasmática de calcio observada durante la respuesta de los astrocitos a este agonista.

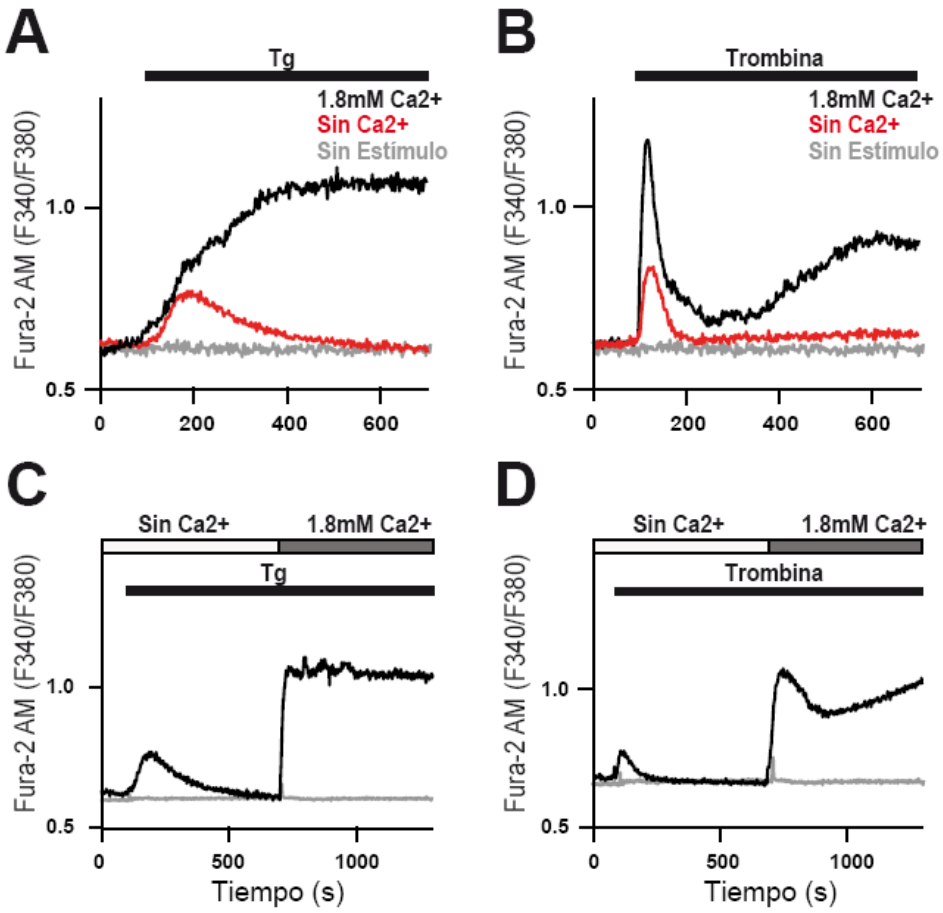

Figura 14. Activación de SOCE en los astrocitos. Trazos de la elevación citoplasmática de calcio inducida por Tg (A) o trombina (B), en presencia (negro) o en ausencia (rojo) de Ca^{2+} extracelular. Para aislar SOCE, las células se estimularon con Tg (C) o trombina (D) siguiendo el protocolo de la Figura 13, salida de calcio desde el RE (primer pico) y entrada de calcio por SOCE (segundo pico).

7.2. LOS ASTROCITOS EXPRESAN ENDÓGENAMENTE STIM1 Y ORAI1

El siguiente paso en nuestro estudio fue determinar la presencia de STIM1 y Orai1 en los astrocitos corticales de rata. Usando anticuerpos dirigidos contra estas dos proteínas nosotros realizamos ensayos de inmunocitoquímica en los cultivos primarios. Los resultados mostraron que ambas proteínas son expresadas endógenamente en los astrocitos. La Figura 15 muestra imágenes representativas de las inmunocitoquímicas realizadas, como se puede evidenciar en las células control no se observó unión inespecífica de los anticuerpos secundarios. El marcaje para STIM1 fue difuso y homogéneo como era de esperarse para esta proteína que se encuentra en el RE. Por su parte, la marca de Orai1 se distribuyó principalmente en la membrana plasmática, aunque varias células mostraron una gran parte de la marca intracelularmente en estructuras vesiculares. Esta distribución vesicular del Orai1 ha sido previamente observada en otros tipos celulares [143, 144].

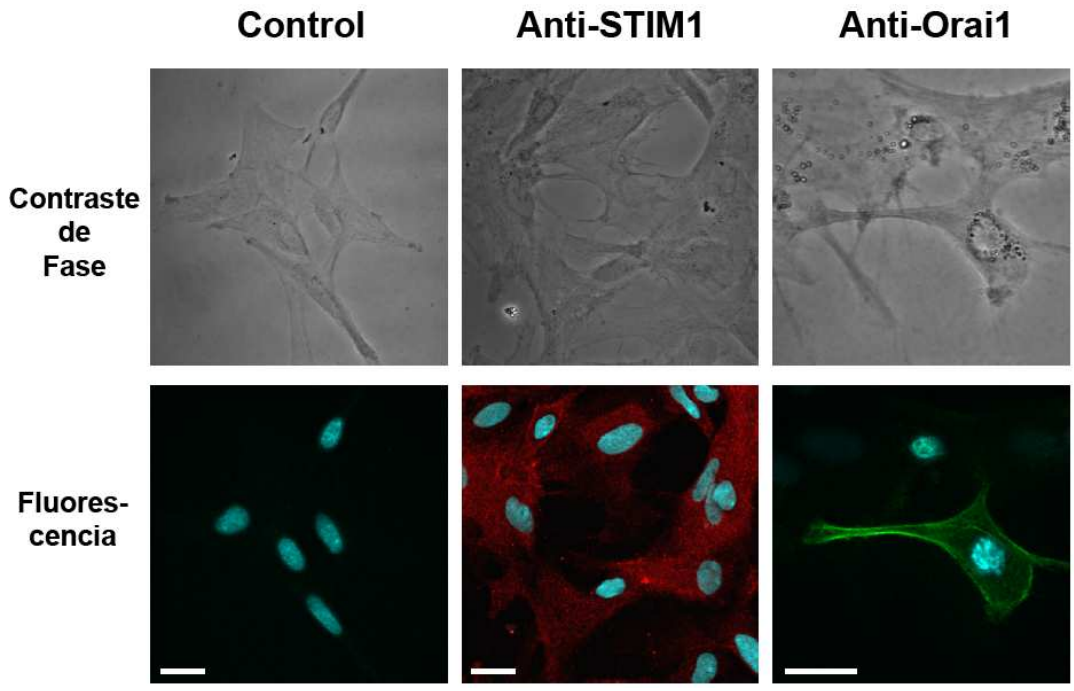

Figura 15. Inmunocitoquímica para la detección de las proteínas STIM1 y Orai1 en los cultivos primarios de astrocitos. Barra: 20 µm.

La especificidad de los anticuerpos fue verificada por la inmunodetección de las proteínas STIM1-YFP y Orai1-YFP transfectadas en los astrocitos. En estos experimentos se determinó el índice de colocalización entre la marca del anticuerpo y la fluorescencia de la proteína YFP. La señal de fluorescencia de los anticuerpos contra STIM1 y Orai1 colocalizó con la de la proteína YFP con un coeficiente de Pearson de 0.78 ± 0.08 y 0.84 ± 0.06, respectivamente (n = 10) (Figura 16).

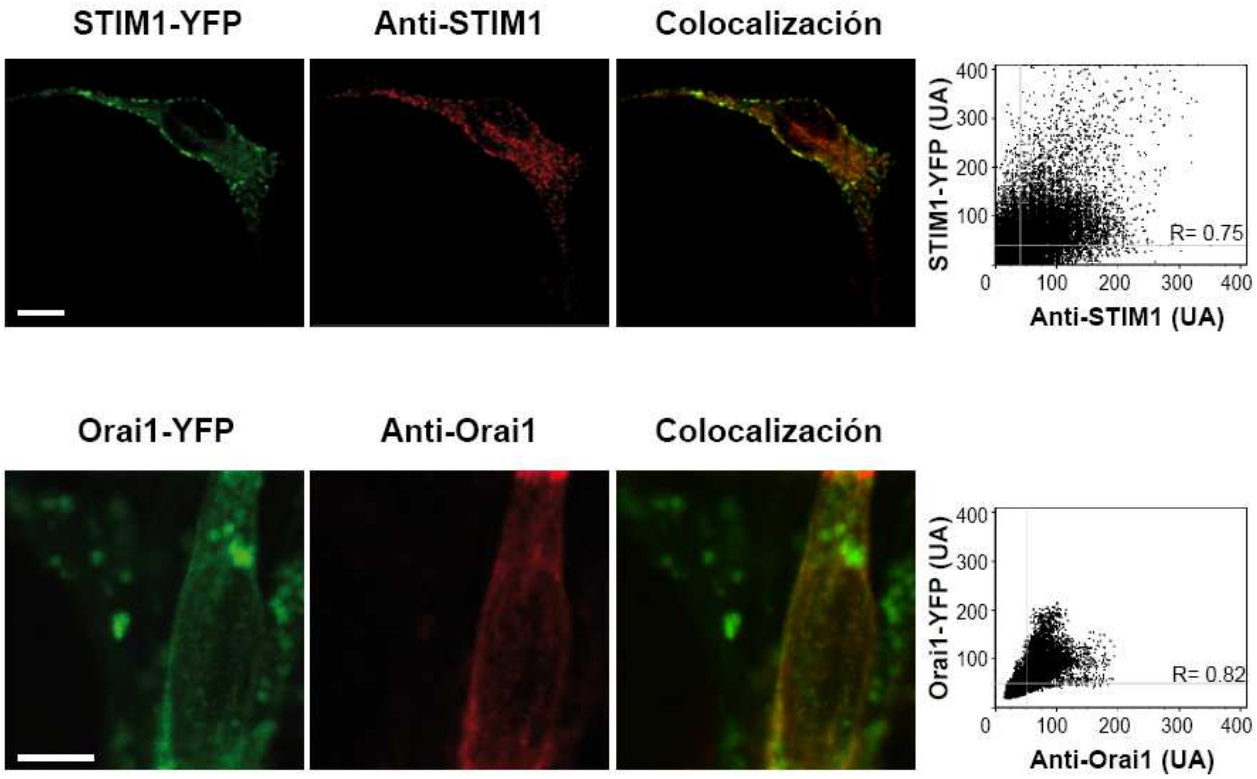

Figura 16. Colocalización de los anticuerpos contra STIM1 y Orai1 (rojo) y las proteínas fluorescentes STIM1-YFP y Orai1-YFP (verde). Barra: 5 µm. R: Coeficiente de Pearson. UA: Unidades arbitrarias de fluorescencia.

Con estos experimentos mostramos que los cultivos primarios de astrocitos corticales de rata expresan a las proteínas STIM1 y Orai1 y que ambas proteínas muestran una localización celular similar a lo que ha sido observado en otros tipos celulares.

7.3. LA ESTIMULACIÓN CON TROMBINA CAUSA LA REORGANIZACIÓN DE STIM1.

Uno de los pasos más característicos durante la activación de SOCE es la oligomerización de STIM1, cuando el RE se vacía esta proteína forma oligómeros que migran hacía las uniones RE-MP y activan al Orai1, esta reorganización fue descubierta gracias al uso de proteínas fluorescente acopladas a STIM1 [72, 145]. La Tg ha sido ampliamente usada para el estudio de la formación de estos cúmulos ya que es un bloqueador irreversible de la SERCA que induce una reorganización permanente de STIM1 [64, 90]. Nosotros empleamos la expresión de la proteína STIM1-YFP y la estimulación con Tg para determinar si esta formación de cúmulos podía ser evidenciada en los astrocitos. En la Figura 17 se puede observar la distribución homogénea y reticular de STIM1 en una célula en condiciones control (10 minutos en solución sin calcio), después de la estimulación con 200 nM de Tg se puede observar la clara formación de cúmulos de STIM1, esta distribución punteada permanece aún después de un lavado con una solución con 1.8 mM de calcio, como era de esperarse por causa de la irreversibilidad de la Tg.

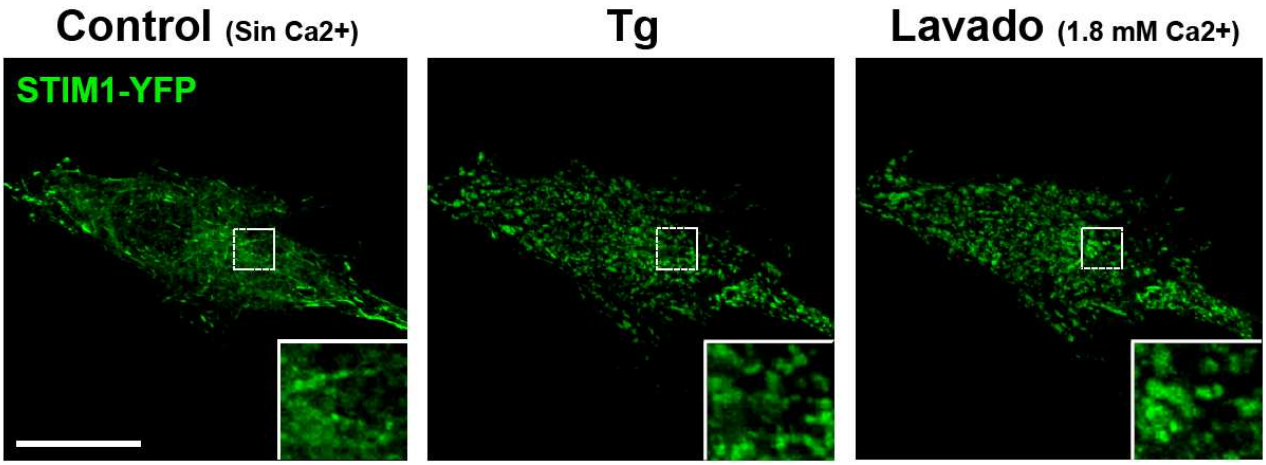

Figura 17. Reorganización de la proteína STIM1-YFP inducida por la aplicación de Tg en astrocitos. Proyecciones en Z de imágenes en microscopía confocal. Barra: 20 µm.

En condiciones fisiológicas, la liberación de calcio desde el RE activa a SOCE, pero SOCE a su vez rellena el RE lo que lleva a su propia inactivación, por ende se esperaría que la formación de

cúmulos de STIM1 fuera transitoria. Nosotros probamos esta hipótesis utilizando la expresión de STIM1-YFP y la estimulación con trombina (5 U/ml). Para estabilizar y hacer visibles estos posibles cúmulos, los astrocitos fueron estimulados en una solución libre de calcio. Como control los astrocitos fueron mantenidos por 10 minutos en la solución libre de calcio sin estímulo para asegurar que la ausencia de calcio no tenía efecto sobre la organización de STIM1. Como se puede observar en la Figura 18, en condiciones control, STIM1 se distribuyó homogéneamente, mientras que tras la estimulación con trombina hubo una evidente formación de cúmulos los cuales se distribuyeron hacia la periferia de la célula. Cuando los astrocitos fueron lavados con una solución que contenía 1.8 mM de $CaCl_2$, los cúmulos de STIM1 desaparecieron y la distribución homogénea fue restablecida. Estos resultados evidenciaron primero, que la trombina es capaz de activar la formación de cúmulos de STIM1, siendo este un paso esencial para SOCE y para la activación de Orai1, y segundo, que ésta reorganización de STIM1 es rápida y transitoria como es de esperarse bajo las condiciones de un estímulo fisiológico.

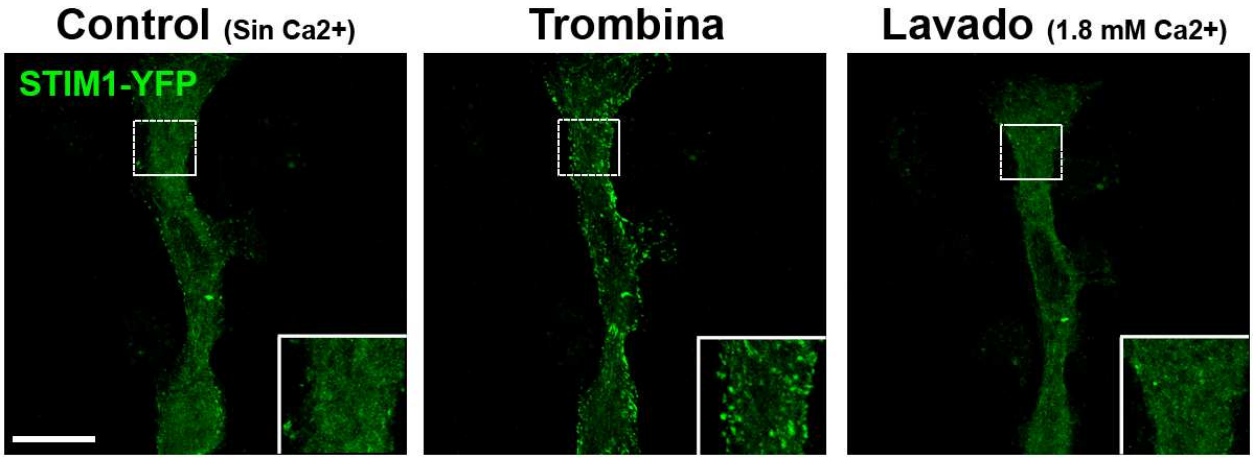

Figura 18. Reorganización de la proteína STIM1-YFP inducida por la aplicación de trombina en astrocitos. Proyecciones en Z de imágenes en microscopía confocal. Barra: 20 µm.

Dada la reorganización observada empleando la proteína STIM1-YFP, quisimos determinar si la proteína endógena también era relocalizada tras la acción de la trombina. Para esto realizamos ensayos de inmunocitoquímica contra STIM1 en células estimuladas por 10 minutos con

trombina (5 U/ml) previo a la fijación, como control las células fueron incubadas por los mismos 10 minutos en solución fisiológica sin ningún estímulo. La Figura 19 muestra que en condiciones de no estimulación el STIM1 endógeno se distribuye homogéneamente, mientras que en la células tratadas con trombina se ve la formación de cúmulos de STIM1, estos cúmulos no fueron tan grandes como los observados en los experimentos de sobreexpresión congruente con la cantidad de proteína endógena.

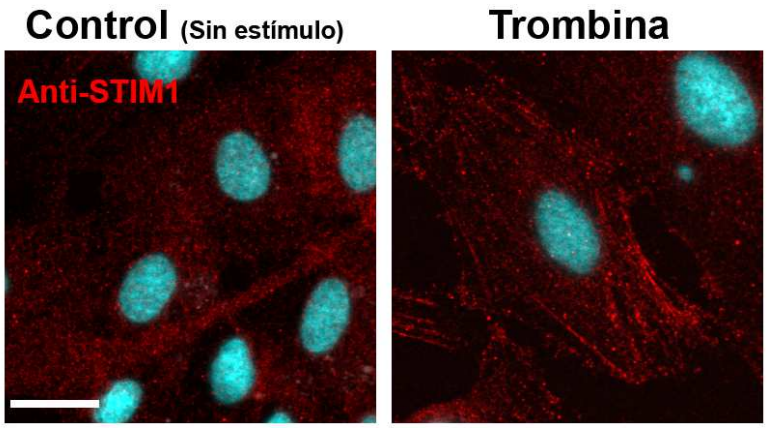

Figura 19. Reorganización de la proteína STIM1 endógena inducida por la aplicación de trombina en astrocitos. Imágenes de microscopía confocal de las células tratadas con el anticuerpo Anti-STIM1. Barra: 20 µm.

Estos resultados muestran que la trombina causa la reorganización del STIM1 endógeno, lo cual sugiere que este proceso hace parte de la activación fisiológica de SOCE y que no se debe a un artefacto de la sobreexpresión de la proteína acoplada a proteínas fluorescentes o del uso de fármacos para vaciar el RE. Ya que la formación de cúmulos de STIM1 es un paso sumamente importante para la activación de SOCE [72, 146, 147], estos experimentos en conjunto son una fuerte evidencia de que SOCE es activada ante la estimulación con trombina en los astrocitos.

Algunos estudios han mostrado que los cúmulos de STIM1 colocalizan con cúmulos en la membrana plasmática de Orai1 [104, 148]. Para determinar si la trombina causa relocalización de Orai1, sobreexpresamos la proteína Orai1-YFP y estimulamos a las células con trombina en

ausencia de calcio extracelular, en estos experimentos observamos una reorganización de Orai1 discreta la cual sólo fue visible en el plano focal de la membrana plasmática (Figura 20).

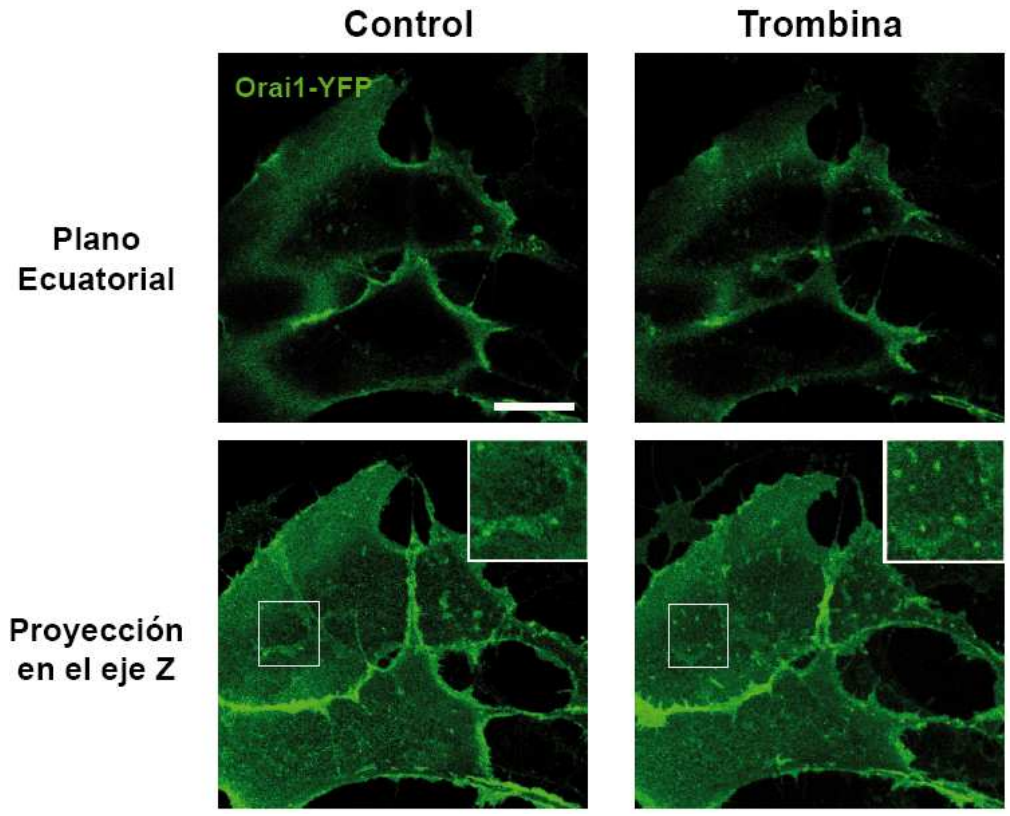

Figura 20. Reorganización de la proteína Orai1-YFP inducida por la aplicación de trombina en astrocitos. Imágenes de microscopía confocal mostrando la relocalización discreta de Orai1 en las células estimuladas. Barra: 20 µm.

