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REGULACIÓN POR CALCIO DE LA EXPRESIÓN DEL GEN *ATP2A2* EN CARDIOMIOCITOS

T E S I S

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RESUMEN

En este trabajo de investigación se exploró la regulación transcripcional del gen *ATP2A2,* que codifica para la proteína SERCA2. En respuesta a concentraciones citosólicas de calcio ($[Ca^{2+}]_c$) elevadas provocadas por tapsigargina (Tg) o por el ionóforo de calcio A23187 se encontró que dichos fármacos aumentan la transcripción del gen y que este aumento se previene mediante el uso de ciclosporina A, inhibidor de la fosfatasa calcineurina (CN). La CN desfosforila al factor transcripcional NFAT para que pueda ser translocado al núcleo y se una a sus secuencias en los genes blanco y aunque se observó que la mutación del sitio de unión a NFAT cercano al promotor proximal del gen (+43) reduce a la mitad su actividad transcripcional, no se previno el aumento inducido por Tg y A23187, concluyendo que NFAT no está directamente relacionado con el aumento de transcripción de *ATP2A2* provocado por el aumento en la $[Ca^{2+}]_c$.

Por tanto, se decidió explorar el papel del elemento de respuesta a estrés del retículo endoplásmico (ERSE, por sus siglas en inglés) presente en el promotor proximal del gen *ATP2A2*. Se descubrió que la secuencia CCAAT presente en el ERSE del gen es la principal responsable del aumento en su transcripción en respuesta a la elevación de $[Ca^{2+}]_c$ inducida por Tg o A23187. Dicha secuencia mostró ser indispensable para la formación de complejos DNA-proteína *in vitro* (mediante ensayos de retardamiento de la movilidad electroforética - EMSAs) en el complejo ERSE del gen *ATP2A2* humano e incluso, el ERSE de dicho gen compitió efectivamente por la formación del complejo con el ERSE del gen *grp78* de rata, un modelo de estrés de retículo endoplásmico ampliamente usado. La similitud entre ambos ERSEs se encontró también cuando ambos complejos dejaron de ser observables en los EMSAs al usar anticuerpos contra el factor transcripcional NF-Y y cuando ninguno de ellos fue modificado al usar anticuerpos contra C/EBPβ. Por último, por medio de ensayos de ChIP se encontró que los factores transcripcionales NF-YB y C/EBPβ se unen *in vivo* al promotor proximal de *ATP2A2*.

Los resultados presentados en esta tesis sugieren que el gen *ATP2A2* es reprimido en condiciones basales por el complejo ERSE y plantean la necesidad de futuros estudios en los que se determine con exactitud la diferencia en la regulación del gen por los factores transcripcionales NF-Y y C/EBPβ.

ABSTRACT

In this research, the transcriptional regulation of the *ATP2A2* gene which codifies SERCA2 protein, was explored. It was found that cytosolic calcium concentrations ($[Ca^{2+}]_c$) increased by thapsigargin (Tg) or calcium ionophore A23187 increase the transcription of the gene and that this increase is prevented by using cyclosporin A, an inhibitor of calcineurin phosphatase (CN). CN dephosphorylates the transcriptional factor NFAT so it can be translocated to the nucleus and bind to its sequences in its target genes. Although it was observed that the mutation of the NFAT binding site near the proximal promoter of the gene (+43), reduces its transcriptional activity by about half; the increase induced by Tg and A2318 was not prevented, concluding that NFAT is not directly related to the *ATP2A2* increased transcription caused by the increase in $[Ca^{2+}]_c$.

Therefore, it was decided to explore the role of the endoplasmic reticulum stress response element (ERSE) present in the proximal promoter of the *ATP2A2* gene. It was discovered that the CCAAT box present in the gene´s ERSE is the main responsible for its increased transcription in response to the elevation of $[Ca^{2+}]_c$ induced by Tg or A23187. The CCAAT box showed to be essential for the formation of DNA-protein complexes *in vitro* (by electrophoretic mobility shift assays - EMSAs) in the ERSE complex of the human *ATP2A2* gene and even, this ERSE effectively competed for the formation of the complex with the ERSE of the rat *grp78* gene, a widely used endoplasmic reticulum stress model.

The similarity between both ERSEs was also found when both complexes disappeared in the EMSAs when using antibodies against the NF-Y transcriptional factor and when none of them was modified when using antibodies against C / EBPβ. Finally, using ChIP assays, it was found that the transcriptional factors NF-YB and C/EBPβ bind *in vivo* to the proximal *ATP2A2* promoter.

The results presented in this thesis suggest that the *ATP2A2* gene is repressed in basal conditions by the ERSE complex and raise the need for future studies in which differences in the regulation of the gene by the transcriptional factors NF-Y and C/EBPβ is accurately determined.

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LISTA DE ABREVIATURAS

A23187	Ionóforo de calcio
AEC	Acoplamiento Excitación-Contracción
AMPc	Monofosfato de adenosina cíclico
ANF	Factor Natriurético Atrial
ATF6	Factor Activador de la Transcripción 6
ATP2A-1, -2, -3	Genes que codifican para las proteínas SERCA1, SERCA2 o SERCA3, respectivamente
BDM	Monoxima de 2,3-butanodiona
BNP	Péptido Natriuurético Cerebral
Brg1	Gen relacionado a Brahma 1 (subunidad catalítica modificadora de cromatina)
CaM	Calmodulina
CaMK	Cinasa dependiente de Calcio-Calmodulina
CASQ2	Calsecuestrina 2
C/EBP	Proteína de Unión al Potenciador CCAAT
ChIP	Inmunoprecipitación de Cromatina
CN	Calcineurina
CN-B1	Subunidad B1 de la Calcineurina
CNP	Péptido Natriurético tip C
CsA	Ciclosporina A
DHPR	Receptor a Di-Hidro-Piridina
Dyrk-1a	Cinasa Regulada por fosforilación de Tirosina de doble especificidad 1a
Egr	Proteína de Respuesta a Crecimiento Temprano (Factor de transcripción)
EMSA	Ensayo de Retardamiento de la Movilidad Electroforética
EPAC	Proteína de Intercambio Activada directamente por AMPc
ER	Retículo Endoplásmico

ERS	Estrés del Retículo Endoplásmico
ERSE	Sitio de Respuesta a Estrés del Retículo Endoplásmico
GAPDH	Deshidrogenasa de Fosfato de Gliceraldehído (Gen de expresión estable)
grp78	Proteína regulada por glucosa (Marcador de ERS)
HC	Hipertrofia Cardiaca
HDAC	Desacetilasa de HistonasS
IC	Insuficiencia Cardiaca
IgG	Inmunoglobulina G
Input	Representación de la cromatina inicial en inmunoprecipitaciones de cromatina
KN-93	Inhibidor de la cinasa CaMK
MAPK	Cinasa Activada por Mitógenos
mCAT	Sitio CCAAT mutado
MEF2	Factor Potenciador de Miocitos 2
mERSF	Sitio ERSF mutado
MHC	Cadena Pesada de Miosina
miRNA	Micro-RNA
mYY1	Sitio YY1 mutado
NADH	Forma reducida del Dinucleótido de Nicotinamida y Adenina
NCX	Intercambiador Na+/Ca2+
ND6	Subunidad 6 de la Deshidrogenasa de NADH
NFAT	Factor Nuclear de células T Activadas
NF-YA	Subunidad A del Factor Nuclear Y
NF-YB	Subunidad B del Factor Nuclear Y
NF-YC	Subunidad C del Factor Nuclear Y
NHE1	Intercambiador Na+/H+

p38	Una Cinasa Activada por Mitógenos
PCR	Reacción e Cadena de la Polimerasa
PDI-6	Isomerasa 6 Asociada a Proteínas Disulfuro
PKA	Proteína Cinasa A
PKC	Proteína Cinasa C
PKD	Proteína Cinasa D
PLB	Fosfolamban
PLC	Fosfolipasa C
PMCA	ATPasa de Calcio de la Membrana Plasmática
PNRC	Co-activador del Receptor Nuclear rico en Prolina
PP1	Fosfatasa 1
RCAN1	Regulador de la Calcineurina-1
RNAm	Ácido Ribonucleico mensajero
RS	Retículo Sarcoplásmico
RyR2	Receptor a Ryanodina 2
SERCA	ATPasa de Calcio del Retículo Sarco(Endoplásmico)
SIRT	Desacetilasa dependiente de NAD sirtuína-1
Sp1	Factor de transcripción ubicuo
Tg	Tapsigargina
Tun	Tunicamicina (inhibidor de glicosilación de proteínas)
TXNIP	Proteína de Interacción con Tiorredoxina
YY1	Ying yang 1 (Factor de transcripción)

INTRODUCCIÓN

En el corazón, los cardiomiocitos forman el tejido muscular estriado que lleva a cabo la contracción y relajación cardiaca, este tejido es una red de células interconectadas entre sí que se sincronizan gracias a las “uniones comunicantes” (gap junctions en inglés) para responder a los estímulos eléctricos que desencadenan la contracción muscular. La red de tejido muscular cardiaco posee la capacidad de responder rápidamente y de manera sincronizada a una despolarización de la membrana plasmática gracias a los túbulos T. Éstos, son inervaciones de la membrana plasmática que están en íntimo contacto con el retículo sarcoplásmico (RS) de la célula. En los túbulos T se encuentran los canales receptores de dihidropiridinas (DHPR), éstos son canales de calcio (Ca^{2+}) tipo L sensibles a voltaje que permiten la entrada de pequeñas corrientes de Ca^{2+} en respuesta a la despolarización de la membrana plasmática (Bers, 2002).

El retículo sarcoplásmico.

El retículo sarcoplásmico (RS) es un organelo que consiste en un sistema de membranas que funciona como un reservorio de Ca^{2+} para la célula y está compuesto por 2 regiones principales: las cisternas terminales y el RS longitudinal. En las cisternas terminales se encuentran los canales de Ca^{2+} llamados receptores de rianodina (RyR2), dichos canales son tetraméricos, y en dicha conformación tienen un peso de aproximadamente 2000 kDa, son los encargados de permitir la salida masiva de Ca^{2+} al citoplasma para iniciar la contracción muscular y se encuentran en gran cercanía a los túbulos T (120 Á) (Bers, 2002; Hong and Shaw, 2017). La región amino-terminal constituye la mayor parte del canal y se encuentra en el citosol, además de controlar las propiedades de apertura de la región carboxilo terminal que se encuentra en el lumen del RS, contiene varios sitios de unión a proteínas reguladoras como calstabina 2, sorcina, proteína cinasa A (PKA), fosfatasas 1 y 2 (PP1 y PP2) y calmodulina (CaM). La regulación de RyR2 durante ejercicio o estrés está mediada por la activación de los receptores β adrenérgicos acoplados a proteínas G (Lanner et al., 2010; Marx et al., 2000).

En el RS, RyR2 está en estrecho contacto con 2 proteínas reguladoras llamadas triadina y junctina. La triadina es una glicoproteína de 95 kDa que se encuentra en la membrana del RS, la mayor parte de la proteína se encuentra en la luz del RS y a pesar de que es capaz de inhibir al RyR uniéndose al dominio citoplásmico de éste, no tiene efecto alguno al unirse al dominio luminal del canal. La junctina es una proteína de 26 kDa que interacciona con RyR2 en el lumen del RS y que por otra parte, al igual que la triadina también es capaz de unirse a la calsecuestrina (CASQ2) para conformar el complejo CASQ2-RyR2-triadina-junctina que es el encargado de regular la liberación de Ca^{2+} del RS al citoplasma (Zhang et al., 1997).

Dentro de la cisterna terminal del RS hay una concentración muy elevada de Ca^{2+} (aproximadamente 20 mM) pero la mayor parte no se encuentra libre (la concentración de Ca^{2+} libre es solo de 1-2 mM) debido a que se halla unido a polímeros de CASQ2, la cual posee una alta capacidad de unión a Ca^{2+} (40-50 moles de Ca^{2+} por mol de proteína) y una afinidad moderada (1 mM) (Slupsky et al., 1987). En condiciones normales, la disociación de la CASQ2 del complejo RyR2-triadina-junctina es suficiente para activar la apertura del canal de RyR2 y se ha demostrado por ensayos *in vitro* (Beard et al., 2002) que la interacción directa CASQ2-RyR2 no permite tal apertura, por lo que se sugiere que la existencia del complejo cuaternario completo es indispensable para que se ejerzan las funciones regulatorias a cargo de la CASQ2 (Zhang et al., 1997).

Los sistemas de transporte que permiten mantener la homeostasis de Ca^{2+} en tejidos musculares, como lo es el músculo cardiaco, son vitales para el correcto funcionamiento y acoplamiento de los ciclos de contracción-relajación muscular. En específico, el acoplamiento excitación-contracción, descrito en la próxima sección, debe ser finamente regulado para mantener concentraciones adecuadas de Ca^{2+} tanto en el RS como en el citosol y dicha regulación depende de una gran cantidad de proteínas que funcionan como receptores, transductores, canales iónicos, moduladores, enzimas sensibles a Ca^{2+}, intercambiadores de iones y ATPasas dependientes de Ca^{2+}.

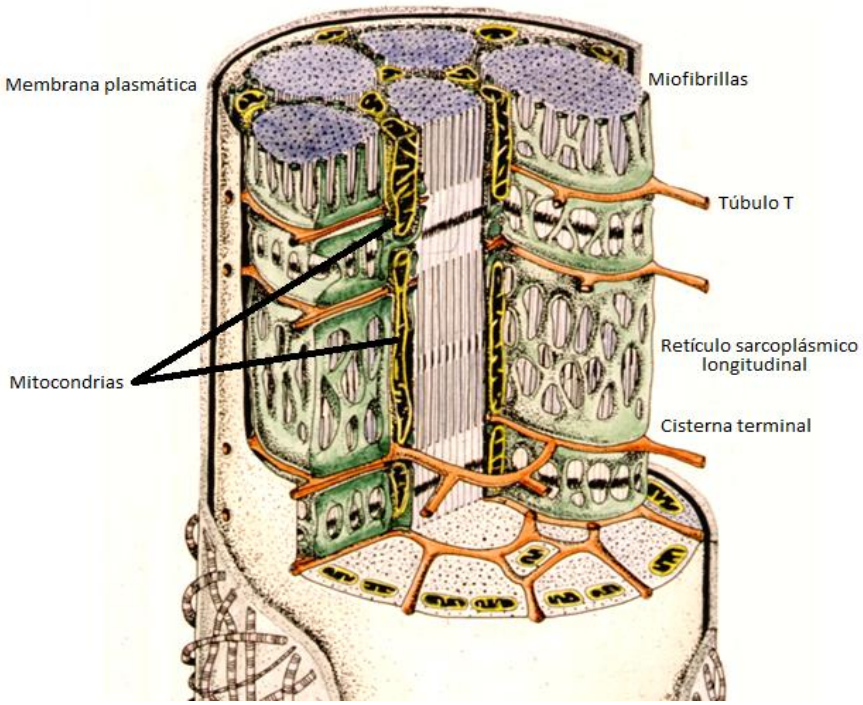

Figura I. La estructura del músculo cardiaco. En la imagen se muestra cómo se aumenta la superficie de la membrana plasmática en el cardiomiocito a través de las invaginaciones conocidas como túbulos T, la gran cercanía de éstos con respecto a la cisterna terminal del RS, la alta cantidad de mitocondrias del músculo cardiaco y la disposición de las miofibrillas que llevan a cabo la contracción.

Acoplamiento excitación-contracción.

El acoplamiento excitación-contracción (AEC) inicia con la despolarización de la membrana del cardiomiocito. Dicha despolarización provoca que los DHPR presentes en los túbulos T permitan la entrada de Ca^{2+} hacia el citoplasma desde el espacio extracelular, donde la concentración de este ion es de 2 a 3 mM (Bers, 2002; Kitazawa, 1984). El aumento en la concentración de Ca^{2+} citoplásmico ($[Ca^{2+}]_c$) que resulta de la apertura de los DHPR induce la apertura de los canales RyR2 y la salida masiva de Ca^{2+} del interior del RS a través de un mecanismo conocido como liberación de calcio inducida por calcio (Fabiato and Fabiato, 1978). Una vez que el calcio citosólico alcanza concentraciones cercanas a 10 µM se puede unir a la troponina C liberando a los miofilamentos de actina de una inhibición que evita su contacto con la miosina. En ausencia del efecto inhibitorio de la troponina C, se puede llevar a cabo la contracción muscular que continúa hasta que

la $[Ca^{2+}]_c$ disminuye principalmente por el transporte activo de Ca^{2+} al interior del RS de SERCA2a y por la salida de Ca^{2+} de la célula por el intercambiador Na^+/Ca^{2+} (NCX) del sarcolema (Schillinger et al., 2003; Xue et al., 1999).

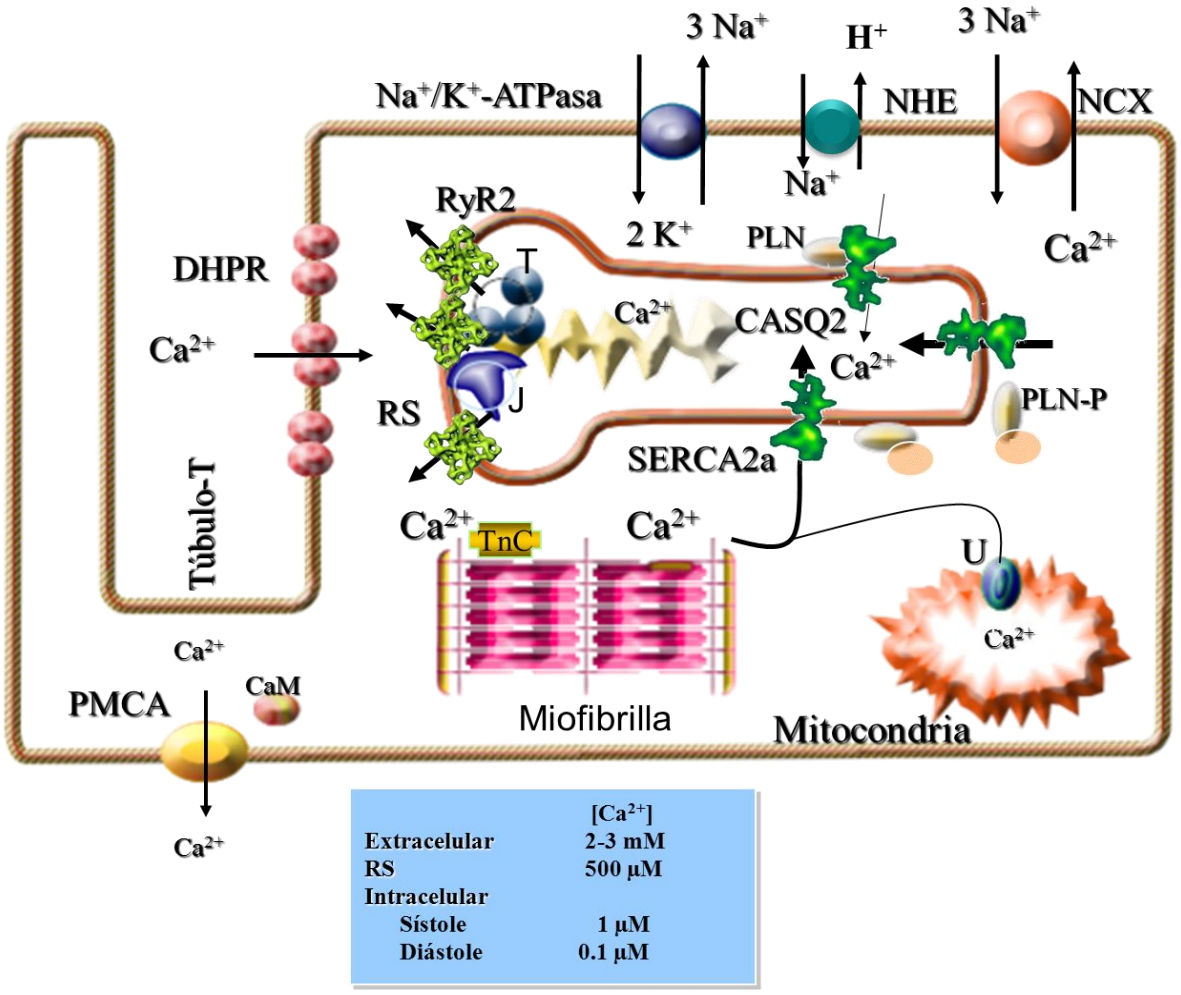

Figura II. Los componentes principales del cardiomiocito y su papel en el ciclo excitación-contracción. De manera esquemática se representa cómo las corrientes de calcio que entran a través de los DHPR alcanzan inmediatamente los canales RyR2 en el retículo sarcoplásmico para que se inicie la liberación masiva de calcio hacia el citosol. En el citosol, la unión del Ca^{2+} a la troponina C desencadena la contracción de las miofibrillas, cuando esto ha ocurrido, las altas concentraciones de Ca^{2+} son regresadas al estado basal principalmente por la acción de SERCA2a y en menor medida por las diversas ATPasas de la membrana plasmática. En el recuadro se observan las concentraciones de los distintos compartimentos involucrados en el proceso (modificado de Zarain-Herzberg. 2006).

El intercambiador NCX permite la salida de 1 ion Ca^{2+} a la matriz extracelular que es intercambiado por 2 iones Na^{+} que entran a la célula, este mecanismo es el responsable de reducir en un 30-40 % la $[Ca^{2+}]_c$ durante la diástole, mientras que el resto y mayor parte del calcio en exceso en el citoplasma es regresado al RS por las bombas SERCA2a. Las bombas de calcio SERCA2a catalizan el transporte activo de Ca^{2+} al interior del retículo endoplásmico y/o sarcoplásmico; donde 2 iones Ca^{2+} son transportados al interior del RS por molécula de ATP hidrolizado (Bers, 1991; Toyoshima et al., 2000).

Aunque su contribución es mínima, existen otros 2 mecanismos por los que las altas concentraciones citosólicas de calcio durante la diástole son reducidas a las cantidades basales, dichos mecanismos los constituyen la entrada de calcio a la mitocondria a través del uniportador de calcio mitocondrial simbolizado como “U” en la **Figura II** y la ATPasa de Ca^{2+} de la membrana plasmática (PMCA), que disminuyen la $[Ca^{2+}]_c$ en menos del 5 % (Brini and Carafoli, 2011) .

Además de los canales mencionados, existen otros tantos que permiten directa o indirectamente la regulación de la $[Ca^{2+}]_c$, tal es el caso de la bomba electrogénica de Na^{+}/K^{+} que es una ATPasa encargada del bombeo de 3 iones sodio hacia el espacio extracelular que son intercambiados por dos iones potasio que entran al citoplasma por cada ATP hidrolizado (Jorgensen, 1974). Este bombeo de iones sodio genera un gradiente de Na^{+} que permite que funcione el intercambiador NCX. Otro actor importante en el funcionamiento del cardiomiocito es el intercambiador de Na^{+}/H^{+} 1 (NHE1), cuya función es transportar un ion sodio desde la matriz extracelular al citoplasma e intercambiarlo por un protón, lo cual además de participar en la regulación del pH celular también es capaz de alterar la homeostasis de calcio indirectamente, ya que únicamente a través de la sobre-expresión de esta proteína se ha detectado la generación de graves problemas cardiacos en animales transgénicos y el desarrollo de hipertrofia en cardiomiocitos neonatales (Nakamura et al., 2008).

Cuando inicia el acople, existe una alta cantidad de Ca^{2+} unido a la CASQ2 y una vez que se activan los canales de RyR2, la mayor parte del Ca^{2+} presente en el RS es liberado al citoplasma. Después que se ha llevado a cabo la contracción, el Ca^{2+} es regresado al RS y vuelve a asociarse con los monómeros de CASQ2

existentes a concentraciones bajas de Ca^{2+} (<1 mM). El rápido incremento del Ca^{2+} en el RS provoca que la CASQ2 vuelva a conformar los polímeros que mantendrán una concentración libre de Ca^{2+} de 1 mM (Bers, 2002; Park et al., 2003).

Una vez que las concentraciones de calcio citoplásmico regresan a niveles basales y que el RS está nuevamente cargado con altas cantidades de este ion, se inicia una vez más el ciclo con la entrada de Ca^{2+} a través de los canales DHPR.

La ATPasa de Ca^{2+} del retículo sarco(endo)plásmico.

La proteína más importante para la regulación de las [Ca^{2+}] citoplásmico e intra-reticular en el cardiomiocito es la bomba SERCA2a. Las SERCAs son ATPasas tipo P, llamadas así por la conservación temporal de la energía del ATP en forma de un intermediario fosforilado de la enzima. Dicho intermediario está formado por el fosfato *γ* del ATP hidrolizado unido a un residuo de ácido aspártico invariable en la secuencia altamente conservada SDKTGT[L/I/V/M][T/I/S]; estas proteínas pesan alrededor de 110 kDa y constituyen la mayor parte de proteína del RS longitudinal (80-90 %).

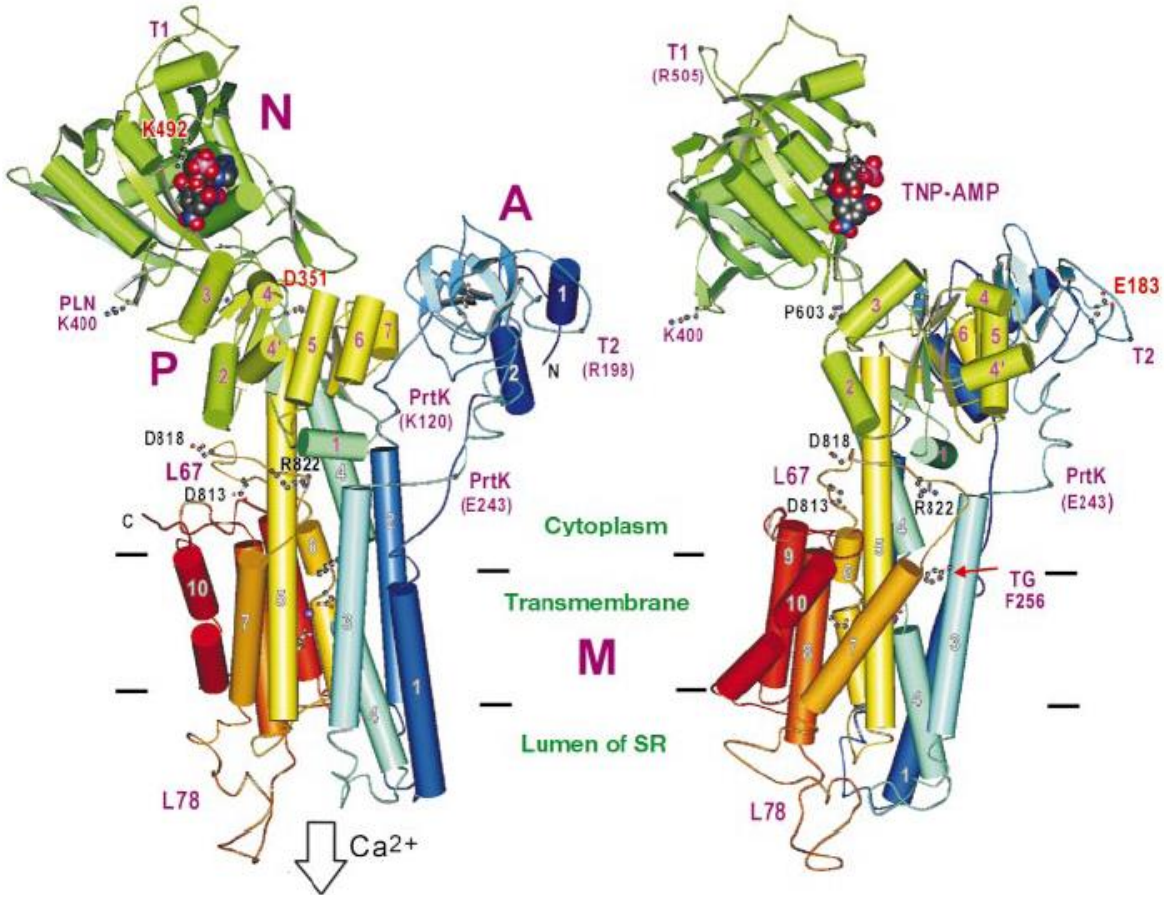

Figura III. Estructura de la bomba de calcio SERCA. En la imagen el color cambia gradualmente desde el azul para la porción amino terminal hasta el rojo para la porción carboxilo terminal. Se muestran los 3 dominios citoplásmicos, A (contiene el motivo TGES característico de las ATPasas tipo P), N (aquí se une el ATP) y P (dominio donde ocurre la fosforilación). Las hélices transmembranales están numeradas al igual que las que se encuentran en los dominios A y P. Las esferas moradas representan 2 iones Ca^{2+}. Se resaltan varios residuos y sitios importantes: TNP-AMP (unión de ATP), E183 (residuo clave para la actividad catalítica), PLN (unión de fosfolamban), D351 (sitio de fosforilación), TG (sitio de unión del inhibidor tapsigargina), PrtK y T (estos dos últimos, sitios susceptibles a digestión por proteinasa K y tripsina respectivamente). *Tomado de Toyoshima, et al. 2000.*

SERCA2a es la principal proteína de la membrana del RS longitudinal del cardiomiocito, se trata de una proteína de 10 pasos transmembranales (Toyoshima et al., 2000) que tiene una alta afinidad por Ca^{2+} (K_m ~ 0.1 µM), por lo que se estima que sólo se necesitan máximo 2 ciclos de transporte para recuperar la [Ca^{2+}] citosólica durante la relajación muscular.

La actividad enzimática de SERCA2a es regulada por fosfolamban (PLB), una proteína pequeña (6.1 kDa, 21 aminoácidos) que en su forma desfosforilada es un inhibidor de SERCA que disminuye la afinidad aparente por Ca^{2+} de la bomba y que puede ser fosforilada por PKA o por la cinasa dependiente de Ca^{2+}/calmodulina II (CaMKII) (Jones et al., 1981; Le Peuch et al., 1979; Morkin and LaRaia, 1974).

Las bombas SERCA poseen una gran diversidad que depende del estado del desarrollo y es tejido-específica (Berridge et al., 2003). En el humano existen 3 genes diferentes para las bombas SERCA llamados *ATP2A1*, *2* y *3*, y cada gen produce varias isoformas a través del proceso de edición (splicing en inglés) alternativa del transcrito primario del RNA pre-mensajero (Martonosi and Pikula, 2003) (**ver Tabla 1**).

Tabla 1. Genes e Isoformas de SERCA.

Gen	Isoformas	Tejidos en que se expresa
ATP2A1	SERCA1a	Músculo esquelético de contracción rápida adulto
	SERCA1b	Músculo esquelético de contracción rápida neonatal
ATP2A2	SERCA2a	Músculos cardiaco y esquelético de contracción lenta
	SERCA2b	Células no musculares y músculo liso (expresión ubicua)
	SERCA2c	Diferenciación de monocitos, corazón
ATP2A3	SERCA3a-f	Sistema nervioso central, células epiteliales y hematopoyéticas

De las 3 isoformas provenientes del gen *ATP2A2* (SERCA2a, b y c), sólo la isoforma SERCA2a se expresa abundantemente en cardiomiocitos y se ha demostrado que los niveles tanto de RNAm como de proteína para dicha isoforma están reducidos en modelos animales con isquemia del miocardio, hipertrofia cardiaca e insuficiencia cardiaca; ocurriendo lo mismo en pacientes con hipertrofia cardiaca severa e insuficiencia cardiaca (Arai et al., 1993; Feldman et al., 1993; Gwathmey et al., 1987; Zarain-Herzberg et al., 1996). En modelos *in vitro* de hipertrofia inducida por el agonista alfa adrenérgico fenilefrina también se han observado bajos niveles de RNAm y proteína para SERCA2a (Prasad et al., 2007a).

La estructura y regulación transcripcional del gen humano que codifica para la proteína SERCA2a ya han sido descritas previamente (Zarain-Herzberg and Alvarez-Fernandez, 2002). El análisis realizado en dicho trabajo se muestra en la **Figura IV**, se debe mencionar la presencia de algunos elementos reguladores de DNA conservados entre ratón, rata, conejo y humano importantes para los objetivos del presente estudio. Como se muestra en la **Figura IV**, existen 3 regiones de homología en la región reguladora del gen *ATP2A2*, la primera región en los primeros 230 pb del promotor basal contiene una caja-TATA, un elemento de respuesta a estrés del retículo endoplásmico (ERSE: Endoplasmic Reticulum Stress Response Element) formado por una caja-E y una secuencia CCAAT, 4 sitios Sp1 en los primeros 200 pb del promotor y un elemento de respuesta a hormona tiroidea (TRE: Thyroid hormone Response Element). La segunda región de homología contiene un sitio TRE y un elemento de respuesta al suero (SRE: Serum Response Element) entre otros. La tercera región de homología entre especies contiene un sitio TRE, un sitio de respuesta al receptor de ácido retinoico (RXR: Retinoic acid Receptor), un sitio de unión al factor potenciador de miocitos-2 (MEF2: Myogenic Enhancer Factor 2), un sitio de unión al factor nuclear de células T activadas (NFAT: Nuclear Factor of Activated T Cells), un sitio Nkx-2.5 y un elemento de unión para el factor de transcripción GATA-4. Aunque fuera de las regiones de homología, el gen humano contiene además varios sitios de unión a RXR, GATA-4, Nkx-2.5 y MEF-2 (Zarain-Herzberg and Alvarez-Fernandez, 2002).

Hipertrofia cardiaca.

La hipertrofia cardiaca (HC) se caracteriza por un aumento en tamaño de los miocitos cardiacos que se presenta como respuesta a un aumento adaptativo en la carga de trabajo del corazón. La hipertrofia cardiaca puede ser una respuesta fisiológica normal como en el caso de los atletas y tiene un efecto benéfico al mejorar la capacidad de trabajo sin provocar daño al miocardio; o puede ser una hipertrofia patológica, como ocurre si el crecimiento es provocado por hipertensión arterial crónica, polimorfismos genéticos, pérdida de cardiomiocitos después de daño isquémico como ocurre en un infarto o por un metabolismo cardiaco alterado. La hipertrofia patológica, en un principio es una respuesta compensatoria pero que al

mantenerse lleva a un decaimiento en la función del miocardio y evoluciona hacia la insuficiencia cardiaca (IC).

En cuanto a la forma del crecimiento, se puede hablar de hipertrofia concéntrica cuando se presenta una sobrecarga de presión crónica que lleva a un volumen reducido del ventrículo izquierdo y aumento en el grosor de la pared del mismo, provocado por la adición de sarcómeros en paralelo. Por otro lado, la sobrecarga de volumen produce hipertrofia excéntrica al provocar dilatación y adelgazamiento de la pared cardiaca, lo que se debe a la adición de sarcómeros en serie.

Una diferencia primordial entre la hipertrofia fisiológica y la patológica es la activación del programa genético fetal, ya que solo en el segundo caso se activa la expresión de genes propios del corazón en desarrollo y que están normalmente reprimidos en corazones adultos. Dicho programa genético tiene por objeto lidiar con la elevada demanda hemodinámica; sin embargo, a pesar de que permite el incremento ordenado del aparato contráctil, también implica la disminución en los niveles de expresión de las isoformas adultas de varios genes que de hecho son usados como biomarcadores de hipertrofia patológica (Barry et al., 2008).

El programa genético fetal está constituido por una gran cantidad de genes que son expresados diferencialmente durante la hipertrofia patológica; sin embargo, existen algunos que representan ejemplos clásicos ya sea por su importancia en la capacidad contráctil del miocito o por la gran diferencia en sus niveles de expresión que se presenta durante la enfermedad como son las isoformas de la cadena pesada de miosina (MHC, por sus siglas en inglés), el factor natriurético atrial (ANF, por sus siglas en inglés), el péptido natriurético cerebral (BNP, por sus siglas en inglés) y la expresión de actina-α esquelética. Uno de los principales marcadores de hipertrofia cardiaca son los niveles de MHC, dicha proteína presenta 2 isoformas en el corazón adulto, β-MHC y α-MHC. Cuando se presenta hipertrofia, se elevan los niveles de la isoforma β-MHC y disminuyen los de α-MHC, con lo que se disminuye la velocidad de la actividad enzimática de la ATPasa de la miosina. Aunque este cambio es bastante característico del crecimiento hipertrófico, se ha reportado que por sí solo pudiera no constituir evidencia de incremento en la hipertrofia (Pandya et al., 2006).

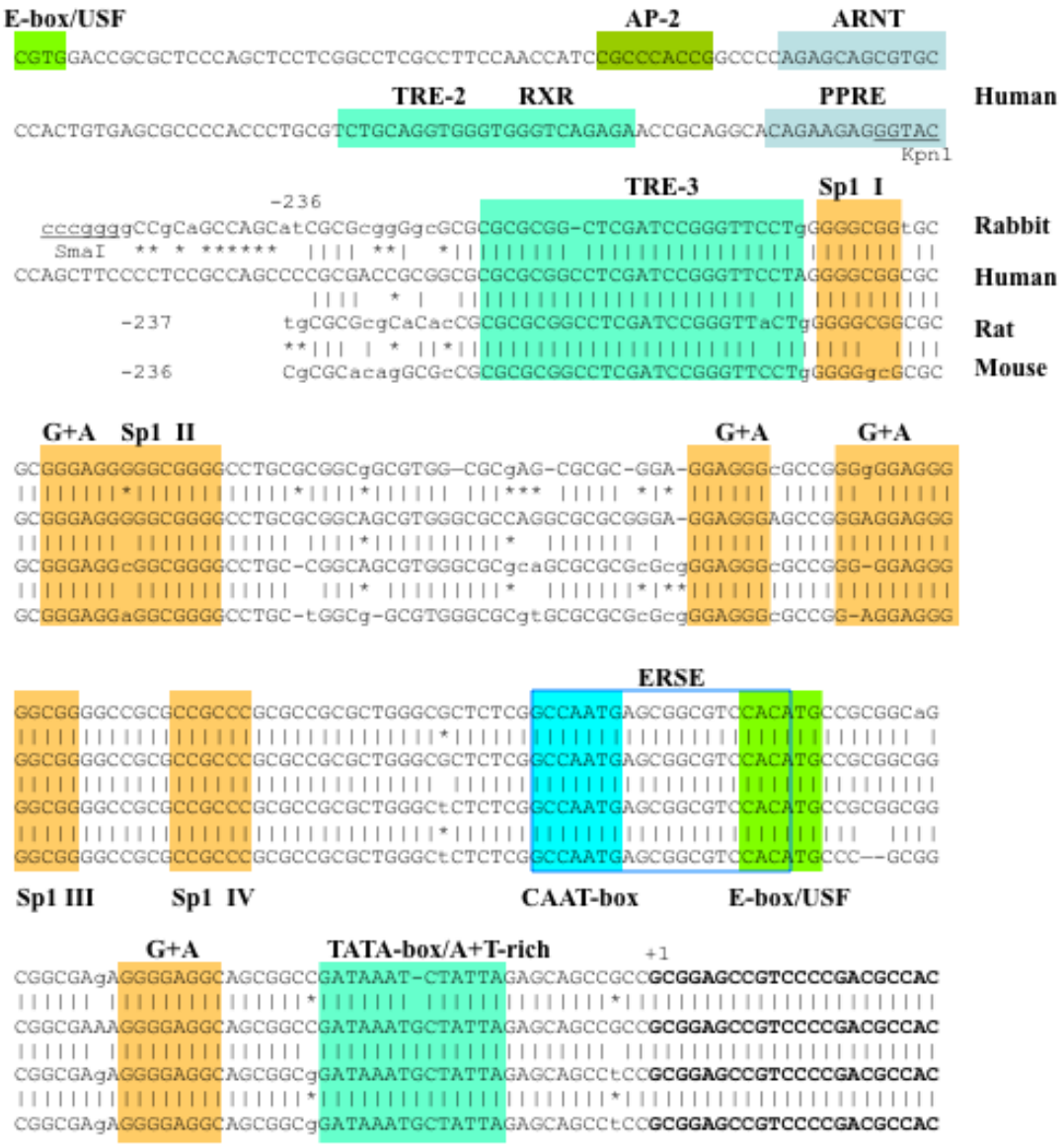

Figura IV. Alineamiento de secuencia de las regiones reguladoras 5´ del gen *ATP2A2*. La homología entre 1.58 kb de la secuencia regulatoria del gen humano para *ATP2A2* y las secuencias del mismo gen para ratón, rata y conejo se muestran con líneas verticales. Un asterisco indica homología sólo entre 2 especies. Las secuencias consenso para varios elementos regulatorios se muestran en áreas coloreadas, el elemento ERSE está encuadrado y la secuencia 5´-no traducida del primer exón está en negritas. (Tomado de (Zarain-Herzberg and Alvarez-Fernandez, 2002).

Figura IV. Continuación.

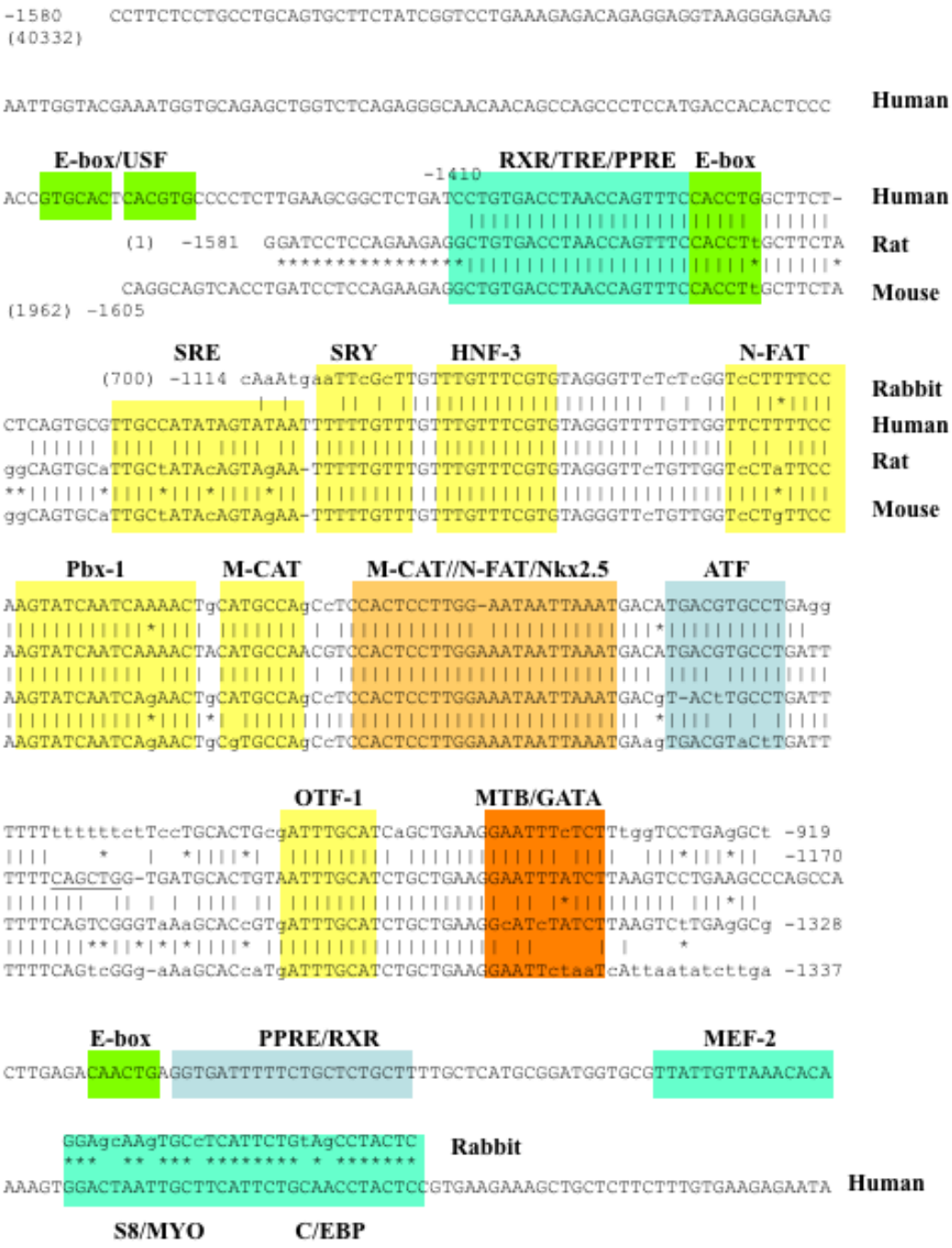

Figura IV. Continuación.

Los péptidos natriuréticos son hormonas que regulan varios procesos fisiológicos del sistema cardiovascular y endocrino y que poseen potentes propiedades inhibitorias sobre la hipertrofia. Existen 3 miembros de estas hormonas, los cuales son ANF, BNP y CNP (Péptido Natriurético tipo C); ANF se expresa principalmente en los atrios, BNP se puede encontrar en atrios y ventrículos, mientras que la distribución de CNP no ha sido descrita en detalle. ANF y BNP se encuentran en altas cantidades en el desarrollo embrionario, pero se encuentran ausentes en el adulto sano. Como consecuencia de un estímulo hipertrófico, se elevan los niveles de ambos péptidos en los cardiomiocitos en un intento de inhibir la respuesta hipertrófica (Barry et al., 2008). Por otra parte, el aumento en la expresión de actina esquelética y de algunas proteínas que tienen que ver con la respuesta a algunas citocinas también se ha asociado con el desarrollo de hipertrofia (Rajan et al., 2006).

ANTECEDENTES

El manejo del calcio en la hipertrofia y la insuficiencia cardiaca.

La contracción y la relajación son los parámetros fisiológicos que se alteran en los miocitos cardíacos de los modelos animales con HC y los pacientes con IC (Heinzel et al., 2011; Morgan et al., 1991; Reyes-Juarez and Zarain-Herzberg, 2006; Sjaastad et al., 2003; Zarain-Herzberg, 2006). Se ha demostrado que una disminución de la velocidad de acortamiento de los miocitos cardiacos durante la contracción se correlaciona con niveles reducidos de la MHC (Alpert et al., 2002), así como con una disminución de $[Ca^{2+}]_c$ (Bers, 2002; Palmer and Kentish, 1998; Vannier et al., 1996). Una disminución de la tasa de extracción de Ca^{2+} del sarcoplasma por el cardiomiocito durante la relajación del miocardio, conduce a un aumento de los niveles de $[Ca^{2+}]_c$ durante la diástole. Lo anterior contribuye al desarrollo de disfunción diastólica, una característica distintiva de la IC, que también se caracteriza por el aumento de la presión diastólica final y por anomalías en el llenado ventricular.

Los transitorios de Ca^{2+} de cardiomiocitos obtenidos de corazones de modelos animales con HC y de pacientes con insuficiencia cardiaca en etapa terminal, muestran una disminución en el pico sistólico de la $[Ca^{2+}]_c$ y una reducida velocidad de bombeo de Ca^{2+} por SERCA2a en el RS. Como consecuencia del pico reducido de $[Ca^{2+}]_c$ derivado de un llenado deficiente con Ca^{2+} del RS, también hay una reducción en la generación de fuerza del miocardio. Se ha demostrado que cardiomiocitos insuficientes presentan un bajo contenido de Ca^{2+} en el RS (Bassani et al., 1994; Teucher et al., 2004), por lo tanto, actualmente se cree que las alteraciones en el manejo de Ca^{2+} del RS son un componente clave que contribuye a la disminución de la contractilidad y la generación de la fuerza en el corazón insuficiente.

Entre las alteraciones que intervienen en la HC e IC están los cambios en los niveles de expresión de las cinasas PKA y CaMKII que actúan como sensores intracelulares de $[Ca^{2+}]$ (Kaprielian et al., 2002). Se ha demostrado que en la IC la expresión y actividad de CaMKII, que desempeña un papel fundamental en la disminución de las propiedades contráctiles del miocardio, se encuentran aumentadas, lo que desestabiliza el AEC y puede conducir a muerte súbita (Soltis and Saucerman, 2010). Estas alteraciones incluyen disminución de los niveles de SERCA2a, con la consiguiente disminución de la recaptura de Ca^{2+} por parte del RS durante la diástole, lo que resulta en una menor cantidad de Ca^{2+} disponible para ser liberado en el ciclo de contracción siguiente. Durante la IC, la relación SERCA2a/PLB disminuye en gran medida por la disminución de SERCA2a, lo que provoca que la cantidad de SERCA2a disociada de PLB disminuya y por lo tanto, también su actividad y capacidad de recuperar el Ca^{2+} liberado durante la contracción (Hoshijima et al., 2006).

La disminución en los niveles de PLB fosforilado hace que la actividad de SERCA2a se encuentre disminuida en la IC; esta disminución no es solo debido a una menor expresión de la proteína SERCA2a, sino también a alteraciones en el nivel de fosforilación de PLB. Cuando la actividad de la proteína cinasa C-α (PKC-α) aumenta, se fosforila el inhibidor (I-1) de la proteína fosfatasa-1 (PP1), que, al no estar así inactivada, desfosforila y activa a PLB. Se ha reportado que la disociación de la proteína calstabina del canal RyR2 lleva a un estado inestable del mismo, lo

que resulta en una mayor probabilidad de apertura del canal. Como resultado, aparecen liberaciones espontáneas de Ca^{2+} durante la diástole (fuga diastólica), que se asocian con arritmias ventriculares y más importantemente, con una disminución de la reserva de Ca^{2+} disponible para la contracción (Bers, 2008; Braunwald and Bristow, 2000).

La fosforilación de PLB, RyR2 y la regulación de corrientes de calcio son blancos funcionales comunes que CaMKII comparte con PKA en la regulación del AEC. Una mayor actividad de CaMKII en la IC contribuye a reducir el contenido de calcio del RS y la función sistólica, causando también fuga diastólica de Ca^{2+} del RS y cambios en las corrientes de Ca^{2+} que pueden ser arritmogénicos. Una de las principales causas de muerte súbita en pacientes con insuficiencia cardiaca es la arritmia ventricular. Aunque la liberación de Ca^{2+} anormal del RS a través de RyR2 se ha relacionado con arritmogénesis, los mecanismos moleculares exactos que median este fenómeno son desconocidos. Estudios recientes sugieren que la fosforilación de RyR2 dependiente de CaMKII está relacionada con la mayor fuga diastólica de Ca^{2+} del RS y el menor llenado con Ca^{2+} del mismo observados durante la IC, lo que contribuiría a las arritmias y la disfunción contráctil presentes en la enfermedad (Ai et al., 2005). La fosforilación de RyR2 en la S2814 por parte de CaMKII juega un papel importante en la arritmogénesis y muerte súbita cardiaca de ratones con insuficiencia cardiaca. Este hecho sugiere que la desestabilización de la actividad de RyR2 debido a su excesiva fosforilación por CaMKII resulta en la reducción de la refractariedad post-descarga, que es un mecanismo común implicado en la arritmogénesis y en la disfunción contráctil del corazón insuficiente. Estos hallazgos podrían conducir al desarrollo de terapias más específicas de drogas que podrían modificar el nivel de fosforilación de RyR2 dependiente de CaMKII, reduciendo así la fuga diastólica de Ca^{2+} y la muerte por arritmias (Belevych et al., 2011; van Oort et al., 2010).

La disfunción contráctil en la IC humana se ha asociado con corrientes de sodio ($[Na^+]_i$) y diastólica de calcio ($[Ca^{2+}]_i$) elevadas. Se ha sugerido que el aumento en la entrada de Na^+ por los canales de sodio tardíos operados por voltaje (INaL) contribuye al aumento de la $[Na^+]_i$ en la IC. Evidencias recientes demuestran que el INaL contribuye a la acumulación del Ca^{2+} intracelular durante la diástole y a su

liberación espontánea en los miocitos ventriculares de un modelo canino de IC crónica, apoyando la idea de que el bloqueo selectivo de los INaL representa una estrategia potencial para el tratamiento de la disfunción diastólica y las arritmias relacionadas con calcio en la IC (Undrovinas et al., 2010). La ranolazina es un fármaco anti-isquémico que inhibe los INaL. En un estudio reciente, la ranolazina redujo el aumento de la tensión diastólica dependiente de frecuencia sin efectos inotrópicos negativos sobre la contractilidad de los músculos de corazones humanos insuficientes en etapa terminal. Por lo tanto, la ranolazina podría ser de beneficio terapéutico para pacientes con disfunción diastólica derivada de $[Na^+]_i$ elevadas o sobrecarga de $[Ca^{2+}]_i$ diastólica, mejorando la función diastólica en la insuficiencia cardiaca (Sossalla et al., 2008).

La sobrecarga de presión cardiaca, como la observada en pacientes hipertensos, es el principal estímulo patológico que desencadena la HC y es el principal factor de riesgo para el desarrollo de IC. Una variedad de cascadas de transducción de señales dependientes de Ca^{2+} se han relacionado con la HC, pero si estas vías son independientes o interdependientes y si hay especificidad entre ellas aún no está claro y se encuentra bajo investigación.

Vías transcripcionales mediadas por calcio en el corazón.

Vía de señalización Calcineurina-NFAT.

La calcineurina (CN) es una fosfatasa de serina/treonina activada por Ca^{2+}-calmodulina que se expresa de forma ubicua y desempeña un papel importante en la transducción de señales dependientes de Ca^{2+}. La CN es un heterodímero compuesto de una subunidad catalítica A de unión a CaM (CN-A) y de una subunidad reguladora B de unión a Ca^{2+} (CN-B) (Diedrichs et al., 2007). En cardiomiocitos, CN desfosforila al factor nuclear de células T activadas-3 (NFAT3 o NFATc4), induciendo la translocación de NFAT3 desde el citosol al núcleo para hacer posible la activación de sus genes blanco.

Varios estudios demuestran que la CN juega un papel muy importante en el desarrollo de la hipertrofia patológica, y su papel como modulador de la HC se ha

estudiado ampliamente usando estrategias tales como fármacos o proteínas inhibidores de la CN o ratones transgénicos que sobre-expresan CN activa.

Aunque la eliminación completa de CN-B conduce a la letalidad embrionaria, en un estudio reciente, la eliminación cardiaco-restringida de CN-B1, permitió que los ratones fueran viables y que se pudiera caracterizar de mejor manera la función de la señalización mediada por CN en el desarrollo del corazón (Schaeffer et al., 2009). Los ratones con deleción cardiaca específica del gen CN-B1 muestran que la CN es necesaria para el crecimiento cardiaco normal después del parto. La baja expresión de CN-B1 provoca que la relación entre el peso ventricular cardiaco y el corporal disminuya, que se desarrolle disfunción sistólica y diastólica, y que aumente la mortalidad alcanzando el 100% a los 7 meses de edad. Curiosamente, las alteraciones en la función cardíaca observadas a los 3 meses de edad utilizando ecocardiografía y mediciones hemodinámicas no fueron acompañadas de anormalidades histológicas en los miocitos cardiacos, como desorganización miofibrilar o fibrosis. Estos datos sugieren que la CN juega un papel importante no sólo en la HC patológica, sino también en el crecimiento normal del corazón. La deficiencia cardiaco-específica de CN provoca cardiomiopatía letal, lo que altera los niveles de proteínas implicadas en el AEC.