Para evaluar la reorganización del Orai1 endógeno tratamos a los astrocitos con trombina durante 10 minutos y luego los procesamos para la inmunocitoquímica. En estos experimentos no observamos evidente relocalización del Orai1 (Figura 21), esto puede deberse a que la señal fluorescente del anticuerpo utilizado era muy débil en el plano focal de la membrana, lo que impidió observar la proteína nativa.

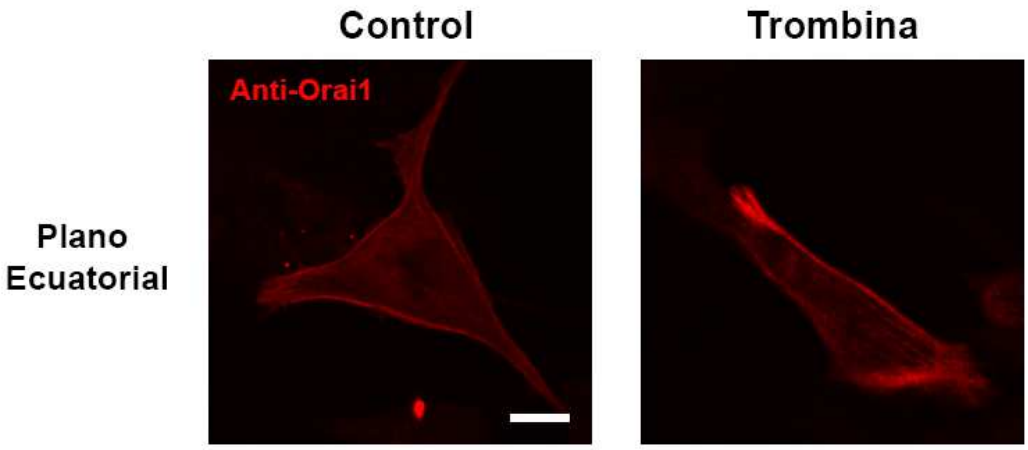

Figura 21. Distribución de la proteína Orai1 endógena en condiciones control y bajo la aplicación de trombina en astrocitos. Imágenes de microscopía confocal de las células tratadas con el anticuerpo Anti-Orai1. Barra: 20 µm.

Aunque la expresión de la proteína Orai1 acoplada a YFP permitió ver una discreta reorganización tras la estimulación con trombina, los resultados no son lo suficientemente concluyentes para afirmar que ésta proteína es relocalizada como parte de la respuesta de los astrocitos a este agonista. Se requerirá de otros estudios utilizando otros anticuerpos para determinar el efecto de la trombina sobre la distribución de Orai1 en los astrocitos.

7.4. EFECTO DE LA SOBREEXPRESIÓN DE STIM1 Y ORAI1 SOBRE LA ENTRADA DE CALCIO INDUCIDA POR TROMBINA.

En varios tipos celulares se ha observado que la sobreexpresión simultánea de STIM1 y Ora1 es suficiente para inducir un aumento de SOCE, comprobando que estas dos proteínas son esenciales para que esta entrada de calcio ocurra [149, 150]. Nosotros estudiamos el efecto de la sobreexpresión de STIM1 y Orai1 sobre SOCE en los astrocitos cuando son tratados con Tg y sobre la respuesta de los astrocitos a la trombina. La sobreexpresión de STIM1 y Orai1 se realizó de manera independiente o simultánea, los cambios en la concentración de calcio citoplasmático se estimaron calculando el área bajo la curva de cada pico de la señal de Fura-2 AM (protocolo

Figura 13). Los resultados mostraron que ninguno de los tratamientos tuvo efecto sobre la liberación de calcio desde el RE (primer pico), pero en lo que respecta a SOCE (segundo pico), sí se observaron diferencias significativas (Figura 22A y B). En primer lugar, la sobreexpresión simultánea de STIM1 y Orai1 incrementó SOCE en los astrocitos en un 75% (259 ± 25, n = 5), comparado con las células control (149 ± 14, n = 5), mientras que la sobreexpresión de STIM1 causó un aumento del 33% en SOCE (196 ± 16, n = 5). Por otro lado, la sobreexpresión de Orai1 causó una disminución del 23% en SOCE (114 ± 13, n = 5), lo cual puede ser atribuido a una alteración en el balance entre el número de canales Orai1 y el número de proteínas STIM1 disponibles para que se dé la activación. Teniendo en cuenta que cada canal Orai1 requiere de 8 moléculas de STIM1 para activarse, un mayor número de canales disponibles llevará a la condición en la que varios canales tendrán menos de 8 moléculas de STIM1 unidas, causando un estado de subconductancia del canal [151, 152].

Si STIM1 y Orai1 participan en la entrada de calcio inducida por la trombina en los astrocitos, sería de esperarse que la sobreexpresión de estas dos proteínas llevara a resultados similares a los obtenidos con la Tg. Para probar esto realizamos experimentos sobreexpresando las proteínas STIM1 y Orai1 en los astrocitos de manera independiente o simultánea y cuantificando los cambios en la concentración citoplasmática de calcio con el protocolo ya mencionado. En cuanto a la entrada de calcio, en la Figura 22C y D se puede observar como la sobreexpresión simultánea de STIM1 y Orai1 incrementó la entrada de calcio activada por la trombina en un 98% (149 ± 6, n = 5) comparado con las células control (75 ± 6, n = 5). La sobreexpresión de STIM1 causó un aumento del 42% (107 ± 6, n = 5), mientras que la sobreexpresión de Orai1 redujo la entrada de calcio en un 32% (52 ± 6, n = 5). Ninguno de los tratamientos tuvo efecto sobre la liberación de calcio desde el RE. El hecho de que la sobreexpresión simultánea de STIM1 y Orai1 haya causado una duplicación de la entrada de calcio inducida por trombina, sumado a las tendencias similares observadas ante la estimulación con Tg, sugiere que estas dos proteínas son esenciales para la respuesta de los astrocitos ante este agonista.

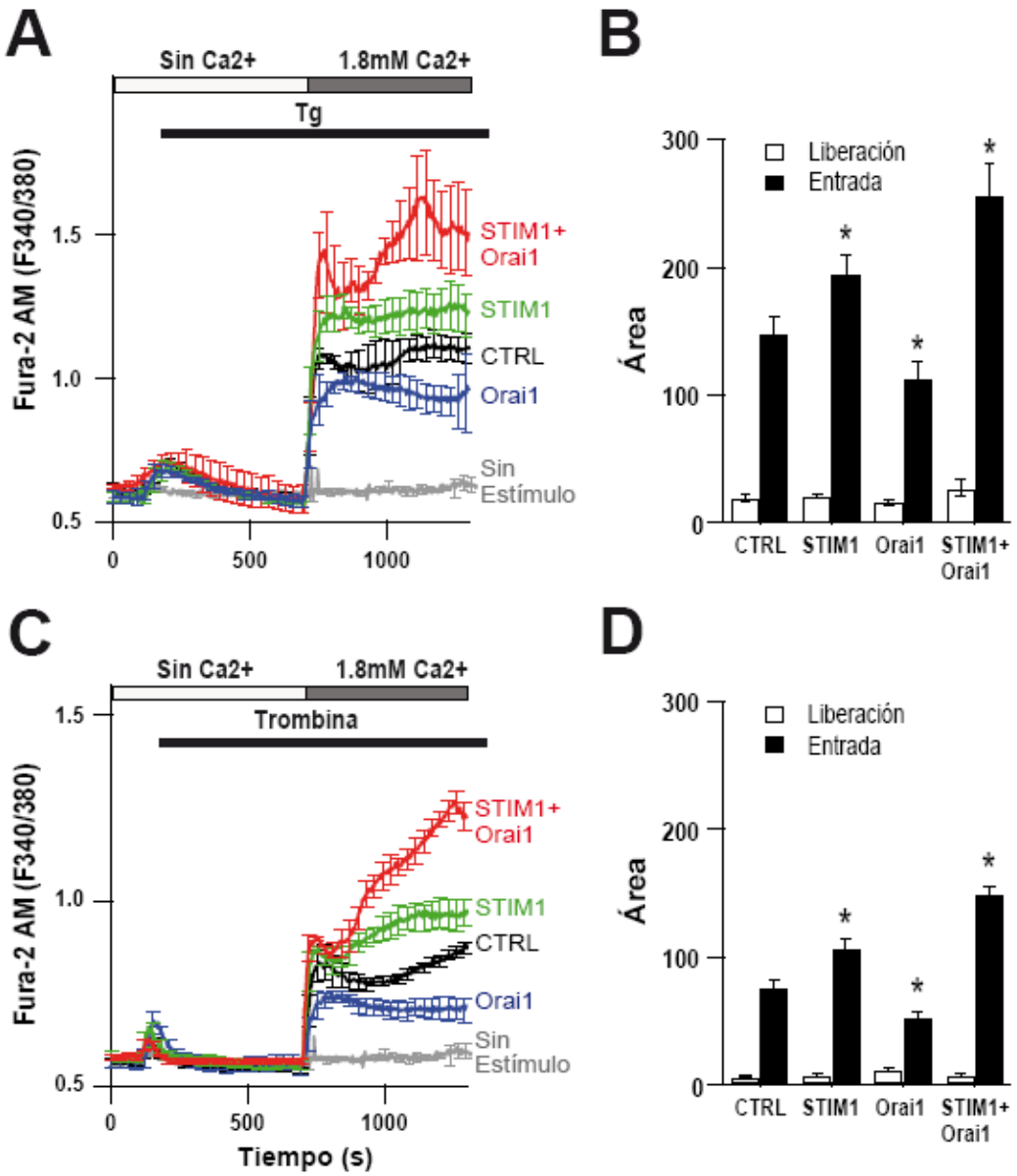

Figura 22. Efecto de la sobreexpresión de STIM1 y Orai1 sobre SOCE inducida por Tg o por la estimulación con trombina. Los astrocitos fueron transfectados con 4 µg de: vector vacío (negro y gris), STIM1 (verde), Orai1 (azul) o 4 µg de STIM1+ 4 µg de Orai1 (rojo). **A.** Células tratadas con 200 nM de Tg. **C**. Células estimuladas con 5 U/ml de trombina. **B y D**. Cuantificación del área bajo la curva de la liberación de calcio desde el RE (barras blancas) y SOCE (barras negras). Los trazos son el promedio ± S.E.M. de 5 experimentos independientes. Cada experimento es el promedio de al menos 50 células. * ($p<0.05$).

7.5. EFECTO DE LA INHIBICIÓN DE LA EXPRESIÓN DE STIM1 Y ORAI1 SOBRE LA ENTRADA DE CALCIO INDUCIDA POR TROMBINA.

Para corroborar que STIM1 y Orai1 son dos proteínas esenciales en la respuesta de los astrocitos a la trombina, realizamos experimentos de RNA de interferencia contra estas dos proteínas. El primer paso en estos experimentos fue determinar la eficiencia de transfección de los siRNAs y la eficiencia de inhibición obtenida en los cultivos primarios de astrocitos. La eficiencia de transfección de los siRNAs se estimó usando el oligonucleótido fluorescente BLOCK-iT, mediante esta técnica se determinó una eficiencia de 85 ± 1% (Figura 23A y B).

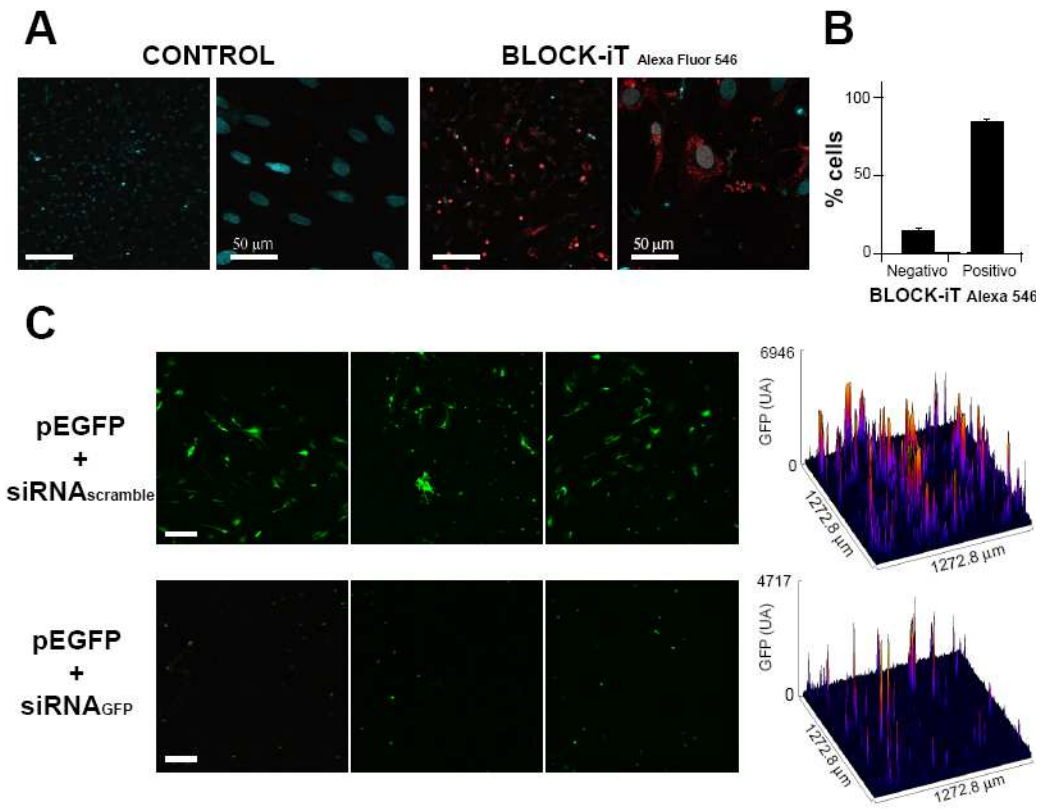

Figura 23. A. Imágenes de microscopía confocal de astrocitos transfectados con 100 pmoles del oligonucleótido fluorescente BLOCK-iT Alexa Fluor 546 (rojo). Núcleos teñidos con DAPI (azul). **B**. Eficiencia de transfección, promedio ± S.E.M., n = 282 células. **C**. RNAi contra la GFP, los astrocitos fueron transfectados con 1µg de pEGFP y 30 pmoles de GFP siRNA. Las gráficas a la derecha representan la fluorescencia promedio de tres experimentos independientes. UA: Unidades arbitrarias de fluorescencia.

La eficiencia de inhibición fue determinada mediante experimentos de RNAi contra la proteína GFP, cotransfectando los astrocitos simultáneamente con el plásmido pEGFP y un siRNA dirigido contra esta proteína. El número de las células que expresaban la proteína GFP, así como la intensidad de la fluorescencia, fueron significativamente disminuidos en las células tratadas con el siRNA, comprobando que el protocolo usado es eficiente para causar la inhibición de proteínas en los cultivos primarios de astrocitos (Figura 23C).

Una vez estandarizado el protocolo para el RNAi, procedimos a realizar la inhibición de STIM1 y Orai1. Para estos experimentos las células fueron tratadas con siRNAs dirigidos contra STIM1 y Orai1 de manera independiente o simultánea y se realizaron las mediciones de calcio ante la estimulación con Tg o con trombina siguiendo el protocolo de la Figura 13. En cuanto a los resultados obtenidos con Tg, observamos que la inhibición simultánea de STIM1 y Orai1 causó una reducción del 58% en SOCE (59 ± 7, n = 4), comparado con los astrocitos transfectados con un siRNA scramble (140 ± 13, n = 4) (Figura 24A y B). Cuando se inhibió la expresión del Orai1 de manera independiente, se obtuvieron resultados muy similares a los obtenidos en la inhibición simultánea, una reducción de SOCE del 62% para Orai1 (53 ± 12, n = 4), lo cual sugiere que el Orai1 podría ser el único canal activado por STIM1 en estas células.

En cuanto a la entrada de calcio inducida por la trombina los resultados fueron similares a los obtenidos con la Tg. Las Figuras 24C y D muestran que la inhibición simultánea de STIM1 y Orai1 causó una disminución del 42% en esta entrada de calcio (39 ± 7, n = 4), comparado con las células transfectadas con el siRNA scramble (68 ± 8, n = 4), mientras que la inhibición de la expresión de Orai1 causó una disminución del 48% (34± 4, n = 4). Estos resultados en conjunto soportan la idea de que STIM1 y Orai1 son dos proteínas necesarias para la respuesta de los astrocitos a la trombina.

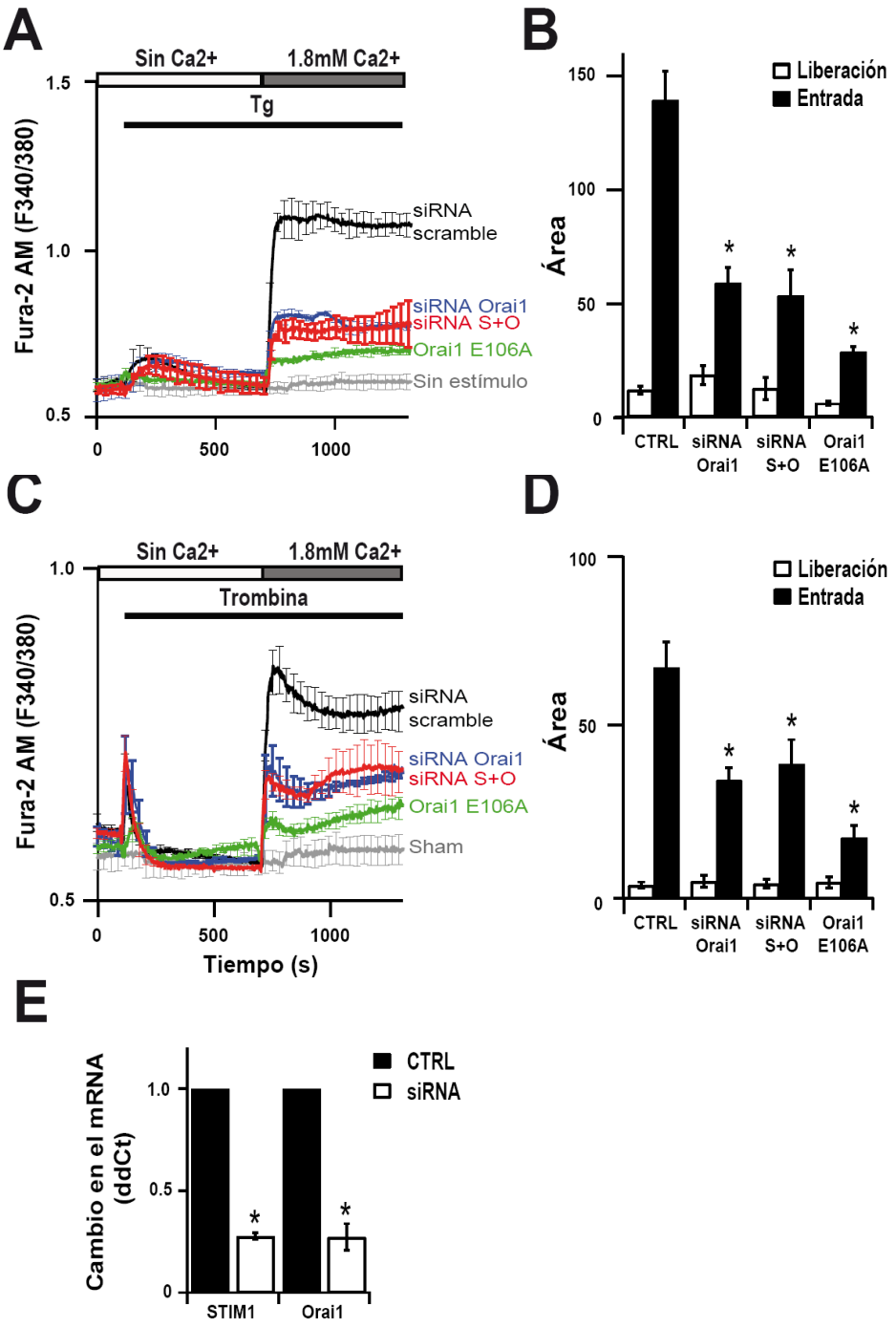

Figura 24. Efecto del RNAi de STIM1 y Orai1 sobre SOCE inducida por **A.** Tg o **C.** por trombina. Los astrocitos fueron transfectados con 100 nM de: siRNA scramble (negro y gris), Orai1 siRNA (azul), STIM1 siRNA + Orai1 siRNA (rojo) o transfectados con el plásmido Orai1E106A (verde, 5 µg). Los trazos son el promedio ± S.E.M. de 4 experimentos independientes. Cada experimento es el promedio de al menos 50 células. **B y D**. Área bajo la curva para los dos picos de aumento de calcio, liberación desde el RE (barras blancas) y SOCE (barras negras). **E**. Cambio en los niveles de RNAm para STIM1 y Orai1 determinados por PCR en tiempo real, usando el método $2^{-\Delta\Delta Ct}$. * ($p<0.05$).

Los niveles de RNAm para STIM1 y Orai1 se cuantificaron mediante experimentos de PCR en tiempo real. Para ambos casos se observó una inhibición en la cantidad de RNAm cercana al 70% en los astrocitos transfectados con los respectivos siRNAs, comparado con aquellos transfectados con el siRNA scramble. El cambio en los niveles de RNAm se cuantificó por el método de $2^{-\Delta\Delta Ct}$ usando el *Gapdh* como gen de referencia interno, se obtuvieron valores de índice de cambio (fold change) de 0.28 ± 0.02 para STIM1 (n = 4) y 0.27 ± 0.06 para Orai1 (n = 4), lo que se interpreta como una diminución de 72% y 73% en el RNAm, respectivamente (Figura 24E).

Como otra aproximación para corroborar la participación del canal Orai1 en la respuesta de los astrocitos a la trombina, empleamos la expresión de la mutante dominante negativa Orai1E106A. Mutaciones en este aminoácido, que funciona como el filtro de selectividad del canal, impiden el flujo de iones calcio y su sobreexpresión causa una inhibición de SOCE [153, 154]. En el caso de que el Orai1 participara en la respuesta de los astrocitos a la trombina, entonces al expresar el Orai1E106A se esperaría que la entrada de calcio se viera disminuida. Primero realizamos los experimentos con Tg para verificar la inhibición de SOCE en los astrocitos al sobreexpresar la mutante. Los resultados mostraron que SOCE fue disminuida en un 79% en los astrocitos transfectados con el Orai1E106A (29 ± 2, n = 3), comparado con las células control (140 ± 13, n = 4) (Figura 24A y B). Estos resultados confirmaron que en los astrocitos el canal Orai1 es el principal responsable de SOCE. En cuanto a la respuesta de la trombina, la expresión del Orai1E106A causó una inhibición de 73% en la entrada de calcio (18 ± 4, n = 4, Figura 24C y D), comparado con el control (68 ± 8, n = 4). Tanto para la Tg como para la trombina se observó que el porcentaje de inhibición fue mayor al obtenido en los experimentos de RNAi (cerca de 15% mayor), esto puede ser explicado por una mejor transfección del plásmido en comparación a los siRNAs o a que la cantidad de proteína remanente tras el RNAi es suficiente para activar algunos canales y permitir que se dé una entrada de calcio mayor. Los experimentos con la mutante negativa muestran entonces que la participación de Orai1 en la respuesta de los astrocitos a la trombina es aún más importante de la que se estimó con el RNAi. Estos resultados sugieren que el flujo de calcio a través de canal Orai1 es la principal vía de entrada de calcio inducida por la trombina en los astrocitos.