También se ha demostrado que la activación de CN o de su factor de transcripción blanco NFAT3 es suficiente para provocar hipertrofia de miocardio *in vivo*. La HC patológica prolongada se asocia con arritmias, descompensación, cardiomiopatía dilatada y muerte súbita. Se ha encontrado que en diferentes etapas de HC humana, la vía de la CN está activa en el miocardio hipertrófico, como lo demuestra el aumento de actividad y expresión de CN y GATA-4, así como el aumento de la translocación de NFAT3 desfosforilado al núcleo. Estos cambios fueron más evidentes en el miocardio hipertrófico descompensado en comparación con el compensado (Diedrichs et al., 2007).

La ruta CN-NFAT es típicamente activada por altas $[Ca^{2+}]_c$ sostenidas, sin embargo, hay evidencia que sugiere señalización co-dependiente con las cinasas MEK1-ERK1/2 (Sanna et al., 2005). Dicha evidencia muestra que la inhibición de CN o NFAT en cultivos de cardiomiocitos neonatales mitiga la respuesta hipertrófica

promovida por MEK1 activada, mientras que la inhibición de la señalización mediada por MEK1-ERK1/2 reduce el crecimiento hipertrófico en respuesta a CN activada.

El papel de la señalización por Ca^{2+} en el desarrollo de hipertrofia se ha investigado en los cardiomiocitos de ratas neonatas *in vitro*. La HC y la reorganización del sarcómero son inducidas por los agonistas de receptores tales como la angiotensina II, la aldosterona y la norepinefrina, y por un aumento en la concentración de KCl extracelular. Todos estos tratamientos aumentaron la frecuencia de los transitorios espontáneos de Ca^{2+}, causaron la translocación nuclear de la proteína de fusión transfectada NFAT-GFP e incrementaron la transcripción de un gen reportero con un promotor sensible a NFAT. La respuesta hipertrófica y la translocación de NFAT fue inhibida por la ciclosporina, inhibidor de la CN A (CsA), pero no por el aumento de la frecuencia de transitorios de Ca^{2+}. Lo anterior sugiere que la vía CN-NFAT puede actuar como un integrador de las señales contráctiles mediadas por calcio y que esta vía puede decodificar alteraciones incluso en la frecuencia de oscilaciones rápidas de Ca^{2+} (Colella et al., 2008).

Vía de señalización CaMK-MEF2.

El factor de transcripción MEF2 (factor potenciador de miocitos 2) es un miembro de la familia de factores de transcripción MADS-box. La familia de factores de transcripción MEF2 es esencial para la expresión de muchos genes específicos de músculo (McKinsey et al., 2002). Existen 4 genes MEF2 (MEF2A, -B, -C, y -D) y todos ellos son capaces de unirse a la secuencia consenso rica en A/T, $YTA(A/T)_4TAR$. Los factores de transcripción MEF2 se expresan principalmente en todos los tipos de músculos, pero también se han encontrado altos niveles de MEF2 en el desarrollo del sistema nervioso central. Durante la diferenciación del músculo esquelético, MEF2D es el primer miembro de la familia en aparecer, seguido por MEF2A y MEF2C (Black and Olson, 1998). MEF2 es capaz de interactuar con los miembros de la familia de proteínas MyoD para controlar la expresión de varios genes músculo-específicos (McKinsey et al., 2002).

MEF2 es un blanco común de varias vías de señalización activadas durante la hipertrofia, aunque su función exacta en el remodelado cardíaco y los genes cardíacos que modula todavía son objeto de investigación de varios grupos. Las proteínas MEF2 son capaces de responder a vías de señalización controladas por calcio tales como CaMK y CN (McKinsey et al., 2002). En el corazón adulto, solo existe actividad transcripcional basal de MEF2, la cual es necesaria para la expresión continua de genes implicados en el mantenimiento de la homeostasis de los cardiomiocitos (el Azzouzi et al., 2010). El efecto de la inhibición de MEF2 en la HC e IC se ha probado en modelos fisiológicos con sobrecarga de presión al someter a ratones transgénicos que expresan una forma dominante negativa de MEF2 a constricción transversal de la aorta. En este modelo, los datos ecocardiográficos demostraron que la expresión de la forma dominante negativa de MEF2 no impide el desarrollo de HC. Sin embargo, la actividad de MEF2 es aumentada por vías pro-hipertróficas dependientes de Ca^{2+} como CN, CaMKII, PKC, PKD, MAPK-1 y p38 MAPK. Además, se encontró una menor expresión de la subunidad 6 de la NADH deshidrogenasa (ND6), que forma parte del Complejo I del sistema de fosforilación oxidativa y que es fuente principal de energía en el músculo cardiaco. La expresión reducida de ND6 produce un aumento de la muerte celular por la sobreproducción de especies reactivas de oxígeno seguido por sobrecarga de presión (el Azzouzi et al., 2010). En contraste con estos hallazgos, reportes anteriores muestran que la actividad disminuida de MEF2 en ratones transgénicos carentes de CN se asocia con una atenuación de la disfunción cardiaca (van Oort et al., 2006). Esto podría explicarse debido a que la activación de la vía de la CN, sucede a la par que la activación de otras cascadas de señalización como p38-MAPK y CaMKII, lo que produce un efecto diferente en el contexto de la sobrecarga de presión (el Azzouzi et al., 2010).

Las CaMKs están involucradas en la regulación de varios factores de transcripción como AP-1, SRF, CREB y MEF2. La activación de MEF2 dependiente de CaMK no se produce por fosforilación directa de MEF2 por la CaMKII, sino que requiere la interacción de las HDACs (desacetilasas de histonas) de clase II (Gordon et al., 2009). Las HDACs de clase II que se expresan en el corazón tienen un dominio de unión a MEF2 en su región N-terminal, que no está presente en las otras

clases de HDACs. Este dominio N-terminal es capaz de unirse a la familia de chaperonas 14-3-3 permitiendo que este complejo se exporte al citoplasma, con la consecuente liberación de la represión de MEF2 (Bers, 2008).

También se ha reportado que mientras que CaMKIIδ$_B$ y CaMKIIδ$_C$ tienen diferentes efectos en la fosforilación de proteínas relacionadas con el manejo del Ca^{2+} como RyR2 y PLB, ambas isoformas afectan la expresión génica de MEF2 mediada por HDACs. La CaMKIIδ$_B$ (nuclear) fosforila a HDAC4 en el núcleo permitiendo la unión de las chaperonas 14-3-3, con la consecuente exportación del complejo fosfo-HDAC4/14-3-3 fuera del núcleo. Como consecuencia de ello, MEF2 es liberado de la represión por parte de HDAC4 y sus genes blanco pueden entonces ser transcritos. Por otro lado, la activación de la CaMKIIδ$_C$ (citoplásmica) permite que fosforile a HDAC4 en el citosol, evitando así su importación desde el citosol al núcleo. Además de que HDAC4 está regulada por Ca^{2+}, HDAC5 también es parcialmente regulada por la $[Ca^{2+}]_c$, ya que su importación al núcleo requiere su dimerización con HDAC4 en forma calcio-dependiente (Backs et al., 2008). Una vez en el núcleo, HDAC5 interactúa con MEF-2 y esta interacción es inhibida por la unión directa de Ca^{2+}/ calmodulina a HDAC5 (Berger et al., 2003). En otras palabras, la CaMKIIδ$_C$ también es capaz de activar la transcripción de los factores MEF2 y otros, además de sus efectos sobre RyR2 y PLB (Zhang et al., 2007). Además, se encontró que una forma constitutivamente activa de CaMKIIδ$_C$ es capaz de fosforilar y por tanto reducir la actividad de CN, resultando en una disminución de la translocación de NFAT al núcleo, mientras que una forma dominante negativa de CaMKIIδ$_C$ indirectamente reduce la fosforilación de NFAT y por tanto promueve su translocación nuclear. Debido al hecho de que, tanto CN como CaMKIIδ$_C$ se activan en forma calcio-dependiente, estos hallazgos sugieren que CaMKIIδ$_C$ podría actuar como un modulador negativo de la actividad de CN (MacDonnell et al., 2009).

Se ha demostrado que las cinasas activadas CaMKI y CaMKIV también inducen respuestas hipertróficas en cardiomiocitos *in vitro*, y que ratones que sobre-expresan CaMKIV desarrollan HC con un diámetro del ventrículo izquierdo al final de la diástole incrementado y una fracción de acortamiento disminuida (Passier et al., 2000). Por otro lado, se demostró que CaMKIV activa al factor de transcripción MEF2 a través de un mecanismo postraduccional en el corazón hipertrófico *in vivo*.

La CN activada es un activador menos eficiente de la transcripción dependiente de MEF2, lo que sugiere que las vías de CN-NFAT y CaMK-MEF2 actúan en paralelo. Estos hallazgos identifican a MEF2 como un blanco para la señalización mediada por CaMK en el corazón hipertrófico y sugieren que las vías CaMK y CN tienen como blancos preferentes diferentes factores de transcripción para inducir HC (Passier et al., 2000). Por lo tanto, la evidencia indica que la activación de MEF2 es indispensable para desencadenar la respuesta hipertrófica fisiológica y patológica, lo que sugiere un mecanismo molecular conservado. Diversos datos apoyan la hipótesis de que la adaptación cardiaca durante la hipertrofia puede ser dependiente de la activación de múltiples factores de transcripción cardiacos como NFAT, GATA-4, Nkx2.5 y MEF2, que emulan el patrón de expresión observado en la morfogénesis del corazón (Kolodziejczyk et al., 1999).

Estrés del retículo endoplásmico, otras vías que regulan concentraciones de calcio citosólico y su interrelación con las vías CN-NFAT y CaMK-MEF2.

El estrés del retículo endoplásmico (ERS, por siglas en inglés) está implicado en diversas enfermedades cardiovasculares y participa en el desarrollo de HC; sin embargo, su mecanismo molecular aún no está totalmente claro. Se ha reportado que MEF2C participa en la HC derivada de ER inducido por calcio (Zhang et al., 2010). La Tg es capaz de inducir ER al igual que hipertrofia en cardiomiocitos, dicho fármaco induce una elevación significativa del nivel de Ca^{2+} intracelular, la activación de CN, y la expresión de MEF2C en forma dosis- y tiempo-dependiente. En el estudio citado, la CsA suprimió la translocación nuclear de MEF2C e inhibió la HC inducida por Tg. En este estudio, los resultados demuestran que la vía CN-MEF2C está involucrada en la HC inducida por el ER en cardiomiocitos (Zhang et al., 2010).

La proteína de intercambio activada directamente por AMPc (EPAC, por sus siglas en inglés) es un sensor de AMPc y es un mecanismo novedoso de transducción para la señalización por AMPc. Datos recientes muestran que EPAC activa una vía de señalización pro-hipertrófica que involucra a PLC, H-Ras, CaMKII y el exporte

nuclear de HDAC4. La activación de EPAC también aumenta los niveles de MEF2 de forma CaMKII-dependiente en miocitos cardiacos primarios (Metrich et al., 2010).

Como se describió anteriormente, CN-NFAT y CaMK-MEF2 son las vías de señalización más importantes en cuanto al control de la expresión de genes cardiacos dependiente de calcio; sin embargo, hay otras señales que pueden regular estas vías en distintos niveles. Además, ambas vías se pueden regular entre sí proporcionando una regulación más compleja y eficiente de la expresión génica. Por ejemplo, en un estudio donde se usó un modelo *in vitro* del estiramiento mecánico de cultivos de cardiomiocitos neonatales para evaluar los mecanismos propuestos para la activación de CN inducida por calcio, se demostró que los canales de Ca^{2+} tipo L, la entrada capacitiva de Ca^{2+} y los intercambiadores NHE y NCX también están involucrados en esta respuesta. Por lo tanto, dichas proteínas son potenciales blancos terapéuticos para la inhibición de la HC (Zobel et al., 2007).

Los microRNAs (miRNAs) están implicados en la hipertrofia y el desarrollo cardiacos, por ejemplo, se ha demostrado que la sobre-expresión de miR-195 puede desencadenar HC e IC; mientras que la hipertrofia mediada por CN constitutivamente activa disminuye los niveles de miR-133 (van Rooij et al., 2006). Recientemente se encontró que miR-1, un miRNA específico de cardiomiocitos, inhibe post-transcripcionalmente y de manera directa la expresión de CaM y MEF2A, e indirectamente la de GATA-4, posicionando a este miRNA como regulador central de la señalización por calcio en HC (Ikeda et al., 2009). Por otro lado, se observó que miR-199b (otro miRNA sobre-expresado durante la hipertrofia) es un blanco de NFAT. Además, miR-199b inhibe a la cinasa regulada por fosforilación de tirosina de doble especificidad 1a (Dyrk1a), cinasa nuclear capaz de fosforilar a NFAT, lo que constituye una retroalimentación patógena (da Costa Martins et al., 2010). En células HEK293T se ha reportado que Dyrk1a y Dyrk3 fosforilan y activan a SIRT1, conocida desacetilasa de histonas dependiente de NAD que promueve la expresión de genes que mejoran la función cardíaca (Guo et al., 2010; Sundaresan et al., 2011).

Se llevaron a cabo análisis de expresión de RNAm por microarreglos con muestras de corazones humanos sanos e insuficientes para encontrar genes que contuvieran conservados motivos de unión a DNA para MEF2, NFAT, NKX2, GATA

y FOX dentro de sus primeros 1000 pb de región promotora, con el fin de investigar la correlación entre estos factores de transcripción. Cuando la expresión diferencial de genes se modela como una función de combinaciones de factores de transcripción presentes en sus regiones promotoras, es posible predecir varias combinaciones de expresión diferencial de genes en el corazón insuficiente. En este estudio se encontró que la mayor cantidad de genes con expresión diferencial contenían sitios de unión tanto a NFAT como a MEF2 en su región promotora. Este hallazgo proporciona evidencia de co-regulación de la expresión génica cardiaca por NFAT y MEF2 en IC humana (Putt et al., 2009). Recientemente se reportó, mediante un modelo animal de IC inducida por CN, que MEF2 activa un programa genético que provoca la dilatación de cavidades, disfunción mecánica, y cardiomiopatía dilatada, pero que afecta mínimamente el crecimiento cardiaco, lo que demuestra que los factores de transcripción cardiacos MEF2 son activados por CN en forma NFAT-dependiente (van Oort et al., 2006).

La regulación por calcio de la expresión de SERCA2a.

En el corazón humano se demostró que la actividad de SERCA2a puede ser regulada en un proceso mediado por la CN (Münch et al., 2002), lo que puso sobre la mesa la pregunta de si también estaba involucrada en su regulación transcripcional. Recientemente, el tratamiento de cardiomiocitos con bajas concentraciones de Tg, dio lugar a la activación de CN, ya que este tratamiento incrementó la expresión de un gen reportero bajo el control del promotor de NFAT y aumentó la translocación nuclear de NFAT (Prasad and Inesi, 2011). La exposición de los miocitos cardiacos a Tg fue seguida por el aumento de la expresión de SERCA2a y aumentó aún más cuando la inactivación de CN por CaMKII se previno con KN93 (un inhibidor de la CaMKII). Además, la expresión de SERCA2a se reduce por la inhibición de CN con CsA. Estos resultados demuestran que la Tg activa la vía CN-NFAT por un aumento de la $[Ca^{2+}]_c$ en reposo, lo que sugiere que el incremento en la expresión de SERCA2a, puede ser mediado a nivel transcripcional, proporcionando un mecanismo homeostático para el control a largo plazo de la $[Ca^{2+}]_c$.

Se ha demostrado que el ERS causado por el vaciamiento de Ca^{2+} es seguido por la translocación de ATF6 al núcleo (Adachi et al., 2008). ATF6 es un factor de transcripción implicado en la regulación de la expresión de proteínas relacionadas con el estrés del retículo endoplásmico como grp78 y Bip, de proteínas implicadas en la homeostasis del calcio como SERCA2, CaM, la ATPasa Na^+/K^+, α-MHC, ATPasas de Ca^{2+} de la membrana plasmática (PMCAs) y la proteína de unión a calcio S100, así como de factores de transcripción involucrados en la expresión de genes cardiacos como Egr-1 y klf15 (Adachi et al., 2008; Belmont et al., 2008; Thuerauf et al., 2001). La activación transcripcional mediada por ATF6 depende del elemento de estrés del retículo endoplasmático (ERSE) en sus genes blanco y requiere de un sitio de unión de alta afinidad a NF-Y con el fin de ser selectivo entre diferentes ERSEs (Li et al., 2000). Como ya se mencionó, se ha demostrado que la expresión del gen *ATP2A2* es activada por Tg, y aunque su promotor proximal tiene un sitio ERSE (posición -78 del gen humano) que es responsable de la activación del gen por ATF6. En estas condiciones, no toda la activación transcripcional se debe a ello, lo que sugiere que otras vías de señalización mediadas por calcio participan en la activación transcripcional del gen inducida por Tg (Thuerauf et al., 2001). Otro gen importante inducido por ATF6 es MCIP-1 (también conocido como regulador de la calcineurina-1 –RCAN1), ya que el estrés del retículo puede ser transducido por esta vía para regular diversas funciones celulares que implican la unión de NFAT, por ejemplo, el crecimiento y el desarrollo (Belmont et al., 2008).

Cardiomiocitos tratados con monoxima de 2,3-butanodiona (BDM), un fármaco que mantiene los flujos de calcio, pero suprime la contracción, o con verapamil, un medicamento que inhibe tanto los flujos de calcio como la contracción, muestran que la BDM aumenta el nivel de RNAm de SERCA2a, lo cual puede ser suprimido por verapamil y reducido por CsA y KN-93 (Vlasblom et al., 2004). En estudios funcionales por transfección transitoria de cardiomiocitos neonatales, la actividad transcripcional del promotor de *ATP2A2* fue estimulada por MEF2C y NFAT3 sólo cuando ambos factores fueron co-transfectados. Esta activación podría ser producto de la estimulación sinérgica de la actividad del promotor de SERCA2 por NFAT3 y MEF2C, a pesar de que una interacción física directa entre ambos

factores no se ha demostrado. De los datos anteriores resulta evidente que la actividad contráctil evita la expresión de SERCA2 a través de vías distintas e independientes. Estos resultados sugieren que en cardiomiocitos con actividad contráctil suprimida con BDM, CN y CaMKII median el aumento en la expresión del RNAm de SERCA2a.

A pesar de que la baja expresión de SERCA2a en HC e IC está bien documentada, hay algunos informes en relación a la regulación positiva calcio-dependiente de la expresión de SERCA2a (Prasad and Inesi, 2011; Vlasblom et al., 2004). De hecho, en modelos *in vitro* hay reportes que muestran una expresión aumentada de SERCA2a en respuesta a tratamientos con fenilefrina, (Anwar et al., 2005; Prasad et al., 2007b; Taigen et al., 2000). Sin embargo, como se discute en dichos trabajos, la estimulación α-adrenérgica activa a la CN y la expresión de NFAT, ambos hechos mediados por alto calcio citoplásmico que también es capaz de promover la transcripción del gen *ATP2A2*. Esta aparente contradicción plantea la necesidad de describir con precisión los mecanismos moleculares que regulan la dependencia de calcio de la expresión de SERCA2a y también de otros mecanismos independientes que participan en el desarrollo de la hipertrofia, que en última instancia conducen a la regulación negativa de la expresión de SERCA2a en la hipertrofia e insuficiencia cardíacas.

Tomando en cuenta las evidencias hasta ahora mencionadas y dada la importancia del ion Ca^{2+} en la regulación de genes expresados en músculo, en este proyecto se estudió la participación del factor transcripcional NFAT y de los factores C/EBPβ y NF-Y en la regulación de la transcripción del gen *ATP2A2* en respuesta a altas concentraciones de Ca^{2+} intracelular. El conocimiento de los mecanismos por los que el calcio regula la transcripción del gen *ATP2A2* en cardiomiocitos normales permitirá un mejor entendimiento de la regulación de este gen bajo condiciones patológicas como la HC.

HIPÓTESIS

La concentración citoplásmica de calcio elevada promueve la unión del (los) factor(es) transcripcional(es) NFAT y/o NF-Y al promotor del gen *ATP2A2,* incrementando su expresión en cardiomiocitos.

OBJETIVO GENERAL

En cultivos primarios de cardiomiocitos de rata neonata, determinar la función de los elementos putativos de unión a los factores transcripcionales NFAT y NF-Y presentes en la región reguladora del gen *ATP2A2* responsables del aumento de su transcripción cuando se eleva la concentración citoplásmica de calcio.

OBJETIVOS PARTICULARES

1. Determinar el efecto que tiene aumentar la concentración de calcio citoplásmico con tapsigargina y el ionóforo A23187 sobre la expresión del gen *ATP2A2* a nivel de RNAm, proteína y actividad transcripcional del promotor del gen.

2. Investigar si el factor transcripcional NFAT es capaz de unirse a su sitio de unión presente en el promotor proximal del gen *ATP2A2.*

3. Investigar la participación del sitio ERSE en la respuesta a elevación de la concentración de calcio citoplásmico y qué factores de transcripción se unen a este sitio.

4. Determinar si el factor de transcripción NF-Y se une *in vivo* en la región del promotor del gen *ATP2A2.*

METODOLOGÍA

Soluciones.

El medio de cultivo DMEM sin glutamina se adicionó con glucosa (4.5 g/l), rojo de fenol (15.9 mg/l), $NaHCO_3$ (1.2 g/l), kanamicina (70 mg/l), piruvato de sodio (0.11 g/l), nistatina al 0.1 % y una mezcla de antibiótico/antimicótico (Invitrogen) al 1 %. El medio de cultivo OPTIMEM (Invitrogen) se adicionó con 2.4 g/l de $NaHCO_3$, los mismos antibióticos usados para el medio DMEM y 5-bromodesoxiuridina (5-BrdU) a una concentración final de 0.1 mM para evitar el crecimiento de fibroblastos en el cultivo como se ha descrito previamente (Simpson and Savion, 1982). El amortiguador ADS contiene las siguientes concentraciones finales: NaCl 116 mM, Hepes 20 mM (pH 7.4), NaH_2PO_4 1 mM, Glucosa 5.5 mM, KCl 5.4 mM y $MgSO_4$ 0.8 mM. El inhibidor específico para SERCA, tapsigargina (SIGMA), el ionóforo de calcio A23187 (Alomone) y la ciclosporina A (SIGMA) fueron disueltos en DMSO y guardados a -20ºC hasta su uso.

Cultivos primarios de cardiomiocitos de rata neonata.

Se usaron ratas neonatas de 1-2 días de edad de la cepa Wistar a las que se extrajeron los ventrículos del corazón. Los ventrículos extraídos se colocaron en amortiguador ADS a 37°C, se fragmentaron y lavaron 3 veces con ADS, se eliminó el sobrenadante (SN) y se incubaron con agitación constante por 15 minutos en ADS adicionado con pancreatina al 0.06% y colagenasa tipo II al 0.03%; después de la incubación se disoció mecánicamente el músculo, se recuperó el SN en medio de cultivo a 4°C (DMEM con 20% de suero fetal bovino inactivado (SFBi) y la pastilla se sometió a 4 nuevas digestiones de 10 minutos con pancreatina y colagenasa, recuperando el SN como en la primera ocasión, como se ha descrito previamente (Harary and Farley, 1960, 1963; Sen et al., 1988). Una vez que se recuperó el total de las células en medio DMEM con 20% de SFBi se pasó dicha suspensión por una malla de tela de aproximadamente 50 µm de diámetro y la suspensión celular recuperada se centrifugó a 800 rpm por 3 minutos a 20°C. El SN fue eliminado y la pastilla se resuspendió en DMEM con 10% de SFBi, lavando una vez más para centrifugar y eliminar nuevamente el SN; la pastilla obtenida se resuspendió en

DMEM con 10 % de SFBi y 5-BrdU 0.1 mM; esta suspensión celular se sembró en placas de cultivo que se incubaron a 37 °C con 5% de CO_2 por 24 horas. Después de la incubación por 24 horas, las células se mantuvieron hasta el aislamiento de proteína o RNA en medio OPTIMEM con 2% de SFBi y 5-BrdU 0.1 mM. Los diferentes fármacos fueron aplicados después de 24h de incubación con medio OPTIMEM por los tiempos indicados en cada caso.

Purificación de RNA total y cuantificación de RNA mensajero por RT-PCR en tiempo real.

Se utilizó la técnica de qRT-PCR en tiempo real para cuantificar los niveles de RNA mensajero (RNAm) para SERCA2a, usando los valores de RNAm de GAPDH para normalizar los datos. A partir de 1 µg de RNA se sintetizó el DNAc usando la enzima reverso-transcriptasa M-MLV (Invitrogen) según las instrucciones del fabricante con algunas modificaciones: se usó una concentración final de oligonucleótidos al azar de 150 ng/µl, de 7.5 ng/µl de oligonucleótido dT20, de 10 mM de dNTPs, de 0.1 M de DTT y 100 unidades de la enzima M-MLV en un volumen final de 20 µl por reacción. La reacción de PCR en tiempo real se llevó a cabo en un equipo Rotor-gene Real-Time PCR System (Qiagen) con el kit Maxima SYBR Green qPCR Master Mix (Thermo Scientific) usando una concentración de 200 nM de cada oligonucleótido y 1 µl de cDNA en un volumen final de 10 µl por reacción. Los resultados numéricos se analizaron con el método propuesto por Pfaffl y colaboradores (Pfaffl, 2001). Los oligonucleótidos fueron diseñados usando MacVector y sintetizados por Sigma-Aldrich; su secuencia se muestra en la **Tabla 2**.

Ensayos funcionales del promotor del gen *ATP2A2.*

Para estudiar el efecto de los diferentes tratamientos en el nivel de transcripción del gen *ATP2A2*, se realizaron transfecciones transitorias con 6 construcciones que contienen al gen reportero luciferasa bajo el control de distintas longitudes del promotor de *ATP2A2* usando como vector al plásmido pGL3-basic (Promega) clonado previamente (Walton et al., 1993). Una construcción que tiene sólo 254 pb de región reguladora-5´ (pGL3-hSERCA2p-254), otra que tiene 2579 pb

(pGL3-hSERCA2p-2579) y 2 intermedias con 1232 y 1741 pb (pGL3-hSERCA2p-1232 y pGL3-hSERCA2p-1714), respectivamente. Adicionalmente se usó una construcción extra que contiene las 2579 pb de la construcción más grande pero que carece de la región -522 a -1005 (pGL3-hSERCA2p-2579Δ) o las distintas versiones mutadas que se especifican en la sección "Mutagénesis dirigida". Los cultivos primarios de cardiomiocitos sembrados en placas de cultivo de 24 pozos con 48 horas de incubación se lavaron 3 veces con medio de cultivo sin suero y sin antibióticos. Se incubaron con 250 µl de este medio por 30 minutos y enseguida se agregaron 250 µl del mismo medio (incubado previamente por 20 minutos a temperatura ambiente) conteniendo 0.5 µg del plásmido correspondiente y 1.25 µl de Lipofectamina 2000 (Invitrogen). Después de agregar la mezcla plásmido-lipofectamina, se incubó por 3 horas a 37°C con CO_2 al 5%. Terminada esta incubación se cambió el medio de cultivo al suplementado con antibióticos, SFB y los fármacos probados en las distintas condiciones. Una vez que pasó el tiempo de incubación con los fármacos correspondientes se usó el amortiguador de lisis pasiva (PLB) de Promega para lisar las células en agitación constante (100 rpm) por media hora. Después de este tiempo se congelaron a -70°C por media hora y se regresaron a temperatura ambiente para recuperar el lisado y centrifugarlo a 11,000 rpm por 2 minutos. Se recuperó el sobrenadante y se midió la actividad de luciferasa (según las instrucciones de PROMEGA, fabricante del sustrato para luciferasa) usando el luminómetro marca Wallac modelo Victor^2. Para normalizar los datos de las transfecciones se midió la concentración de proteína usando el reactivo de Bio-Rad basado en el método de Bradford.