8. DISCUSIÓN

La unión de la trombina a sus receptores PAR en la membrana de los astrocitos lleva a una elevación del calcio intracelular induciendo una gran variedad de respuestas que van desde la proliferación hasta la muerte celular [110, 118, 155]. La entrada de calcio operada por las reservas (SOCE) es un componente importante de esta elevación de calcio inducida por la trombina [119]. En muchos tipos celulares ha sido visto que existen dos proteínas claves para el funcionamiento de SOCE: STIM1 y Orai1, STIM1 censa el vaciamiento del RE y activa al canal Orai1 para permitir la entrada de calcio [9, 90, 129]. Hasta la fecha no existían estudios que evaluaran la participación de estas dos proteínas en los astrocitos, ni su papel en la respuesta que estas células presentan ante la trombina. El principal objetivo de este trabajo fue precisamente investigar el papel de STIM1 y Orai1 en la entrada de calcio inducida por la trombina en los astrocitos.

El primer paso en este estudio fue determinar la presencia de STIM1 y Orai1 en los astrocitos corticales de rata. Nuestros resultados muestran que los astrocitos expresan ambas proteínas, lo que se ajusta a lo ya encontrado previamente en astrocitos de ratón, en los cuales se detectó el RNAm de estas proteínas [156, 157]. En cuanto a su distribución, ambas proteínas mostraron una localización que coincide con lo observado en otros tipos celulares [9, 63, 65]. Al estudiar la reorganización de STIM1, nosotros encontramos que en condiciones control la proteína se distribuye de manera homogénea en el RE, mientras que tras la estimulación con trombina, es rápidamente redistribuida formando cúmulos en la periferia celular. Esta reorganización sólo pudo ser observada cuando las células fueron estimuladas en ausencia de calcio extracelular y se revirtió completamente tras la adición de calcio. Esto muestra que la reorganización de STIM1 inducida por la trombina es rápida y transitoria, como es de esperarse para un estímulo fisiológico; la salida de calcio desde el RE causa la oligomerización de STIM1 y la activación de SOCE para rellenar el RE, una vez lleno el RE se da la inactivación de SOCE y el desensamblaje de STIM1 [55, 97], contrario a lo que se observa con fármacos irreversibles como la Tg. La formación de cúmulos de STIM1 tras la estimulación con trombina fue también observada para la

proteína nativa lo cual muestra que este proceso ocurre también endógenamente y que no es un efecto de la sobreexpresión de la proteína. Ya que la reorganización de STIM1 es un paso crucial en la activación de SOCE [72, 90], estos resultados sugieren que STIM1 es activada en respuesta a la trombina y participa en la entrada de calcio inducida por este agonista en los astrocitos.

La sobreexpresión de STIM1 en los astrocitos fue suficiente para incrementar la entrada de calcio inducida por trombina, lo cual sugiere que la presencia de un mayor número de proteínas STIM1 permite la activación de más SOCs endógenos disponibles, funcionado STIM1 como el limitante de la activación. Cuando STIM1 se sobreexpresó simultáneamente con Orai1, la entrada de calcio inducida por trombina se incrementó aún más, alcanzando casi el doble de su magnitud comparado con los astrocitos no transfectados. Esto soporta la idea que la entrada de calcio inducida por la trombina en los astrocitos es debida a la activación de los canales Orai1 activados por STIM1, lo que concuerda con lo observado en plaquetas, en donde STIM1 y Orai juegan un papel fundamental en la respuesta a la trombina [138].

Los experimentos de RNAi contra el Orai1 mostraron que la inhibición de su expresión causa una disminución del 48% en la entrada de calcio inducida por la trombina, este porcentaje fue muy similar al obtenido cuando se inhibió simultáneamente la expresión de STIM1 y Orai1, lo cual sugiere que Orai1 podría ser el único SOC activado por STIM1 que participa en la entrada de calcio durante la respuesta a la trombina en los astrocitos. La expresión de la mutante dominante negativa Orai1E106A, causó una reducción aún mayor de la entrada de calcio inducida por la trombina, alcanzando el 70% de inhibición, lo cual sugiere que la entrada de calcio a través del Orai1 es la principal vía de flujo de calcio que se da durante la respuesta a la trombina en los astrocitos.

Sin embargo, nuestro estudio no excluye la posibilidad de que otros SOCs puedan estar siendo activados en esta respuesta. Ya que la inhibición mediante los siRNA no fue completa (70% de reducción de RNAm), es posible que el nivel de proteínas expresadas tras la inhibición sea suficientes para sostener cierto porcentaje de la entrada de calcio. Por otro lado, los otros

miembros de la familia Orai, el Orai2 y el Orai3, podrían estar participando en esta entrada de calcio. Ya que ha sido visto que los astrocitos de rata expresan todos los miembros de la familia de los TRPCs [134], otra posibilidad es que en los astrocitos se esté dando la activación de alguno de estos canales durante la repuesta a la trombina. Ha sido reportado que en astrocitos el TRPC1 es responsable del 40% de la entrada de calcio inducida por el fármaco CPA y de casi el 100% de la entrada que se da tras la estimulación purinérgica [158].

Otra posibilidad interesante es que la trombina esté activando simultáneamente dos entradas de calcio diferentes: SOCE y ROCE (*receptor-operated calcium entry*), como ha sido visto en plaquetas [140]. En este caso el TRPC3 podría estar participando en esta vía de calcio independiente de STIM1 [159]. Respecto a ROCE en los astrocitos se ha visto que, por lo menos en la estimulación purinérgica, esta entrada de calcio no se activa a pesar de la presencia del TRPC3 [160]. Sin embargo, no hay estudios acerca de la participación de ROCE en la respuesta a la trombina, por lo que se requiere más investigación en este campo para dilucidar el papel de los canales TRPC en esta entrada de calcio en los astrocitos.

Como respuesta a la trombina los astrocitos experimentan cambios en su morfología, en su proliferación, en la transcripción génica y en la exocitosis de gliotransmisores [23]. Muchos de los cambios inducidos por la trombina en los astrocitos dependen del aumento del calcio citoplasmático [118, 121, 161], por lo que la identificación de las proteínas involucradas en esta señalización es fundamental para el entendimiento de los efectos que la trombina tiene en el cerebro. Nuestros resultados muestran que STIM1 y Orai1 son responsables de una gran parte del aumento de calcio inducido por esta proteasa, lo que sugiere que estas dos proteínas podrían ser claves para que estos cambios ocurran. La entrada de calcio a través del Orai1 en respuesta a la trombina podría estar implicada en la remodelación del citoesqueleto causada por este agonista [122], provocando la pérdida de los contactos del astrocito con las neuronas y la vasculatura, lo cual conllevaría a alteraciones fisiológicas en la homeostasis iónica, la regulación sináptica y la modulación de la presión sanguínea. Por otro lado, esta entrada de calcio podría ser blanco de moléculas pro-inflamatorias como las prostaglandinas y el TNF-α, que causan aumentos en los

niveles basales de calcio e inducen el proceso de inflamación del cerebro [162], sin embargo estas son preguntas que aún faltan por explorar.

Una de las principales causas del daño cerebral durante un accidente cerebrovascular es la excito-toxicidad inducida por el glutamato [115, 163, 164]. La entrada de calcio en los astrocitos, activada por la trombina o por otros agonistas, es la principal fuente de calcio para que se dé la liberación vesicular de glutamato [18, 126, 165, 166]. Nosotros mostramos que en lo que respecta a la trombina, el Orai1 es el canal responsable de esta entrada de calcio, lo que sugiere que esta proteína juega un papel importante en la gliotransmisión y en el daño inducido por el glutamato en el cerebro. Al inducir la liberación de glutamato, esta entrada de calcio también podría estar participando en el estrés oxidativo que se genera en el cerebro ante la acción de este agonista [164].

Otro aspecto importante en la señalización de calcio de los astrocitos son las ondas intercelulares características del estado patológico del cerebro [167]. Estas ondas contribuyen a la muerte del tejido nervioso y han sido observadas en la enfermedad de Alzheimer [168], en la depresión [169] y en accidentes cerebrovasculares [170]. Ya que se ha visto que SOCE es fundamental para la generación de ondas y de oscilaciones de calcio en otras células [171-173], es posible que STIM1 y Orai1 tengan un papel importante en la propagación de estas ondas en los astrocitos. Posibles alteraciones en la regulación de estas proteínas o en sus niveles de expresión podrían ser las responsables de los cambios en la señalización de calcio de los astrocitos durante los estados patológicos del cerebro.

Por último, se sabe que la trombina causa la activación de los astrocitos, en un proceso conocido como astrogliosis, que implica una serie de cambios morfológicos, moleculares y funcionales que dan lugar a la formación de la cicatriz glial [114]. La astrogliosis es una característica de casi todas las patologías cerebrales, incluyendo los traumas, los accidentes cerebrovasculares, la isquemia, el cáncer y las enfermedades neuroinflamatorias y neurodegenerativas [18, 109]. En este trabajo nosotros mostramos que STIM1 y Orai1 subyacen a la mayor parte de la entrada de

calcio activada por la trombina en los astrocitos, por lo que estas proteínas podrían ser blancos potenciales para la terapia de diversas enfermedades neurológicas.

Con la identificación de STIM1 y Orai1 como las proteínas más importantes en la entrada de calcio activada por la trombina en los astrocitos, abrimos las puertas para la investigación del papel que estas proteínas puedan tener en el cerebro, no solo ante la acción de este agonista, sino ante la amplia gama de estímulos a los cuales los astrocitos están expuestos durante diferentes estados fisiológicos y patológicos.

9. CONCLUSIONES

- Los astrocitos responden a la trombina con una elevación en la concentración de calcio citoplasmática cuyo principal componente es la entrada de calcio operada por las reservas intracelulares (SOCE).

- Los astrocitos expresan endógenamente tanto al sensor de calcio del RE STIM1, como al canal operado por las reservas Orai1.

- La estimulación con trombina en los astrocitos causa que el STIM1 se reorganice formando cúmulos cercanos a la membrana plasmática, los cuales son fundamentales para la activación de SOCE.

- La sobreexpresión de STIM1 y Orai1 en los astrocitos duplica la entrada de calcio activada por la trombina.

- La inhibición de la expresión de STIM1 y Orai1 causa una disminución significativa de la entrada de calcio activada por la trombina en los astrocitos.

- El canal Orai1, el cual es activado por STIM1, es responsable de más del 70% de la entrada de calcio inducida por la trombina en los astrocitos.

- STIM1 y Orai1 son dos proteínas esenciales para que los astrocitos respondan a la estimulación con trombina.

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STIM1 and Orai1 mediate thrombin-induced Ca^{2+} influx in rat cortical astrocytes

Claudia Moreno[a], Alicia Sampieri[a], Oscar Vivas[b], Claudia Peña-Segura[c], Luis Vaca[a,*]

[a] Departamento de Biología Celular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 México, DF, Mexico
[b] Departamento de Fisiología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 México, DF, Mexico
[c] Departamento de Neurodesarrollo y Fisiología, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria, C.P. 04510, México DF, Mexico

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ABSTRACT

In astrocytes, thrombin leads to cytoplasmic Ca^{2+} elevations modulating a variety of cytoprotective and cytotoxic responses. Astrocytes respond to thrombin stimulation with a biphasic Ca^{2+} increase generated by an interplay between ER-Ca^{2+} release and store-operated Ca^{2+} entry (SOCE). In many cell types, STIM1 and Orai1 have been demonstrated to be central components of SOCE. STIM1 senses the ER-Ca^{2+} depletion and binds Orai1 to activate Ca^{2+} influx. Here we used immunocytochemistry, overexpression and siRNA assays to investigate the role of STIM1 and Orai1 in the thrombin-induced Ca^{2+} response in primary cultures of rat cortical astrocytes. We found that STIM1 and Orai1 are endogenously expressed in cortical astrocytes and distribute accordingly with other mammalian cells. Importantly, native and overexpressed STIM1 reorganized in puncta under thrombin stimulation and this reorganization was reversible. In addition, the overexpression of STIM1 and Orai1 increased by twofold the Ca^{2+} influx evoked by thrombin, while knockdown of endogenous STIM1 and Orai1 significantly decreased this Ca^{2+} influx. These results indicate that STIM1 and Orai1 underlie an important fraction of the Ca^{2+} response that astrocytes exhibit in the presence of thrombin. Thrombin stimulation in astrocytes leads to ER-Ca^{2+} release which causes STIM1 reorganization allowing the activation of Orai1 and the subsequent Ca^{2+} influx.

1. Introduction

Astrocytes are active elements in the physiology and pathology of the brain. They are involved in several functions such as ion homeostasis, uptake of neurotransmitters, metabolic support of neurons, synaptic modulation and memory formation [1–4]. Astrocytes also enwrap blood vessels and contact the blood–brain barrier, protecting the brain from being exposed to endogenous molecules and/or neurotoxins that might be present in the blood. Astrocytes exhibit a form of excitability that is based on cytoplasmic Ca^{2+} elevations [5]. The Ca^{2+} inside the endoplasmic reticulum (ER) is the main source for cytoplasmic Ca^{2+} signaling in these cells and its mobilization depends mainly on activation of IP_3 receptors downstream the activation of a vast repertoire of G-protein coupled receptors [6].

Under cerebrovascular accidents, blood-derived factors extravasate and perfuse into the brain. Thrombin, a serine-protease known for its role in thrombosis and inflammation, is one of these factors which has important effects in astrocytes and neurons [7]. Thrombin can be generated in the brain during ischemia or can enter this organ after an intracerebral hemorrhage or a blood–brain barrier breakdown [8,9]. Astrocytes are the primary thrombin-responsive cells in the brain [10,11] and they express the three members of the Protease Activated Receptor (PAR) family that mediate the intracellular signaling cascade triggered by thrombin [12]. PARs are coupled to $G_{\alpha q/11}$ leading to PLCβ activation and IP_3-mediated Ca^{2+} release [13]. Thrombin, via PARs, can induce a variety of responses in astrocytes which go from cytoprotective to cytotoxic [14]. These responses include proliferation [15,16], reversal of astrocyte stellation [17], changes in cell motility [11], secretion [18] and cell death [19]. Astrocytes respond to sustained thrombin stimulation with a biphasic Ca^{2+} increase, characterized by an initial peak followed by a sustained plateau phase [20]. This Ca^{2+} response is generated by an interplay between ER-Ca^{2+} release and store-operated Ca^{2+} entry (SOCE).

SOCE is an orchestrated process which activates in response to Ca^{2+} release from the ER, it involves signaling between the ER and the plasma membrane to convey the depleted state of the stores and to activate Ca^{2+} influx [21,22]. This route of Ca^{2+} entry is critical to Ca^{2+} homeostasis and controls many functions in a variety of mammalian cells [23–25], where astrocytes seem not to be the exception [26,27]. In astrocytes and astrocytomas, SOCE activates in response to many metabotropic receptor agonists including glutamate [28], histamine [29] and thrombin [20], suggesting that these cells express the necessary machinery for this form of Ca^{2+} entry. Two central components are necessary for SOCE to occur, the first one is STIM1, a Ca^{2+} sensor that communicates the depleted state of

* Corresponding author. Tel.: +52 55 56225654; fax: +52 55 56225611.
E-mail address: lvaca@ifc.unam.mx (L. Vaca).

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the ER to the plasma membrane and activates the store-operated channels (SOCs) [30,31]; the second one is SOC itself, the plasma membrane channels that allow Ca^{2+} entry from the extracellular space. There are two different families of channels underlying SOCE, the Orai [32,33] and the TRPC [34–37]. Orai1 functions as an unconditional SOC while the members of TRPC family seem to act as conditional SOCs, being activated by ER-depletion only under specific conditions [38]. However, in astrocytes the studies about the molecular machinery underlying SOCE have focused mainly in the role of TRPCs. There is evidence supporting the participation of TRPC1 in SOCE in mouse cortical astrocytes treated with CPA [39], and in rat cortical astrocytes stimulated with glutamate [40]. Moreover, TRPC3 underlies SOCE in rat spinal astrocytes when activated by substance P [41]. TRPC3 is so far the only molecule that has been demonstrated to participate in the store operated Ca^{2+} entry activated by thrombin in astrocytes [42,43].

STIM1 and Orai1 are central players in SOCE in many cell types. Under ER-Ca^{2+} release, STIM1 oligomerizes and migrates to the plasma membrane activating Orai1 and TRPC1 to allow Ca^{2+} entry [25,31,44–46]. Recently, it has been shown that Orai1 is an essential component of CPA-induced SOCE in mouse cortical astrocytes [47]. Furthermore, there is evidence that in platelets and endothelial cells STIM1 and Orai1 are required for SOCE activation under thrombin stimulation [48–52]. Nevertheless, the role of STIM1 and Orai1 in the thrombin-induced Ca^{2+} response in astrocytes remains unexplored. In the present work, we have examined the involvement of endogenous STIM1 and Orai1 in the thrombin-induced Ca^{2+} response in primary cultures of rat cortical astrocytes. We show here that cortical astrocytes express STIM1 and Orai1 and that these proteins are responsible of a significant part of the Ca^{2+} influx elicited by thrombin. Moreover, we show that thrombin stimulation in astrocytes causes a transient reorganization of endogenous STIM1. The overexpression of STIM1 and Orai1 causes a twofold increase in the Ca^{2+} influx evoked by thrombin, while the knockdown of STIM1 and Orai1 (via RNAi) leads to a significant reduction in SOCE. Similar changes were observed when SOCE was activated by thapsigargin, supporting the idea that maneuvers leading to ER depletion are the triggering mechanism responsible for SOCE in astrocytes. These findings show that, thrombin stimulation activates STIM1 and initiates Ca^{2+} entry through Orai1, playing these proteins an important role in the response that cortical astrocytes exhibit in the presence of thrombin.

2. Materials and methods

2.1. Cell culture and transfection

All procedures for maintaining the rats and for isolation of astrocytes were approved by the Animal Care Committee of the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. Animal care was performed according to the "International Guiding Principles for Biomedical Research Involving Animals" (Council for International Organizations of Medical Sciences, 2010). Cortical astrocytes cultures were obtained from Wistar 1-day-old rat pups. Briefly, the brain cortex from six to eight pups were separated from the meninges, placed in Krebs solution and cut in small slices. The tissue was dissociated with a Krebs solution containing trypsin (4800 U/ml, Sigma, St. Louis, MO) for 10 min. Then the cells were mechanically dissociated by passage through 80 μm nylon mesh and resuspended in culture medium. The culture medium consisted of basal Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Gibco), 2 mM glutamine (Sigma), 50 U/ml penicillin (Gibco), and 50 μg/ml streptomycin (Gibco). The dissociated cells were plated on 10 cm plastic petri dishes and incubated at 37 °C in humidified 5% CO_2, 95% air atmosphere. After 2–3 weeks in culture cells were plated on poly-L-lysine coated glass coverslips to perform the experiments. For Ca^{2+} measurements astrocytes at a density of 0.5×10^6 cells/dish were grown on 10 mm × 40 mm coated coverslips set over 60 mm petri dishes.

Astrocytes cultures of 80% confluence in 35 mm or 60 mm plates were transfected for overexpression experiments using Lipofectamine PLUS™ Reagent (Invitrogen, Carlsbad, CA.). The quantity of DNA used is described in figure legends. Briefly, for 35 mm plates 1 μg DNA STIM1-YFP (Addgene, plasmid 18857) [53], Orai1-YFP (Addgene, plasmid 19756) [33], or YFP empty vector was mixed with 6 μl PLUS into 100 μl Opti-MEM reduced serum medium (Gibco), and incubated at room temperature for 15 min. After that, the mix was added to a second tube containing 4 μl Lipofectamine (Invitrogen) diluted into 100 μl Opti-MEM and incubated for another 15 min. The culture medium of the plates was replaced with 800 μl fresh Opti-MEM medium and the mixture was added. The cells were incubated at 37 °C at 5% CO_2, after 6 h the medium was replaced with normal growth medium without antibiotics. Protein expression and function was assayed 24–36 h post-transfection. For 60 mm plates the quantities were scaled to surface area. Orai1 E106A mutant was a generous gift from Dr. Shenyuan Zhang (Texas A&M University) and it was transfected using Lipofectamine 2000 following the protocol detailed in the siRNA section.

2.2. Immunofluorescence and confocal microscopy

Astrocytes cultured on 9 mm × 9 mm coverslips were washed with PBS and immediately fixed in methanol at −20 °C for 10 min. After fixation, cells were incubated in PBS 50 mM glycine and blocked in 5% BSA PBS for 1 h at room temperature. The cells were incubated overnight at 4 °C with the primary antibodies using a dilution 1:40 in 1% BSA PBS. The antibody against STIM1 was purchased from BD Biosciences (San Jose, CA) and Anti-Orai1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After 3 washes with PBS, the bound antibodies were detected using DyLight-549 donkey anti-mouse IgG and DyLight-649 donkey anti-goat IgG from Jackson ImmunoResearch Labs Inc. (West Grove, PA) at 1:200 dilution. The cells were washed three times with PBS and incubated with DAPI (Molecular Probes, Eugene, OR), at 1:1000 dilution for 5 min. Images were collected with an Olympus FV10i confocal microscope and analyzed using Fluoview 10-ASW2.1 software. YFP-STIM1 overexpression experiments were assessed in an Olympus FV1000 confocal microscope.

2.3. $[Ca^{2+}]_i$ measurement

Ca^{2+} measurement experiments were performed using control or Ca^{2+}-free solutions. Control solution contained: 120 mM NaCl, 4.75 mM KCl, 1.2 mM $MgCl_2$, 20 mM HEPES, 1.8 mM $CaCl_2$, 10 mM glucose, 1.17 KH_2PO_4 and 0.05% BSA (adjusted to 300 mOsm, pH 7.4). In Ca^{2+}-free solution $CaCl_2$ was omitted and 200 μM EGTA was added.

To estimate changes in the cytoplasmic Ca^{2+} concentration, astrocytes cultured on 10 mm × 40 mm coverslips were loaded with Fura-2 AM (Molecular Probes) at 5 μM final concentration in control solution and incubated for 30 min at room temperature. The coverslips were then gently washed to remove the extracellular dye and were placed at a 50° angle relative to the excitation light path in a cuvette filled with pre-warmed (37 °C) control solution or Ca^{2+}-free solution in an Aminco-Bowman luminescence spectrometer (Thermo Electron, Madison, WI). Excitation wavelength was alternated between 340 nm and 380 nm and the emitted fluorescence was collected at 510 nm. Thapsigargin (Calbiochem, San Diego, CA) was used at 200 nM and thrombin (BioPharm Labs, Bluffdale, UT)

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at 5 U/ml. Chemicals were diluted in control or Ca^{2+}-free solution depending on the experiment.

Results are presented as the ratio of 340/380 fluorescence. As the Ca^{2+} measurements were performed in astrocytes adherent cultures and all the light emitted in the pathway was collected, the Ca^{2+} response observed per experiment corresponds to a cell population response (at least 50 cells). The values of the change in cytosolic Ca^{2+} concentration due to ER-Ca^{2+} release (first phase of Ca^{2+} increase) and Ca^{2+}-influx (second phase of Ca^{2+} increase) were determined estimating the total area under the curve of each phase for all independent measurements. Integration time for each phase was 600 s. Data presented are the mean ± SE of the number of experiments indicated for each figure legend.

2.4. siRNA

Set of three oligonucleotides of Stealth siRNAs for rStim1 (RSS324019, RSS360174, RSS324020) and for rOrai1 (RSS357633, RSS357634, RSS357635) were purchased from Invitrogen. A Stealth RNAi scramble negative control duplex (Invitrogen) was used in parallel experiments as a negative control. siRNAs transfection was carried out on primary astrocytes using Lipofectamine 2000 and Opti-MEM reduced serum medium according to the manufacturer's instructions (Invitrogen). Briefly, in a first tube 200 pmoles of the siRNA set or siRNA negative control was diluted into 500 μl Opti-MEM, in a second tube 15 μl Lipofectamine was diluted into 500 μl Opti-MEM, the tubes were incubated at room temperature for 5 min. The dilutions were mixed and incubated for 25 min. The culture medium of a 60 mm 70% confluent dish was replaced with 1 ml fresh Opti-MEM medium and the mixture solution containing the siRNA was added, leading to a siRNA final concentration of 100 nM. The cells were incubated at 37 °C at 5% CO_2 overnight. The next day the medium was replaced with normal growth medium without antibiotics. Protein expression and function was assayed 36 h post-transfection. Transfection efficiency was determined using the BLOCK-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen). For siRNA experiments against GFP, the GFP-22 siRNA (1022064, Qiagen) and negative control siRNA (1022076, Qiagen) were used using the same protocol detailed above. The efficiency of the siRNAs was determined by quantitative RT-PCR.

2.5. Total RNA extraction, synthesis of first strand cDNA and quantitative PCR analysis

Total RNA was isolated and purified by applying Trizol reagent (Invitrogen) to astrocyte primary cultures according to the manufacturer's instructions. For complementary DNA (cDNA) synthesis, 2.5 μg of total RNA was reverse transcribed using the SuperScript III RT (Invitrogen) and Oligo dT primers (Invitrogen). For quantitative PCR, 2 μl of RT product (total cDNA) was amplified using SYBR Green PCR Mastermix (Applied Biosystems, Warrington, UK) under the following conditions: initial denaturation for 10 min at 95 °C, followed by 40 cycles consisting of 15 s at 95 °C and 30 s at 60 °C. Table 1 shows the sets of primers designed for each gene. Primer Express software (Applied Biosystems) was used for data analysis. Fold change in the expression of mRNA for rStim1 and rOrai1was calculated using the $2^{-\Delta\Delta CT}$ method taking rGapdh and rHprt as control reference genes.

2.6. Data analysis

All data presented in this study represent the mean ± S.E.M. obtained from the different number of independent experiments indicated in each figure legend. The statistical significances of differences between groups were determined by unpaired *t*-student test. Data were plotted using Igor Pro 6 (WaveMetrics, Lake Oswego, OR).

3. Results

STIM1 and Orai1 are essential components of store-operated Ca^{2+} entry (SOCE), which is activated in response to Ca^{2+} release from endoplasmic reticulum (ER). Thrombin, a blood-derived protease, induces a Ca^{2+} response in astrocytes characterized by two components that include ER-Ca^{2+} release and SOCE [20]. However, the role of STIM1 and Orai1 in the store-operated Ca^{2+} entry in astrocytes and specifically in their response to thrombin remains unexplored. In the present study, we employed overexpression and siRNA experiments and showed that STIM1 and Orai1 are responsible of the store-operated Ca^{2+} entry activated by thrombin in rat cortical astrocytes.

3.1. Tg-induced ER depletion and thrombin stimulation activate store-operated Ca^{2+} entry (SOCE) in cortical astrocytes cultures

In order to study SOCE activation, we performed cell-population Ca^{2+} measurements in adherent cultures of cortical astrocytes. In these experiments, the increase in Fura-2 fluorescence was quantified. As control of SOCE activation, we used thapsigargin (Tg) to induce ER-Ca^{2+} depletion. Fig. 1A shows representative Ca^{2+} rise traces induced by Tg in the absence or presence of Ca^{2+} into the extracellular solution. In the absence of Ca^{2+}, the Ca^{2+} rise was diminished by about 80% compared to the cells stimulated in a solution containing 1.8 mM $CaCl_2$, showing that Tg-induced response is mainly due to the Ca^{2+}-influx that follows ER-Ca^{2+} depletion. When astrocytes were stimulated with thrombin in the presence of extracellular Ca^{2+}, a biphasic response was observed, characterized by a fast initial transient followed by a sustained elevation (Fig. 1B). In the absence of Ca^{2+}, the second phase was abolished reducing the total response by about 80%, similar to that observed for Tg stimulation, showing that a prominent component of thrombin-induced response is also due to Ca^{2+} influx.

ER-Ca^{2+} release and SOCE were clearly separated when astrocytes were first stimulated in a Ca^{2+}-free solution and then a solution containing 1.8 mM $CaCl_2$ was added. Fig. 1C shows the ER-Ca^{2+} release (first phase of Ca^{2+} increase) and the Ca^{2+} influx (second phase of Ca^{2+} increase) in astrocytes stimulated with Tg. Ca^{2+} influx induced by thrombin, similar to the induced by Tg, was clearly separated under this protocol, supporting that in astrocytes thrombin activates SOCE (Fig. 1D). Together, these results show that under thrombin stimulation, cortical astrocytes exhibit a Ca^{2+} response that mainly depends on SOCE.

Table 1
Oligonucleotide sequences of primers used for quantitative RT-PCR.

Gene	Accession no.	Size (bp)	Primers		Location
rOrai1	NM_001013982.1	75	FW: 5-CATGGTAGCGATGGTGGAAGT-3	RV: 5-GGCGCTGAAGACGATGAGTAAC-3	303–378
rStim1	NM_001108496.2	104	FW: 5-AGCTGAAGGCTCTGGACACAGT-3	RV: 5-CACCCACACCAATCACGATAGA-3	572–676
rGapdh	NM_017008.3	91	FW: 5-ACTCTACCCACGGCAAGTTCAA-3	RV: 5-CGCTCCTGGAAGATGGTGAT-3	143–233
rHprt1	NM_012583.2	93	FW: 5-TTGCTCGAGATGTCATGAAGGA-3	RV: 5-CCAGCAGGTCAGCAAAGAACT-3	146–238

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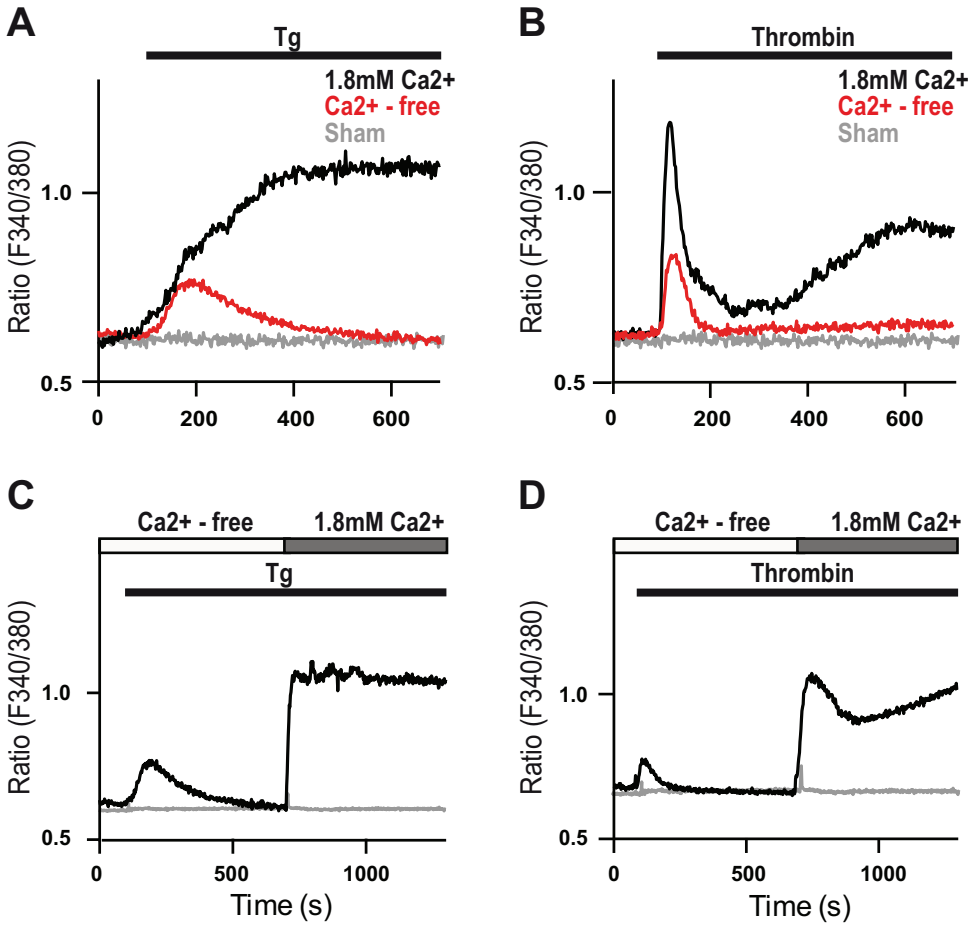

Fig. 1. Store-operated Ca^{2+} entry activation in cortical astrocytes. Representative Ca^{2+} rise traces of the response of astrocytes to thapsigargin, Tg (A) and to thrombin (B) in the presence (black traces) or in the absence of extracellular Ca^{2+} (red traces). To isolate the store-operated Ca^{2+} entry, the cells were stimulated with Tg (C) or with thrombin (D) in a Ca^{2+}-free solution in order to deplete the ER Ca^{2+} stores (first phase of Ca^{2+} increase), after 10 min a solution containing 1.8 mM $CaCl_2$ was added to evoke Ca^{2+} influx (second phase of Ca^{2+} increase). In sham traces (gray) the same exchange of solutions was performed using only the vehicle solution without Tg or thrombin. Astrocytes were loaded with Fura-2 AM for 30 min and stimulated with 200 nM thapsigargin (Tg) or with 5 U/ml thrombin. Each trace shows the average for at least 50 cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. *Native expression of STIM1 and Orai1 in cortical astrocytes primary cultures*

We used immunocytochemistry assays to determine the presence and localization of STIM1 and Orai1 in primary cultures of cortical astrocytes. The specificity of the STIM1 and Orai1 antibodies used in this study was assessed by immunodetection of transfected STIM1-YFP and Orai1-YFP proteins (Fig. S1). Colocalization between the respective fluorescent secondary antibodies and YFP was determined for both proteins. The immunofluorescence signal of STIM1 and Orai1 antibodies colocalized with the YFP signal in astrocytes overexpressing STIM-YFP or Orai1-YFP, with a Pearson's coefficient of 0.78 ± 0.08 and 0.84 ± 0.06, respectively ($n = 10$). Besides the detection of expressed YFP-coupled proteins, native proteins were also clearly identified (labeled only with the antibody). This was more evident in astrocytes stimulated with Tg where STIM1 puncta formation was induced. In Fig. S1, STIM1-YFP puncta and native STIM1 puncta can be clearly observed. Fig. 2 shows the immunodetection of native STIM1 and Orai1 proteins in astrocytes under resting conditions. Unspecific binding of the secondary antibodies was not observed as it is shown in the control panel. Staining for native STIM1 was diffused and homogeneously distributed, mapping the ER. The localization of native Orai1 was restricted to the plasma membrane, although some cells showed a high portion of Orai1 protein in what appeared to be intracellular vesicles (data not shown). This intracellular distribution for Orai1 has been previously observed in different cell types [54,55]. Here, we showed that primary cultures of rat cortical astrocytes express STIM1 and Orai1 proteins and that they show typical cellular localizations, as seen in other cells.

3.3. *Thrombin stimulation induces a transient reorganization of STIM1 in astrocytes*

STIM1 reorganizes rapidly under SOCE activation forming puncta near the plasma membrane to gate SOC [56,57]. Inhibitors of SERCA pumps, such as Tg, are commonly used to visualize STIM1 reorganization since they deplete irreversibly the ER-stores and cause a sustained Ca^{2+} influx and permanent STIM1 puncta formation [53,58]. Astrocytes expressing the STIM1-YFP plasmid were transferred to a Ca^{2+}-free solution and treated with Tg to visualize STIM1 puncta formation. As expected, Tg treatment caused a clear reorganization of STIM1-YFP (Fig. 3A). The distribution of STIM1 in puncta remained after washout with a solution containing 1.8 mM $CaCl_2$, as expected, since the binding of Tg to the ATPase is very difficult to wash.

Under physiological conditions, in which Ca^{2+} release is counterbalanced with recapture, influx and extrusion, a transient

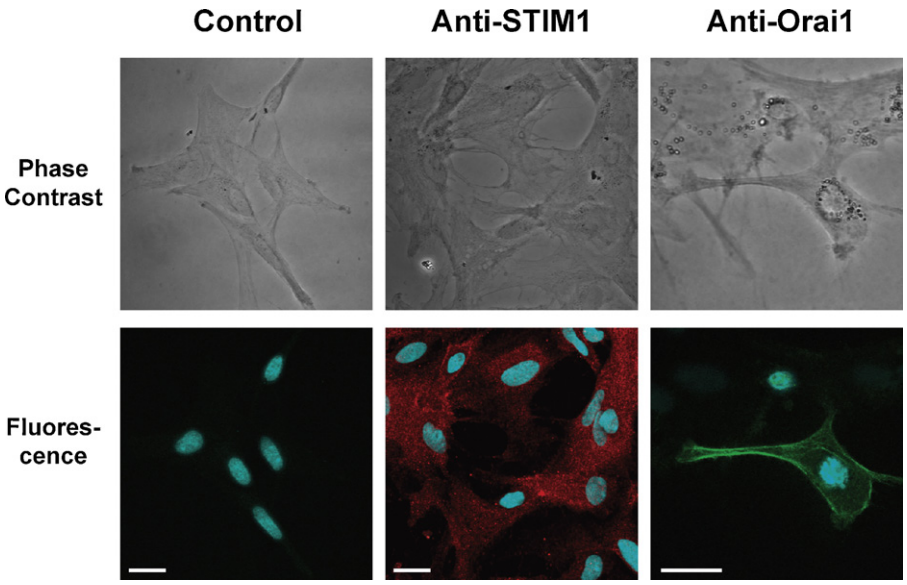

Fig. 2. Expression of STIM1 and Orai1 in cortical astrocytes. Astrocytes primary cultures were fixed and immunostained with STIM1 (red) or Orai1 (green) antibodies. Nuclei were stained with DAPI (blue). Control cells were treated in the absence of primary antibodies. Upper panels correspond to contrast phase images of cultures and lower panels to confocal fluorescence microscope images. Scale bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

reorganization of STIM1 would be expected. Astrocytes stimulated with thrombin allowed us to test this idea. To stabilize STIM1 organization and to allow puncta visualization, astrocytes stimulation was assessed in a Ca^{2+}-free solution (to prevent the rapid refilling of the ER by Ca^{2+} influx and the disassembly of the STIM1 puncta). We found that STIM-YFP was distributed diffusely at rest (without thrombin), when astrocytes were incubated in the Ca^{2+}-free solution for 10 min, demonstrating that the absence of Ca^{2+} in the extracellular solution alone was not enough to promote STIM1 reorganization. After thrombin addition, STIM1 clearly reorganized in puncta and distributed in the periphery of the cell. When the cells where washed with a solution containing 1.8 mM $CaCl_2$, STIM1 puncta disappeared and the diffuse distribution of STIM1 observed in resting conditions was recovered. Data of these experiments are summarized in Fig. 3B and Movie S1. Given the reorganization of STIM1 under thrombin stimulation in astrocytes expressing the STIM-YFP construct, we examined the effect of thrombin stimulation over the distribution of native STIM1. Astrocytes were stimulated with thrombin in a Ca^{2+}-free solution and after 10 min cells were fixed for immunostaining to identify endogenous STIM1. In astrocytes without stimulation STIM1 was distributed diffusely, while under thrombin stimulation astrocytes exhibited discrete STIM1 puncta (Fig. 3C). These results show that thrombin caused a transitory puncta reorganization of STIM1 that could be visualized under Ca^{2+}-free conditions and which is rapidly reversed in the presence of Ca^{2+}. Moreover, the evidence of native STIM1 reorganization under thrombin stimulation shows that this phenomenon occurs under physiological conditions. Since puncta reorganization is a hallmark of SOCE activation, this is a strong evidence of the participation of SOCE in the thrombin-induced Ca^{2+} response in cortical astrocytes. As far as we know, this is the first demonstration that an agonist can induce the reorganization of endogenous STIM1 into puncta, demonstrating that puncta formation is not the result of STIM1 overexpression in combination with drugs that deplete irreversibly the ER (such as Tg).

Similar experiments overexpressing the Orai1-YFP construct in astrocytes showed a discrete reorganization of Orai1 under thrombin stimulation, which was only visible in the membrane focal plane (Fig. S3A). To evaluate native Orai1 reorganization, astrocytes were treated with thrombin for 10 min prior to fixation and then processed for Orai1 immunodetection. We did not observe evident reorganization of the native Orai1 (Fig. S3B), this could be attributed to the weak fluorescence signal in the membrane focal plane of the secondary antibody used. Further experiments are required to determine if Orai1 is reorganized in response of thrombin stimulation in astrocytes.

3.4. Effect of overexpression of STIM1 and Orai1 on the thrombin induced store-operated Ca^{2+} entry in astrocytes

The simultaneous coexpression of STIM1 and Orai1 has been found to be sufficient to increase Ca^{2+} influx [59,60]. In this study, we evaluated the effect of STIM1 and Orai1 overexpression in the response of cortical astrocytes to thrombin stimulation. Fura-2 was loaded to perform Ca^{2+} measurements in cortical astrocytes overexpressing STIM1 and Orai1, either independently or simultaneously. The cells were transferred to a Ca^{2+}-free solution and after 100 s the stimulus (Tg or thrombin) was added to induce ER-Ca^{2+} release. The solution was changed after 600 s with a solution containing 1.8 mM $CaCl_2$ to initiate Ca^{2+} influx. Fig. 4A shows the fluorescence traces obtained for control cells and cells overexpressing STIM1, Orai1 or both under treatment with Tg. None of the overexpression conditions had any significant effect over ER-Ca^{2+} release, indicating that STIM1 and/or Orai1 overexpression affect primarily Ca^{2+} influx. On the other hand, the quantification of the second peak area showed that when compared to control cells (149 ± 14, $n = 5$), the overexpression of Orai1 caused a 23% decrease in Ca^{2+} influx (114 ± 13, $n = 5$), while the overexpression of STIM1 increased Ca^{2+} influx by near 33% (196 ± 16, $n = 5$). The simultaneous expression of STIM1 and Orai1 caused a 75% increase in SOCE compared to control (259 ± 25, $n = 5$) (Fig. 4B).

If STIM1 and Orai1 participate in the response evoked by thrombin in astrocytes it would be expected that their overexpression lead to similar results to that obtained with Tg. In fact, similar tendencies on the Ca^{2+} influx were observed in cells overexpressing

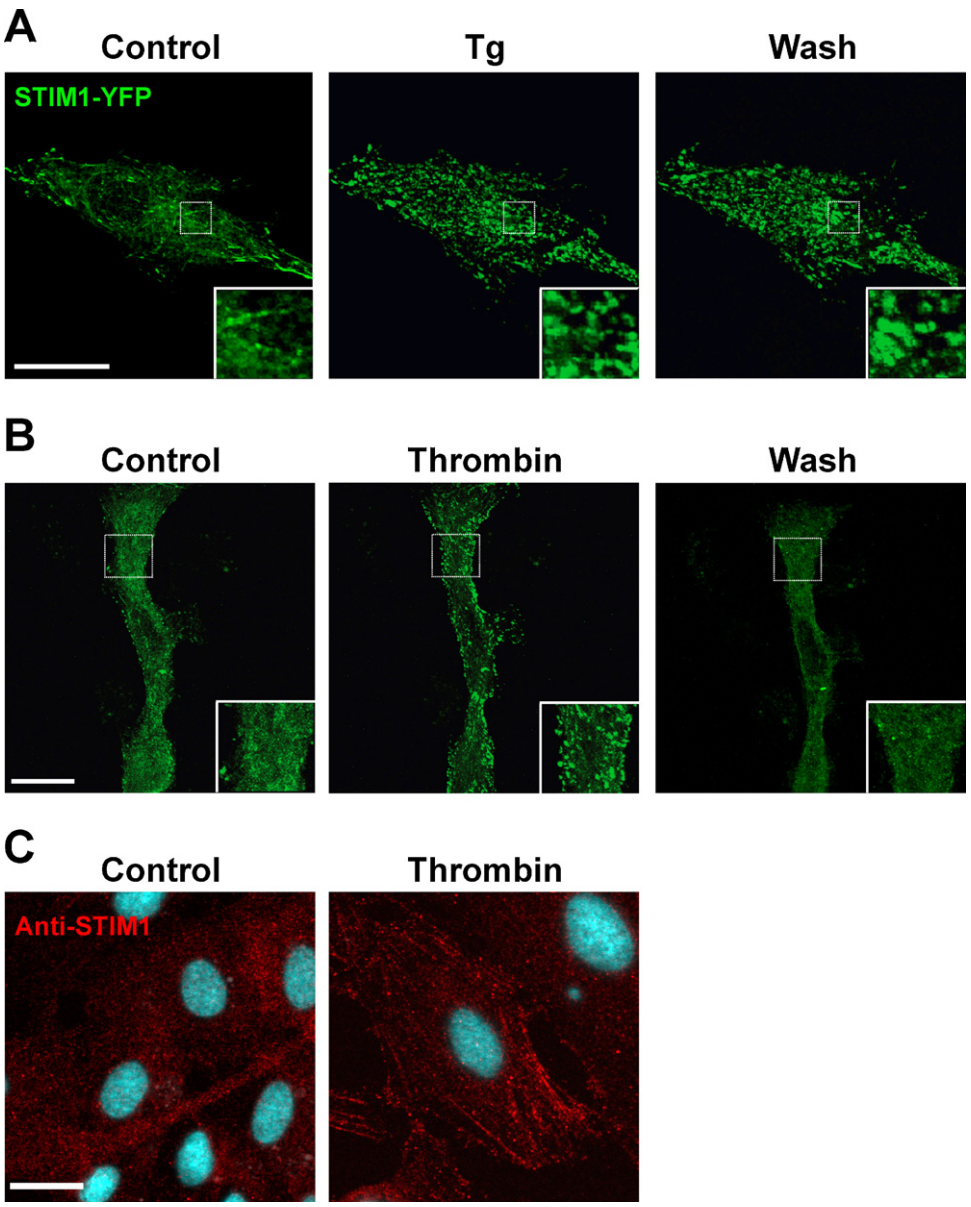

Fig. 3. Thrombin stimulation induces STIM1 puncta reorganization in cortical astrocytes. Astrocytes were transfected with 1 μg of STIM1-YFP and experiments were assessed 24 h after transfection. In control, cells were incubated in Ca^{2+}-free solution for 10 min. To induce ER Ca^{2+} release astrocytes were treated with 200 nM thapsigargin (Tg) (A) or stimulated with 5 U/ml thrombin (B) for 10 min in Ca^{2+}-free solution. Subsequently the cells were washed with a solution containing 1.8 mM $CaCl_2$ to induce Ca^{2+} influx. In thrombin-treated cells STIM1 puncta reorganization was reversed after washout. The images are Z projections of confocal image stacks. (C) Endogenous STIM1 is also reorganized under thrombin stimulation. Astrocytes were stimulated with thrombin in Ca^{2+}-free solution, after 10 min astrocytes were fixed and processed for STIM1 immunodetection. In control, the cells were incubated for 10 min in Ca^{2+}-free solution without thrombin prior to fixation. Scale bar, 20 μm.