Ensayos de retardamiento de la movilidad electroforética (EMSAs).

Para determinar si los factores de transcripción NF-Y y C/EBPβ se unían a la secuencia CCAAT presente en el promotor del gen *ATP2A2*, se extrajeron núcleos de cardiomiocitos en cultivo. Dichos núcleos fueron incubados con oligonucleótidos sintéticos (marcados con el isótopo radiactivo ^{32}P) con las secuencias mostradas en la **Tabla 3**. Posteriormente, se llevaron a cabo separaciones electroforéticas en geles de acrilamida de los núcleos incubados con las sondas marcadas en presencia o ausencia de excesos molares de oligonucleótidos sin marcar para

determinar la especificidad de las uniones DNA-proteína. Los extractos nucleares también fueron incubados con anticuerpos contra los factores de transcripción NF-Y (FL-207, sc13045, Santa Cruz Biotechnology, TX, USA) y C/EBPβ (C-19, sc-150, Santa Cruz Biotechnology, TX, USA) para confirmar si la movilidad electroforética de los complejos observados en los geles de acrilamida era afectada por la unión de los anticuerpos a sus proteínas blanco. Las condiciones de incubación/corrida de los ensayos se indican en los pies de figura.

Inmunoprecipitación de la cromatina.

Con el fin de verificar si los factores transcripcionales mencionados se unían *in vivo* a la región promotora del gen *ATP2A2,* cultivos de 48 h de cardiomiocitos de rata neonata fueron fijados con formaldehído al 1 % por 15 min. y la reacción de fijado se detuvo con glicina para alcanzar una concentración final de 0.125 M. Posteriormente el medio de cultivo fue retirado y las células se lavaron con PBS 1X conteniendo PMSF 1 mM para luego agregar *buffer* de lisis (Tris-HCl 50 mM pH 8.0, EDTA 10 mM, SDS 1 % y coctel inhibidor de proteasas Sigma Fast [Sigma-Aldrich, MO, USA]). Las células lisadas se sometieron a 5 ciclos de sonicación de 60 segundos encendido/60 segundos apagado en un sonicador Biorruptor Pico (Diagenode, NJ, USA). La inmunoprecipitación se llevó a cabo con el "One-day ChIP kit" (Número de catálogo C01010081, Diagenode, NJ, USA) siguiendo las instrucciones del fabricante: la cromatina sonicada fue incubada con 4 µg de anticuerpo contra NF-YB (FL-207, sc13045, Santa Cruz Biotechnology, TX, USA), contra C/EBPβ (C-19, sc-150, Santa Cruz Biotechnology, TX, USA) o contra Sp1 (PEP2, sc-59X, Santa Cruz Biotechnology, TX, USA) como control positivo. Se usó IgG de conejo (kch 504-250, Diagenode, NJ, USA) como control negativo. Se sometieron a PCR 5 microlitros de DNA obtenido por muestra usando 2 µl de cada uno de los primer 10 µM del gen *ATP2A2* (Tabla 2) y 10 µl de Zymo Taq PreMix (Zymo Research, USA) en un volumen final de 20 µl con una concentración de DMSO de 5 %. Los parámetros de la reacción fueron: activación a 94 °C por 4 min, 40 ciclos de amplificación a 94 °C por 30 segundos seguidos de hibridación a 68 °C por 1 min; amplificación final a 72 °C por 5 min. El DNA de estas reacciones se

separó por electroforesis en gel de agarosa al 2%, se tiñó con bromuro de etidio y fue fotografiado para el análisis densitométrico con el software Image J.

Tabla 2. Secuencias de los oligonucleótidos usados para qRT-PCR y ChIP.

Oligonucleótido	Secuencia	Producto
SERCA2a F	5´-GGCTGATGGTGCTGAAAATCTC-3´	115
SERCA2a R	5´-CAATGTTTAGGAAGCGGTTACTCC-3´	
GAPDH F	5´-GGAGAAACCTGCCAAGTATGATGA-3´	126
GAPDH R	5´-TGGGAGTTGCTGTTGAAGTCG-3´	
ChIP F	5´-CAGCGAGCACAGCGAGGAC-3´	326
ChIP R	5´-GACAGCGGCGGAGGAAACTG -3´	

Tabla 3. Secuencias de los oligonucleótidos usados para EMSAs.

Oligonucleótido	Secuencia (5´ - 3´)
ERSE WT (-98) de Grp78 de rata F	GTAGCGAGTTCACCAATCGGAGGCCTCCACGACGGGGCTG
ERSE WT (-98) de Grp78 de rata R	CAGCCCCGTCGTGGAGGCCTCCGATTGGTGAACTCGCTAC
ERSE WT (-78) de *SERCA2* humano F	GGGCTCTCTCGGCCAATGAGCGGCGTCCACATGCCGCGGC
ERSE WT (-78) de *SERCA2* humano R	GCCGCGGCATGTGGACGCCGCTCATTGGCCGAGAGAGCCC
ERSE CATm (-78) de *SERCA2* humano F	GGGCTCTCTCGGCC**CC**TGAGCGGCGTCCACATGCCGCGGC
ERSE CATm (-78) de *SERCA2* humano R	GCCGCGGCATGTGGACGCCGCTCA**GG**GGCCGAGAGAGCCC
ERSE ERSFm (-78) de *SERCA2* humano F	GGGCTCTCTCGGCCAATGAGC**TT**CGTCCACATGCCGCGGC
ERSE ERSFm (-78) de *SERCA2* humano R	GCCGCGGCATGTGGACG**AA**GCTCATTGGCCGAGAGAGCCC
ERSE YY1m (-78) de *SERCA2* humano F	GGGCTCTCTCGGCCAATGAGCGGCGT**TACA**ATGCCGCGGC
ERSE YY1m (-78) de *SERCA2* humano R	GCCGCGGCAT**TGTA**ACGCCGCTCATTGGCCGAGAGAGCCC
ERSE TRIm (-78) de *SERCA2* humano F	GGGCTCTCTCGGCC**CC**TGAGC**TT**CGT**TACA**ATGCCGCGGC
ERSE TRIm (-78) de *SERCA2* humano R	GCCGCGGCAT**TGTA**ACG**AA**GCTCA**GG**GGCCGAGAGAGCCC

Las bases mutadas en los oligonucleótidos originales están subrayadas en la secuencia de los mutados.

Mutagénesis dirigida.

Se usaron 200 ng del plásmido hSERCA2-luc con -254 pb del promotor de SERCA2 que se hicieron reaccionar con 125 ng de cada uno de los dos oligonucleótidos correspondientes para mutar el sitio NFAT presente en la posición +43 del gen, la secuencia CCAAT en la posición -78, el sitio ERSF en la posición -72 o el sitio YY1 en la posición -73 (**Tabla 4**). La reacción contenía dNTPs a una concentración final de 200 µM y la enzima *Pfu* X DNA polimerasa (Jena Biosciences, Alemania) en el buffer proporcionado por el fabricante. El programa del termociclador fue el siguiente: 5 min a 95 °C; 18 ciclos con 50 seg a 95 °C, 50 seg a 75 °C y 5 min a 68 °C; y finalmente 7 min a 68 °C. El producto de la reacción de PCR se sometió a digestión enzimática con 10 U de la enzima de restricción DpnI por 2 horas para eliminar el plásmido original. El producto de la digestión enzimática se usó para transformar bacterias *E. coli* DH5α y obtener así los plásmidos mutados en las posiciones deseadas.

Tabla 4. Secuencias de los oligonucleótidos usados para las reacciones de mutagénesis dirigida.

Oligonucleótido	Secuencia (5´ - 3´)
NFATm (+43) de *SERCA2* humano F	CTTTTCCTTCGCCGCAGTTT**AAG**CCGCCGCTGTC
NFATm (+43) de *SERCA2* humano F	GACAGCGGCGG**CTT**AAACTGCGGCGAAGGAAAAG
CATm (-78) de *SERCA2* humano F	GGGCGCTCTCGG**CCCCT**GAGCGGCG
CATm (-78) de *SERCA2* humano R	CGCCGCTC**AGGGG**CCGAGAGCGCCC
ERSFm (-72) de *SERCA2* humano F	CTCGGCCAAT**GAGCGGCGT**CCACATGCCGCGG
ERSFm (-72) de *SERCA2* humano R	CCGCGGCATGTGG**ACGCCGCTC**ATTGGCCGA
YY1m (-63) de *SERCA2* humano F	GAGCGGCGT**TACAA**TGCCGCGGCG
YY1m (-63) de *SERCA2* humano R	CCGCCGCGGCA**TTGTA**ACGCCGCTC

Los sitios mutados se muestran en negritas y están subrayados.

Análisis estadístico.

Los resultados son expresados como la media de al menos 3 experimentos independientes +/- error estándar, los valores fueron comparados con análisis de varianza (ANOVA) aplicando la prueba de Holm-Sidak para comparaciones múltiples mediante el software SigmaPlot 11. El valor de $p < 0.05$ fue considerado como estadísticamente significativo. En las gráficas, el asterisco denota valores estadísticamente significativos comparados con los respectivos controles de cada experimento, en caso de haberse realizado comparaciones entre otros valores que no fuesen el control, se indican con líneas horizontales y las comparaciones con respecto a controles diferentes al principal se denotan con símbolos de numeral. Si ninguna de las comparaciones hechas fue estadísticamente significativa, se omiten los símbolos.

Aumento de la concentración citosólica de calcio y la expresión de SERCA2a.

El análisis bioinformático de la región reguladora del gen *ATP2A2* mostró la presencia de 6 sitios de unión al factor transcripcional NFAT y 2 para MEF2 además de los sitios ERSE y Sp1 mencionados en la introducción (**Figura V**).

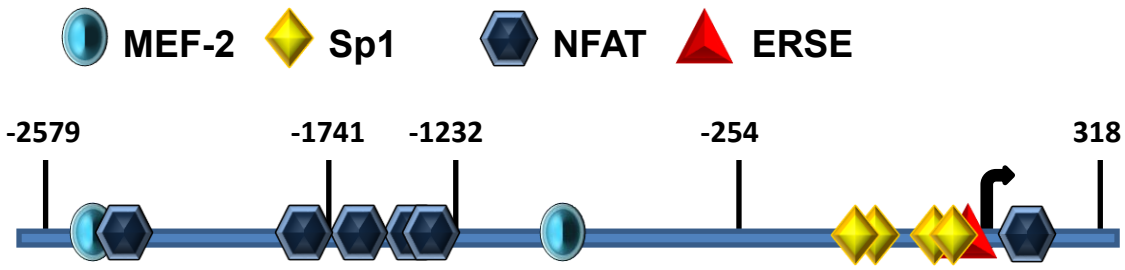

Figura V. Representación de algunos sitios de unión a factores de transcripción en el promotor proximal del gen *ATP2A2*.

Basados en los reportes de la literatura y en resultados previos del laboratorio, primero se decidió analizar la expresión del gen *ATP2A2* en respuesta a aumentos en la concentración citoplásmica de calcio. Al ensayar la abundancia del RNAm para SERCA2a en respuesta a los tratamientos con Tg y el ionóforo A23187, se encontró que se requería una concentración 200 nM de Tg o 500 nM del ionóforo para aumentar en un 50-60 % la abundancia mencionada (**Figura VI**).

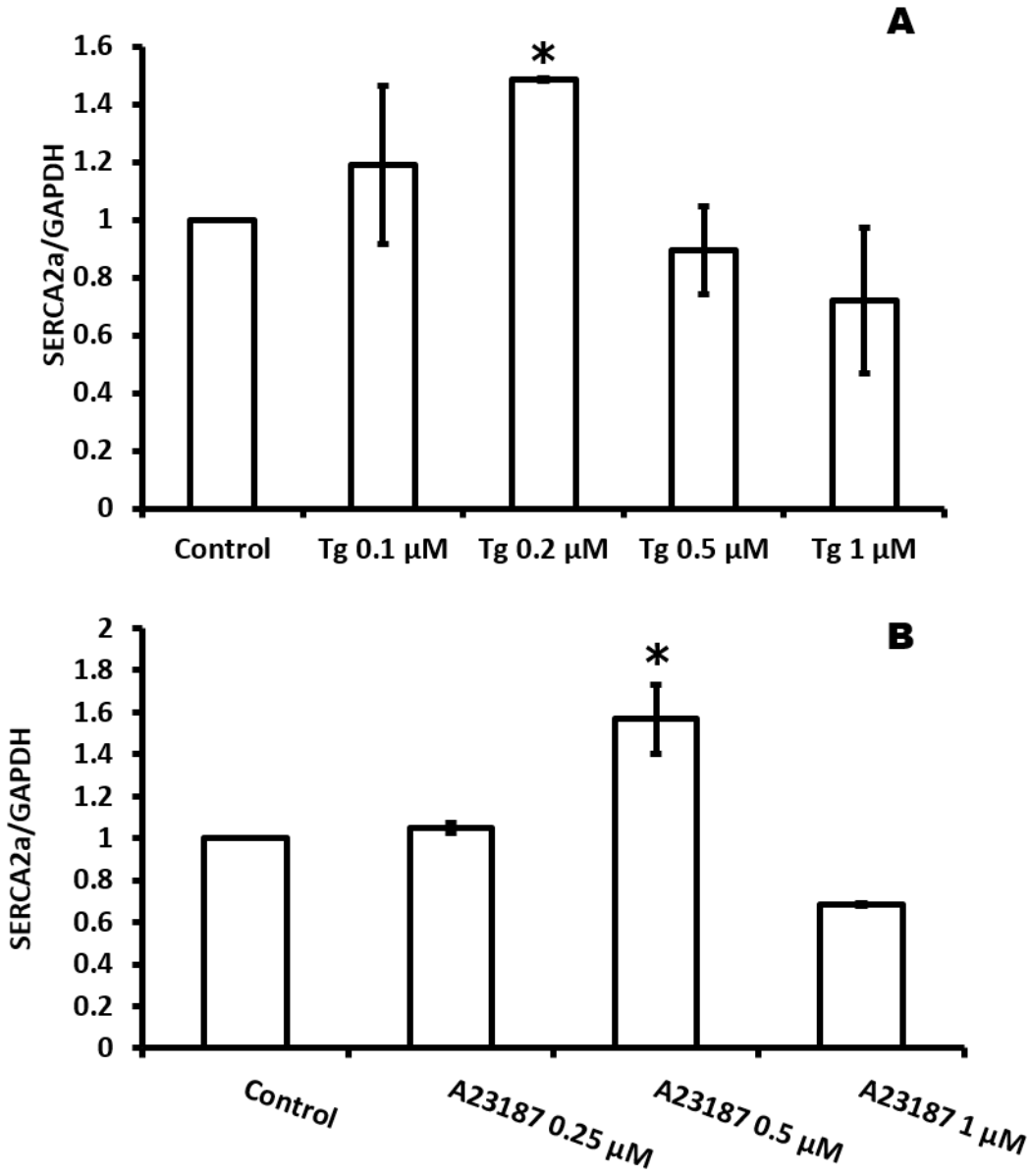

Figura VI. Curvas dosis-respuesta para la tapsigargina y el ionóforo A23187.

Abundancia relativa del RNAm de SERCA2a en respuesta a varias concentraciones de tapsigargina (A) o A23187 (B). A 48 horas de haber sido sembrados, los cardiomiocitos en cultivo fueron expuestos a los fármacos por 12 h. 1 µg de RNA total fue sometido a retrotranscripción y la abundancia del RNAm fue medida por qRT-PCR en tiempo real usando el nivel del RNA m de GAPDH para normalizar los datos. Media +/- error estándar, (p<0.05).

Como se muestra en la **Figura VII**, la estimulación por 12 h con Tg desde una concentración 50 nM aumentó la actividad transcripcional tanto de la construcción de 254 pb (denominada de aquí en adelante construcción corta o hSERCA2p-254) como de la de 2579 pb (identificada como construcción larga o hSERCA2p-2579) en 3.2 y 4.3 veces, respectivamente. Por otro lado, el ionóforo a una concentración 500 nM elevó la actividad transcripcional de la construcción corta 2.2 veces y la actividad de la construcción larga 3.6 veces. La ciclosporina A (CsA) disminuyó la inducción de la actividad transcripcional provocada por tapsigargina.

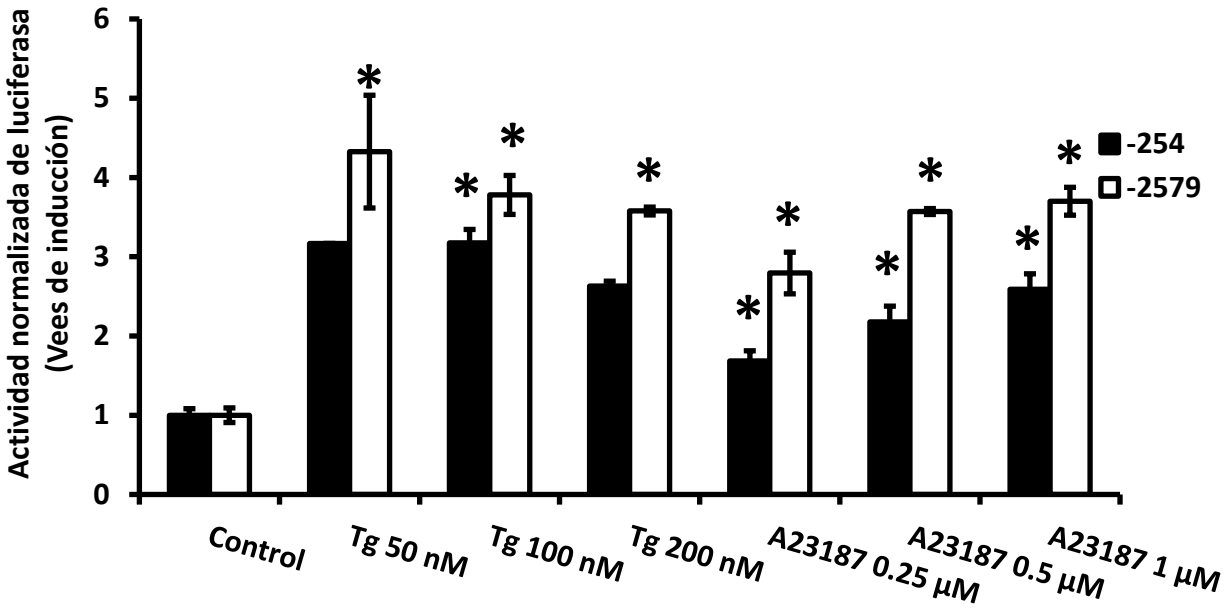

Figura VII. La tapsigargina y el ionóforo A23187 aumentan la actividad transcripcional de 2 construcciones de la región proximal del promotor de *ATP2A2.* Después de 48 horas de sembrar los cardiomiocitos en placas de 24 pozos, fueron transfectados con 0.5 µg del plásmido hSERCA2-luc conteniendo 254 pb o 2579 pb de la región promotora 5´ no traducida del gen *ATP2A2*. La actividad de luciferasa se midió después que se aplicaron los fármacos por 12 h y fue normalizada utilizando la concentración de proteína de los extractos. Media +/- error estándar, ($p<0.05$).

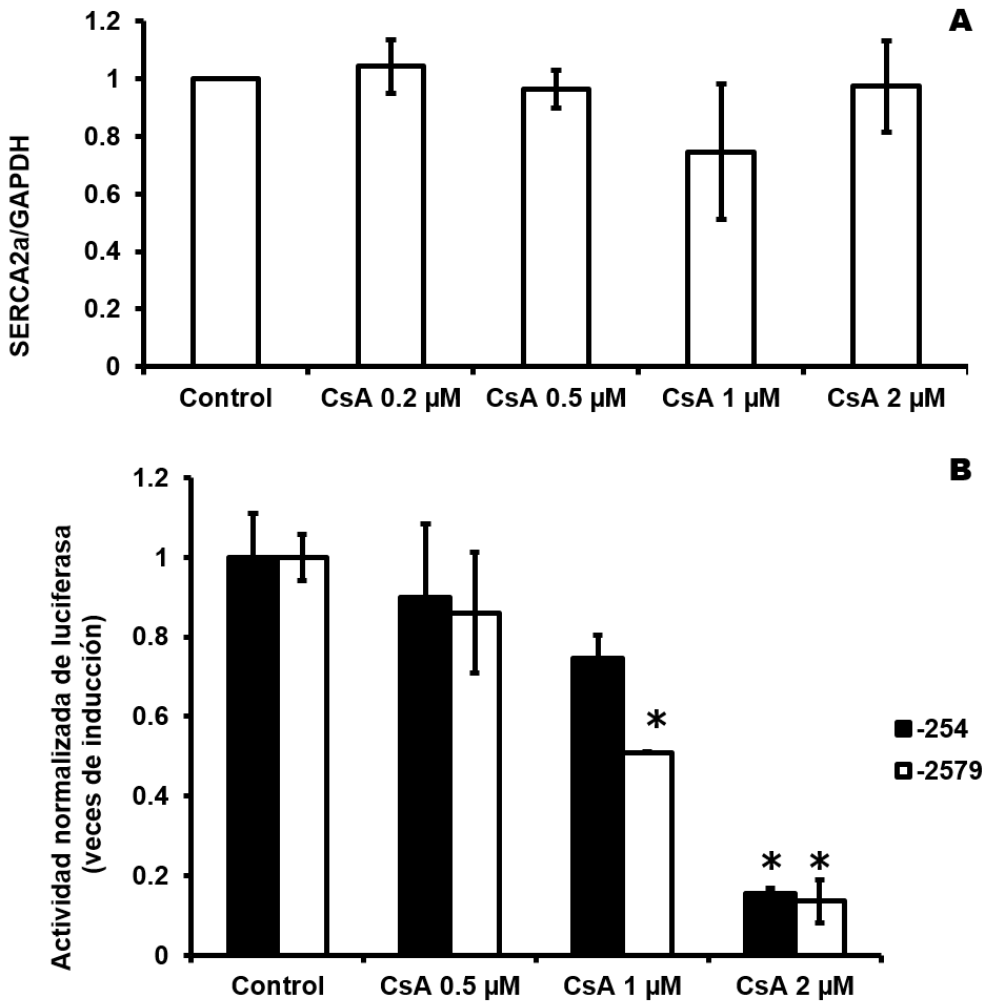

Figura *VIII*. La ciclosporina A disminuye la actividad transcripcional del gen *ATP2A2* pero no la cantidad de transcrito para SERCA2a. A) Después de 48 horas de haber sido sembrados, los cardiomiocitos en cultivo fueron expuestos a los fármacos por 12 horas. 1 µg de RNA total fue sometido a retrotranscripción y la abundancia del RNAm fue medida por qRT-PCR usando el nivel del RNAm de GAPDH para normalizar los datos B) Curva dosis-respuesta para la actividad transcripcional de 2 construcciones de la región proximal del promotor de *ATP2A2* en respuesta a idénticos tratamientos. 48 h después de sembrar los cardiomiocitos en placas de 24 pozos, fueron transfectados con 0.5 µg del plásmido hSERCA2-luc conteniendo 254 pb o 2579 pb de la región promotora 5´ no traducida del gen *ATP2A2*. La actividad de luciferasa fue medida después que se aplicaron los fármacos por 12 h y fue normalizada utilizando la concentración de proteína de los extractos. Media +/- error estándar, ($p<0.05$).

Por otro lado, como se observa en la **Figura VIII**, varias concentraciones de CsA no cambiaron la abundancia del RNAm para SERCA2a, pero sí la actividad transcripcional de ambas construcciones, por lo que se decidió ocupar la concentración de 2 µM para los siguientes experimentos.

Una vez establecidas las concentraciones adecuadas para los ensayos, se investigó si bajo estas condiciones el incremento en la actividad transcripcional del promotor del gen *ATP2A2* o la expresión del gen endógeno provocado por la Tg o por el ionóforo A23187 podía ser prevenido por el bloqueo de la ruta CN-NFAT con ciclosporina A. En la **Figura IX** se muestra que el incremento de la actividad transcripcional provocado por Tg o por el ionóforo A23187 es evitado por el co-tratamiento con CsA 2 µM, mientras que a nivel de RNAm, la CsA también evita que la Tg induzca la expresión del gen.

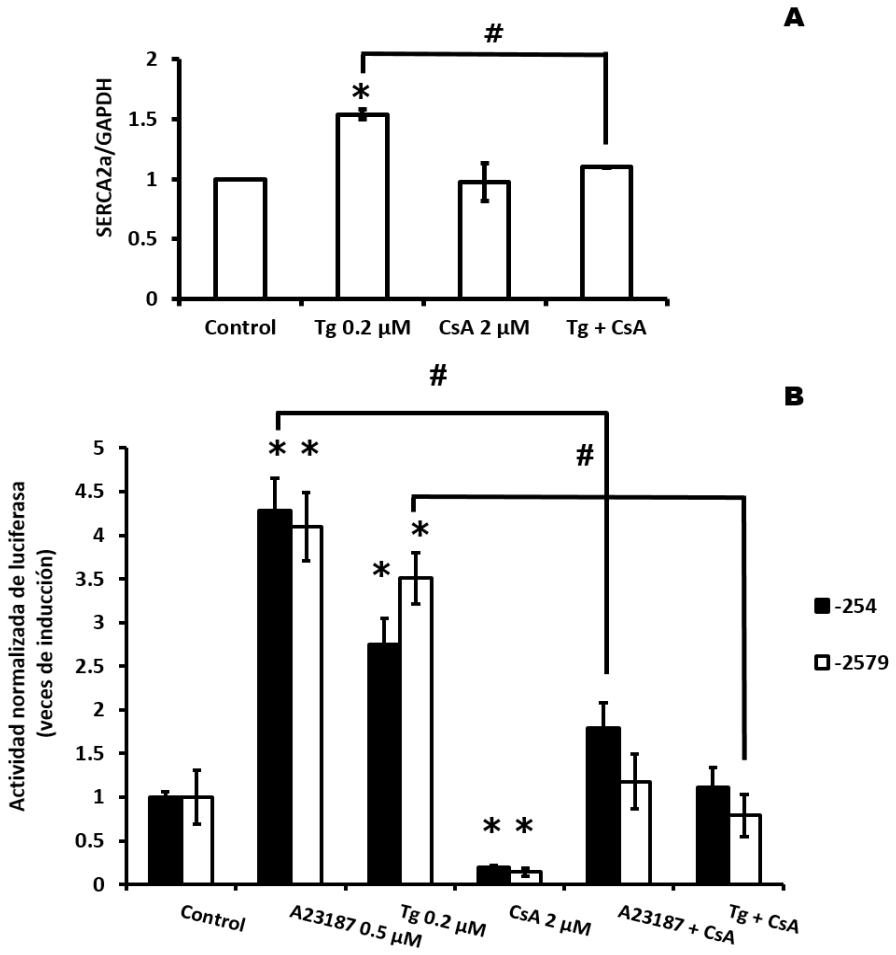

Figura IX. La CsA previene el aumento en la transcripción de *ATP2A2* y abundancia de RNA mensajero para SERCA2a. A) Abundancia relativa del RNAm de SERCA2a en respuesta a Tg y CsA. A 48 horas de haber sido sembrados, los cardiomiocitos en cultivo fueron expuestos a los fármacos por 18 h. Se extrajo el RNA total y 1 µg del mismo fue sometido a retrotranscripción y la abundancia del RNAm se midió por qRT-PCR usando el nivel del RNAm de GAPDH para normalizar los datos. B) Respuesta transcripcional de 2 construcciones de la región proximal del promotor de *ATP2A2* a Tg, al ionóforo A23187 o a CsA. 48 h después de sembrar los cardiomiocitos en placas de 24 pozos, fueron transfectados con 0.5 µg del plásmido hSERCA2-luc conteniendo 254 pb o 2579 pb de la región promotora 5´ no traducida del gen *ATP2A2*. La actividad de luciferasa fue medida después que se aplicaron los fármacos por 12 horas y fue normalizada utilizando la concentración de proteína de los extractos. Media +/- error estándar, ($p<0.05$).