STIM1 and/or Orai1 after thrombin stimulation. Fig. 4C shows the traces obtained for thrombin-induced Ca^{2+} responses of cortical astrocytes in control conditions and overexpressing STIM1, Orai1 or both. Notice that under thrombin stimulation no differences in the amplitude of ER-Ca^{2+} release were observed for the different experimental conditions, similarly to the results obtained for Tg. Orai1 overexpression significantly diminished Ca^{2+} influx induced by thrombin by about 32% (52 ± 6, $n = 5$), while STIM1 overexpression increased the Ca^{2+} influx by about 42% (107 ± 6, $n = 5$) compared to control conditions (75 ± 6, $n = 5$). The simultaneous overexpression of STIM1 and Orai1 increased the Ca^{2+} influx induced by thrombin by about 98% (149 ± 6, $n = 5$) (Fig. 4D). This twofold increase in the thrombin-induced Ca^{2+} influx, in astrocytes overexpressing both STIM1 and Orai1, suggests that these two proteins are directly involved in the Ca^{2+} response that astrocytes elicit in the presence of thrombin.

3.5. *Effect of knockdown of STIM1 and Orai1 on the thrombin induced store-operated Ca^{2+} entry in astrocytes*

In order to confirm the role of endogenous STIM1 and Orai1 in the response of astrocytes to thrombin, we performed RNAi experiments to knockdown the expression of these two proteins. Cortical astrocytes cultures had a siRNA transfection efficiency of $85 \pm 1\%$, estimated by means of the red fluorescent oligo BLOCK-iT (Fig. S2A and B). As a control of the capacity to perform gene knockdown in primary astrocytes cultures, we cotransfected the cells with the EGFP plasmid in the absence or presence of a siRNA against GFP. The number of GFP-positive cells and the intensity of GFP fluorescence were noticeably reduced in cells transfected with GFP-siRNA (Fig. S2C). These results confirmed the high transfection efficiency obtained with astrocytes and showed the significant reduction of GFP protein synthesis in the presence of siRNAs.

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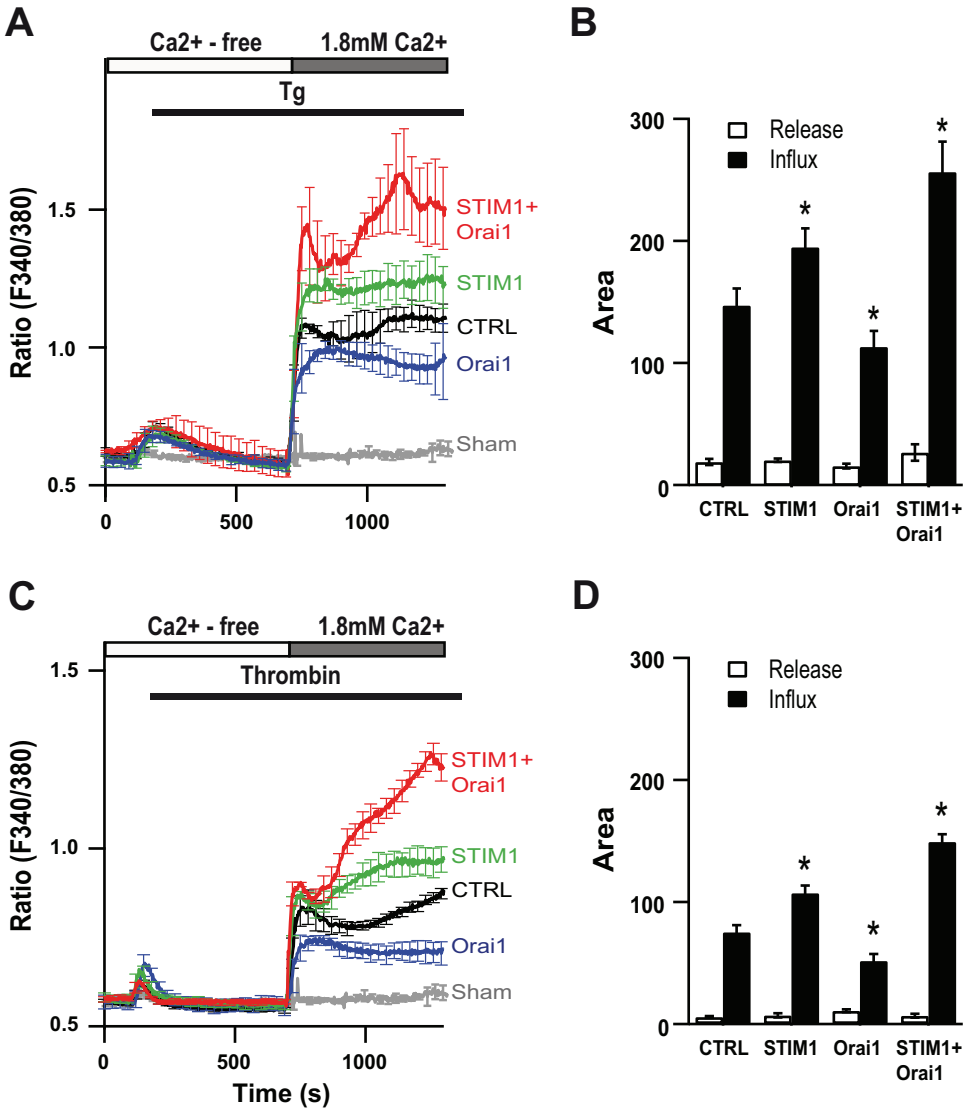

Fig. 4. Effect of overexpression of STIM1 and Orai1 on the thapsigargin-induced Ca^{2+} influx and on the thrombin-induced Ca^{2+} response in cortical astrocytes. Astrocytes were transfected with 4 μg empty vector (black and gray traces), STIM1 (green traces), Orai1 (blue traces) or cotransfected with 4 μg STIM1 and 4 μg Orai1 (red traces). The cells were used 36 h after transfection for Ca^{2+} measurements. Cells were incubated with Fura-2 AM for 30 min. Cells were treated with 200 nM thapsigargin (Tg) (A) or stimulated with 5 U/ml thrombin (C) in Ca^{2+}-free solution to induce ER Ca^{2+} release (first phase of Ca^{2+} increase) and exposed to a solution containing 1.8 mM $CaCl_2$, utilized to initiate Ca^{2+} influx (second phase of Ca^{2+} increase). In sham traces (gray) the same exchange of solutions was performed using only the vehicle solution without Tg or thrombin. The traces are the mean ± S.E.M. of five independent experiments. Each experiment is the average of at least 50 cells. Data summarized for the total area of the first phase of Ca^{2+} increase for ER-Ca^{2+} release (white bars) and the total area of the second phase of Ca^{2+} increase for Ca^{2+} influx (black bars), expressed as mean ± S.E.M. (B and D). Asterisk denotes a significant difference ($p < 0.05$) compared to control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Once we established that the siRNAs entered the cells and that the knockdown of a control gene (GFP) in primary cultures of astrocytes was successful, we proceeded to knockdown simultaneously the expression of STIM1 and Orai1 in cortical astrocytes. Population Ca^{2+} measurements were performed with the same protocol used in overexpression experiments. Fig. 5A shows the traces of Tg-induced Ca^{2+} response in cortical astrocytes transfected with Orai1-siRNA or STIM1/Orai1-siRNAs. The simultaneous knockdown of STIM1 and Orai1 caused a 58% reduction in SOCE under Tg stimulation (59 ± 7, $n = 4$) when compared to cells transfected with a scramble siRNA (140 ± 13, $n = 4$). When astrocytes were treated only with the Orai1 siRNA, Ca^{2+} influx was reduced by about 62% (53 ± 12, $n = 4$) (Fig. 5B). Knockdown of STIM1 alone inhibited Ca^{2+} influx to a similar extent (data not shown). Fig. 5C shows the thrombin-induced Ca^{2+} response for control, Orai1-siRNA and STIM1/Orai-siRNAs. The results obtained for thrombin stimulation were similar to that obtained for Tg. The simultaneous knockdown of STIM1 and Orai1 caused a large reduction in the thrombin-induced Ca^{2+} influx and this reduction was also evident when studying individual cells with confocal microscopy using the Ca^{2+} fluorescent indicator Fluo-4 (Movie S2). In the Fura-2 experiments, we observed a 42% reduction of the Ca^{2+} influx evoked by thrombin stimulation (39 ± 7, $n = 4$) when compared to astrocytes transfected with a scramble siRNA (68 ± 8, $n = 4$) (Fig. 5D). The knockdown of Orai1 alone caused a similar reduction in Ca^{2+} influx (48%, 34 ± 4, $n = 4$) with no significant differences compared to simultaneous knockdown with STIM1. Knockdown of STIM1 alone caused similar percentage of inhibition (data not shown). None of the treatments under Tg or thrombin stimulation caused significant changes in the ER-Ca^{2+} release. These results support the involvement of STIM1 and Orai1 in the Ca^{2+} influx induced by thrombin in cortical astrocytes.

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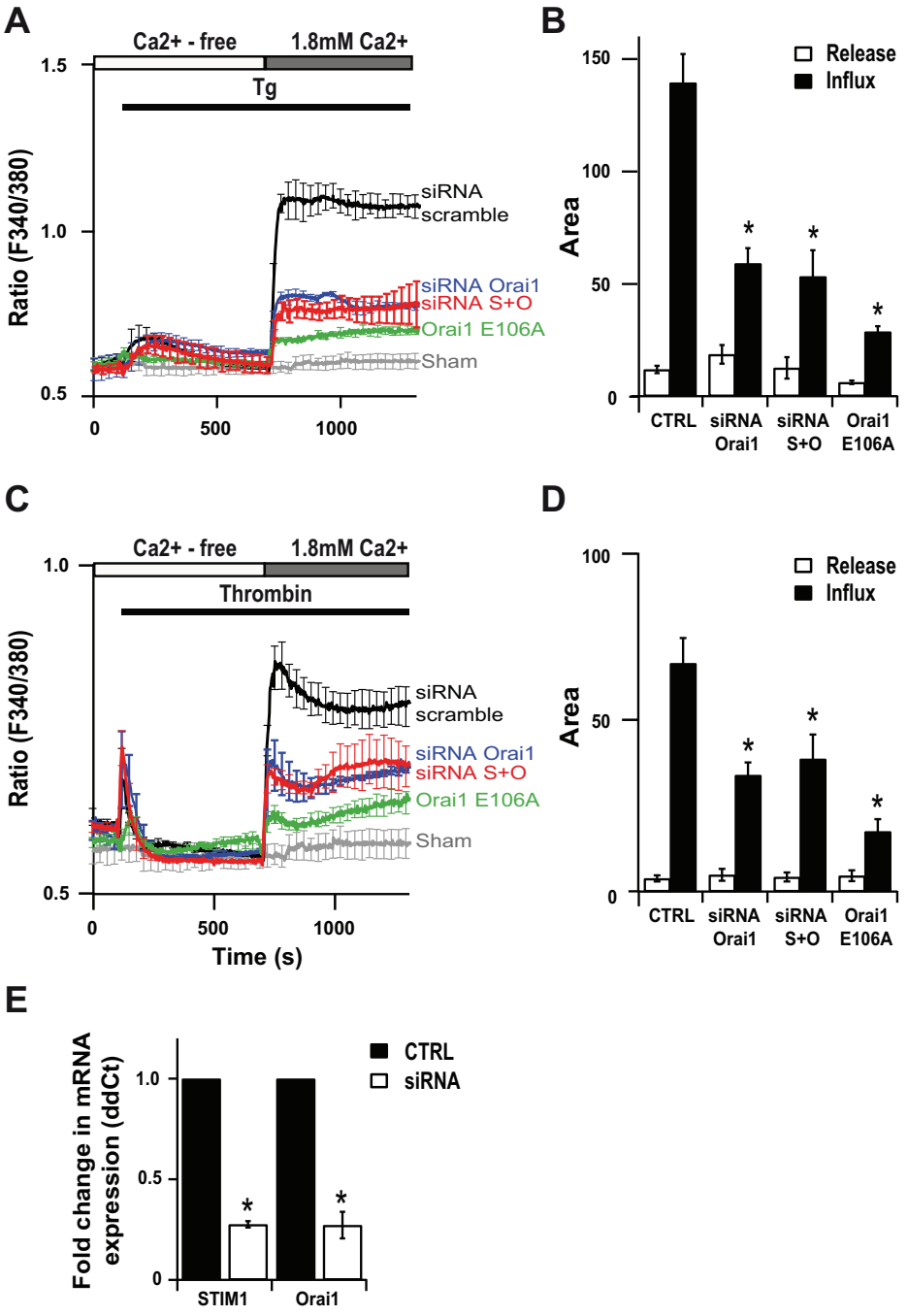

Fig. 5. Effect of STIM1 and Orai1 knockdown on the thapsigargin-induced Ca^{2+} influx and on the thrombin-induced Ca^{2+}response in cortical astrocytes. Astrocytes were treated with scrambled siRNA (black and gray traces), Orai1 siRNA (blue traces), simultaneously with STIM1 siRNA and Orai1 siRNA (red traces) or transfected with the Orai1E106A construct (green traces, 5 μg) overnight as detailed in Section 2. After 36 h, the cells were used for Ca^{2+} measurements. Cells were incubated with Fura-2 AM for 30 min. Then cells were treated with 200 nM thapsigargin (Tg) (A) or stimulated with 5 U/ml thrombin (C) in Ca^{2+}-free solution to induce ER Ca^{2+} release (first phase of Ca^{2+} increase) and exposed to a solution containing 1.8 mM $CaCl_2$ to evaluate Ca^{2+} influx (second phase of Ca^{2+} increase). In sham traces (gray) the same exchange of solutions was performed using only the vehicle solution without Tg or thrombin. The traces are the mean ± S.E.M. of four independent experiments. Each experiment is the average of at least 50 cells. Data summarized for the area of the first peak for Ca^{2+} release (white bars) and the area of the second peak for Ca^{2+} influx (black bars), expressed as mean ± S.E.M. (B and D). The efficiency of the Orai1 and STIM1siRNA probes was determined by quantitative RT-PCR in astrocytes (E). Data are expressed as the mean ± S.E.M. fold change relative to cells treated with scramble siRNA (CTRL) for four independent experiments. Fold change was calculated by means of the $2^{-\Delta\Delta Ct}$ method using GAPDH as a reference. Asterisk denotes a significant difference ($p < 0.05$) compared to control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

mRNA levels for STIM1 and Orai1 were monitored by quantitative RT-PCR to determine the effect of RNAi on the mRNA concentration. The mRNA levels of STIM1 and Orai1 were reduced by about 70%, in astrocytes transfected with the siRNAs against STIM1 or against Orai1, respectively, when compared to cells transfected with a scramble siRNA (fold change STIM1 0.28 ± 0.02, $n = 4$, fold change Orai1 0.27 ± 0.06, $n = 4$) (Fig. 5E). Fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method using GAPDH as a reference. Similar results on the fold change were obtained when compared with HPRT as a second reference gene (data not shown).

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In order to test if Orai1 was functioning as the pore of the channel responsible for SOCE in astrocytes and therefore if the decrease in SOCE observed under Orai1 silencing was due to a decrease in the flow of Ca^{2+} ions through this channel, we performed experiments using a non-conducting mutant channel of Orai1 (Orai1-E106A). For these experiments astrocytes were transfected with 5 μg of Orai1-E106A. Under Orai1-E106A expression Ca^{2+} influx in astrocytes stimulated with Tg was reduced by about 79% (29 ± 2, $n = 3$, Fig. 5A and B). On the other hand, Ca^{2+} influx evoked by thrombin stimulation under Orai1 E106A expression was reduced by about 73% (18 ± 4, $n = 4$, Fig. 5B and C). In both cases, the percentage of SOCE reduction under Orai1-E106A expression was approximately 15% higher compared to the results obtained in the siRNA experiments. These results suggest that Orai1 is indeed functioning as a SOC in astrocytes and that Ca^{2+} flowing through its pore is necessary for the Ca^{2+} influx induced by Tg and thrombin in astrocytes. The higher inhibition observed when expressing the Orai1-E106A mutant might be the result of better transfection for the plasmid DNA than for the siRNA.

4. Discussion

In astrocytes, thrombin leads to cytoplasmic Ca^{2+} elevations, which modulate a variety of responses ranging from proliferation to cell death [7,13,14]. Store-operated Ca^{2+} entry (SOCE) is an important component of this Ca^{2+} elevation evoked by thrombin [20]. In many cell types, STIM1 and Orai1 are central components of SOCE [30,32,33,53]. In this study, we found that STIM1 and Orai1 play an essential role in SOCE in rat cortical astrocytes. We used thapsigargin to induce Ca^{2+} release from ER, a general pharmacological approach to study SOCE without activation of G-protein coupled receptors [31,35]. The overexpression of STIM1 and Orai1 increased the thapsigargin-induced Ca^{2+} influx, while the knockdown of these proteins diminished it, suggesting that STIM1 and Orai1 are necessary components of SOCE in rat cortical astrocytes.

However, the main goal of the present work was to assess the role of endogenous STIM1 and Orai1 in the response of astrocytes to thrombin, a blood-derived factor released under cerebrovascular accidents. In the present study, we used several experimental approaches to determine the role of STIM1 and Orai in SOCE in astrocytes. Firstly, we showed the presence of STIM1 and Orai1 proteins in primary cultures of rat cortical astrocytes. Secondly, we showed that overexpression of STIM1–Orai1 results in a large increment of Ca^{2+} influx evoked by thrombin without affecting the release from the ER; thirdly, using RNAi studies to knockdown STIM1/Orai1 we obtained a significant reduction in SOCE induced by thrombin. Furthermore, we showed for the first time that agonist stimulation could induce puncta formation on both transfected and endogenous STIM1, similarly to what has been previously reported for Tg. This work provides the first evidence of the involvement of endogenous STIM1 and Orai1 in the thrombin-induced Ca^{2+} response in astrocytes.

The presence of STIM1 protein in rat cortical astrocytes is in line with previous reports showing the detection of STIM1 mRNA in an astrocytoma cell line [29] and the expression of the protein in mouse astrocytes [47,61]. Moreover, we showed that STIM1 reorganizes from a diffusely distribution to a transient puncta organization after thrombin stimulation. This transient organization was visualized only when the cells were stimulated in the absence of extracellular Ca^{2+}, a condition that prevents ER-Ca^{2+} refilling and therefore stabilizes STIM1 oligomers. The reversibility of STIM1 puncta under thrombin fits well to what is expected for a physiological stimulation, in which the agonist causes a transiently ER-Ca^{2+} release that, in turn, activates SOCE to rapidly replenish the ER, resulting in the concomitant disassembly of the store-operated Ca^{2+} influx complex (SOCIC) [22,62]. STIM1 puncta formation under thrombin stimulation was also evident for the endogenous protein, showing that it was not an effect of the overexpression. Since STIM1 reorganization is a crucial step for SOCE activation [57,58], our result strongly support that STIM1 is involved in the response of astrocytes to thrombin.

The overexpression of STIM1, without overexpression of Orai1, was sufficient to increase significantly the thrombin-induced Ca^{2+} response, suggesting that there are more endogenous Orai channels available to STIM1. When STIM1 was coexpressed with Orai1, the Ca^{2+} influx induced by thrombin was further increased; supporting the notion that Ca^{2+} entry through Orai1 channels is a downstream event of thrombin binding in astrocytes. In platelets, STIM1 and Orai also underlie thrombin response [48]; here, we show that these proteins are key components of the astrocytes response to thrombin. Overexpression of Orai1 alone diminished Ca^{2+} influx in astrocytes stimulated with both, Tg and thrombin. Similar results in previous reports have been attributed to alterations in the STIM1:Orai1 ratio due to an excess of available Orai1, leading to a reduction in the activity of the channel [63,64].

Since its identification in T-lymphocytes of a patient with a severe immunodeficiency syndrome, Orai1 has been identified as a major store-operated channel (SOC) in many of the cell types studied [25,32,33]. Here, we showed that rat cortical astrocytes express Orai1 and that it localizes mainly at the plasma membrane. In the RNAi experiments against Orai1, the percent of mRNA reduction correlated well with a 62% reduction of SOCE induced by Tg, supporting the idea that Orai1 is an essential SOC in rat cortical astrocytes as it has been shown recently in mouse cortical astrocytes [47]. Under thrombin stimulation, Orai1 knockdown caused a 48% reduction of the Ca^{2+} influx, a similar percent of reduction obtained after simultaneous knockdown of STIM1 and Orai1, suggesting that Orai1 might be the only STIM1-activated channel involved in the thrombin induced response.

The expression of the mutant Orai1-E106A, which corresponds to a mutation in the pore of Orai1 that prevents Ca^{2+} permeation, resulted in a significant reduction of SOCE under Tg or thrombin stimulation. The fact that the percentage of reduction in SOCE observed under Orai1-E106A expression was greater than that observed in our siRNA experiments (around 15% greater) confirms that Ca^{2+} entry through Orai1 accounts for a significant percentage of SOCE in astrocytes. In fact, these data show that Orai1 underlies more than 70% of the Ca^{2+} influx induced by thrombin. These results together suggest that astrocytes require the presence and the ion-conducting function of Orai1 for the Ca^{2+} mobilization after thrombin stimulation.

Nevertheless, our studies do not rule out the possibility that other SOCs may play a role in SOCE in astrocytes, since our RNAi and Orai1-E106A expression experiments did not prevent completely SOCE initiated by Tg or thrombin. Given the partial efficiency in mRNA reduction (70%) with siRNAs for STIM1 and Orai1, it is difficult to determine if other channels may be involved in SOCE in this cell type. In addition, the residual STIM1 that remains after the knockdown could be sufficient to activate other SOCs, such as TRPCs. Rat astrocytes express all the members of the TRPC family [40]. In astrocytes, the TRPC1 is responsible of 40% of the CPA-induced SOCE and almost all the Ca^{2+} influx evoked by purinergic stimulation [65]. Moreover, the expression of TRPC1 and Orai1 is simultaneously downregulated in astrocytes from mice lacking amyloid precursor protein causing a dysregulation of SOCE, suggesting that TRPC1 acts as an important SOC in these cells [47]. Finally, we cannot rule out the role of Orai2 and Orai3 in SOCE after thrombin stimulation.

In addition, although the TRPC3 functions as a SOC in astrocytes and its expression is upregulated under chronic thrombin stimulation [43] it has been seen that TRPC3 could also function as

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a receptor-operated channel (ROC) [66]. Therefore, another interesting possibility is that thrombin in astrocytes could be activating simultaneously a receptor-operated calcium entry (ROCE) by means of DAG production, as it has been seen in platelets [50]. In that case TRPC3 and TRPC6 could be underlying this calcium entry in a STIM1-independent manner. There is evidence showing that ROCE is not activated under purinergic stimulation in astrocytes in spite of TRPC3 expression [67]. However, there are no studies exploring thrombin-induced ROCE activation in astrocytes. Further studies are required to elucidate the role of the members from the TRPC family to identify a putative role as SOC or ROC in the thrombin-induced Ca^{2+} response in astrocytes.

Since we show that STIM1 and Orai1 underlie a significant portion of the Ca^{2+}elevation induced by thrombin in astrocytes and thrombin is released under cerebrovascular accidents then, these proteins could play a relevant role in glutamate exocytosis and astrogliosis, two determinant factors for the brain damage caused by cerebrovascular accidents [16,68].