Para determinar si la presencia o ausencia de distintas regiones del promotor con sitios de unión a NFAT y/o MEF2 afectaba la respuesta transcripcional ante estímulos que modifican las concentraciones citoplásmicas de calcio o que bloquean alguna de las vías de respuesta a estos cambios, se usaron tres construcciones más del promotor del gen *ATP2A2*. Las longitudes de las construcciones están representadas por las líneas numeradas en la **Figura V** y van desde la posición 318 del gen hasta la -254, la -1232, la -1741 o la -2579 respectivamente. También se usó una quinta que contiene hasta la posición -2579 pero con una deleción de -522 a -1005 (-2579Δ) para eliminar exclusivamente el primer sitio de unión a MEF2 en el promotor sin prescindir del resto.

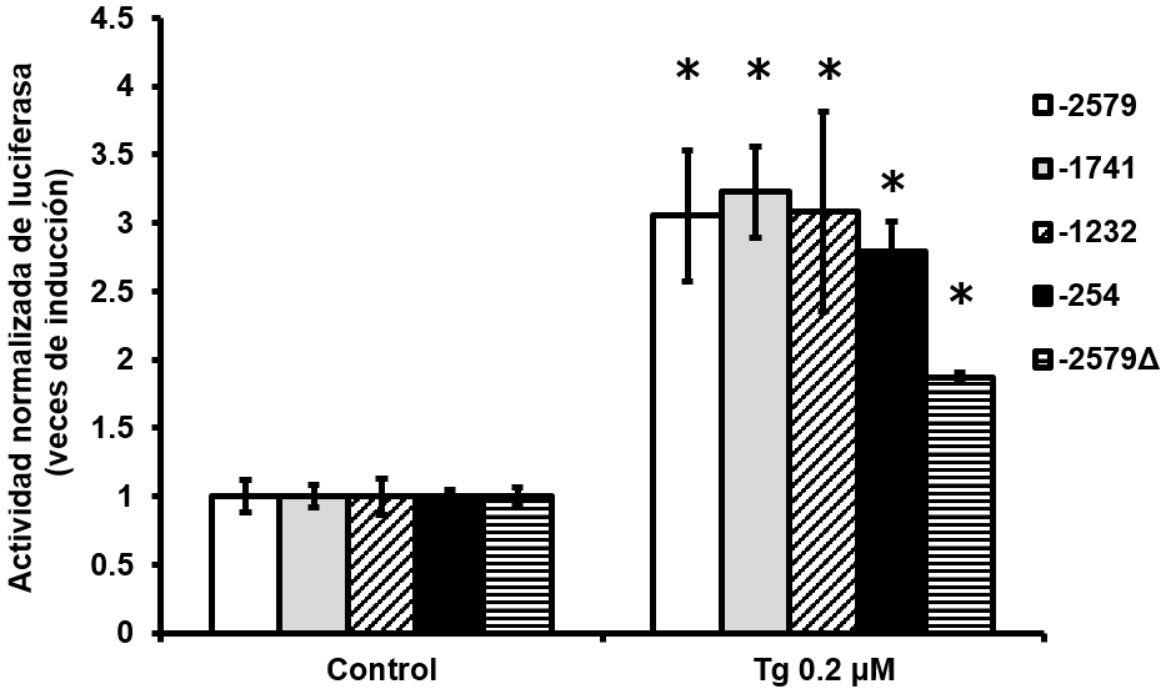

Figura X. Respuesta transcripcional de 5 construcciones de la región proximal del promotor *ATP2A2* a tapsigargina. Después de 48 h de sembrar los cardiomiocitos en placas de 24 pozos, se transfectaron con 0.5 µg del plásmido hSERCA2-luc correspondiente (-254, -1232, -1741, -2579 ó -2579Δ). La actividad de luciferasa se midió después que se aplicaron los fármacos por 12 h y se normalizó utilizando la concentración de proteína de los extractos. Media +/- error estándar, ($p<0.05$).

En la **Figura X** se observa que salvo la construcción -2579Δ en la que hay una ligera menor inducción, en todas se observa que la tapsigargina aumenta aproximadamente en la misma proporción la actividad transcripcional. Por este motivo se concluyó que era muy probable que el o los sitios que median la respuesta del gen a los tratamientos con Tg o ionóforo estuvieran en la zona más cercana al sitio de inicio de la transcripción y dado que la inducción había mostrado ser sensible al tratamiento con CsA, algún sitio de unión a NFAT podía ser el responsable.

El sitio putativo de unión a NFAT no media la activación de la transcripción del gen *ATP2A2* por tapsigargina.

El análisis bio-informático había mostrado que el único sitio de unión a NFAT en la construcción corta era el de la posición +43 y por ello se procedió a mutarlo para verificar si la actividad transcripcional de la construcción mutante dejaba de ser inducida por Tg. En la **Figura XI** se muestra que, aunque la actividad basal disminuye alrededor de un 60 %, la construcción mutada sigue siendo inducida por el tratamiento con Tg y A23187.

Como se menciona en la introducción, las altas $[Ca^{2+}]_c$ son capaces de inducir estrés del RE. Para valorar dicha posibilidad en nuestro sistema, se midieron los niveles del RNAm para la proteína grp78, indicador de dicho estrés. Como se muestra en la **Figura XII**, los tratamientos con Tg y A23187 aumentaron entre 4 y 8 veces los niveles basales de expresión de dicho marcador, indicando la existencia de estrés del retículo en los cultivos de cardiomiocitos.

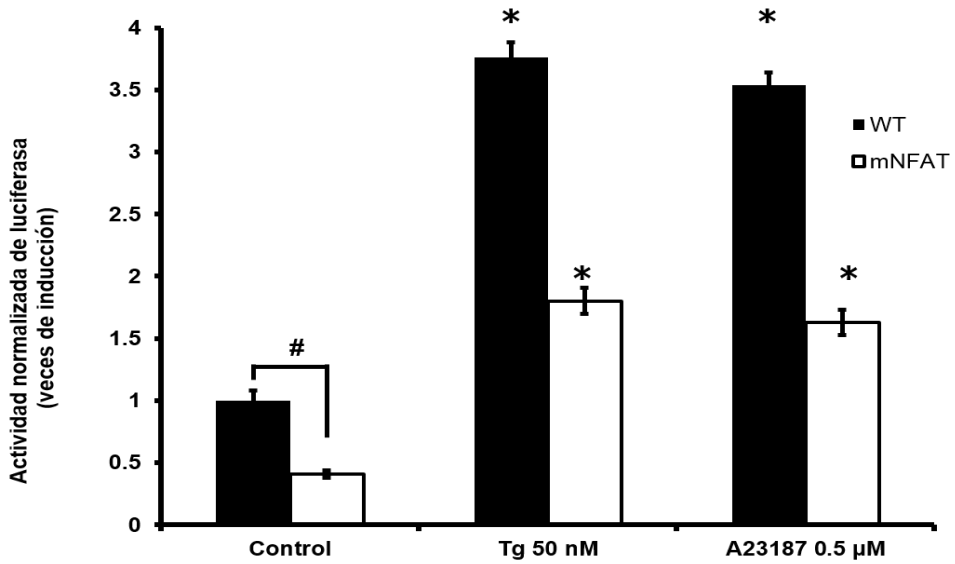

Figura XI. La mutación del sitio de unión a NFAT en el promotor del gen *ATP2A2* no evita que éste aumente su expresión en respuesta a tapsigargina. Después de 48 h de sembrar los cardiomiocitos en placas de 24 pozos, fueron transfectados con 0.5 µg del plásmido hSERCA2-luc correspondiente (-254 silvestre -WT- o su versión en la que el sitio de unión a NFAT de la posición +43 fue mutado -mNFAT-). La actividad de luciferasa fue medida después que se aplicaron los fármacos mostrados por 12 h y fue normalizada utilizando la concentración de proteína de los extractos. Media +/- error estándar, ($p<0.05$).

La tapsigargina activa la transcripción del gen *ATP2A2* a través del sitio de Respuesta a Estrés del Retículo Endoplásmico (ERSE). Debido a que los tratamientos usados mostraron inducir ERS, se decidió explorar si la mutación de cada uno de los 3 componentes del sitio ERSE modificaba la inducción de la actividad transcripcional por Tg o A23187. Los resultados de dicho experimento demostraron que la inducción de estrés por Tg, A23187 o tunicamicina, que se usó como control de la inducción de estrés, era evitada si se mutaban la secuencia CCAAT o el sitio YY1 del ERSE pero no cuando se mutaba el sitio ERSF (**Figura XIII**).

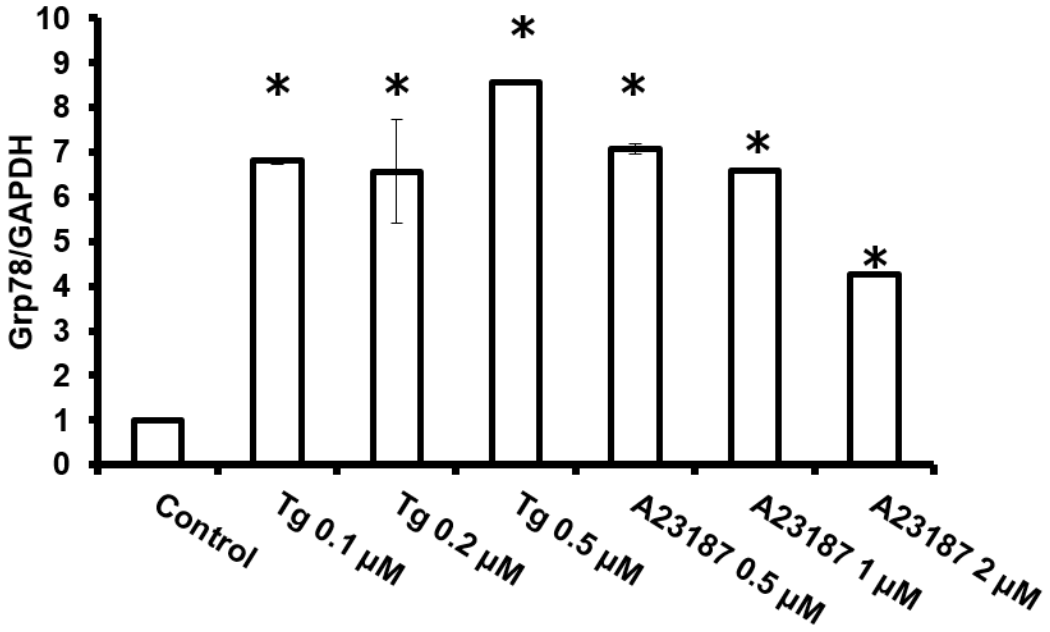

Figura XII. La Tg y el ionóforo A23187 desencadenan la respuesta de estrés del retículo endoplásmico. Después de 48 horas de haber sido sembrados, los cardiomiocitos en cultivo fueron expuestos a los fármacos por 12 h. 1 µg de RNA total fue sometido a retrotranscripción y la abundancia del RNAm fue medida por qRT-PCR usando el nivel del RNAm de GAPDH para normalizar los datos. Media +/- error estándar, ($p<0.05$).

Habiendo identificado 2 sitios potencialmente responsables de la activación por tapsigargina de la actividad transcripcional de *ATP2A2*, se decidió explorar si estos sitios intactos o modificados eran capaces de unir al factor transcripcional NF-Y. Para este propósito se obtuvieron extractos nucleares de cardiomiocitos de rata neonata y se llevaron a cabo ensayos de retardamiento de la movilidad electroforética (EMSAs) marcando radiactivamente sondas del sitio ERSE del gen *ATP2A2* humano y de un sitio de respuesta a estrés usado ampliamente como modelo de estudio, el sitio ERSE del gen *grp78* de rata.

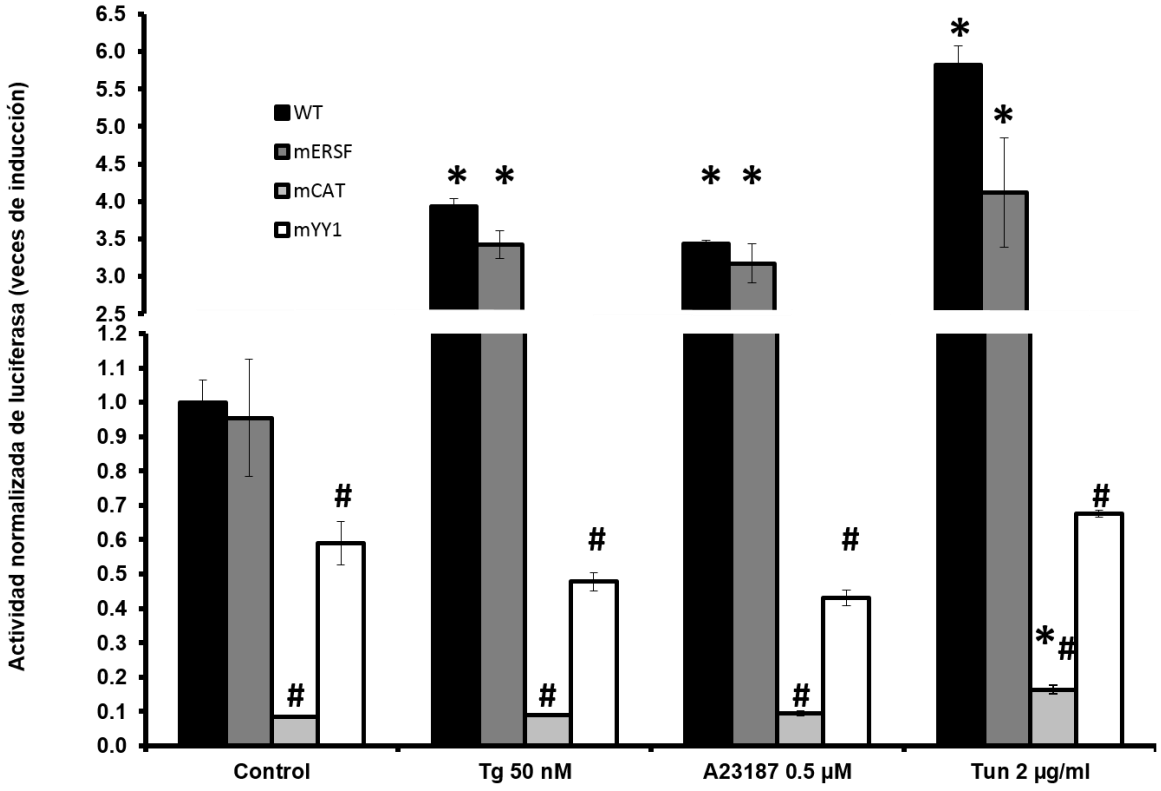

Figura XIII. La mutación del sitio ERSE en el promotor del gen *ATP2A2* previene que éste aumente su expresión en respuesta a tapsigargina. Después de 48 h de sembrar los cardiomiocitos en placas de 24 pozos, fueron transfectados con 0.5 µg del plásmido hSERCA2-luc correspondiente (-254 silvestre -WT- o su versión en la que el sitio de unión a NFAT de la posición +43 fue mutado -mNFAT-). La actividad de luciferasa fue medida después que se aplicaron los fármacos mostrados por 12 h y fue normalizada utilizando la concentración de proteína de los extractos. Media +/- error estándar, ($p<0.05$).

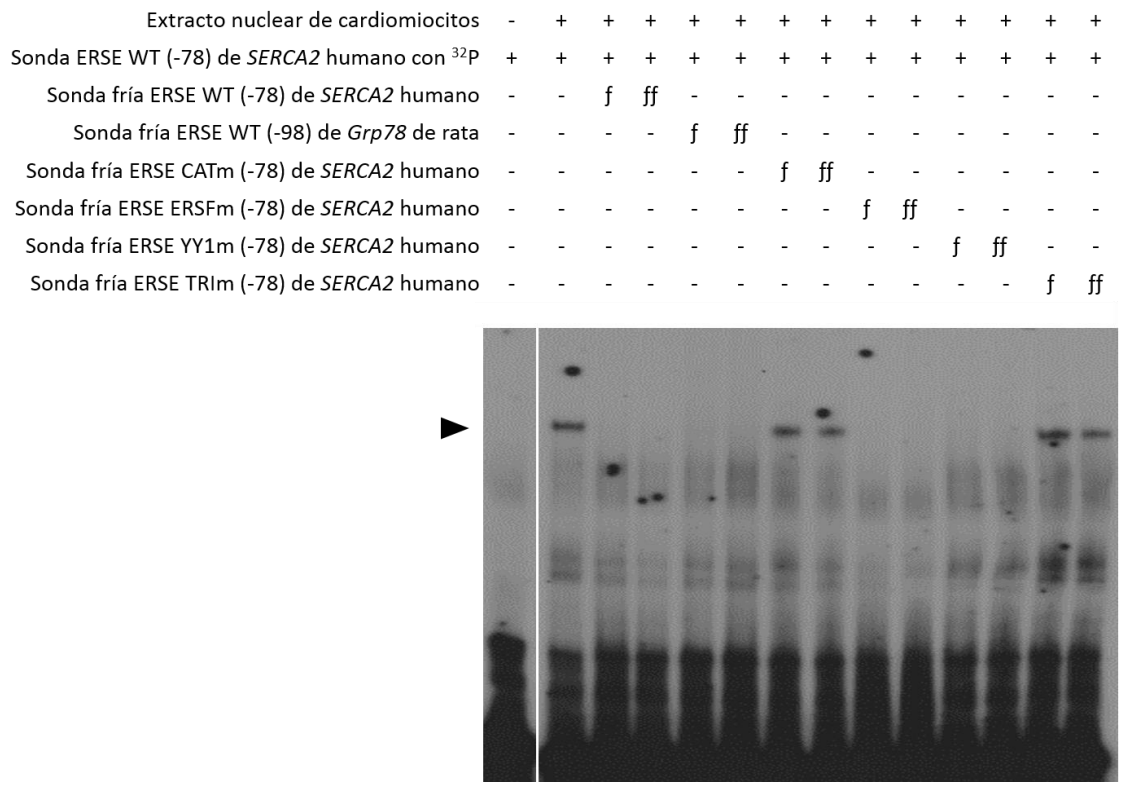

Figura XIV. La secuencia CCAAT presente en el sitio ERSE del gen *ATP2A2* es necesaria para formar un complejo específico DNA-proteína. Los ensayos de retardamiento de la movilidad electroforética se realizaron con cardiomiocitos de rata neonata usando oligonucleótidos marcados con [γ-^{32}P] ATP del sitio ERSE silvestre (WT) del gen *ATP2A2* (sonda con ^{32}P) y los excesos molares indicados de oligonucleótidos sin marcar (sondas frías: f= 100 veces y ff= 200 veces) correspondientes a: sitio ERSE silvestre (WT) de *ATP2A2* (carriles 3 y 4), sitio ERSE silvestre (WT) de grp78 de rata (carriles 5 y 6), sitio ERSE de *ATP2A2* con la secuencia CCAAT en -78 mutada (carriles 7 y 8), sitio ERSE de *ATP2A2* con el sitio ERSF en -72 mutado (carriles 9 y 10) sitio ERSE de *ATP2A2* con el sitio YY1 en -63 mutado (carriles 11 y 12) o sitio ERSE de *ATP2A2* con los 3 sitios mutados (carriles 13 y 14). El complejo específico se señala con una flecha. Imagen representativa de 3 experimentos independientes.

La secuencia CCAAT es necesaria para la formación del complejo ERSE.

Los resultados de los experimentos mostrados en la **Figura XIV** demostraron que la secuencia CCAAT intacta del ERSE de *ATP2A2* es necesaria para la formación de un complejo específico entre los extractos nucleares de cardiomiocitos y la sonda marcada radiactivamente. Posteriormente se verificó que la secuencia CCAAT compite eficientemente por la formación del complejo ERSE del gen *grp78* de rata (**Figura XV**). Una vez identificada la importancia y funcionalidad del sitio, se verificó si la unión era específica para el factor transcripcional NF-Y, por lo que se incubaron extractos nucleares con anticuerpos contra las 3 subunidades del factor NF-Y y se verificó si el complejo específico se seguía observando.

El factor transcripcional NF-Y se une al ERSE del gen *ATP2A2.*

Los experimentos mostraron que el complejo desapareció cuando se usaron los anticuerpos contra NF-Y pero no contra C/EBPβ o su forma fosforilada -factor transcripcional cuya interacción con la secuencia CCAAT ha sido documentada ampliamente (Ramji and Foka, 2002). Adicionalmente se encontró que la unión preferencial al factor NF-Y ocurría igual para el ERSE del gen *grp78* de rata (**Figura XVI**).

Como es sabido que las conformaciones de distintos factores transcripcionales pueden ser modificadas en respuesta a distintos estímulos, se verificó si la unión del sitio ERSE a los extractos nucleares tratados con Tg se modificaba o si el tratamiento de los cardiomiocitos con dicho fármaco hacía posible la interacción de las sondas con el factor transcripcional C/EBPβ.

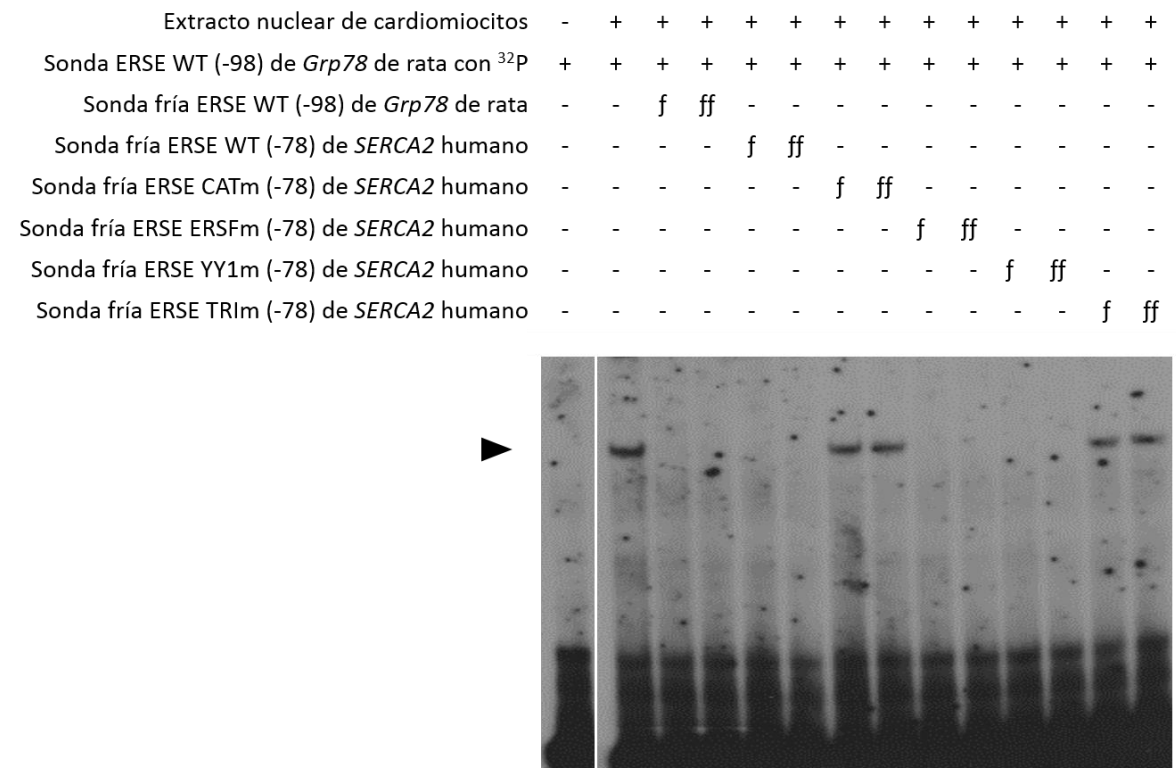

Figura XV. La secuencia CCAAT presente en el sitio ERSE del gen *ATP2A2* humano compite por la formación del complejo ERSE del gen *grp78* de rata. Los ensayos de retardamiento de la movilidad electroforética se realizaron con cardiomiocitos de rata neonata usando oligonucleótidos marcados con [γ-^{32}P]-ATP del sitio ERSE silvestre (WT) de grp78 (sonda con ^{32}P) y los excesos molares indicados de oligonucleótidos sin marcar (sondas frías: f= 50 veces y ff= 100 veces) correspondientes a: sitio ERSE silvestre (WT) de grp78 de rata (carriles 3 y 4), sitio ERSE silvestre (WT) de *ATP2A2* (carriles 5 y 6), sitio ERSE de *ATP2A2* con la secuencia CCAAT en posición -78 mutada (carriles 7 y 8), sitio ERSE de *ATP2A2* con el sitio ERSF en -72 mutado (carriles 9 y 10) sitio ERSE de *ATP2A2* con el sitio YY1 en posición -63 mutado (carriles 11 y 12) o sitio ERSE de *ATP2A2* con los 3 sitios mutados (carriles 13 y 14). El complejo específico se señala con una flecha. Imagen representativa de 3 experimentos independientes.

La tapsigargina modifica la interacción de NF-Y con el ERSE.

Como se observa en la **Figura XVII**, los tratamientos con Tg disminuyeron la cantidad de complejo observada pero no modificaron la interacción de la sonda con los extractos nucleares.

También se encontró que este comportamiento frente a la Tg ocurría de igual manera para el gen *grp78* de rata (**Figura XVIII**).

Los factores NF-Y y C/EBPβ se unen *in vivo* al promotor del gen *Atp2a2* de rata.

Por último, se usó cromatina fragmentada de cardiomiocitos de rata neonata para comprobar si al inmunoprecipitarla con anticuerpos contra los factores transcripcionales estudiados, se podía detectar la interacción con el promotor del gen *ATP2A2* donde está contenido el sitio ERSE. En la **Figura XIX** se puede observar que la inmunoprecipitación tanto con anti NFY-B como con anti-C/EBPβ permitió detectar un enriquecimiento de dichos factores transcripcionales en el promotor del gen *Atp2a2* de rata.