Conflict of interest

There are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ceca.2012.08.004.

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Critical Review

SOC AND NOW ALSO SIC: Store-operated and Store-inhibited Channels

Claudia Moreno and Luis Vaca

Departamento de Biología Celular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria, Del. Coyoacán, 04510 México DF, México

Summary

There is a specialized form of calcium influx that involves a close communication between endoplasmic reticulum and the channels at the plasma membrane. In one side store depletion activates channels known as store-operated channels (SOC), which are responsible of the well-studied store-operated calcium entry (SOCE). SOC comprises two different types of channels. Orai, which is exclusively activated by store depletion being the channel responsible of the calcium release-activated calcium current, and transient receptor potential canonical channel, which in contrast, is activated by store depletion only under specific conditions and carries nonselective cationic currents. On the other hand, it has been recently shown that store depletion also inhibits calcium channels. The first member identified, of what we named as store-inhibited channels (SIC), is the L-type voltage-gated calcium channel. Stores control both SOC and SIC by means of the multifunctional protein STIM1. The identification of SOC and SIC opens a new scenario for the role of store depletion in the modulation of different calcium entry pathways, which may satisfy different cellular processes.

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Keywords store depletion; calcium entry; Orai; TRPC; L-type VGCC; STIM1.

Abbreviations 2-APB, 2-aminoethyldiphenyl borinate; CAD, CRAC activation domain; DAG, diacylglycerol; ER, endoplasmic reticulum; ERM, ezrin-radixin-moesin domain; GPCR, G-protein coupled receptors; I_{crac}, calcium release activated calcium current; IP3, inositol trisphosphate; PIP2, Phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PM, plasma membrane; ROC, receptor-operated channel; SIC, store-inhibited channels; SOAR, STIM1-Orai activating region; SOC, store-operated channels; SOCE, store-operated calcium entry; STIM1, stromal-interacting molecule 1; TG, thapsigargin; TM, transmembrane domain; TRPC, transient receptor potential canonical channel; VGCC, voltage-gated calcium channel.

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Address correspondence to: Luis Vaca, Departamento de Biología Celular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria, Del. Coyoacán, 04510 México DF, México. Tel: +52-55-56225654; Fax: +52-55-56225611.
E-mail: lvaca@ifc.unam.mx.

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STORE-OPERATED CALCIUM ENTRY

Calcium is one of the most important second messengers in the cell, playing a pivotal role in multiple physiological and pathological processes, including cell division, differentiation, apoptosis, and cancer (*1, 2*). On account of its central role, cells maintain low calcium levels in the cytoplasm and have developed accurate mechanisms to handle the intracellular calcium concentration. One of these mechanisms is the calcium storing inside specific cell organelles. The endoplasmic reticulum (ER) is the most important calcium store in the cell, reaching a calcium concentration 10,000 times greater inside than in the cytoplasm and having specific mechanisms to release and recapture this ion (*3*).

Calcium is typically released from the ER in response to the activation of those G-protein coupled receptors (GPCR), which stimulate phospholipase C (PLC) and induce inositol trisphosphate (IP3) production. Binding of IP3 to its receptor at the ER membrane induces a massive efflux of calcium from the ER lumen and into the cytosol (*4*). This event results in a fast elevation of the cytoplasmic calcium concentration. Putney in 1985 showed that calcium response elicited by different GPCR agonists actually has two components, an initial peak corresponding to calcium release from the ER and a sustained calcium entry through plasma membrane (PM; *5*).

It was seen that this calcium entry depends directly on the ER-filling state, being activated in response to ER depletion and deactivated after ER refilling (*5*). This was the reason why this phenomenon was originally named capacitative calcium entry now also known as store-operated calcium entry (SOCE). Moreover, it was found that store depletion activates at least two dif-

ferent classes of ionic currents, a highly calcium-selective current identified by Hoth and Penner in mast cells (*6*), better known as calcium release-activated calcium current (I_{crac}) and nonselective cationic currents (*7–9*). Although some of these currents were characterized more than 20 years ago, elucidating the molecular identity of the store-operated channels (SOC) and other proteins underlying SOCE has been challenging.

The study of SOCE for the last two decades has revealed many important aspects of its functioning (*10, 11*). SOCE comprises at least four fundamental steps, the first one is the sensing of ER depletion followed by the activation of SOC, then the sensing of ER-refilling and finally SOC deactivation. Surprisingly, there is a single protein involved in communicating all these processes. Stromal-interacting molecule 1 (STIM1), which we will discuss further on, resides in the ER-membrane and has a calcium binding domain facing the lumen. This domain allows this protein to sense calcium changes inside the ER (*12*). When luminal calcium decreases, STIM1 undergoes a conformational change followed by a translocation to so-called ER-PM (endoplasmic reticulum-plasma membrane) junctions, leading to the activation of the two SOC identified so far, transient receptor potential canonical channel (TRPC) and Orai (*13*). Once the ER has been refilled, calcium reassociates to the EF (E and F type helices found in the helix-helix domain) hand from STIM1, and this molecule detaches from TRPC1 and Orai, resulting in channel closure. Many studies show that Orai is the channel responsible of the I_{crac} (*14, 15*) while TRPCs appear to explain many of the nonselective cationic currents that have been described as activated in response to ER-depletion (*16*).

STIM1 appears to be a multifunctional protein, controlling the opening of SOC on one hand, and the inactivation of other channels. It has been recently shown that ER-depletion inactivates L-type voltage-gated calcium channels (VGCC) by a STIM1-mediated mechanism (*17, 18*). These results open a new scenario for the role of SOCE in the modulation of different signaling pathways as well as in the manner by which cells handle their different calcium entry pathways.

STIM1: MANY FUNCTIONS, ONLY ONE PROTEIN

STIM1 could be considered one of the most important components of the SOCE machinery. The role of STIM1 in SOCE was found by means of RNAi screenings in S2 *Drosophila* cells (*19, 20*). This key protein has multiple functions in SOCE, in summary STIM1 senses the ER calcium depletion, migrates to ER-PM junctions and activates both Orai1 and TRPCs, senses ER refilling and unbinds from SOC, deactivating calcium entry through these channels.

STIM1 has a single-pass transmembrane domain and resides mainly in the ER membrane (*21*). Its N-terminal is facing the ER lumen and contains an EF-hand, which binds calcium with a low affinity (200–600 nM), that causes calcium to be bound to STIM1 when the ER is full (near 1 mM) and unbound when stores are depleted (*22, 23*). In the ER-full state, STIM1 distributes homogeneously at the ER-membrane, while under ER depletion STIM1 oligomerizes, forming what has been described as puncta (*24–26*). STIM1 oligomerization occurs by interactions between sterile α-motifs in its N-terminal and also between coiled-coils localized in the C-terminal domains of the protein (*25, 27, 28*).

Once oligomerizated, STIM1 is translocated to regions where the ER is in close proximity to PM, although the exact mechanism controlling this translocation is still unknown, it has been reported that polybasic domains in the C-terminal of STIM1 are necessary to this relocation (*29*). The localization inside ER-PM junctions allows STIM1 to recruit SOC. The recruitment of Orai1 and TRPC1 precedes their activation (*26*). In addition, the recruitment of TRPC1 by STIM1 seems to be a requirement for the store-operated activity of this channel (*30, 31*). The ezrin-radixin-moesin domain (ERM) domain in the C-terminal of STIM1 seems to be the region that associates with TRPC1, allowing its clustering (*32*). STIM1 activates both Orai1 and TRPC1 by different mechanisms involving different domains, supporting the idea that these two channels function independently. STIM1 mediates TRPC1 gating by means of an electrostatic interaction between two lysines (K684–K685) of its lysine-rich domain and two aspartates (D639–D640) in the C-terminal of the channel (*33*). On the other hand, STIM1 activates Orai1 by a region identified simultaneously by two groups and named as STIM1-Orai activating region (SOAR; *34*) or CRAC activation domain (CAD)(*27*). This region comprises amino acids 342–448, located inside the ERM domain of STIM1 and appears to bind both the C-terminal and the N-terminal of Orai1 (*27, 34*).

One of the most important functions of SOCE is to refill the ER. Once calcium concentration inside ER has been restored, calcium binds to STIM1 via its EF hand domain. Then, STIM1 disaggregates and detaches from SOC, leading to channel closure and SOCE deactivation (*29, 35*).

Unexpectedly, STIM1 appears to have even more functions. It was recently described that this protein could bind L-type VGCC through the SOAR domain and cause inhibition and internalization of these channels in both neurons (*17*) and muscle cells (*18*). These results position STIM1 as the key protein that convey the depleted state of the ER to different classes of ionic channels at the PM. Therefore, STIM1 could be considered as the orchestrator of the activity of a variety of channels that are modulated by intracellular calcium stores.

STORE-OPERATED CHANNELS

As we mentioned before, store depletion activates two different types of currents. The first one is known as I_{crac} and is a highly calcium-selective current, showing a selectivity for calcium over sodium close to 1,000 ($P_{Ca}/P_{Na} \sim 1{,}000$), other well-known characteristics of this current are its small single channel conductance ($\sim$ 24 fS) and its strong inward rectification (*6, 36, 37*). The second current described is a nonselective cationic current, in contrast to I_{crac}, this current appears to be produced by channels with poor selectivity to calcium ions, showing calcium

over sodium ratios between 10 and 40 ($P_{Ca}/P_{Na} \sim 10–40$) and even in some of them showing a preference to sodium ($P_{Ca}/P_{Na} = 1$). Another characteristic of the channels carrying these currents is a greater single channel conductance ranging between 25 and 60 pS (*7, 38–42*).

There are two families of channels that have been related with SOCE, Orai and TRPC (*43*). While Orai channels recapitulate most of the properties of the I_{crac} current (*44–47*), TRPCs appear to explain many of the nonselective cationic currents activated after store depletion (*16, 48, 49*). Both Orai and TRPC1 are activated directly by STIM1 (*27, 33, 34*), a property that defines them as SOC.

The exact contribution of these two types of channels to SOCE appears to change from cell to cell. For example, in T-lymphocytes, a single mutation in Orai is enough to abolish completely SOCE (*44*), suggesting that in these cells, Orai is the only SOC. While in salivary gland cells, knockdown of TRPC1 causes a reduction of >60% in SOCE (*50, 51*), suggesting that in these cells TRPC1 accounts for most of the SOCE.

These differences found between diverse cells in terms of the participation of Orai and TRPC in SOCE, have generated a controversy about the operation and possibly cooperation of these two channels (*23, 52, 53*). The existence of cells exhibiting only I_{crac} with no evidence of other type of current diminishes the role of TRPC in SOCE (*54*). While reports of cells where store depletion causes the activation of nonselective cationic currents but not of I_{crac} support TRPC participation and arise questions about Orai function in these particular cells (*7, 50, 55*). Even more, there are studies showing that the inhibition of Orai expression in turn inhibits calcium entry through TRPC1 channels, leading these researchers to propose the hypothesis that Orai may function as a regulatory subunit of TRPC (*52, 56–58*).

Many of these discrepancies could be attributed to the experimental approach used by different groups. There are reports showing that I_{crac} could be masked behind nonselective cationic currents depending on experimental conditions such as the composition of intracellular solutions in patch clamp experiments (*59*). This could explain why in some cells I_{crac} cannot be detected. In addition, an interesting work recently published by Cheng et al.(*60*) shows that in human salivary gland cells, calcium entry through Orai1 initiated by store depletion causes the activation of a nonselective cationic current carried by TRPC1. Thus, Orai1 activation is required for the STIM1-mediated activation of TRPC1. This result could explain the fact that inhibition of Orai expression completely abolishes SOCE in some cells without excluding TRPC participation. These results provide a conciliatory point of view in the field, providing evidence of the relevance of both Orai and TRPC in SOCE.

ORAI IS THE UNCONDITIONAL SOC

Identified in 2006 by gene mapping on SOCE-lacking lymphocytes obtained from a patient with a severe combined immunodeficiency syndrome (*44*), Orai appears to be the channel responsible for $I_{crac.}$ Orai expression inhibition and point mutations diminish SOCE and modify SOC current properties, respectively (*47, 61–63*). And coexpression of Orai with STIM1 produces large currents in a wide variety of cells (*45, 64*).

Orai is expressed in all multicellular animals, from nematodes to human. In vertebrates, there are three members of the Orai family (Orai1-3; *44*). All of them are exclusively activated by store depletion and share many structural and functional characteristics (*65*). Orai protein has four transmembrane domains (TM), both its C-terminal and N-terminal are facing to the cytoplasm and are important for Orai/STIM1 interaction (*44, 47*). Open Orai channels appear to be tetramers, composed by four Orai monomers (*66*), where TM1 is believed to form the pore region (*67, 68*). Two glutamate residues, one in the pore region (E106) and one in TM3 (E190) are involved in the calcium selectivity of the channel (*46, 47*), while conserved glutamates in the C-terminal are involved in channel inactivation (*65*). Orai opening is activated directly by STIM1-SOAR domain which binds the C-terminal (*34*) and the N-terminal of the channel (*27*).

The current carried by Orai was described almost 15 years before than the channel was discovered. I_{crac}, which was the first store depletion-activated calcium current to be described, was first identified in rat mast cells (*6*) and later in a number of cell types (*37, 69–73*). This current is not voltage activated. In contrast, its activation depends on active or passive ER-depletion. The fact that I_{crac} can be activated by different experimental manipulations, such as intracellular application of IP3, sarcoendoplasmic reticulum calcium ATPase blocking, ionomycin, and intracellular perfusion with calcium chelators, represents an unequivocal evidence that it is the store depletion, more than second messenger production, the process that activates this current (*6, 36, 70, 72, 74*). I_{crac} has a high selectivity for calcium over monovalent cations with a reversal potential greater than +50mV and a characteristic inward rectification. Moreover, this current carries twice calcium than Sr^{2+} or Ba^{2+}. Characteristically, it exhibits a particular small unitary conductance of $\sim$ 24 fS (*37*). The current amplitude ranges between 0.5 and 1.1 pA/pF, and the time constant for activation, which is a measure from the time taken for the current to reach the 63% of the maximal steady-state level, ranges between 20 and 30 s. Furthermore the current is inhibited in a concentration-dependent manner by a variety of divalent cations in the following order, $Zn^{2+} > Cd^{2+} > Be^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+} > Sr^{2+} > Ba^{2+}$. However, the most potent blockers of this current are the trivalent cations La^{3+} and Gd^{3+} and high concentrations of 2-aminoethyldiphenyl borinate (2-APB; *75*). I_{crac} also exhibits a calcium dependent inactivation (*36*).

TRPCS ARE CONDITIONALS SOCS

The other channels activated by store depletion are some members of the TRPC family. TRPC are nonselective calcium permeable cation channels which are in mammals the most related to the

Trp channel originally described in Drosophila (*76*). In fact, *Trp* was the first SOC to be described with the demonstration that it could be activated after ER-depletion with the application of thapsigargin (TG; *77*). Since this report was published, *Trp* homologues have been identified in a wide variety of organisms, resulting in a new superfamily of channels (*25, 26*).

TRPC subfamily is composed of seven members (TRPC 1–7). Although only TRPC1 has been shown to be operated by store depletion, there is evidence showing that other members could be part of SOC depending on heteromultimerization properties (*49*). TRPCs are constituted by four monomers, each one having six transmembrane domains (1TM–6TM), where TM5 and TM6 may associate to form the conducting pore, in analogy to potassium channels.

TRPC associate to produce homomultimers or heteromultimers of four subunits but with certain restrictions. For example, TRPC1 associates only with TRPC3, TRPC4, and TRPC5 (*78, 79*). As heteromultimerization could lead to changes in the properties of the channels (*79*), it may influence also the sensitivity to STIM1.

TRPC genes produce nonselective cationic channels poorly selective to calcium with P_{Ca}/P_{Na} between 0 and 9 (*80, 81*). Conductances of TRPC channels range between 16 and 75 pS depending on subunit composition. TRPC1 unitary conductance is estimated to be 16 pS (*9*), while TRPC4 has a conductance of 41 pS and TRPC5 of 63 pS (*81, 82*). TRPC are inhibited by low micromolar concentrations of 2-APB, by SKF96365 and by high concentrations of Gd^{3+} (*49*).

In contrast with Orai, which are exclusively activated by STIM1, under certain conditions TRPC could be activated also by G protein-coupled receptors in a STIM1-independent manner (*59, 83, 84*). In this form of activation, the channels are named as receptor-operated channel (ROC) as opposed to SOC. For example, channels lacking the TRPC1 subunit could be activated directly by diacylglycerol (DAG), a product of phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis (*85*). While channels containing TRPC1 may be activated by DAG (*79*), PKC-dependent phosphorylation (*86, 87*) and direct PIP2 interaction (*88*). These multiple forms of activation are closely related with channel subunit composition (*89*) and have made extremely difficult to demonstrate convincingly that TRPCs may function as SOC. However, it has been demonstrated that the interaction with STIM1 (*30, 31*) and Orai1 (*57, 58*) converts the TRPC operation mode from ROC to SOC. This is the reason why we would classify this channel as a conditional SOC.

The relevance of TRPC family in SOCE was established by experiments in which the overexpression of different TRPC increased calcium entry, while the expression of antisense cDNA of the same channels diminishes it (*90*). Zitt and coworkers identified TRPC1 as the first SOC in mammals (*9*), they demonstrated that TRPC1 was activated by depletion of intracellular stores after intracellular application of IP3 or by TG (*9*). Subsequently, manipulations of TRPC1 expression showed its key role in SOCE in salivary gland cells (*29, 50, 91*). There is also experimental evidence positioning TRPC4 and TRPC5 as SOC in excitable cells (*92, 93*). Other members of the TRPC family have been reported to act as SOC; in fact, only TRPC2 and TRPC6 have not been related to SOCE (*49, 55, 94*).

Perhaps one of the most conclusive evidence about the SOC activity of TRPC1 was the identification of the mechanism by which STIM1 activates this channel. Gating of TRPC1 requires an electrostatic interaction between two aspartates in its C-terminal (D639–D640) and two lysines (K684–K685) of the lysine-rich domain from STIM1 (*33*). It has been suggested by the authors that TRPC1 activation implies a removal of a preexisting channel-inhibition mediated by the intermolecular electrostatic interaction with STIM1.

There are many reports showing the activation of nonselective cationic currents in response to store depletion. Cationic currents with P_{Ca}/P_{Na} ratios between 1 and 40 and conductances ranging between 25 and 60 pS have been found in vascular smooth muscle cells (*8, 38, 95*), airway smooth muscle cells (*40, 41*), human submandibular gland cells, and parotid gland cells (*42*). Although not all these preparations have been tested for the participation of TRPC in these currents, TRPC channels have been detected in all these tissues (*50, 96, 97*). In the case of pulmonary arterial smooth muscle, it has been demonstrated that TRPC1 is the channel carrying this nonselective cationic current (*98*). Many experimental evidence in a wide variety of cells and tissues point to TRPC as channels responsible for the nonselective cationic currents activated after ER depletion (*16, 49, 51, 56, 94, 99, 100*). Differential expression of TRPC isoforms and formation of heterotetrameric channels could lead to the different cationic currents observed in diverse tissues.

STORE-INHIBITED CHANNELS

Channels that are activated in response to ER-depletion have been studied for the last two decades. However, a new class of channels that are inhibited by the same phenomenon has been recently discovered. The first member identified, of what could be named as store-inhibited channels (SIC), is the L-type VGCC. It was demonstrated that in cortical rat neurons (*17*) and vascular smooth muscle cells (*18*), the depolarization-induced opening of L-type VGCC is inhibited by store depletion in a STIM1-dependent way. The mechanism by which STIM1 inhibits L-type calcium currents appears to be mediated by the SOAR domain, the same region responsible for Orai activation. Coimmunoprecipitation experiments show that SOAR binds to the C-terminal of L-type VGCC, and that this domain is necessary and sufficient to cause channel inhibition. Furthermore, STIM1 seems to induce L-type VGCC internalization as a second mechanism to control these channels in a longer lasting mode.

An interesting finding by Wang and coworkers (*18*), is a possible participation of Orai1 in the mechanism of L-type VGCC inhibition by STIM1. They found that Orai1 colocalizes with STIM1 and L-type VGCC in ER-PM junctions after store depletion. Moreover, inhibition of STIM1 expression alone fails

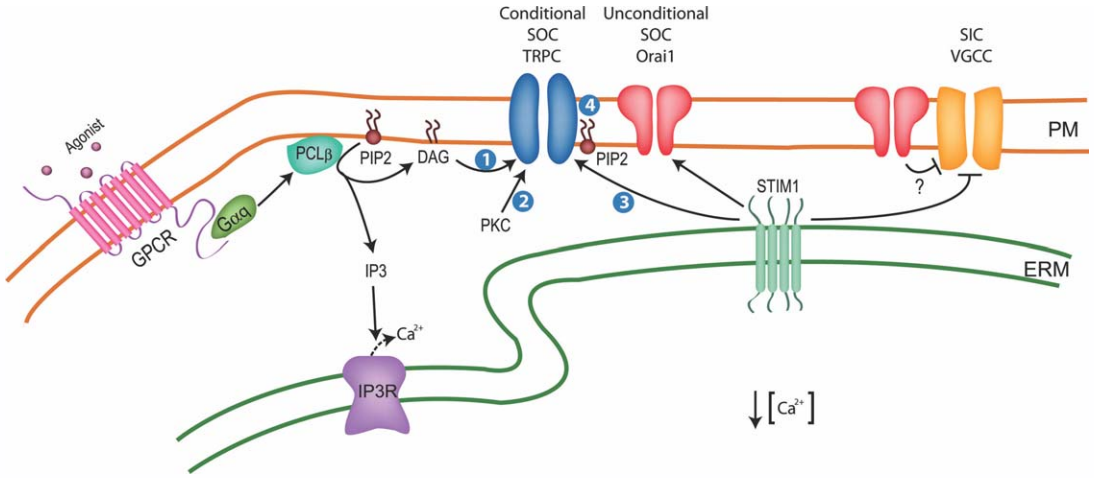

Figure 1. Channels regulated by store depletion. Agonist binding to Gαq-coupled receptors activates PLCβ, resulting in the production of IP3 and DAG from PIP2. In turn, IP3 induces calcium release from intracellular calcium stores. Depletion of the ER is followed by oligomerization of STIM1 and translocation of this protein to ER-PM junctions. Store depletion has two different effects over PM channels, both of them mediated by STIM1, it activates SOCs (TRPCs and Orai1) while inhibits SIC (L-type VGCC). Orai1 acts as an *Unconditional SOC,* since it is exclusively activated by STIM1 while TRPCs operate as *Conditional SOCs,* activated by different effectors such as (1) DAG, (2) PKC, (3) STIM1 and (4) PIP2. ERM, endoplasmic reticulum membrane. PM, plasma membrane.

to abolish L-type VGCC inhibition while simultaneous inhibition of STIM1 and Orai1 results in a reduction of L-type VGCC inhibition by store depletion. Orai seems to be necessary for STIM1/L-type VGCC interaction, since a SOAR mutant (which cannot interact with Orai1) is unable to inhibit L-type VGCC, strongly suggesting that Orai-STIM1 interaction is required for the STIM1-mediated inhibition of VGCC. Given the fact that nonconducting mutants of Orai1 still participate in the inhibition of VGCC, it could be suggested that Orai1 may be working as a scaffolding or coupling component, bringing STIM1 and L-type VGCC closer or interacting directly with the calcium channel to inhibit it. Interestingly, it has been recently shown that Orai functions as a regulator of TRPC channel activity (*52, 57*).

The elucidation of SIC has important implications for calcium signaling in excitable and nonexcitable cells. VGCC are the most important calcium carriers in excitable cells, such as neurons or muscle cells, whereas SOC are expressed in a lesser level. On the other hand, nonexcitable cells have a reduced (or absent) number of VGCC and in counterpart express high levels of SOC, which causes SOCE to be the main calcium entry in many of these cells. Then, reciprocal regulation of SOC and SIC by STIM1 could function as a way to fine tune calcium entry in cells expressing the two types of channels.

Since store depletion reciprocally regulates SOC and SIC, it could be expected that alterations in store depletion signaling lead to pathologies related with the calcium pathways downstream these channels. In fact, mutations in both STIM1 and Orai1 cause severe immunodeficiency syndromes associated with the lack of SOCE. However, the effect on processes related with L-type VGCC, such as synaptic transmission or muscle contraction, remains to be investigated.

CONCLUSIONS

Store depletion has two (apparently) opposite effects on PM channels. On one hand, it causes opening of SOC leading to SOCE, whereas on the other hand, it inhibits SIC modulating the voltage-dependent calcium entry. As we discussed here, both Orai and TRPC function as SOC with differences in store operation exclusivity and in biophysical properties. While these channels are opened, L-type VGCCs channels are inhibited by the same signaling mechanism (store depletion). Thus, store depletion acts as a switch between these two forms of calcium entry. The orchestrator of these two processes is the protein STIM1, which interacts directly with both SOC and SIC. In both forms of interaction Orai protein seems to play a crucial role, apparently working as a channel (SOC) or a linker (SIC). Figure 1 summarizes how store depletion differentially regulates these channels. The reciprocal control of SOC and SIC by stores may have functional implications in cells expressing both types of channels, as SOCE and VGCC activate different signaling cascades. Further studies about other channels modulated by store depletion may enhance our understanding of cell calcium signaling.

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Microdomain Organization and the Role of Second Messengers

7

Microdomain Organization of SOCE Signaling

Claudia Moreno and Luis Vaca

7.1 Introduction

Since the original model proposed by Putney (1986) about the so called store-operated Ca^{2+} entry (SOCE), the existence of specialized regions where endoplasmic reticulum (ER) and plasma membranes (PM) are closely apposed seems a natural requirement. The study for the last 25 years of this Ca^{2+} entry has clarified some of the mechanisms underlying this process (Potier and Trebak 2008; Putney 2007, 2009). Many studies have confirmed that in fact SOCE occurs in ER-PM junctions. These junctions function as specialized microdomains where several molecules coordinate Ca^{2+} entry in a highly regulated fashion (Vaca 2010). However, many of these components have been described only recently.

The functional unit of SOCE could be envisioned as a macromolecular complex that assembles in response to ER depletion, allowing Ca^{2+} influx from the extracellular space. Activators, effectors and regulators that intimately interact to circumscribe SOCE spatiotemporally compose this complex. We have named recently this complex as SOCIC (Store-operated Calcium Influx Complex) (Vaca 2010). Considering that under physiological conditions Ca^{2+} entry is transient, SOCIC should be a dynamic structure that goes through assembly and disassembly states depending on cell requirements. After store depletion, many SOCIC components are assembled, in an organized fashion, associating at discrete regions on the cell, forming microdomains of SOCE signaling. Thus, it is possible to define SOCE microdomains as restricted ER-PM junctions where SOCIC components assemble and converge in response to ER depletion, in order to facilitate and optimize Ca^{2+} influx.

C. Moreno • L. Vaca (✉)
Departamento de Biología Celular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México city, DF, Mexico
e-mail: lvaca@ifc.unam.mx

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In this chapter we will discuss the evidence supporting the idea that SOCE occurs in restricted microdomains in the cell. Firstly, we will introduce the SOCIC components that have been identified until now. Then, we will illustrate some ideas on how the complex is assembled and disassembled. We will then address the evidence pointing at the role of molecules and events that trigger SOCIC assembly as well as its disassembly. We will present evidence supporting the key role of lipid rafts in the formation of these microdomains and finally, we will discuss some physiological and pathological implications of the microdomain organization of SOCE.

7.2 SOCIC, the Store-operated Ca^{2+} Influx Complex

The restriction of SOCE to specific cell microdomains in the PM implies recruitment of the molecules underlying the process and generation of fast and localized Ca^{2+} elevations near the PM. In order to show that SOCE is spatially restricted, we performed experiments in HEK293 cells in which Ca^{2+} elevations near the plasma membrane were monitored using a fluorescent acetoxymethyl ester (FFP-18-AM) that associates preferentially to the PM. We explored Ca^{2+} elevations at two regions, one enriched in STIM1-YFP and other where there was no STIM1-YFP in the vicinity of the PM (Fig. 7.1a).

Activation of SOCE after thapsigargin administration induced a higher and faster Ca^{2+} elevation at the region where STIM1 was near the PM, as compared to regions where both membranes were apart (Fig. 7.1a). Kinetic analyses of Ca^{2+} rise at these regions showed that in fact Ca^{2+} elevations outside the microdomains were delayed by almost 20s after SOCE activation (Fig. 7.1b), while in the STIM1-enriched microdomains, Ca^{2+} increase reached its maximal level in only 2s (Fig. 7.1a). Removing Ca^{2+} from bath solution prevented the fluorescence increments observed when extracellular Ca^{2+} was present. These results indicate that the increments in fluorescence reflect more significantly Ca^{2+} entry, and were not due to Ca^{2+} release from the ER (Fig. 7.1d, e). An important aspect is that for the proper function of SOCE all molecular components responsible for the Ca^{2+} entry should assemble at these microdomains.

The study of SOCE in the last five years has led to the conclusion that the process depends on the association of several molecules (Vaca 2010). These molecules converge spatiotemporally in response to ER depletion at specific microdomains, visualized as puncta near the PM (Liou et al. 2005). Inside microdomains these molecules appeared to be arranged as many copies of a common complex (Deng et al. 2009; Lee et al. 2009a; Luik et al. 2006; Ong et al. 2007). This complex, that we have recently named SOCIC, has been proposed to be the functional unit of SOCE (Vaca 2010).

SOCIC consists of the ER- Ca^{2+} sensor and channel activator STIM1, and the PM- Ca^{2+} channels TRPC1 and Orai1 (Putney 2007). Although it has been demonstrated that these three proteins are necessary and sufficient to produce SOCE, more SOCIC components have been identified. They include regulators such as

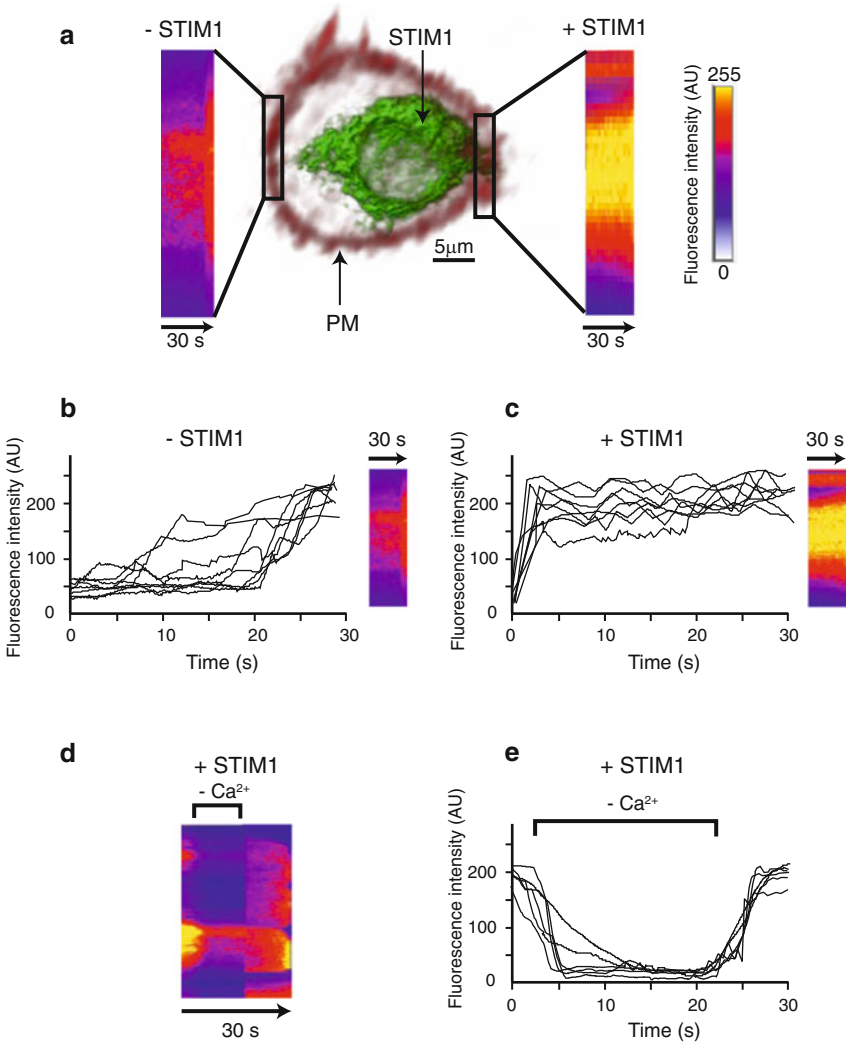

Fig. 7.1 Visualization of Ca^{2+} entry into microdomains in living cells. (**a**) Simultaneous measurement of STIM-YFP, FM-464 (PM fluorescent marker) and Ca^{2+} by confocal microscopy in HEK293 cells. Rapid Ca^{2+} increments near the PM were monitored using the membrane-delimited Ca^{2+} indicator FFP-18 AM. Fluorescence was monitored in line-scan mode to rapidly measure Ca^{2+} increments near the PM. Ca^{2+} increments were induced by stimulation with thapsigargin (TG) 200nM. The lateral upper panel shows subplasmalemmal Ca^{2+} elevation at a region corresponding to a SOCE microdomain where STIM1 is in close proximity to the PM (*right*), and at a region outside the microdomain, where STIM1 is far from the PM (*left*). The pseudo-color scale shows fluorescence values. (**b**) Time courses of Ca^{2+} elevations plotted in fluorescence arbitrary units (AU) outside SOCE microdomain (−STIM) and (**c**) inside SOCE microdomain (+STIM1). Each line represents an independent measurement obtained from a different cell. Notice the rapid fluorescence increment observed inside microdomains and the delayed increment outside the domain (**d**) Representative experiment illustrating the effect of removing extracellular Ca^{2+} in

calmodulin (CaM) (Mullins et al. 2009), CRACR2A (Srikanth et al. 2010) and the golli protein (Walsh et al. 2010), and proteins related with SOCE physiological functions such as SERCA (Sampieri et al. 2009) and adenylyl cyclase (Lefkimmiatis et al. 2009). Moreover, there is evidence showing that more components could be involved (Varnai et al. 2007). Figure 7.2 illustrates a proposed model of SOCE microdomains organization in the cell. The SOCIC components identified thus far are arranged in the model, accordingly to their functional associations reported in the literature. Within the next sections we will discuss the evidence supporting the role of the different SOCIC components in the assembly and modulation of Ca^{2+} microdomains.

7.2.1 STIM1, Communicating the Depleted State of the ER to the PM

STIM1 (stromal interacting molecule 1) could be considered one of the most important components of SOCIC. The key role of STIM1 in SOCE was found by means of RNAi screenings in S2 *Drosophila* cells (Roos et al. 2005). STIM1 has two determinant functions in SOCE, the first one is to sense the ER Ca^{2+} depletion and the other is to activate the store-operated channels (SOC) (Zhang et al. 2005). Both functions convey the depleted state of the ER to the PM.

STIM1 is a single-pass transmembrane protein, which resides mainly in ER membrane. Until now several domains within the STIM1 structure, which are important for its function and for the interaction with other SOCIC components, have been identified (Lee et al. 2009a; Dziadek and Johnstone 2007). The STIM1 N-terminal is facing the ER lumen and contains classical and hidden Ca^{2+} binding EF- hands (67–96) and a sterile α-motif (SAM, 132–200). The classical EF-hand binds Ca^{2+} with low affinity (200–600 nM), therefore when the ER is full (near 1 mM) Ca^{2+} is bounded to STIM1 and the protein is in its inactive state (dissociated from the SOCIC). Ca^{2+} unbinding causes a conformational change in STIM1 resulting in the oligomerization of this protein, and initiates a process that leads to SOCE activation (Liou et al. 2005; Williams et al. 2001). The hidden EF-hand and SAM domain interact to facilitate STIM1 clustering (see also Chap. 2, Stathopulos et al. 2008).

STIM1 C-terminal includes a serine/proline-rich domain (600–629) possibly involved in cytoskeleton interactions (Grigoriev et al. 2008) and a lysine-rich

Fig. 7.1 (continued) the fluorescent signals at a SOCE microdomain (area with STIM1 in close proximity to the PM). Notice that this maneuver results in rapid decrement of the fluorescence, indicating that most of the signal results from Ca^{2+} influx. (**e**) Time courses of Ca^{2+} elevations obtained from at least seven independent cells at SOCE microdomains illustrating the effect of removing extracellular Ca^{2+} in the fluorescent signal

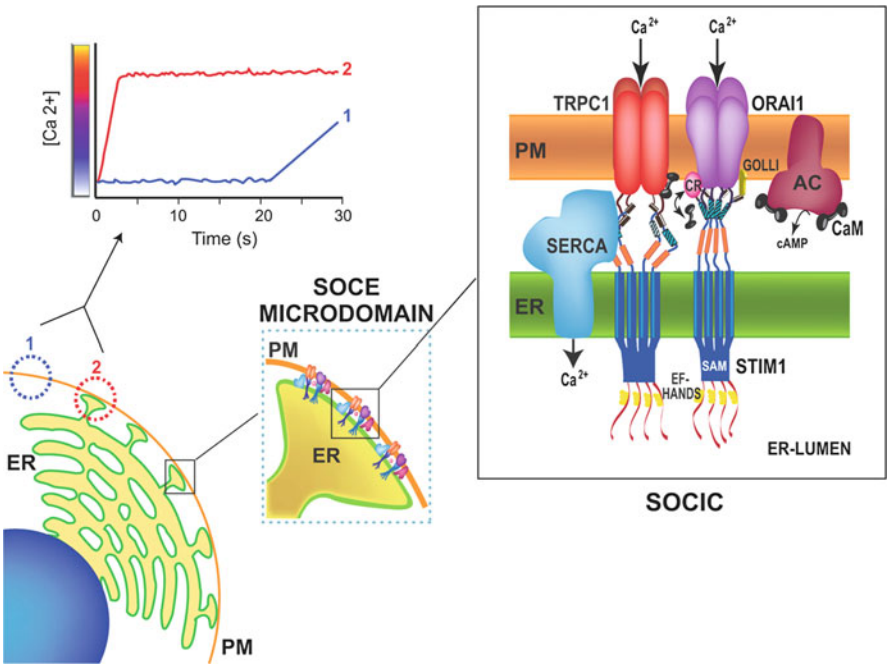

Fig. 7.2 A model of SOCE microdomain organization. Inside the cell, endoplasmic reticulum (ER) is organized in such a way that some regions are in close proximity to the plasma membrane (PM). Under ER depletion, these regions function as SOCE microdomains where all molecules underlying Ca^{2+} entry converge. Inside microdomains these molecules are arranged as many copies of a store-operated Ca^{2+} influx complex (SOCIC). The inset shows a model of SOCIC organization taking into account the possible components identified so far. This complex include the Ca^{2+}-sensor and channel activator STIM1, the PM-channels TRPC1 and Orai1, the regulator proteins calmodulin (CaM), CRACR2A (CR) and golli and the proteins related with SOCE physiological functions, such as SERCA and adenylyl cyclase (AC). Restriction of SOCE machinery to microdomains causes localized Ca^{2+} elevations (*top-left*), thus inside microdomains, Ca^{2+} concentration increases faster than outside as illustrated in experiments of Fig. 7.1. This SOCIC localization may favor also the generation of gradients for Ca^{2+}, cAMP and possibly other second messengers, as shown in different reports (Golovina 2005; Willoughby and Cooper 2007; Willoughby et al. 2010)

domain (672–685) mediating STIM1 anchoring to the PM (Liou et al. 2007) and TRPC gating (Zeng et al. 2008). These polybasic domains are also important for STIM1 translocation to the ER-PM junctions (Liou et al. 2007). Moreover, there is an ERM domain (251–535) encompassing three adjacent alpha-helical coiled-coils. Inside this large domain there is the region responsible for Orai activation (known as SOAR or CAD) (Park et al. 2009; Yuan et al. 2009) and a region related with Ca^{2+}-dependent inactivation of Orai (470–491) (Mullins et al. 2009).

7.2.2 Orai1 and TRPC1, the Store-operated Ca^{2+} Channels

Another important component of SOCIC is the channel that allows Ca^{2+} influx through the PM. The evidence gathered so far strongly suggests that there are at least two channels underlying SOCE, the TRPC1 and the Orai.

Since the finding that TRP channels from Drosophila can be activated after depleting intracellular Ca^{2+} stores (Vaca et al. 1994), some members of this family were considered the most likely candidates for the elusive SOC. The Chaps. 5 and 6 review the role of the TRPC subfamily in SOCE. Many members from the TRPC subfamily have been shown to function as SOC (Salido et al. 2009). However, the role of these channels in SOCE has been controversial, because there are contradictory reports on their activation by ER depletion (Salido et al. 2009). In spite of this controversy there is accumulated evidence over the last 10 years that positions TRPC1 as a strong candidate to form SOC (Ambudkar et al. 2007). A conciliatory point of view is that TRPC1 could have at least two possible configurations, one as a SOCIC component and another as a SOCIC-independent channel (Alicia et al. 2008).

TRPC1 is constituted by four monomers, each one has six transmembrane domains (1TM-6TM), TM5 and TM6 may associate to form the conducting pore, in analogy to potassium channels. Inside the TRPC1 structure there have been found some important domains for the interaction with several of the SOCIC components (Rychkov and Barritt 2007; Zeng et al. 2008). The C-terminus of TRPC1 contains two aspartates (639 and 640) that interact with the lysine-rich domain of STIM1 (Zeng et al. 2008), two CaM binding sites (719–751 and 758–793) (Singh et al. 2002) and SERCA associating regions (Golovina 2005).

The other channel involved in SOCE is the Orai, a novel protein discovered by gene mapping on lymphocytes obtained from a patient with a severe combined immunodeficiency syndrome (Feske et al. 2006). In vertebrates, there are three homologues named Orai1, 2 and 3. Although these proteins share most of their structural and functional characteristics (Gwack et al. 2007), Orai1 has been the most studied (see also Chap. 3) and for this reason we will only refer to it throughout this chapter. There is solid evidence accumulated over the last few years strongly suggesting that Orai1 is the molecular entity responsible for the Ca^{2+}-release activated current identified by Hoth and Penner (1992) and that it is an essential component of SOCE (Feske et al. 2006; Vig et al. 2006a, 2006b). Point mutations in Orai1 alter SOC current properties (Gwack et al. 2007; Yeromin et al. 2006), and co-expression with STIM1 produce large currents in a wide variety of cells (Peinelt et al. 2006). Orai1 has four transmembrane domains and is located at the PM, it has been suggested that Orai1 forms dimers at rest and tetramers following STIM1 interaction after ER depletion (Penna et al. 2008). About the most important structural domains for Orai1 function, two glutamate residues (106 and 190) are involved in Ca^{2+} selectivity of the channel (Prakriya et al. 2006; Vig et al. 2006b) while its C-terminus is important for recruitment into the SOCIC (Muik et al. 2008) and for STIM1 activation (Muik et al. 2008; Yuan et al. 2009) (see also Chap. 4). Conserved glutamates in the C-terminus are involved in channel inactivation

(Lee et al. 2009b). On the other hand, the N-terminus contains a proline-rich region that appears to be involved in a negative regulation mediated by STIM1 (Yuan et al. 2009) and a CaM-binding site (Mullins et al. 2009).

Recently, it has been demonstrated by FRET experiments that an association between TRPC1 and Orai occurs after ER-depletion (Vaca 2010). Although it has been suggested that Orai1 and TRPC1 could bind to form a functional SOC (Liao et al. 2007), new data suggest that both channels may also work independently (Hong et al. 2011).

7.2.3 CaM, CRACR2A and Other SOCE Regulators

The first report showing SOC modulation by CaM came from studies with endothelial cells (Vaca 1996). CaM is a small Ca^{2+}-binding protein involved in many biological processes. It has four EF-hands separated in two globular domains with a short linker domain between them. Ca^{2+} binding to EF-hands causes a conformational change in CaM allowing exposure of hydrophobic residues. These residues are important for the interaction of CaM with other proteins (Chin and Means 2000).

The role of CaM as a SOCIC component is confirmed by its interaction with many components of this complex. CaM interacts with the lysine-rich domain of STIM1. It is believed that this interaction could be involved in the control of STIM1 translocation to ER-PM junctions or in STIM-Orai1 disassembling (Bauer et al. 2008). It has been reported that CaM binds to a region in the C-terminal of TRPC1 (758–793) thus regulating Ca^{2+}-dependent inactivation of this channel (Singh et al. 2002). Furthermore, CaM-TRPC1 interaction appears to have an inhibiting effect modulating the delay period between ER depletion and SOC activation (Vaca and Sampieri 2002). CaM interacts with Orai1 also. CaM binds to a region (68–91) in the N-terminus of Orai1 and mediates the Ca^{2+}-dependent inactivation of this channel too (Mullins et al. 2009; Litjens et al. 2004). Thus, it is widely accepted now that CaM exerts a negative control over SOCE, via interactions with TRPC1 (Singh et al. 2002), Orai1 (Mullins et al. 2009; Litjens et al. 2004) or both.

Moreover, CaM binds to the N and C-terminal of adenylyl cyclase 8 (AC8) governing its Ca^{2+}-sensibility (Gu and Cooper 1999). This fact deserves consideration here because, as we will discuss later, there is recent evidence positioning AC8 as a novel member of the SOCIC.

Using immunoaffinity protein purification in Jurkat T cells, Srikanth et al. (2010) identified CRACR2A, another SOCIC component. This protein acts as a SOCE regulator stabilizing STIM1-Orai1 interaction. CRACR2A is a cytoplasmic Ca^{2+}-binding protein that contains two EF-hand domains in its N-terminus. This protein binds to the N-terminus of Orai1 and the polybasic-rich domains of STIM1, forming a ternary complex that dissociates at high Ca^{2+} concentrations. Ca^{2+} binding to the EF-hand motifs from CRACR2A reduces the interaction of this protein with Orai1 and STIM1. siRNA assays showed that CRACR2A is important for clustering of Orai1 and STIM1 at ER-PM junctions after ER depletion.

CRACR2A is the first described regulator of STIM1-Orai1 interaction. However, it is not yet known if CRACR2A is involved in translocation of Orai1 and STIM1 to ER-PM junctions or if it is recruited after STIM1-Orai1 assembly.