Extracto nuclear de cardiomiocitos	-	+	+	+	+	+	+	+	+	+	+
Sonda ERSE WT (-78) de *SERCA2* humano con ^{32}P	-	-	-	-	-	-	-	+	+	+	+
Sonda ERSE WT (-98) de *Grp78* de rata con ^{32}P	+	+	+	+	+	+	+	-	-	-	-
Sonda fría ERSE WT (-98) de *Grp78* de rata	-	-	+	-	-	-	-	-	-	-	-
Sonda fría ERSE WT (-78) de *SERCA2* humano	-	-	-	+	-	-	-	-	-	-	-
Anticuerpos contra NF-YA+B+C	-	-	-	-	+	-	-	-	+	-	-
Anticuerpo contra C/EBPβ	-	-	-	-	-	+	-	-	-	+	-
Anticuerpo contra C/EBPβ –P	-	-	-	-	-	-	+	-	-	-	+

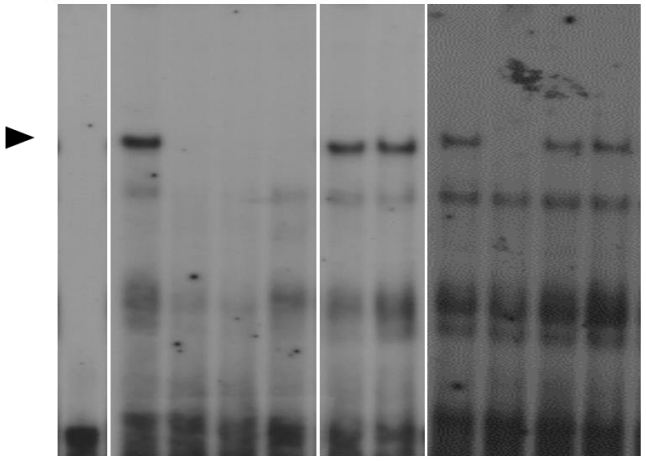

Figura XVI. Las secuencias CCAAT presentes en los sitios ERSE de los genes *ATP2A2* y *grp78* se unen a NF-Y *in vitro*. Se incubaron extractos nucleares de cardiomiocitos de rata neonata con oligonucleótidos marcados con [γ-^{32}P] ATP del sitio ERSE silvestre (WT) de *grp78* de rata (sonda marcada con ^{32}P) (carriles 2-7) y competidos con exceso molar de 200 veces de oligonucleótidos sin marcar del sitio ERSE silvestre (WT) de *grp78* de rata (carril 3), sitio ERSE silvestre (WT) de *ATP2A2* (carril 4), 6 µg de anticuerpos contra NF-Y (2 µg de NF-YA + 2 µg de NF-YB + 2 µg de NF-YC) (carril 5), 2 µg de anticuerpo contra C/EBPβ (carril 6) o 2 µg de anticuerpo contra C/EBPβ fosforilado (carril 7). En los carriles 8 a 11 los extractos nucleares fueron incubados con oligonucleótidos marcados con [γ-^{32}P]-ATP del sitio ERSE silvestre (WT) de *ATP2A2* y competidos con 6 µg de anticuerpos contra NF-Y (2 µg de NF-YA + 2 µg de NF-YB + 2 µg de NF-YC) (carril 9), 2 µg de anticuerpo contra C/EBPβ (carril 10) o 2 µg de anticuerpo contra C/EBPβ fosforilado (carril 11). El complejo específico se señala con una flecha. Imagen representativa de 4 experimentos independientes. Los carriles 8-11 son de una película diferente a la de los carriles 1-7.

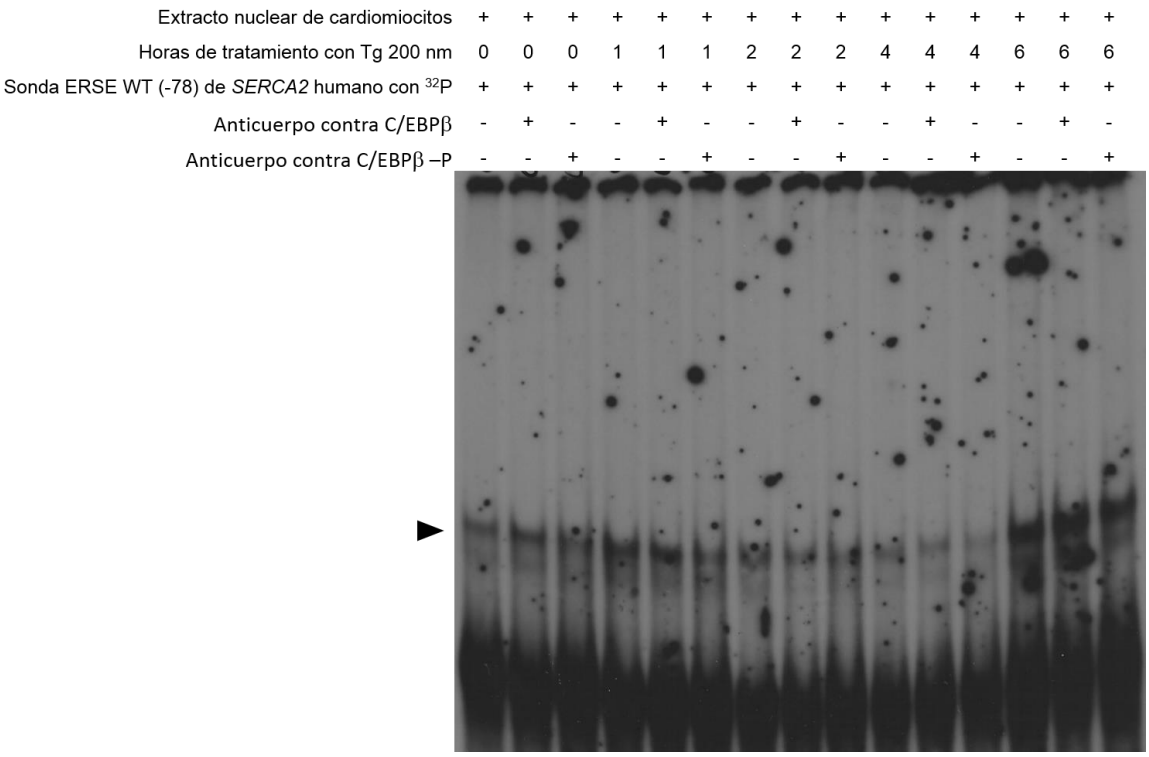

Figura XVII. La tapsigargina modifica la formación del complejo ERSE del gen *ATP2A2*. Los ensayos de retardamiento de la movilidad electroforética se realizaron con cardiomiocitos de rata neonata tratados con Tg 200 nM por los tiempos indicados, incubados con oligonucleótidos marcados con [γ-^{32}P] ATP del sitio ERSE silvestre (WT) de *ATP2A2* (sonda con ^{32}P) y competidos con 2 µg de anticuerpo contra C/EBPβ (carriles 2, 5, 8, 11 y 14) o 2 µg de anticuerpo contra C/EBPβ fosforilado (carriles 3, 6, 9, 12 y 15). El complejo específico se señala con una flecha. Imagen representativa de 4 experimentos independientes.

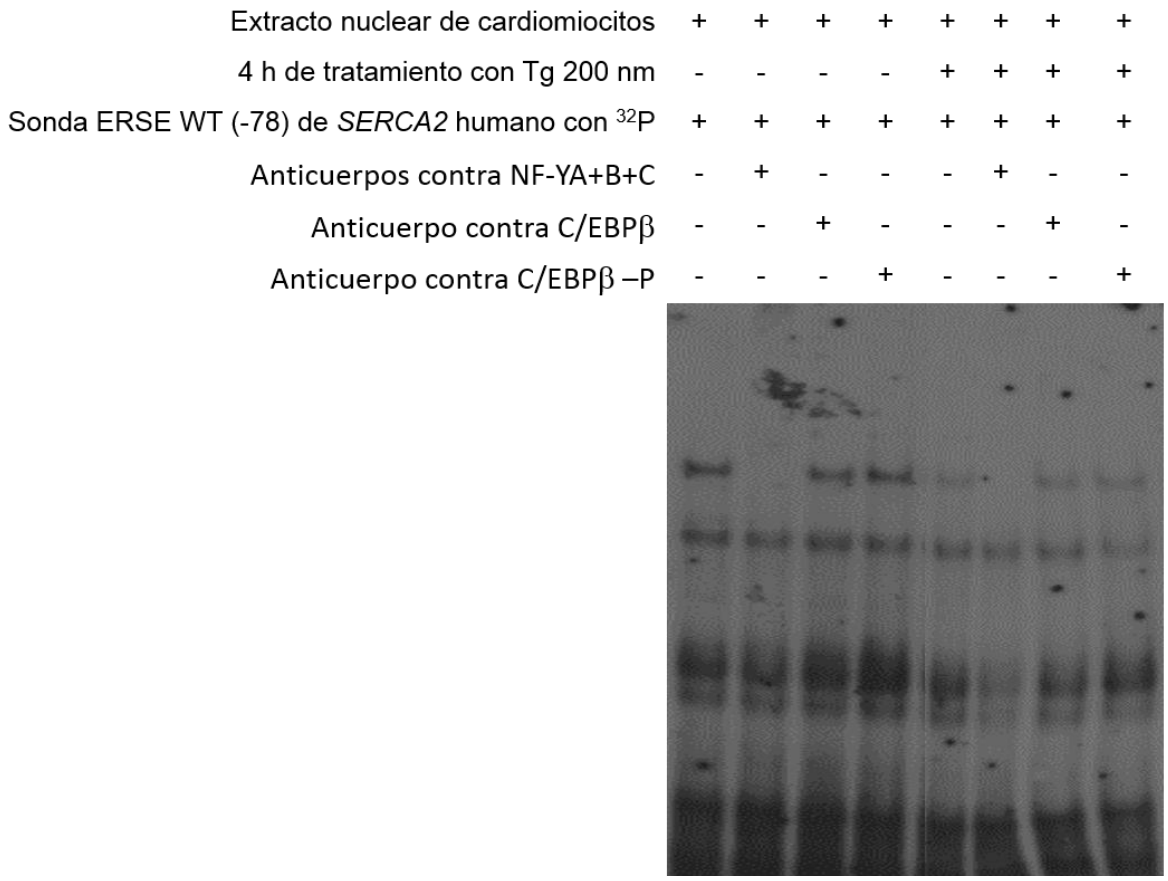

Figura XVIII. La tapsigargina disminuye la formación del complejo ERSE del gen *ATP2A2*. Extractos nucleares de cardiomiocitos de rata neonata sin tratamiento (carriles 1-4) o tratados con Tg 200 nM por 4 h (carriles 5-8) fueron incubados con oligonucleótidos marcados con [γ-^{32}P]-ATP del sitio ERSE silvestre (WT) de *ATP2A2* (sonda marcada con ^{32}P) y competidos con 6 µg de anticuerpos contra NF-Y (2 µg de NF-YA + 2 µg de NF-YB + 2 µg de NF-YC) (carriles 2 y 6), 2 µg de anticuerpo contra C/EBPβ (carriles 3 y 7) o 2 µg de anticuerpo contra C/EBPβ fosforilado (carriles 4 y 8). El complejo ERSE específico se señala con una flecha. Imagen representativa de 4 experimentos independientes.

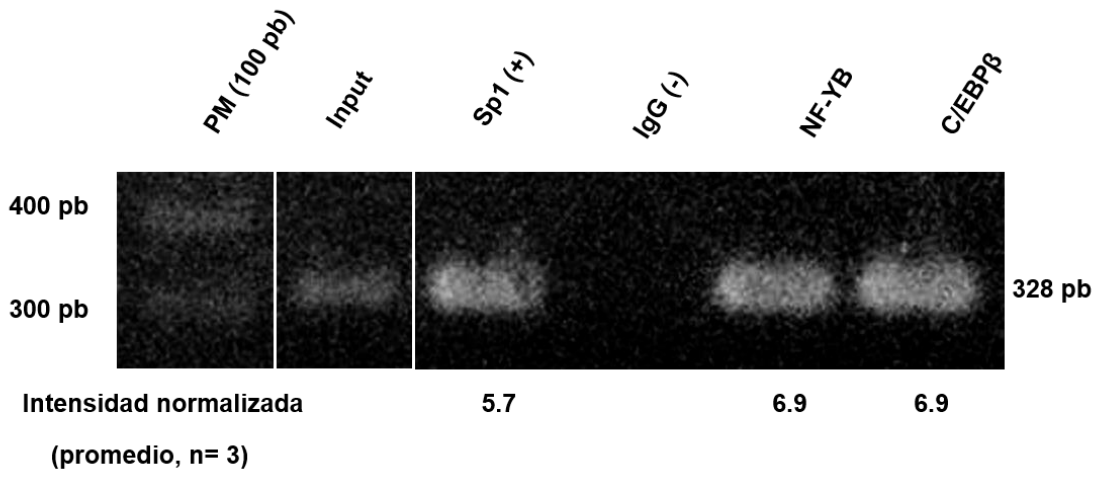

Figura XIX. NF-YB y C/EBPβ se unen in vivo al promotor proximal del gen *Atp2a2* de rata. Imagen representativa de inmunoprecipitaciones de cromatina (ChIPs) donde una región de 328 pb que contiene -268 pb del promotor proximal de *Atp2a2* de rata y 60 pb de la región 5´-no traducida, fue amplificada. Se obtuvo cromatina fragmentada de cardiomiocitos de rata neonata que fue inmunoprecipitada con anticuerpos contra NF-YB o C/EBPβ. Se usaron IgG y anticuerpo contra Sp1 como controles negativo y positivo, respectivamente. El experimento se realizó por triplicado y cada PCR fue llevado a cabo dos veces. El incremento en el producto de PCR con respecto al "Input", se muestra en la imagen.

DISCUSIÓN

Los datos encontrados respecto a la inducción de la expresión del gen a nivel de RNAm por tapsigargina y el ionóforo A23187 muestran aumentos de 30 a 60% a las 12 h que se usaron como tiempo base, sin embargo, este nivel de inducción concuerda con algunos datos previamente reportados (Prasad and Inesi, 2009) obtenidos con menor concentración pero a un mayor tiempo. Con respecto al efecto de la ciclosporina A (que como se mencionó es un inhibidor de la calcineurina que a su vez promueve la translocación de NFAT al núcleo) sobre la abundancia de RNAm para SERCA2a no existen reportes sobre su efecto directo. Sin embargo, el grupo mencionado anteriormente también cuantificó su efecto sobre la inducción que ejerce la tapsigargina, encontrando igualmente que la ciclosporina A evita que la tapsigargina induzca la expresión de SERCA2a.

La ciclosporina A evita que la tapsigargina y el ionóforo A23187 aumenten la actividad transcripcional del gen *ATP2A2*.

A nivel de la actividad transcripcional de las construcciones del promotor proximal del gen *ATP2A2*, se observaron cambios más pronunciados, pero con tendencias muy parecidas a los resultados encontrados a nivel de RNAm. Sin embargo, las diferencias en cuanto a magnitud de las respuestas observadas se deban muy probablemente a lo indirecto de las mediciones de la actividad de luciferasa. Es importante señalar la diferencia entre las respuestas de ambas construcciones a la estimulación por calcio, ya que casi por regla se observó una mayor inducción de la actividad transcripcional en la construcción más larga con tapsigargina o el ionóforo A23187. Asimismo, la disminución de actividad transcripcional causada por ciclosporina A también fue ligeramente mayor para la construcción larga.

Las diferencias mencionadas podrían deberse a los distintos elementos de unión a factores transcripcionales responsivos a calcio encontrados a lo largo del promotor proximal de *ATP2A2*, como se muestra en la **Figura V**. En la construcción corta se encuentran contenidos sólo un elemento ERSE y un sitio NFAT en

comparación con los elementos contenidos en el resto del promotor proximal donde se concentran varios sitios NFAT y 2 sitios MEF2. El hecho de que la CsA prevenga el aumento de la transcripción de *ATP2A2* incluso usando una construcción con apenas -254 pb del promotor, sugirió que los sitios principalmente responsables de dicha inducción estaban presentes en la región más proximal del gen.

El sitio putativo de unión a NFAT en la posición +43 del gen *ATP2A2* no media su respuesta a tapsigargina.

Los resultados mostrados en la **Figura XI** resultan intrigantes pues a pesar de haber observado que la CsA impide la inducción del RNAm de SERCA2a por Tg y sabiendo que uno de los principales efectos de la CsA es evitar que se active la transcripción mediada por NFAT, la mutación del sitio de unión a NFAT presente en el gen, no modificó la inducción por Tg. Este hecho plantea la posibilidad de que la inhibición de la calcineurina en cardiomiocitos afecte principalmente alguna ruta de señalización distinta a la translocación de NFAT al núcleo ya que los blancos de la calcineurina, además de NFAT son diversos y también pueden afectar otros aspectos de la fisiología del cardiomiocito (Maillet et al., 2010; Wang et al., 2011). Por otro lado, es posible que el sitio mutado no sea funcional y por lo tanto no permita la unión de NFAT al promotor.

Si esta última hipótesis es correcta, los resultados plantearían la pregunta de la ruta afectada pues, aunque la construcción mutada siguió respondiendo a la inducción por Tg, su actividad basal fue disminuida en ~60 %. Estudios posteriores en los que se sobre-exprese al factor NFAT para verificar si la actividad de dicha construcción permanece constante y ChIPs en los que se defina si NFAT se une *in vivo* al promotor de *Atp2a2* de rata, permitirían discernir si es un sitio NFAT funcional.

Una de las rutas de control de la expresión génica recientemente estudiadas es la remodelación de la cromatina (revisado en (Badeaux and Shi, 2013)) y algunos estudios se han centrado en la influencia de las concentraciones de calcio sobre la remodelación de la cromatina y su impacto sobre la expresión génica (Brignall et al.,

2017; Phengchat et al., 2016). Por ejemplo, se encontró que cromosomas expuestos a altas concentraciones de calcio forman estructuras globulares a diferencia de aquellos expuestos a medios sin calcio en los que la estructura encontrada es de fibras (Phengchat et al., 2016).

Sobre el factor de transcripción NFAT y su activación por altas concentraciones de calcio se ha encontrado que el tratamiento de células Jurkat con ionomicina (A23187) promueve la activación de distintos genes y que a pesar de encontrarse presentes sitios de unión a NFAT en muchos de ellos, la activación de dichos genes es muy pequeña (Brignall et al., 2017). De hecho, previamente se ha reportado que la activación de los genes blanco de NFAT requiere en su mayoría la presencia de otros factores incluyendo AP-1, Oct-1, c-Maf, GATA-4, Sp1 y EGR; proponiendo que la función principal del factor es reclutar complejos remodeladores de la cromatina para hacer accesibles algunos sitios (Johnson et al., 2004). Incluso, se ha demostrado que uno de los blancos de la calcineurina es Brg1, una ATPasa parte de la enzima remodeladora de la cromatina SWI/SNF (Nasipak et al., 2015).

En conjunto, los datos reportados respecto a las funciones de la calcineurina y de NFAT parecen indicar que la caída de la actividad transcripcional del promotor de *ATP2A2* sin el sitio putativo de unión a NFAT en la posición +43 y el efecto de la CsA, son resultado de una alteración generalizada en la accesibilidad del gen para la maquinaria transcripcional y no de una respuesta específica a concentraciones de calcio.

El sitio de respuesta a estrés presente en el gen *ATP2A2* es responsable de la activación por tapsigargina.

La tapsigargina y el ionóforo A23187 son conocidos agentes inductores de estrés del retículo endoplásmico (Gilmore and Kirby, 2004; Vitadello et al., 2003) y debido a los resultados con la construcción mutante para el sitio NFAT, se verificó si los tratamientos farmacológicos usados causaban estrés del retículo endoplásmico en los cardiomiocitos midiendo el RNAm para el gen *grp78* y los resultados permitieron explorar si el ERSE de *ATP2A2* podía ser el mediador de la inducción de la expresión de SERCA2a por Tg y A23187.

La participación de ATF6 en la transcripción de *ATP2A2* inducida por estrés del RE fue previamente demostrada (Thuerauf et al., 2001). En ese estudio, una forma constitutivamente activa de ATF6 se utilizó para aumentar la actividad transcripcional de una construcción que contiene 324 pb del promotor de *ATP2A2* de rata. Mediante el vaciado del calcio del RS de cardiomiocitos de rata, se produjo estrés y se demostró la participación de ATF6 en este proceso. El ERSE en la posición -78 del promotor de *ATP2A2* de rata se describió como el sitio de respuesta a ATF6 y vaciado de calcio. Además, se demostró que la mutación del sitio YY1 en la posición -63 evita completamente la respuesta a ATF6 constitutivamente activo, pero previene solo parcialmente el aumento debido al vaciado de calcio del RS.

Los resultados de la **Figura XIII** demuestran que la mutación de la secuencia CCAAT en la posición -78 del promotor de *ATP2A2* evitó completamente la respuesta al aumento de calcio citosólico inducido por Tg y A23187. Además, las transfecciones transitorias y los estudios de interacción DNA-proteína destacaron la importancia del factor de transcripción NF-Y y de la secuencia CCAAT en la transcripción basal de *ATP2A2* y la inducida por Tg, ya que la presencia de la secuencia CCAAT es esencial para promover la formación de un complejo transcripcional en el ERSE proximal de *ATP2A2*. Los ensayos funcionales mostraron claramente que la Tg fue incapaz de aumentar la actividad transcripcional de la construcción corta cuando la secuencia CCAAT o los sitios YY1 estaban mutados.

Como se muestra en las **Figuras XIV-XVI**, el comportamiento del ERSE en la posición -98 del promotor de *grp78* se parece mucho al del ERSE en la posición -78 del gen *ATP2A2*. Esta similitud podría ayudar a extrapolar algunas observaciones previas sobre la regulación transcripcional de *grp78* a la de *ATP2A2* ya que el promotor de *grp78* es un modelo extensamente utilizado para explicar la activación transcripcional a través de sitios ERSE.

La represión basal a través del ERSE en el gen *ATP2A2* regula la expresión de SERCA2a.

NF-Y es un conocido factor de transcripción ubicuo que controla la transcripción de varios genes inducibles por estrés (Dolfini et al., 2012) y se ha demostrado que otros promotores de genes que unen NF-Y a la secuencia CCAAT, son reprimidos transcripcionalmente en condiciones basales (Vekich et al., 2012; Zhang et al., 2008). De hecho, se ha demostrado que la actividad de *grp78* es reprimida por la interacción de NFY con Sp1 y HDAC1 en la secuencia ERSE de la posición -163 en células sin estímulo de estrés (Baumeister et al., 2005).

Por ejemplo, en células HepG2, se demostró la unión específica de NF-Y a la secuencia CCAAT presente en el promotor del Co-activador del Receptor Nuclear rico en Prolina (PNRC) humano. Además, la sobreexpresión de NF-Y reprimió la actividad transcripcional del promotor de PNRC cuando la secuencia CCAAT estaba intacta, pero no cuando la secuencia CCAAT fue mutada (Zhang et al., 2008). En cardiomiocitos, se encontró que NF-Y se une a una secuencia CCAAT aislada presente en el promotor de la Isomerasa 6 Asociada a Proteínas Disulfuro (PDI-6) y media la represión basal de PDI-6 (Vekich et al., 2012). La capacidad de NF-Y (A, B y C) y C/EBPβ para unirse a la secuencia CCAAT ha sido bien documentada en muchos sistemas (Groenendyk et al., 2013); sin embargo, su influencia directa en la transcripción de *ATP2A2* no ha sido descrita. Como se muestra en la **Figura XVI**, los anticuerpos contra NF-Y impidieron totalmente la interacción entre los extractos nucleares de cardiomiocitos y el ERSE en la posición -78 del gen *ATP2A2* humano, mientras que los anticuerpos contra C/EBPβ no lo hicieron.

Previamente se ha descrito la unión de NF-Y al promotor de la acetilcolinesterasa humana en respuesta al tratamiento de las células HeLa con A23187 o Tg (Zhu et al., 2007a). Además, en un segundo estudio, se encontró una intensidad reducida para el complejo NF-Y cuando se trataron células con A23187 (Zhu et al., 2007b). Por otro lado, la reducción de la concentración de calcio intracelular por el bloqueador de primera generación de canales de calcio, verapamil, redujo la expresión cardiaca de la proteína pro-apoptótica que interactúa con la tiorredoxina (TXNIP) a través de NF-Y (Cha-Molstad et al., 2012). Además,

ese estudio demostró que tanto el inhibidor de verapamil como el de calcineurina, ciclosporina A, indujeron la unión de NF-Y al promotor de TXNIP, lo que sugiere que la unión de NF-Y a sus blancos en el corazón aumenta por la disminución del calcio intracelular y viceversa. Por lo tanto, la unión de NF-Y al promotor de *ATP2A2* confirma la importancia de la secuencia CCAAT en la regulación de SERCA2 mientras que plantea la posibilidad de un papel represor para el complejo ERSE en condiciones basales.

Resulta interesante que el anticuerpo contra C/EBPβ no afectó la formación del complejo ERSE como se muestra en los experimentos EMSA (**Figura XVI**), pero los ensayos ChIP mostraron que *in vivo,* C/EBPβ es reclutado al promotor proximal del gen *ATP2A2* tan efectivamente como NF-YB (**Figura XVII**). Ambos factores de transcripción se han asociado con la maquinaria transcripcional basal y la respuesta al estrés. Algunos estudios han encontrado la coordinación entre estos dos factores de transcripción, demostrando que pueden actuar de forma sinérgica en las líneas celulares suprarrenales y de tipo ameloblástico de ratón (Xu et al., 2006; Yu et al., 1995). Específicamente, la cooperación mencionada se encontró en células J774.2 similares a macrófagos murinos para activar el gen mdr1b y en la línea celular similar a ameloblastos LS8 para aumentar la expresión del gen amelogenina. En este último estudio, se propuso un mecanismo para la actividad sinérgica de los factores de transcripción que sugiere que C/EBPα no activaría la expresión génica directamente a través de su interacción con el DNA, si no a través de su asociación con NF-Y unido al DNA.

Dado que se ha reportado que co-reguladores como Sp1 y HDAC1 interactúan con NF-Y y modifican la expresión de sus genes blanco (Potluri et al., 2013) y teniendo en cuenta que la correcta transcripción basal de *ATP2A2* depende sitios de unión a Sp1 (Lu et al., 2011), es altamente probable que la transcripción basal de *ATP2A2* involucre la interacción de ambos factores y que el tratamiento con Tg modifique esta interacción. De hecho, en el gen *ATP2A2*, 22 pb río arriba de la secuencia CCAAT, hay un sitio de unión Sp1.

Una posible explicación para el hecho de que la Tg disminuya la cantidad de complejos ERSE observados mediante EMSA sería que el estímulo de estrés disminuya la cantidad de factores de transcripción necesarios para formar el complejo ERSE. Sin embargo, diversos grupos de investigación han reportado que el estrés del retículo endoplásmico o sarcoplásmico producido por Tg incrementa la transcripción y síntesis de diversos factores que interactúan con la secuencia ERSE (revisado en (Oslowski and Urano, 2011)). Ya que el tratamiento con Tg incrementa la expresión de CHOP, ATF6 y XBP-1, la baja cantidad de complejos observados en cardiomiocitos tratados con Tg sugiere que el complejo ERSE reprime basalmente la transcripción de *ATP2A2* y que la Tg libera al promotor de esta represión. Es probable que el aumento en el calcio citosólico desencadene alguna ruta de señalización que modifique la afinidad de los factores de transcripción por la secuencia ERSE ya que el tiempo en el cual se observan los cambios (mínimo a las 4h), no permitiría la síntesis de nuevos factores de transcripción.

Las diferencias y similitudes específicas entre el control transcripcional a través de NF-Y o C/EBPβ aún no se han investigado por completo, y ciertamente, su elucidación ayudará a comprender mejor la regulación de la expresión de SERCA2a en el corazón normal y las diferentes patologías en las que está involucrado el ERS.

CONCLUSIONES

La hipótesis de esta tesis doctoral es que elementos reguladores presentes en el promotor del gen *ATP2A2* son responsables de los cambios en la transcripción del gen observados por cambios en la $[Ca^{2+}]_c$ inducidos por tapsigargina y el ionóforo A23187. Uno de los candidatos para este aumento fue el sitio de unión a NFAT presente en la región del promotor proximal. Por tanto, primero se exploró la participación del sitio putativo de unión NFAT en posición +43. Se encontró que este sitio NFAT está involucrado en la transcripción basal del gen pero que no modifica su respuesta a altas concentraciones de calcio.

El otro candidato del aumento en la transcripción del gen en respuesta a $[Ca^{2+}]_c$ es el elemento de respuesta a estrés del retículo endoplásmico (ERSE) presente en la posición -78 de promotor y que contiene una secuencia CCAAT. Se encontró que esta secuencia resulta indispensable para la correcta transcripción basal del gen y también para su respuesta ante las concentraciones elevadas de calcio que provoca la tapsigargina. En específico, se encontró que los factores transcripcionales NF-YB y C/EBPβ se unen *in vivo* a la región promotora de *ATP2A2* donde se encuentra la secuencia. Particularmente, se comprobó que el ERSE del gen *ATP2A2* tiene un comportamiento prácticamente idéntico al del gen *grp78* de rata ante el tratamiento con Tg.

El gen *grp78* es un modelo clásico de respuesta a estrés y la similitud encontrada de su ERSE con el de *ATP2A2* complementa los resultados de un estudio previamente publicado en el que se estudió uno de los mecanismos de inducción de la expresión de la bomba de calcio SERCA2 por estrés. Aunque previamente se habían reportado algunos ejemplos en los que los ERSEs funcionan como mediadores de represión transcripcional, los resultados obtenidos en esta tesis sugieren que el gen *ATP2A2* en corazón es reprimido basalmente y que el desencadenamiento de estrés del retículo provocado por aumento de calcio citoplásmico libera dicha represión permitiendo un aumento en la expresión del gen. En esta represión podría estar involucrada alguna vía de señalización mediada por la fosfatasa calcineurina cuya elucidación permitirá una mejor descripción de la regulación del gen.

Adicionalmente, se demostró por ChIP que el factor transcripcional C/EBPβ se une al promotor proximal del gen que contiene el ERSE aunque no se determinó con precisión si se une directamente a la secuencia CCAAT.

PERSPECTIVAS

Dado el alcance de la presente investigación, distintos aspectos quedan como oportunidades para nuevos proyectos, entre ellos, determinar cómo la sobre-expresión o bloqueo de expresión del factor NF-Y modificará la expresión de SERCA2 y si esto modificará la formación de los complejos en el sitio ERSE. Por otro lado, la evaluación del efecto de la Tg en presencia de un inhibidor de la producción de estrés permitiría definir la participación de las vías de señalización relacionadas con la calcineurina independientes de la producción de estrés. Por ejemplo, el uso de modelos en los que ATF6 ha sido inactivado, permitiría cuantificar parcialmente la participación de las vías dependientes de calcineurina sobre la expresión de SERCA2. Igualmente, investigar si la producción de estrés mediante otros métodos promueve la formación de los mismos complejos en el promotor de *ATP2A2* permitiría diferenciar mejor la contribución del estrés de la posible contribución de las rutas controladas por la fosfatasa calcineurina.

Otro aspecto que plantea posibilidades para continuar la investigación es determinar los mecanismos que permiten la unión diferencial de NF-Y y C/EBPβ al promotor del gen *ATP2A2*. Ya que no fue posible detectar la unión de C/EBPβ al sitio ERSE *in vitro* pero sí *in vivo*, las diferencias específicas que favorecen dicha unión mejorarán considerablemente el entendimiento de la inducción/represión del gen.

Por último, la evaluación de distintas corrientes de calcio desde y hacia el retículo sarcoplásmico bajo las condiciones de nuestro estudio mejorarían el entendimiento de las cascadas de señalización que llevan a la activación del gen.

REFERENCIAS

1 Adachi, Y., Yamamoto, K., Okada, T., Yoshida, H., Harada, A., and Mori, K. (2008). ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. Cell Struct Funct *33*, 75-89.

2 Ai, X., Curran, J.W., Shannon, T.R., Bers, D.M., and Pogwizd, S.M. (2005). Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure. Circ Res *97*, 1314-1322.

3 Alpert, N.R., Brosseau, C., Federico, A., Krenz, M., Robbins, J., and Warshaw, D.M. (2002). Molecular mechanics of mouse cardiac myosin isoforms. American journal of physiology Heart and circulatory physiology *283*, H1446-1454.

4 Anwar, A., Taimor, G., Korkususz, H., Schreckenberg, R., Berndt, T., Abdallah, Y., Piper, H.M., and Schluter, K.D. (2005). PKC-independent signal transduction pathways increase SERCA2 expression in adult rat cardiomyocytes. J Mol Cell Cardiol *39*, 911-919.

5 Arai, M., Alpert, N.R., MacLennan, D.H., Barton, P., and Periasamy, M. (1993). Alterations in sarcoplasmic reticulum gene expression in human heart failure. A possible mechanism for alterations in systolic and diastolic properties of the failing myocardium. Circ Res *72*, 463-469.

6 Backs, J., Backs, T., Bezprozvannaya, S., McKinsey, T.A., and Olson, E.N. (2008). Histone deacetylase 5 acquires calcium/calmodulin-dependent kinase II responsiveness by oligomerization with histone deacetylase 4. Mol Cell Biol *28*, 3437-3445.

7 Badeaux, A.I., and Shi, Y. (2013). Emerging roles for chromatin as a signal integration and storage platform. Nat Rev Mol Cell Biol *14*, 211-224.

8 Barry, S.P., Davidson, S.M., and Townsend, P.A. (2008). Molecular regulation of cardiac hypertrophy. Int J Biochem Cell Biol *40*, 2023-2039.

9 Bassani, J.W., Qi, M., Samarel, A.M., and Bers, D.M. (1994). Contractile arrest increases sarcoplasmic reticulum calcium uptake and SERCA2 gene expression in cultured neonatal rat heart cells. Circ Res *74*, 991-997.

10 Baumeister, P., Luo, S., Skarnes, W.C., Sui, G., Seto, E., Shi, Y., and Lee, A.S. (2005). Endoplasmic reticulum stress induction of the Grp78/BiP promoter: activating mechanisms mediated by YY1 and its interactive chromatin modifiers. Mol Cell Biol *25*, 4529-4540.

11 Beard, N.A., Sakowska, M.M., Dulhunty, A.F., and Laver, D.R. (2002). Calsequestrin is an inhibitor of skeletal muscle ryanodine receptor calcium release channels. Biophys J *82*, 310-320.

12 Belevych, A.E., Terentyev, D., Terentyeva, R., Nishijima, Y., Sridhar, A., Hamlin, R.L., Carnes, C.A., and Gyorke, S. (2011). The relationship between arrhythmogenesis and impaired contractility in heart failure: role of altered ryanodine receptor function. Cardiovascular research *90*, 493-502.

13 Belmont, P.J., Tadimalla, A., Chen, W.J., Martindale, J.J., Thuerauf, D.J., Marcinko, M., Gude, N., Sussman, M.A., and Glembotski, C.C. (2008). Coordination of growth and endoplasmic reticulum stress signaling by regulator of calcineurin 1 (RCAN1), a novel ATF6-inducible gene. J Biol Chem *283*, 14012-14021.

14 Berger, I., Bieniossek, C., Schaffitzel, C., Hassler, M., Santelli, E., and Richmond, T.J. (2003). Direct interaction of Ca2+/calmodulin inhibits histone deacetylase 5 repressor core binding to myocyte enhancer factor 2. J Biol Chem *278*, 17625-17635.

15 Berridge, M.J., Bootman, M.D., and Roderick, L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. Molecular cell biology *4*, 517-529.

16 Bers, D.M. (1991). Species differences and the role of sodium-calcium exchange in cardiac muscle relaxation. Ann N Y Acad Sci *639*, 375-385.

17 Bers, D.M. (2002). Cardiac excitation-contraction coupling. Nature *415*, 198-205.

18 Bers, D.M. (2008). Calcium cycling and signaling in cardiac myocytes. Annu Rev Physiol *70*, 23-49.

19 Black, B.L., and Olson, E.N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu Rev Cell Dev Biol *14*, 167-196.
20 Braunwald, E., and Bristow, M.R. (2000). Congestive heart failure: fifty years of progress. Circulation *102*, IV14-23.
21 Brignall, R., Cauchy, P., Bevington, S.L., Gorman, B., Pisco, A.O., Bagnall, J., Boddington, C., Rowe, W., England, H., Rich, K.*, et al.* (2017). Integration of Kinase and Calcium Signaling at the Level of Chromatin Underlies Inducible Gene Activation in T Cells. J Immunol *199*, 2652-2667.
22 Brini, M., and Carafoli, E. (2011). The plasma membrane Ca(2)+ ATPase and the plasma membrane sodium calcium exchanger cooperate in the regulation of cell calcium. Cold Spring Harb Perspect Biol *3*.
23 Colella, M., Grisan, F., Robert, V., Turner, J.D., Thomas, A.P., and Pozzan, T. (2008). Ca2+ oscillation frequency decoding in cardiac cell hypertrophy: role of calcineurin/NFAT as Ca2+ signal integrators. Proc Natl Acad Sci U S A *105*, 2859-2864.
24 Cha-Molstad, H., Xu, G., Chen, J., Jing, G., Young, M.E., Chatham, J.C., and Shalev, A. (2012). Calcium channel blockers act through nuclear factor Y to control transcription of key cardiac genes. Mol Pharmacol *82*, 541-549.
25 da Costa Martins, P.A., Salic, K., Gladka, M.M., Armand, A.S., Leptidis, S., el Azzouzi, H., Hansen, A., Coenen-de Roo, C.J., Bierhuizen, M.F., van der Nagel, R.*, et al.* (2010). MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signalling. Nat Cell Biol *12*, 1220-1227.
26 Diedrichs, H., Hagemeister, J., Chi, M., Boelck, B., Muller-Ehmsen, J., and Schneider, C.A. (2007). Activation of the calcineurin/NFAT signalling cascade starts early in human hypertrophic myocardium. J Int Med Res *35*, 803-818.
27 Dolfini, D., Gatta, R., and Mantovani, R. (2012). NF-Y and the transcriptional activation of CCAAT promoters. Crit Rev Biochem Mol Biol *47*, 29-49.
28 el Azzouzi, H., van Oort, R.J., van der Nagel, R., Sluiter, W., Bergmann, M.W., and De Windt, L.J. (2010). MEF2 transcriptional activity maintains mitochondrial adaptation in cardiac pressure overload. Eur J Heart Fail *12*, 4-12.
29 Fabiato, A., and Fabiato, F. (1978). Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned cells from adult human, dog, cat, rabbit, rat, and frog hearts and from fetal and new-born rat ventricles. Ann N Y Acad Sci *307*, 491-522.
30 Feldman, A.M., Weinberg, E.O., Ray, P.E., and Lorell, B.H. (1993). Selective changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with chronic aortic banding. Circ Res *73*, 184-192.
31 Gilmore, W.J., and Kirby, G.M. (2004). Endoplasmic reticulum stress due to altered cellular redox status positively regulates murine hepatic CYP2A5 expression. J Pharmacol Exp Ther *308*, 600-608.
32 Gordon, J.W., Pagiatakis, C., Salma, J., Du, M., Andreucci, J.J., Zhao, J., Hou, G., Perry, R.L., Dan, Q., Courtman, D.*, et al.* (2009). Protein kinase A-regulated assembly of a MEF2{middle dot}HDAC4 repressor complex controls c-Jun expression in vascular smooth muscle cells. J Biol Chem *284*, 19027-19042.
33 Groenendyk, J., Agellon, L.B., and Michalak, M. (2013). Coping with endoplasmic reticulum stress in the cardiovascular system. Annu Rev Physiol *75*, 49-67.
34 Guo, X., Williams, J.G., Schug, T.T., and Li, X. (2010). DYRK1A and DYRK3 promote cell survival through phosphorylation and activation of SIRT1. J Biol Chem *285*, 13223-13232.
35 Gwathmey, J.K., Copelas, L., MacKinnon, R., Schoen, F.J., Feldman, M.D., Grossman, W., and Morgan, J.P. (1987). Abnormal intracellular calcium handling in myocardium from patients with end-stage heart failure. Circ Res *61*, 70-76.
36 Harary, I., and Farley, B. (1960). In vitro studies of single isolated beating heart cells. Science *131*, 1674-1675.

37 Harary, I., and Farley, B. (1963). In vitro studies on single beating rat heart cells. II. Intercellular communication. Exp Cell Res *29*, 466-474.
38 Heinzel, F.R., MacQuaide, N., Biesmans, L., and Sipido, K. (2011). Dyssynchrony of Ca2+ release from the sarcoplasmic reticulum as subcellular mechanism of cardiac contractile dysfunction. J Mol Cell Cardiol *50*, 390-400.
39 Hong, T., and Shaw, R.M. (2017). Cardiac T-Tubule Microanatomy and Function. Physiol Rev *97*, 227-252.
40 Hoshijima, M., Knoll, R., Pashmforoush, M., and Chien, K.R. (2006). Reversal of calcium cycling defects in advanced heart failure toward molecular therapy. J Am Coll Cardiol *48*, A15-23.
41 Ikeda, S., He, A., Kong, S.W., Lu, J., Bejar, R., Bodyak, N., Lee, K.H., Ma, Q., Kang, P.M., Golub, T.R*., et al.* (2009). MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. Mol Cell Biol *29*, 2193-2204.
42 Johnson, B.V., Bert, A.G., Ryan, G.R., Condina, A., and Cockerill, P.N. (2004). Granulocyte-macrophage colony-stimulating factor enhancer activation requires cooperation between NFAT and AP-1 elements and is associated with extensive nucleosome reorganization. Mol Cell Biol *24*, 7914-7930.
43 Jones, L.R., Maddock, S.W., and Hathaway, D.R. (1981). Membrane localization of myocardial type II cyclic AMP-dependent protein kinase activity. Biochim Biophys Acta *641*, 242-253.
44 Jorgensen, P.L. (1974). Isolation and characterization of the components of the sodium pump. Q Rev Biophys *7*, 239-274.
45 Kaprielian, R., del Monte, F., and Hajjar, R.J. (2002). Targeting Ca2+ cycling proteins and the action potential in heart failure by gene transfer. Basic research in cardiology *97 Suppl 1*, I136-145.
46 Kitazawa, T. (1984). Effect of extracellular calcium on contractile activation in guinea-pig ventricular muscle. J Physiol *355*, 635-659.
47 Kolodziejczyk, S.M., Wang, L., Balazsi, K., DeRepentigny, Y., Kothary, R., and Megeney, L.A. (1999). MEF2 is upregulated during cardiac hypertrophy and is required for normal post-natal growth of the myocardium. Curr Biol *9*, 1203-1206.
48 Lanner, J.T., Georgiou, D.K., Joshi, A.D., and Hamilton, S.L. (2010). Ryanodine receptors: structure, expression, molecular details, and function in calcium release. Cold Spring Harb Perspect Biol *2*, a003996.
49 Le Peuch, C.J., Haiech, J., and Demaille, J.G. (1979). Concerted regulation of cardiac sarcoplasmic reticulum calcium transport by cyclic adenosine monophosphate dependent and calcium--calmodulin-dependent phosphorylations. Biochemistry *18*, 5150-5157.
50 Li, M., Baumeister, P., Roy, B., Phan, T., Foti, D., Luo, S., and Lee, A.S. (2000). ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. Mol Cell Biol *20*, 5096-5106.
51 Lu, P., Hankel, I.L., Hostager, B.S., Swartzendruber, J.A., Friedman, A.D., Brenton, J.L., Rothman, P.B., and Colgan, J.D. (2011). The developmental regulator protein Gon4l associates with protein YY1, co-repressor Sin3a, and histone deacetylase 1 and mediates transcriptional repression. J Biol Chem *286*, 18311-18319.
52 MacDonnell, S.M., Weisser-Thomas, J., Kubo, H., Hanscome, M., Liu, Q., Jaleel, N., Berretta, R., Chen, X., Brown, J.H., Sabri, A.K*., et al.* (2009). CaMKII negatively regulates calcineurin-NFAT signaling in cardiac myocytes. Circ Res *105*, 316-325.
53 Maillet, M., Davis, J., Auger-Messier, M., York, A., Osinska, H., Piquereau, J., Lorenz, J.N., Robbins, J., Ventura-Clapier, R., and Molkentin, J.D. (2010). Heart-specific deletion of CnB1 reveals multiple mechanisms whereby calcineurin regulates cardiac growth and function. J Biol Chem *285*, 6716-6724.
54 Martonosi, A.N., and Pikula, S. (2003). The structure of the Ca2+-ATPase of sarcoplasmic reticulum. Acta Biochimica Polonica *50*, 337-365

55 Marx, S.O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblit, N., and Marks, A.R. (2000). PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. Cell *101*, 365-376.
56 McKinsey, T.A., Zhang, C.L., and Olson, E.N. (2002). MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends Biochem Sci *27*, 40-47.
57 Metrich, M., Laurent, A.C., Breckler, M., Duquesnes, N., Hmitou, I., Courillau, D., Blondeau, J.P., Crozatier, B., Lezoualc'h, F., and Morel, E. (2010). Epac activation induces histone deacetylase nuclear export via a Ras-dependent signalling pathway. Cell Signal *22*, 1459-1468.
58 Morgan, J.P., Perreault, C.L., and Morgan, K.G. (1991). The cellular basis of contraction and relaxation in cardiac and vascular smooth muscle. Am Heart J *121*, 961-968.
59 Morkin, E., and LaRaia, P.J. (1974). Biochemical studies on the regulation of myocardial contractility. N Engl J Med *290*, 445-451.
60 Münch, G., Bölck, B., Karczewski, P., and Schwinger., R.H.G. (2002). Evidence for Calcineurin-mediated Regulation of SERCA 2a Activity in Human Myocardium. J Mol Cell Cardiol *34*, 321-334.
61 Nakamura, T.Y., Iwata, Y., Arai, Y., Komamura, K., and Wakabayashi, S. (2008). Activation of Na+/H+ exchanger 1 is sufficient to generate Ca2+ signals that induce cardiac hypertrophy and heart failure. Circ Res *103*, 891-899.
62 Nasipak, B.T., Padilla-Benavides, T., Green, K.M., Leszyk, J.D., Mao, W., Konda, S., Sif, S., Shaffer, S.A., Ohkawa, Y., and Imbalzano, A.N. (2015). Opposing calcium-dependent signalling pathways control skeletal muscle differentiation by regulating a chromatin remodelling enzyme. Nat Commun *6*, 7441.
63 Oslowski, C.M., and Urano, F. (2011). Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods Enzymol *490*, 71-92.
64 Palmer, S., and Kentish, J.C. (1998). Roles of Ca2+ and crossbridge kinetics in determining the maximum rates of Ca2+ activation and relaxation in rat and guinea pig skinned trabeculae. Circ Res *83*, 179-186.
65 Pandya, K., Kim, H.S., and Smithies, O. (2006). Fibrosis, not cell size, delineates beta-myosin heavy chain reexpression during cardiac hypertrophy and normal aging in vivo. Proc Natl Acad Sci U S A *103*, 16864-16869.
66 Park, H., Wu, S., Dunker, A.K., and Kang, C. (2003). Polymerization of calsequestrin. Implications for Ca2+ regulation. J Biol Chem *278*, 16176-16182.
67 Passier, R., Zeng, H., Frey, N., Naya, F.J., Nicol, R.L., McKinsey, T.A., Overbeek, P., Richardson, J.A., Grant, S.R., and Olson, E.N. (2000). CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. J Clin Invest *105*, 1395-1406.
68 Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res *29*, e45.
69 Phengchat, R., Takata, H., Morii, K., Inada, N., Murakoshi, H., Uchiyama, S., and Fukui, K. (2016). Calcium ions function as a booster of chromosome condensation. Sci Rep *6*, 38281.
70 Potluri, V., Noothi, S.K., Vallabhapurapu, S.D., Yoon, S.O., Driscoll, J.J., Lawrie, C.H., and Vallabhapurapu, S. (2013). Transcriptional repression of Bim by a novel YY1-RelA complex is essential for the survival and growth of Multiple Myeloma. PLoS One *8*, e66121.
71 Prasad, A.M., and Inesi, G. (2009). Effects of thapsigargin and phenylephrine on calcineurin and protein kinase C signaling functions in cardiac myocytes. Am J Physiol Cell Physiol *296*, C992-C1002.
72 Prasad, A.M., and Inesi, G. (2011). Silencing calcineurin A subunit reduces SERCA2 expression in cardiac myocytes. American journal of physiology Heart and circulatory physiology *300*, H173-180.
73 Prasad, A.M., Ma, H., Sumbilla, C., Lee, D.I., Klein, M.G., and Inesi, G. (2007a). Phenylephrine hypertrophy, Ca^{2+}-ATPase (SERCA2), and Ca^{2+} signaling in neonatal rat cardiac myocytes. AJP Cell Physiology *292*, C2269-C2275.

74 Prasad, A.M., Ma, H., Sumbilla, C., Lee, D.I., Klein, M.G., and Inesi, G. (2007b). Phenylephrine hypertrophy, Ca2+-ATPase (SERCA2), and Ca2+ signaling in neonatal rat cardiac myocytes. Am J Physiol Cell Physiol *292*, C2269-2275.
75 Putt, M.E., Hannenhalli, S., Lu, Y., Haines, P., Chandrupatla, H.R., Morrisey, E.E., Margulies, K.B., and Cappola, T.P. (2009). Evidence for coregulation of myocardial gene expression by MEF2 and NFAT in human heart failure. Circ Cardiovasc Genet *2*, 212-219.
76 Rajan, S., Williams, S.S., Jagatheesan, G., Ahmed, R.P., Fuller-Bicer, G., Schwartz, A., Aronow, B.J., and Wieczorek, D.F. (2006). Microarray analysis of gene expression during early stages of mild and severe cardiac hypertrophy. Physiol Genomics *27*, 309-317.
77 Ramji, D.P., and Foka, P. (2002). CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J *365*, 561-575.
78 Reyes-Juarez, J.L., and Zarain-Herzberg, A. (2006). [Function and role of the sarcoplasmic reticulum in heart disease]. Arch Cardiol Mex *76 Suppl 4*, S18-32.
79 Sanna, B., Bueno, O.F., Dai, Y.S., Wilkins, B.J., and Molkentin, J.D. (2005). Direct and indirect interactions between calcineurin-NFAT and MEK1-extracellular signal-regulated kinase 1/2 signaling pathways regulate cardiac gene expression and cellular growth. Mol Cell Biol *25*, 865-878.
80 Schaeffer, P.J., Desantiago, J., Yang, J., Flagg, T.P., Kovacs, A., Weinheimer, C.J., Courtois, M., Leone, T.C., Nichols, C.G., Bers, D.M*., et al.* (2009). Impaired contractile function and calcium handling in hearts of cardiac-specific calcineurin b1-deficient mice. American journal of physiology Heart and circulatory physiology *297*, H1263-1273.
81 Schillinger, W., Fiolet, J.W., Schlotthauer, K., and Hasenfuss, G. (2003). Relevance of Na+-Ca2+ exchange in heart failure. Cardiovasc Res *57*, 921-933.
82 Sen, A., Miller, J.C., Reynolds, R., Willerson, J.T., Buja, L.M., and Chien, K.R. (1988). Inhibition of the release of arachidonic acid prevents the development of sarcolemmal membrane defects in cultured rat myocardial cells during adenosine triphosphate depletion. J Clin Invest *82*, 1333-1338.
83 Simpson, P., and Savion, S. (1982). Differentiation of rat myocytes in single cell cultures with and without proliferating nonmyocardial cells. Cross-striations, ultrastructure, and chronotropic response to isoproterenol. Circ Res *50*, 101-116.
84 Sjaastad, I., Wasserstrom, J.A., and Sejersted, O.M. (2003). Heart failure -- a challenge to our current concepts of excitation-contraction coupling. J Physiol *546*, 33-47.
85 Slupsky, J.R., Ohnishi, M., Carpenter, M.R., and Reithmeier, R.A. (1987). Characterization of cardiac calsequestrin. Biochemistry *26*, 6539-6544.
86 Soltis, A.R., and Saucerman, J.J. (2010). Synergy between CaMKII substrates and beta-adrenergic signaling in regulation of cardiac myocyte Ca(2+) handling. Biophys J *99*, 2038-2047.
87 Sossalla, S., Wagner, S., Rasenack, E.C., Ruff, H., Weber, S.L., Schondube, F.A., Tirilomis, T., Tenderich, G., Hasenfuss, G., Belardinelli, L*., et al.* (2008). Ranolazine improves diastolic dysfunction in isolated myocardium from failing human hearts--role of late sodium current and intracellular ion accumulation. J Mol Cell Cardiol *45*, 32-43.
88 Sundaresan, N.R., Pillai, V.B., and Gupta, M.P. (2011). Emerging roles of SIRT1 deacetylase in regulating cardiomyocyte survival and hypertrophy. J Mol Cell Cardiol *51*, 614-618.
89 Taigen, T., De Windt, L.J., Lim, H.W., and Molkentin, J.D. (2000). Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci U S A *97*, 1196-1201.
90 Teucher, N., Prestle, J., Seidler, T., Currie, S., Elliott, E.B., Reynolds, D.F., Schott, P., Wagner, S., Kogler, H., Inesi, G*., et al.* (2004). Excessive sarcoplasmic/endoplasmic reticulum Ca2+-ATPase expression causes increased sarcoplasmic reticulum Ca2+ uptake but decreases myocyte shortening. Circulation *110*, 3553-3559.
91 Thuerauf, D.J., Hoover, H., Meller, J., Hernandez, J., Su, L., Andrews, C., Dillmann, W.H., McDonough, P.M., and Glembotski, C.C. (2001). Sarco/endoplasmic reticulum calcium ATPase-2 expression is regulated by ATF6 during the endoplasmic reticulum stress response: intracellular signaling of calcium stress in a cardiac myocyte model system. J Biol Chem *276*, 48309-48317.