Another protein that seems to have a regulating role in SOCE is golli, a member of the MBP (myelin basic protein) family. Golli regulates negatively Ca^{2+} influx after store depletion in T-lymphocytes (Feng et al. 2006). Immunoprecipitation experiments have recently shown that golli directly interacts with the C-terminus of STIM1 (Walsh et al. 2010). This interaction appears to be modulated by the intracellular Ca^{2+} concentration. It is suggested that after SOCE activation, and once high Ca^{2+} levels are reached, golli interacts with STIM1-Orai1 complexes to reduce Ca^{2+} influx, but the molecular mechanism underlying this interaction has not been defined yet.

7.2.4 SERCA, Refilling the ER

One of the most important functions of SOCE is to refill the depleted ER. It has been seen that SOCE activation occurs at ER-PM junctions where Ca^{2+} concentration transiently increases, avoiding a global Ca^{2+} elevation (Golovina 2005). This spatial restriction relies in the efficient ER Ca^{2+} recapture mediated by SERCA (Jousset et al. 2007). Indeed, it has been recently demonstrated that SERCA is a SOCIC component recruited to ER-PM junctions after store depletion (Sampieri et al. 2009). SERCA might be an important component of the complex since ER refilling is necessary to sustain cell response to repetitive stimulation by agonists.

SERCA transports Ca^{2+} ions to the lumen of the ER in order to keep low Ca^{2+} concentration at the cytoplasm and to concentrate Ca^{2+} inside the ER. SERCA is composed of one transmembrane domain consisting of 10 helices wherein Ca^{2+} binds, and three cytoplasmic domains classified in an actuator domain, a phosphorylation domain and a nucleotide-binding domain (Wuytack et al. 2002).

There are three known isoforms of SERCA. Concerning to interaction of SERCA with other SOCIC components, it has been shown that SERCA2 and SERCA3 associate with STIM1 probably by an interaction with its C-terminus (Lopez et al. 2008; Sampieri et al. 2009). Coimmunoprecipitation experiments have shown interactions between SERCA-TRPC1 and SERCA-Orai1 in human platelets, reinforcing the idea that all of these proteins are part of SOCIC (Redondo et al. 2008b).

7.2.5 Adenylyl Cyclase, SOCE Downstream

Although ER-refilling is one of the most important functions of SOCE, Ca^{2+} elevation in microdomains could activate different Ca^{2+}-dependent signaling pathways, giving SOCE the capacity to generate a variety of downstream effects. That is the case for cAMP-signaling, which under certain conditions is specifically stimulated by SOCE (Cooper et al. 1994). SOCE may increase cAMP synthesis by

means of recruiting adenylyl cyclase 8 (AC8) into the SOCIC, even though this has not been explicitly shown (Fagan et al. 2000; Fagan et al. 1996; Lefkimmiatis et al. 2009; Willoughby et al. 2010). Ca^{2+} entry through SOC then stimulates AC8 and potentiates cAMP production. As Ca^{2+} and cAMP are two fundamental second messengers of G-protein-coupled receptor signaling, regulation of cAMP by SOCE could be a way to govern a vast array of cellular functions.

AC8 is one of the nine transmembrane isoforms of adenylyl cyclases. The protein has two transmembrane domains containing six helices and a large cytoplasmic catalytic loop (Sunahara and Taussig 2002; Willoughby and Cooper 2007). AC8 is stimulated via Ca^{2+}-CaM, which bind to the N and C-terminal binding-sites (Gu and Cooper 1999). It has been recently reported that the regulation of AC8 by SOCE requires STIM1 clustering, although direct interaction between these proteins has not been detected thus far (Lefkimmiatis et al. 2009). AC8 also colocalizes with Orai1 and that this colocalization occurs in lipid rafts (Martin et al. 2009), the relevance of these lipid domains in SOCIC assembly will be discussed later. Stimulation of AC8 causes an increment in cAMP production and activation of PKA being the most important link between SOCE and different signaling pathways downstream SOCE.

7.3 Microdomains Assembly

Physiological activation of SOCE (via agonists and hormones, as opposed to the use of SERCA blockers like thapsigargin) is transient and occurs when an agonist binds to a GPCR-coupled receptor, inducing IP_3 production and consequently Ca^{2+} release from the ER. As we mentioned before, SOCE requires SOCIC assembly at microdomains but SOCIC components appear to be spatially distant under resting conditions (Baba et al. 2006; Deng et al. 2009; Ong et al. 2007; Sampieri et al. 2009). STIM1 and SERCA reside at the ER membrane while Orai1, TRPC1 and AC localize in the PM. Other components as CRACR2A and CaM are freely distributed in the cytoplasm. Since physical interaction of these proteins is necessary to induce SOCE, a tightly controlled assembly mechanism is required. Although this mechanism is not completely understood, a logical step sequence of SOCIC assembly has been recently proposed (Vaca 2010). (i) STIM1 senses ER-Ca^{2+} depletion. (ii) STIM1 oligomerizes. (iii) STIM1 translocates to ER-PM junctions. (iv) STIM1 recruits SOC to microdomains. (v) SOC are activated by STIM1 and Ca^{2+} influx starts. (vi) CRAC2RA stabilizes STIM1-SOC interaction. (vii) SERCA and AC8 arrive to microdomains and assemble into SOCIC. (viii) CaM and golli integrate to SOCIC and modulate Ca^{2+} entry.

We can envision STIM1 as the first SOCIC component involved in triggering SOCE. When the ER is full, STIM1 distributes homogeneously at the ER-membrane and exhibits constitutive movements. However, when ER is depleted STIM1 stops moving and clusters (Liou et al. 2005; Baba et al. 2006; Sampieri et al. 2009). The EF-hand domain in the N-terminus enables STIM1 to sense changes in Ca^{2+} concentration inside the ER (Williams et al. 2001). For instance mutations altering

Ca^{2+}-binding capacity cause STIM1 clustering and constitutive SOCE activation (Liou et al. 2005; Zhang et al. 2005). When the ER is full, the Ca^{2+}-bound EF-hand establishes intimate hydrophobic interactions with the SAM domain. Ca^{2+} unbinding destabilizes these interactions and leads to the exposure of hydrophobic residues (see also Chap. 2). As interaction of these residues with the aqueous environment is unfavorable, then oligomerization of STIM1 is promoted (Stathopulos et al. 2008). Coiled-coils at the C-terminal of STIM1 are also required for oligomerization, showing that the initial change induced by Ca^{2+} releasing has also an effect in the C-terminus of the protein (Park et al. 2009). As we stated before, SOCE microdomains correspond to the experimentally observed puncta. It is important to emphasize that STIM1 oligomerization is an initial step of microdomain assembly and it occurs before puncta is evident. Indeed, FRET experiments have shown that STIM1 oligomerization precedes its translocation to the ER-PM junctions (Liou et al. 2007).

Once STIM1 oligomerizes, it migrates to ER-PM junctions (Liou et al. 2005; Luik et al. 2006; Wu et al. 2006; Zhang et al. 2005). The mechanism underlying STIM1-oligomers translocation is one of the less understood steps in the microdomain assembly. However, it has been reported that polybasic-domain removal prevents STIM1 translocation. Therefore it has been proposed that oligomerization causes a conformational change resulting in the polybasic domain exposure, and allowing an interaction with unknown proteins in charge of STIM1 translocation to ER-PM junctions (Liou et al. 2007). It is known that microdomain assembly occurs at regions where the ER and PM are in close proximity (10–25 nm) (Golovina 2005; Jaconi et al. 1997; Luik et al. 2006; Wu et al. 2006). Some of the ER-PM junctions already exist before ER-depletion and seem to be formed by specialized compartments of the ER (Lur et al. 2009), most likely interacting with specialized cytoskeleton. However it has been reported that at least one third of ER-PM junctions are formed after ER depletion (Orci et al. 2009). STIM1 appears to play an important role in remodeling the ER and then in the establishment of these new junctions, apparently by the interaction with the microtubule-plus-end tracking protein EB1 (Grigoriev et al. 2008). In fact, depletion of STIM1 and EB1 decreases ER-PM junction formation (Grigoriev et al. 2008). Interestingly, it has been found that STIM1 binds EB1 through its polybasic-rich domain (Grigoriev et al. 2008) and that this interaction is lost as long as puncta are evident (Sampieri et al. 2009). This evidence suggests that EB1 might be one of the STIM1-translocators. When STIM1 arrives at the ER-PM junction, dissociation from EB1 may be necessary for its interaction with other SOCIC components. ER remodeling mediated by the interaction between ER and microtubule-associated proteins could be a possible mechanism for EB1 to bring STIM1 closer to the PM.

Once STIM1 is at ER-PM junctions, the recruitment of Orai1, TRPC1 or both is needed to form a functional channel. The incorporation of these channels into microdomains is mediated by STIM1 and precedes their activation (Sampieri et al. 2009). Recruitment of TRPC1 by STIM1 seems to be a requirement for the operation of TRPC1 as a SOC (Alicia et al. 2008; Lee et al. 2010). The ERM domain of the C-terminal of STIM1 seems to be the region that associates with

TRPC1, allowing its clustering (Huang et al. 2006). Anchoring of TRPC1 with caveolin before STIM1 binding seems to be also important for this interaction (Pani et al. 2009). Concerning Orai1, it has been reported that in resting conditions this protein arranges in dimers homogeneously distributed at the PM, after ER depletion Orai1 forms tetramers that co-cluster with STIM1 at microdomains (Luik et al. 2006; Penna et al. 2008; Sampieri et al. 2009). The fact that Orai1 clustering requires STIM1 co-expression suggests that it is recruited at microdomains by binding to STIM1 (Xu et al. 2006). Indeed, it has been shown that amino acids 342–440 inside the ERM domain of STIM1 are responsible of Orai1 clustering, since expression of this region leads to Orai1 clustering without channel activation (Park et al. 2009). The fact that SOC clustering is not sufficient to initiate Ca^{2+} influx implies that these two events may be independent.

STIM1 mediates through different mechanisms TRPC1 and Orai1 activation, a fact that supports the idea that these proteins may function as independent channels. Gating of TRPC1 requires an electrostatic interaction between two aspartates in its C-terminal (639–640) and two lysines (684–685) of the lysine-rich domain of STIM1 (Zeng et al. 2008). The authors propose a model for TRPC1 activation that implies that STIM1 removes a preexisting channel-inhibition by means of this intermolecular electrostatic interaction, allowing channel opening. On the other hand, Orai1 is activated by a region comprising amino acids 342–448, located inside the ERM domain of STIM1 (Park et al. 2009; Yuan et al. 2009). This activating domain, also known as SOAR or CAD, seems to bind both the C-terminal (Yuan et al. 2009) and the N-terminal strands of Orai1 (Park et al. 2009).

Even though STIM1-SOC complex is sufficient to allow Ca^{2+} influx, the concomitant arrival of the other components of SOCIC is important for the functionality and regulation of the complex. Based on one of its proposed functions, the next protein that should arrive to microdomains is CRACR2A. The evidence shows that CRACR2A could be recruited after STIM1-Orai1 association and that it is responsible of its stabilization by an interaction with the N-terminal of Orai1 (Srikanth et al. 2010). Moreover, the fact that CRACR2A unbinds from STIM1-Orai1 when Ca^{2+} concentration increases, suggests that this protein arrives early to the complex. However, more studies are required to understand the scenario where CRACR2A acts.

Once Ca^{2+} influx is established, proteins considered as SOCE effectors incorporate into SOCIC. Because one of the most important functions of SOCE is to refill the ER (see also Chap. 11), the arrival of SERCA guarantees efficient Ca^{2+} reuptake (Redondo et al. 2008b). It has been reported that STIM1 participates also in this recruitment (Lopez et al. 2008), functioning as the molecule that orchestrate SOCIC assembly. Association between SERCA and STIM1 occurs after STIM1–Orai1 interaction (Sampieri et al. 2009). Moreover, SERCA seems to be located at the periphery of microdomains forming a ring structure surrounding STIM1 clusters (Sampieri et al. 2009). The mechanism for the recruitment of the other effector, AC8, is still unknown. There is evidence that AC8 co-localizes with Orai1 and requires STIM1 clustering to be modulated by SOCE (Lefkimmiatis et al. 2009; Martin et al. 2009). Lipid rafts could be the connection between AC8 and the other

SOCIC components, since it has been seen that raft integrity is necessary for the modulation of AC8 by SOCE (Pagano et al. 2009).

Finally, there are mechanisms regulating Ca^{2+} influx before SOCIC disassembly. Inhibition of the current flowing through SOC is mediated by STM1, CaM and golli. Fast Ca^{2+}-dependent inactivation (CDI) is one of the mechanisms that decreases Ca^{2+} influx, and it has been shown that the negatively charged residues 474–485 of STIM1 mediates in part CDI (Derler et al. 2009; Mullins et al. 2009). Also, it has been demonstrated that CaM also mediates CDI (Litjens et al. 2004; Moreau et al. 2005; Singh et al. 2002), apparently by an interaction with the CaM-binding site (68–91) in the N-terminal of Orai1 (Mullins et al. 2009). Based in the proximity of CaM and CRACR2A-binding sites, it has been proposed that these proteins compete for Orai1 binding and because of their opposite effects it could involve an exclusion mechanism (Srikanth et al. 2010). Golli, a poorly studied protein, seems to inhibit SOCE by an interaction with the C-terminal of STIM1, a region needed to interact with other SOCIC components (Walsh et al. 2010). As we have stated, STIM1 is essential in the establishment and maintenance of microdomains, therefore the binding of golli could destabilize many of the STIM1 interactions causing SOCE attenuation via favoring SOCIC disassembly.

An interesting question is if microdomains assemble randomly in the cell or instead if assembly occurs at specific and predetermined areas in the PM. The use of ML-9 to reverse microdomains assembly has shown that upon a new stimulation, microdomains reassemble in the same regions where they were initially located before ML-9 treatment (Smyth et al. 2008). This very important result strongly suggests that location of microdomains assembly is predetermined and that other structural components may take part in this arrangement.

Another aspect that requires further studies is the identification of other SOCIC components. Studies using an elegant methodology for chemical induction of bridges between ER and PM suggest the existence of unidentified components required for initial steps of SOCIC assembly (Varnai et al. 2007). Indeed, recent co-immunoprecipitation assays have shown that Orai1-interacting protein complex contains at least three unidentified components of SOCIC (Srikanth et al. 2010). For example, one of these missing components could be the IP_3R, since it has been shown that IP_3R associates with TRPC1 (Redondo et al. 2008a; Rosado and Sage 2000) and Orai1 after ER depletion (Hong et al. 2011). Although we describe here several components of this complex, the existing gaps in the assembly mechanism of SOCIC lead to the logical assumption that other elements might be required.

7.4 Lipids Rafts as Centers of Microdomains Assembly

We have emphasized the restriction of SOCE signaling to specialized microdomains where many SOCIC are assembled, but this raises the important question of where these microdomains are assembled in the PM. In this regard, it has been shown in different cells that SOCIC components reside in lipid rafts (Alicia et al. 2008; Liao et al. 2009; Pagano et al. 2009; Pani et al. 2008). Lipid rafts are plasma

membrane microdomains which contain high concentration of cholesterol and sphingolipids, and function as centers for the assembly of signaling complexes (Lingwood and Simons 2010). In this way, lipid rafts allow close proximity between associated molecules in the PM and facilitate their interactions and operation, specificity and regulation of signaling events related to these molecules (Simons and Toomre 2000).

The evidence shows that lipid rafts play a relevant role in SOCE, maybe serving as coordinating centers where SOCIC components converge and assemble (Pani and Singh 2009). Indeed, it has been seen that lipid rafts destabilization causes a decrease in SOCE (Murata et al. 2007; Pani et al. 2008; Prakash et al. 2007). Moreover, many of the SOCIC components appear to be associated to lipid rafts. For example Pani et al. (2008) demonstrated that clustering of STIM1 at ER-PM junctions depends on lipid rafts, since removing cholesterol from the PM with methyl-β-cyclodextrin decreases STIM1 clustering, even in the constitutively activated mutant STIM1-D76A. In the same direction, it has been reported that TRPC1 is assembled in lipid rafts domains, specifically in caveolae (Lockwich et al. 2000). This specificity depends on an interaction between caveolin and a domain in the N-terminus of TRPC1 (322–349) (Brazer et al. 2003). Lipid raft association of TRPC1 seems to be mediated by STIM1, which determines TRPC1 function as a SOCIC component (Alicia et al. 2008). Co-immunoprecipitation assays have shown that Orai1 is also associated with caveolin (Martin et al. 2009). Furthermore, Orai1 recruitment to the complex depends on lipid rafts (Jardin et al. 2008). Likewise, it has been shown that AC8 is associated with caveolin and that disruption of raft integrity inhibits SOCE-mediated AC8 stimulation (Martin et al. 2009; Pagano et al. 2009).

An important aspect on the role of lipid rafts in SOCE is that lipid rafts are necessary for the activation of SOCE but not for its maintenance, this suggests a role of these lipid domains in the initial steps of SOCIC formation (Galan et al. 2010). The fact that lipid rafts are highly dynamic suggests that they might allow a continuous exchanging of components with surrounding membrane regions (Shaw 2006). However, this dynamics together with the small sizes of lipid rafts have make difficult the study of the role of these lipids domains in SOCE microdomain assembly.

7.5 Microdomains Disassembly

A relevant aspect of the physiological role of SOCE is that this process must be reversible. If not, the permanent activation of SOCE would cause a non-controlled elevation of intracellular Ca^{2+}, leading to cell damage or death. Microdomains disassembly is perhaps the least understood step in SOCE signaling. One reason is that most of the studies in this field use irreversible SOCE activators such as the SERCA inhibitor, thapsigargin.

However there are some clues about how SOCIC disassembly occurs. As ER depletion is the signal for SOCE activation, SOCE-dependent ER refilling appears

to be the event that induces SOCE termination (Smyth et al. 2008; Varnai et al. 2007). Indeed, it has been seen using reversible SERCA blockers, that after store depletion SERCA reactivation is not sufficient to initiate SOCIC disassembly, but Ca^{2+} influx is required in order to initiate this process (Sampieri et al. 2009). Comparison of ER-refilling and SOCE kinetics shows that SOCE termination occurs rapidly and without any delay regarding ER-refilling (Edwards et al. 2010; Malli et al. 2007). Moreover, ER refilling induces SOCIC disassembly at different steps since Ca^{2+} addition reverses both STIM1 oligomerization and STIM1-Orai1 interactions (Liou et al. 2007; Muik et al. 2008). A secondary factor, which could induce microdomains disassembly, is the cytoplasmic Ca^{2+} elevation. It has been shown that Ca^{2+} elevation causes dissociation of CRACR2A from STIM1-Orai1 and this could lead to SOCIC destabilization (Srikanth et al. 2010).

There are few studies showing how SOCIC components dissociate. The study of the time course of STIM1-SERCA association shows that SERCA is recruited to the SOCIC after STIM1 oligomerization and translocation to ER-PM junctions (Sampieri et al. 2009). In contrast, STIM1-SERCA dissociation occurs faster (Sampieri et al. 2009). Furthermore, it has been shown that reversal of STIM1 clustering is also faster than its assembly (Smyth et al. 2008). All this evidence suggests that microdomain disassembly could be faster and could require fewer steps than microdomain assembly.

7.6 Physiological and Pathological Implications of Microdomain Organization of SOCE

Microdomain organization of SOCE produces spatiotemporally restricted Ca^{2+} signals, which have been shown to be important for several functions, in a wide variety of cell and tissues. SOCE deregulation has also been related to multiple pathological conditions. In this book there are several chapters dealing with (patho) physiology implications of SOCE. Thus, we will only mention a few examples where SOCE microdomain organization appears to have a central role.

SOCE is the main Ca^{2+} entry pathway into the cells of the periphery immune system (Hogan et al. 2010). For instance, following antigen recognition T-lymphocytes need SOCE activation to initiate signaling cascades related to diverse functions, such as gene expression (Negulescu et al. 1994), cell motility and formation of the immunological synapse (Negulescu et al. 1996). In T-lymphocytes, microdomains are assembled into the entire cell periphery when ER is depleted by thapsigargin application, in contrast under a physiological condition such as T-cell contact with a dendritic cell, microdomains assemble only at the immunological synapse (Lioudyno et al. 2008). Both STIM1 and Orai1 are recruited to the synapse, resulting in a localized increment in the Ca^{2+} concentration (Lioudyno et al. 2008). Then SOCE restriction to microdomain has been related with immunological synapse maintenance, allowing a sustained Ca^{2+} entry that enables the long-term physiological function of T-lymphocytes. Indeed, a mutation in Orai1 or STIM1 abrogation results in

severe combined immunodeficiency (SCID) in humans (Feske et al. 2006; Picard et al. 2009).

Another physiological example of SOCE microdomains arrangement is observed in skeletal muscle. At resting conditions STIM1 is localized in regions where the t-tubule system of sarco-endoplasmic reticulum (SR) is closely apposed to the PM, allowing a rapid activation of SOC under SR depletion (Stiber et al. 2008). Contrary to T-lymphocytes which show a delay of approximately 1 min between ER depletion and SOC activation, in skeletal muscle SOCE is activated in less than 1s after SR depletion (Launikonis and Rios 2007). This fast activation depends on the pre-existence of SOCE microdomains in t-tubules. Interestingly, skeletal muscle cells are probably the first case in which a constitutive establishment of SOCE microdomains has been observed, showing how microdomains could arrange based on cell-specific requirements. The relevance of SOCE in skeletal muscle becomes evident from data obtained in mice lacking STIM1 or Orai1. These animals developed several myophaties (Stiber et al. 2008; Vig et al. 2008). Furthermore, muscular hyphotonia has been observed in SCID patients with the STIM1 or Orai1 deficiencies mentioned above (Feske et al. 2006; Picard et al. 2009).

A very interesting example about the functional significance of SOCE microdomains has been recently described in secretory epithelial cells (Hong et al. 2011). These cells show polarized Ca^{2+} signals that originate at the apical membrane and spread into the cell. In order to generate these localized signals, a polarization of all Ca^{2+} signaling-related proteins is required (Kiselyov et al. 2006). The apical pole is enriched in several SOCIC components as STIM1, Orai1, TRPC1 and SERCA (Hong et al. 2011; Lee et al. 1997) which have been shown to be spatially located at particular regions of the membrane (Hong et al. 2011). Most interestingly, SOCICs containing Orai1-TRPC1 are selectively located at the apical membrane, while SOCICs at the basolateral membrane lack Orai1 (Hong et al. 2011). The presence of both channels might enhance Ca^{2+} signaling at the apical pole, facilitating important processes such as exocytosis. Indeed, epithelial cells lacking STIM1, Orai1 or TRPC1 have impairments in Ca^{2+} influx and in Ca^{2+} oscillations needed for the correct function of epithelium (Hong et al. 2011).

Conclusions

A wealth of information about SOCE has been produced since the original model proposed by Putney was published (Putney 1986). In the last 20 years we discovered the first members from a new superfamily of ionic channels (TRP), and encountered more questions than answers. Only until recently the complexity of this mechanism is beginning to unveil, thanks to the identification of novel proteins involved in SOCE. We have postulated that, rather than simplifying this process as a mechanism that requires a couple of proteins to function properly, a more elaborated system involving the orderly and timely association of several proteins into a complex might be needed. We proposed the name of store-operated Ca^{2+} influx complex (SOCIC) for such orchestrated system. In this chapter we attempted to review the evidence for such complex, and elaborate on

some of the participants and its function in SOCIC. Finally, we have discussed recent evidence strongly suggesting that the existence of microdomains modulating Ca^{2+} influx is not a curiosity of how cells organize their proteins, but may have important implications for spatiotemporal organization of Ca^{2+} influx and signaling.

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