92 Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000). Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. Nature *405*, 647-655.
93 Undrovinas, N.A., Maltsev, V.A., Belardinelli, L., Sabbah, H.N., and Undrovinas, A. (2010). Late sodium current contributes to diastolic cell Ca2+ accumulation in chronic heart failure. J Physiol Sci *60*, 245-257.
94 van Oort, R.J., McCauley, M.D., Dixit, S.S., Pereira, L., Yang, Y., Respress, J.L., Wang, Q., De Almeida, A.C., Skapura, D.G., Anderson, M.E*., et al.* (2010). Ryanodine receptor phosphorylation by calcium/calmodulin-dependent protein kinase II promotes life-threatening ventricular arrhythmias in mice with heart failure. Circulation *122*, 2669-2679.
95 van Oort, R.J., van Rooij, E., Bourajjaj, M., Schimmel, J., Jansen, M.A., van der Nagel, R., Doevendans, P.A., Schneider, M.D., van Echteld, C.J., and De Windt, L.J. (2006). MEF2 activates a genetic program promoting chamber dilation and contractile dysfunction in calcineurin-induced heart failure. Circulation *114*, 298-308.
96 van Rooij, E., Sutherland, L.B., Liu, N., Williams, A.H., McAnally, J., Gerard, R.D., Richardson, J.A., and Olson, E.N. (2006). A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci U S A *103*, 18255-18260.
97 Vannier, C., Chevassus, H., and Vassort, G. (1996). Ca-dependence of isometric force kinetics in single skinned ventricular cardiomyocytes from rats. Cardiovascular research *32*, 580-586.
98 Vekich, J.A., Belmont, P.J., Thuerauf, D.J., and Glembotski, C.C. (2012). Protein disulfide isomerase-associated 6 is an ATF6-inducible ER stress response protein that protects cardiac myocytes from ischemia/reperfusion-mediated cell death. J Mol Cell Cardiol *53*, 259-267.
99 Vitadello, M., Penzo, D., Petronilli, V., Michieli, G., Gomirato, S., Menabo, R., Di Lisa, F., and Gorza, L. (2003). Overexpression of the stress protein Grp94 reduces cardiomyocyte necrosis due to calcium overload and simulated ischemia. Faseb J *17*, 923-925.
100 Vlasblom, R., Muller, A., Musters, R.J., Zuidwijk, M.J., Van Hardeveld, C., Paulus, W.J., and Simonides, W.S. (2004). Contractile arrest reveals calcium-dependent stimulation of SERCA2a mRNA expression in cultured ventricular cardiomyocytes. Cardiovascular research *63*, 537-544.
101 Walton, D.K., Gendel, S.M., and Atherly, A.G. (1993). DNA sequence and shuttle vector construction of plasmid pGL3 from Plectonema boryanum PCC 6306. Nucleic Acids Res *21*, 746.
102 Wang, J.X., Jiao, J.Q., Li, Q., Long, B., Wang, K., Liu, J.P., Li, Y.R., and Li, P.F. (2011). miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1. Nat Med *17*, 71-78.
103 Xu, Y., Zhou, Y.L., Luo, W., Zhu, Q.S., Levy, D., MacDougald, O.A., and Snead, M.L. (2006). NF-Y and CCAAT/enhancer-binding protein alpha synergistically activate the mouse amelogenin gene. J Biol Chem *281*, 16090-16098.
104 Xue, X.H., Hryshko, L.V., Nicoll, D.A., Philipson, K.D., and Tibbits, G.F. (1999). Cloning, expression, and characterization of the trout cardiac Na(+)/Ca(2+) exchanger. Am J Physiol *277*, C693-700.
105 Yu, L., Wu, Q., Yang, C.P., and Horwitz, S.B. (1995). Coordination of transcription factors, NF-Y and C/EBP beta, in the regulation of the mdr1b promoter. Cell Growth Differ *6*, 1505-1512.
106 Zarain-Herzberg, A. (2006). Regulation of the sarcoplasmic reticulum Ca2+-ATPase expression in the hypertrophic and failing heart. Can J Physiol Pharmacol *84*, 509-521.
107 Zarain-Herzberg, A., Afzal, N., Elimban, V., and Dhalla, N.S. (1996). Decreased expression of cardiac sarcoplasmic reticulum Ca(2+)-pump ATPase in congestive heart failure due to myocardial infarction. Mol Cell Biochem *163-164*, 285-290.
108 Zarain-Herzberg, A., and Alvarez-Fernandez, G. (2002). Sarco(endo)plasmic reticulum Ca2+-ATPase-2 gene: structure and transcriptional regulation of the human gene. ScientificWorldJournal *2*, 1469-1483.

109 Zhang, L., Kelley, J., Schmeisser, G., Kobayashi, Y.M., and Jones, L.R. (1997). Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor. Proteins of the cardiac junctional sarcoplasmic reticulum membrane. J Biol Chem *272*, 23389-23397.
110 Zhang, T., Kohlhaas, M., Backs, J., Mishra, S., Phillips, W., Dybkova, N., Chang, S., Ling, H., Bers, D.M., Maier, L.S*., et al.* (2007). CaMKIIdelta isoforms differentially affect calcium handling but similarly regulate HDAC/MEF2 transcriptional responses. J Biol Chem *282*, 35078-35087.
111 Zhang, Y., Chen, B., Li, Y., Chen, J., Lou, G., Chen, M., and Zhou, D. (2008). Transcriptional regulation of the human PNRC promoter by NFY in HepG2 cells. J Biochem *143*, 675-683.
112 Zhang, Z.Y., Liu, X.H., Hu, W.C., Rong, F., and Wu, X.D. (2010). The calcineurin-myocyte enhancer factor 2c pathway mediates cardiac hypertrophy induced by endoplasmic reticulum stress in neonatal rat cardiomyocytes. American journal of physiology Heart and circulatory physiology *298*, H1499-1509.
113 Zhu, H., Gao, W., Jiang, H., Jin, Q.H., Shi, Y.F., Tsim, K.W., and Zhang, X.J. (2007a). Regulation of acetylcholinesterase expression by calcium signaling during calcium ionophore A23187- and thapsigargin-induced apoptosis. Int J Biochem Cell Biol *39*, 93-108.
114 Zhu, H., Gao, W., Shi, Y.F., and Zhang, X.J. (2007b). The CCAAT-binding factor CBF/NF-Y regulates the human acetylcholinesterase promoter activity during calcium ionophore A23187-induced cell apoptosis. Biochim Biophys Acta *1770*, 1475-1482.
115 Zobel, C., Rana, O.R., Saygili, E., Bolck, B., Diedrichs, H., Reuter, H., Frank, K., Muller-Ehmsen, J., Pfitzer, G., and Schwinger, R.H. (2007). Mechanisms of Ca2+-dependent calcineurin activation in mechanical stretch-induced hypertrophy. Cardiology *107*, 281-290.

ANEXO: LISTA DE ARTÍCULOS PUBLICADOS

1. Zarain-Herzberg A., Fragoso-Medina J. and Estrada-Avilés R. (2011). "Calcium-regulated Transcriptional Pathways in the Normal and Pathologic Heart". IUBMB Life 63(10): 847–855.

2.Zarain-Herzberg A., Estrada-Avilés R. and Fragoso-Medina J. (2012). "Regulation of Sarco(endo)plasmic Reticulum Ca2+-ATPase and Calsequestrin Gene Expression". Canadian Journal of Physiology and Pharmacology 90(8):1017-1028.

3. Fragoso-Medina J. and Angel Zarain-Herzberg (2014). "SERCA2a: its role in the development of heart failure and as a potential therapeutic target". Research Reports in Clinical Cardiology 5: 43–55.

4. Fragoso-Medina J., Rodriguez G. and Zarain-Herzberg A. "The CCAAT box in the proximal SERCA2 gene promoter regulates basal and stress-induced transcription in cardiomyocytes" (2018). Mol Cell Biochem 442(1-2):19-28.

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Critical Review

Calcium-regulated Transcriptional Pathways in the Normal and Pathologic Heart

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Summary

The cytosolic calcium concentration ($[Ca^{2+}]_c$) is key for the regulation of many cellular processes, such cell signaling and proliferation, metabolism, and muscle contraction. In cardiomyocytes, Ca^{2+} is an important regulator in many cellular functions such electrophysiological processes, excitation-contraction coupling, regulation of contractile proteins activity, energy metabolism, cell death, and transcriptional regulation by the activation of Ca^{2+}-dependent transcriptional pathways. In cardiomyocytes, the two main Ca^{2+}-dependent pathways are the Ca^{2+}/calmodulin-calcineurin-NFAT and the Ca^{2+}/calmodulin-dependent kinases-MEF2. Both pathways are involved in the transcriptional control of many cardiac genes. Cardiac hypertrophy (CH) and heart failure (HF) are characterized by alterations in calcium handling such a low sarcoplasmic reticulum Ca^{2+} content, decreased rate of Ca^{2+} removal from the sarcoplasm, increased diastolic $[Ca^{2+}]_c$, and decreased systolic $[Ca^{2+}]_c$, all of them contributing to diminished contractibility and force generation in failing heart. At gene expression level, there are also many changes such decreased levels of SERCA2a and activation of a fetal gene expression program in cardiomyocytes. A variety of Ca^{2+}-dependent signaling pathways have been implicated in CH and HF, but whether these pathways are interrelated and whether there is specificity among them are still unclear and under investigation. The focus of this review is to make an analysis of the current knowledge about the role of Ca^{2+} signaling pathways in the regulation of cardiac gene expression making special emphasis in novel strategies to correct Ca^{2+} handling alterations by means of SERCA2a gene therapy.

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Keywords calcium; signaling; heart; transcription; gene expression; NFAT; MEF2.

Abbreviations BDM, 2,3-butanedione monoxime; CaMKII, Ca^{2+}-calmodulin-dependent protein kinase II; CH, cardiac hypertrophy; Casq2, calsequestrin; CN, calcineurin; CaM, calmodulin; CsA, cyclosporine A; ERK, extracellular signal-regulated kinase; ERS, endoplasmic reticulum stress; ERSE, endoplasmic reticulum stress element; EPAC, exchange protein directly activated by cAMP; ECC, excitation-contraction coupling; ERK1/2, extracellular signal-regulated kinase 1/2; HF, heart failure; HDACs, histone deacetylases; I-1, inhibitor 1 of PP1; LVAD, left ventricular assist devices; MEF2, myocyte enhancer factor 2; MAP, mitogen-activated protein; MEK1 MAP Kinase/ERK kinase 1; miRNAs, MicroRNAs; NCX, Na^+/Ca^{2+} exchanger; ND6, NADH dehydrogenase subunit 6; NFAT, nuclear factor of activated T-cells; p38-MAPK, p38 mitogen-activated protein kinase; PMCAs, plasma membrane Ca^{2+}-ATPases; PLB, phospholamban; PKA, protein kinase A; PKC-α, protein kinase C-α; PP1, protein phophatase-1; RyR2, ryanodine receptor; SR, sarcoplasmic reticulum; SERCA2a, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; siRNA, short interference RNA; Tg, thapsigargin; Dirk1a, tyrosine-(Y)-phosphorylation-regulated kinase-1a.

INTRODUCTION

In the cardiac myocyte, the excitation-contraction coupling (ECC) process begins when a depolarizing stimulus reaches the sarcolemmal voltage dependent L-type calcium (Ca^{2+}) channels that allow the entrance of small amounts of Ca^{2+} into the cell sarcoplasm at the triadic junction. This Ca^{2+} entrance induces opening of the sarcoplasmic reticulum (SR) Ca^{2+} release channel also known as ryanodine receptor (RyR2), resulting in a rapid and massive Ca^{2+} release from the interior of the SR lumen to the sarcoplasm. The sudden rise in cytosolic calcium concentration ($[Ca^{2+}]_c$) from 20 to 50 nM to $\sim$ 1 μM activates troponin C that triggers myofibril contraction by crossbridge cycling of actin and myosin. During muscle relaxation, the α- and β-myosin heavy chain (MHC) ATPase activities use the energy from ATP to relax the myofibril. During muscle relaxation,

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Ca^{2+} is transported from the sarcoplasm into the SR lumen by the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a; 60–80% of total), although Ca^{2+} is also removed to the extracellular milieu by the Na^{+}/Ca^{2+} exchanger (NCX; 20–40% of total) to maintain cellular Ca^{2+} homeostasis (*1, 2*).

The SR is the main organelle that regulates uptake, storage, and release of Ca^{2+} in cardiac myocytes. The proteins responsible for Ca^{2+} uptake into the SR are SERCA2a and its regulatory protein phospholamban (PLB); inside the SR, there is a protein with high Ca^{2+} binding capacity (40 mol Ca^{2+}/mol protein) and moderate affinity for Ca^{2+} (Kd $\sim$ 1 mM)-named calsequestrin (Casq2) that is responsible for storage of Ca^{2+} and also participates in Ca^{2+}-release by RyR during contraction (*3*; Fig. 1). Therefore, it is evident that a precise control of Ca^{2+} cycling plays a key role to preserve normal contractile beat-to-beat activity of the cardiac myocyte, and that abnormal Ca^{2+} handling significantly contributes to the contractile dysfunction observed in cardiac hypertrophy (CH) and heart failure (HF).

Calcium signaling serves as a common mechanism to couple membrane excitation to intracellular functions in most tissues. In cardiac muscle, variations of $[Ca^{2+}]_c$ are involved in several signaling functions including contraction and activation of gene transcription. Two main calcium-dependent signaling pathways have been identified in cardiomyocytes, the calcineurin-NFAT pathway and the CaMK-MEF2 pathway, which involves histone deacetylases (HDACs) nuclear export (*2*). Both pathways participate in normal heart development and are altered in CH. One of the main features of CH and HF is downregulation of SERCA2a expression, leading to alterations in Ca^{2+} signaling pathways. Ca^{2+} signaling fluctuations and contractile function alterations have been demonstrated following specific inhibition of SERCA2a transport activity with thapsigargin (Tg), reduction of expression by a SERCA2 gene null mutation and SERCA2 gene silencing with short interference RNA (siRNA) (*4*).

In this article, we review the current status of literature in the knowledge of the main calcium signaling pathways and their role in the regulation of cardiac gene transcription, and analyze future perspectives of correcting calcium signaling by gene therapy.

ALTERATIONS IN CALCIUM HANDLING IN CARDIAC HYPERTROPHY AND HEART FAILURE

Contraction and relaxation are physiological parameters that are altered in cardiac myocytes of animal models with CH and patients with HF (*5, 6*). It has been demonstrated that a decreased shortening velocity of cardiac myocytes during contraction correlates with the reduced myofibrillar MHC-ATPase activity (*7*), as well with a diminished $[Ca^{2+}]_c$ (*1, 8–10*). A decreased rate of Ca^{2+} removal from the sarcoplasm by the failing cardiomyocyte during myocardial relaxation leads to increased diastolic $[Ca^{2+}]_c$ levels, contributing to diastolic dysfunction development, a distinctive feature of HF, which is also characterized by increased end-diastolic pressure and ventricular filling abnormalities.

The Ca^{2+} transients from cardiomyocytes obtained from hearts of animal models with CH and from patients with end-stage HF, exhibit decreased peak systolic $[Ca^{2+}]_c$ concentration and reduced velocity of Ca^{2+} pumping by SERCA2a into the SR (*5, 6*). As a consequence of the reduced peak $[Ca^{2+}]_c$ concentration, derived from reduced load of the SR with Ca^{2+}, there is also a reduction of force development by the myocardium. It has been demonstrated that a low SR Ca^{2+} content in failing cardiomyocytes (*11, 12*), therefore, is currently believed that altered Ca^{2+} handling by the SR is a key component contributing to the diminished contractility and force generation in the failing heart.

Cardiac ECC is a highly coordinated process controlled by several signaling pathways, including protein kinase A (PKA) and Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII). Among the alterations involved in CH and HF are changes in the expression levels of these regulatory proteins which act as sensors of intracellular $[Ca^{2+}]$ (*13*). In HF has been shown an increased expression and activity of CaMKII, which plays a critical role in the decreased contractile properties of the myocardium, which destabilizes ECC and may lead to sudden death (*14*). These alterations also include decreased levels of SERCA2a, with a subsequent decrease of the recapture of Ca^{2+} to the SR during diastole, resulting in a smaller amount of Ca^{2+} available to be released in the following contraction cycle. Closely related to SERCA2a is PLB, a peptide of 52 amino acids peptide, which is the main regulator of SERCA2a activity. PLB inhibits the pump in its native state, and dissociates from the pump when is phosphorylated by CaMKII or the protein kinase A (PKA) in response to β-adrenergic stimulation, in turn increasing the Ca^{2+} transport velocity by SERCA2a. During HF, the SERCA2a/PLB ratio is diminished to a great extent by the decrease of SERCA2a, which causes that the amount of SERCA2a that is dissociated from PLB diminishes and therefore, its activity and capacity to recapture the released Ca^{2+} during contraction (*15*).

The decrease in phosphorylated PLB functional levels causes the SERCA2a activity to be decreased during HF; this decrease is not only due to a smaller expression of the SERCA2a protein but also to alterations in the PLB phosphorylation level. As a result of an increase of protein kinase C-α (PKC-α) activity, the inhibitor (I-1) of the protein phophatase-1 (PP1) is phosphorylated, relieving the PP1 inactivation, in turn causing PLB dephosphorylation by PP1 which results in PLB activation. The SR Ca^{2+} release channel of the SR (RyR2) has been reported in an unstable state, dissociated from the protein calstabin, which results in a higher channel opening probability. As a result, spontaneous Ca^{2+} liberations appear during the diastole (diastolic leak), which are associated with ventricular arrhythmias and more importantly with a decrease of the Ca^{2+} reserve available for contraction (*2, 16*).

CaMKII shares common functional targets with PKA regulating ECC, such as PLB and RyR2 phosphorylation and calcium currents. An increased CaMKII activity in HF contributes

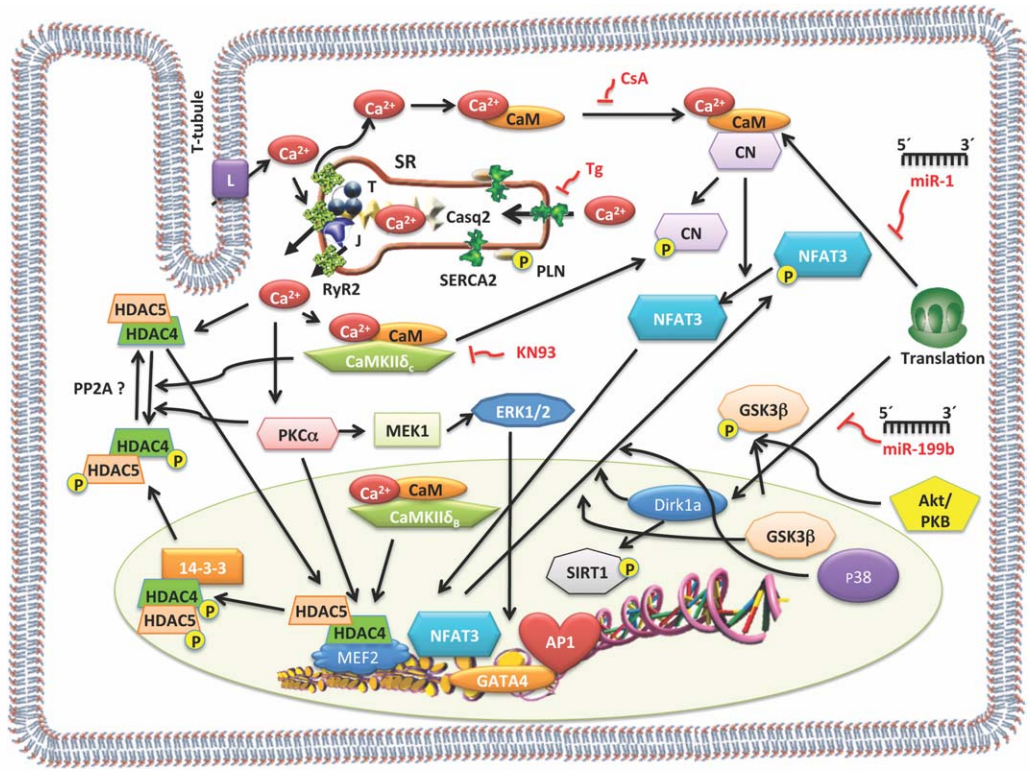

Figure 1. Calcium signaling pathways involved in cardiac gene expression. The main cardiac calcium-dependent transcriptional regulation pathways are shown. Ca^{2+}/CaM-Calcineurin activates the nuclear factor of activated T cells (NFAT), allowing its translocation to nucleus with the consequent NFAT-dependent transcriptional activation. Ca^{2+}-Calmodulin-dependent kinases (CaMKIIδb and CaMKIIδc) phosphorylate HDAC4 allowing its interaction with the chaperone protein 14-3-3 resulting in HDAC4 export from the nucleus to the cytoplasm (CaMKIIδb) or avoiding its nuclear import (CaMKIIδc). HDAC4 cytoplasmic location permits MEF2-dependent transcription activation. HDAC5 nuclear import is achieved by its dimerization with HDAC4 and avoided by CaMKIIδc or PKCα phosphorylation. NFAT-dependent MicroRNA miR-199b inhibits the translation of nuclear NFAT kinase dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase-1a (Dirk1a) which is involved in the regulation of NFAT nuclear export. MicroRNA miR-1 inhibits the translation of calmodulin (CaM) with the consequent reduction of NFAT activation. ERK1/2 and nuclear active GSK3β phosphorylate NFAT3 and promote its cytoplasmic location. J, Junctin; L, voltage-dependent L-type Ca^{2+}-channel; SR, sarcoplasmic reticulum; T, triadin; CN, calcineurin; CaM, calmodulin; PLB, phospholamban; RyR2, cardiac SR Ca^{2+}-release channel; SERCA2, cardiac sarcoendoplasmic reticulum Ca^{2+}-ATPase; cyclosporine A; MEF2, myocyte enhancer factor 2; HDAC4, histone deacetylase class II-4; HDAC5, histone deacetylase class II-5; PP2A, protein phosphatase-2A; PKCαprotein kinase C-α; PKB, protein kinase B; p38, p38 mitogen-activated protein kinase; SIRT1, silent mating type information regulation 2 homolog-1; Tg, Thapsigargin; CsA.

to reduced SR calcium content and systolic function, and also cause diastolic SR Ca^{2+} leak and Ca^{2+} current changes that may be arrhythmogenic. One of the main causes of sudden death in patients with HF is ventricular arrhythmia. Although abnormal Ca^{2+} release from the SR through RyR2 has been linked to arrhythmogenesis, the exact molecular mechanisms are unknown. Recent studies suggest that CaMKII-dependent phosphorylation of RyR2 is involved in enhanced SR diastolic Ca^{2+} leak and reduced SR Ca^{2+} load in HF, and contributes to arrhythmias and contractile dysfunction in HF (*17*). CaMKII phosphorylation of RyR2 at S2814 plays an important role in arrhythmogenesis and sudden cardiac death in mice with HF. This fact suggests that destabilization of RyR2 activity due to excessive CaMKII phosphorylation results in reduced postrelease refractoriness which is a common mechanism involved in arrhythmogenesis and contractile dysfunction in the failing heart. These findings could lead to the development of more specific drug therapies that could modify the level of CaMKII phosphorylation of RyR2, thus reducing diastolic Ca^{2+} leak and death from arrhythmias (*18, 19*).

Contractile dysfunction in human HF has been associated with increased $[Na^+]_i$ and elevated diastolic $[Ca^{2+}]_i$. Increased Na^+ influx through late voltage-gated Na^+ channels (INaL) has been suggested to contribute to elevated $[Na^+]_i$ in HF. Recent evidence demonstrates that INaL contributes to the diastolic intracellular Ca^{2+} accumulation and spontaneous release in ven-

tricular myocytes from a dog model of chronic HF, supporting the idea that selective blockade of INaL represents a potential strategy to treat calcium-related diastolic dysfunction and arrhythmias in HF (*20*). Ranolazine, is an anti-ischemic drug that inhibits INaL. In a recent study, ranolazine reduced the frequency-dependent increase in diastolic tension without negative inotropic effects on contractility of muscles from end-stage failing human hearts. Therefore, ranolazine may be of therapeutic benefit in patients with diastolic dysfunction due to elevated $[Na^+]_i$ and diastolic $[Ca^{2+}]_i$ overload, improving diastolic function in HF (*21*).

Cardiac pressure overload, as the one seen in hypertensive patients, is the major pathologic stimulus for CH and the main risk factor for HF development. Initially, hypertrophic growth is an adaptive response of the heart to diverse pathological stimuli and is characterized by cardiomyocyte enlargement, sarcomere assembly and activation of a fetal cardiac gene expression program (*6*). A variety of Ca^{2+}-dependent signal transduction pathways have been implicated in CH, but whether these pathways are independent or interdependent and whether there is specificity among them are still unclear and under investigation.

TRANSCRIPTIONAL PATHWAYS MEDIATED BY CALCIUM IN THE HEART

Calcineurin-NFAT Pathway

Calcineurin (CN) is a Ca^{2+}-calmodulin-activated serine/threonine phosphatase that is ubiquitously expressed and plays an important role in transducing Ca^{2+}-dependent signals. CN is a heterodimer composed of a calmodulin (CaM) binding catalytic subunit A (CN-A) and a Ca^{2+}-binding regulatory subunit B (CN-B; *22*). In cardiomyocytes, CN dephosphorylates the nuclear factor of activated T-cells-3 (NFAT3 or NFATc4), inducing NFAT3 translocation from the cytosol to the nucleus then making possible the activation of its target genes (Fig. 1).

Several studies demonstrate that CN plays a very important role in the development of pathological hypertrophy, and its role as a modulator of CH has been extensively studied using different strategies such as the use of CN inhibitors, transgenic mice overexpressing active CN or CN inhibiting proteins. Although global CN-B deletion leads to embryonic lethality, in a recent study, the cardiac-restricted CN-B1 deletion, allowed viable mice to be born and further characterization of the CN signaling role in heart development (*23*). Mice with cardiac-specific disruption of the CN-B1 gene show that CN is necessary for normal postnatal cardiac growth. Reduced expression of CN-B1 results in reduced cardiac ventricular to body weight ratio, systolic and diastolic dysfunction, and increased mortality reaching 100% by 7 months of age. Interestingly, the alterations in cardiac function observed at 3 months of age using echocardiography and hemodynamic measurements were not accompanied by any histological abnormalities in cardiac myocytes, such as myofibrillar disarray and fibrosis. These data suggest that CN plays an important role, not only in pathological CH but also in normal heart growth. The cardiac-specific CN deficiency leads to a lethal cardiomyopathy, which alters levels of proteins involved in ECC.

It also has been shown that activation of CN or its target transcription factor NFAT-3 is sufficient to evoke myocardial hypertrophy *in vivo*. Prolonged pathologic CH is associated with arrhythmias, decompensation, dilated cardiomyopathy, and sudden death. It has been found that at different stages of human CH, the CN pathway is activated in the hypertrophic myocardium, as demonstrated by increased CN and GATA-4 activity and expression, and increased translocation of dephosphorylated NFAT-3 into the nucleus. These changes were more evident in decompensated compared with compensated hypertrophic myocardium (*22*).

The CN-NFAT pathway is typically activated by sustained high $[Ca^{2+}]_c$, however, there are some evidence regarding codependent signaling with MEK1-ERK1/2 kinases (*24*); showing that CN or NFAT inhibition in cultured neonatal cardiomyocytes blunts the hypertrophic response driven by activated MEK1, while targeted MEK1-ERK1/2 signaling inhibition reduces the hypertrophic growth response directed by activated CN (Fig. 1).

The role of Ca^{2+} signaling in triggering hypertrophy has been investigated in neonatal rat cardiomyocytes *in vitro*. CH and sarcomere reorganization is induced by receptor agonists such as Angiotensin II, aldosterone, and norepinephrine and by a rise in extracellular KCl concentration. All these treatments increased the frequency of spontaneous Ca^{2+} transients, caused nuclear translocation of a transfected NFAT-GFP fusion protein, and increased the transcription of a NFAT-sensitive-promoter/reporter gene. The hypertrophic response and NFAT translocation was inhibited by the CN inhibitor cyclosporine A (CsA), but not by the increased frequency of Ca^{2+} transients, suggesting that the CN-NFAT pathway can act as an integrator of the contractile Ca^{2+} signals, and that this pathway can decode alterations in the frequency even of rapid Ca^{2+} oscillations (*25*).

Ca^{2+}-Calmodulin-dependent Kinases-MEF2 Pathway

The transcription factor MEF2 (myocyte enhancer factor 2) is a member of the MADS-box family of transcription factors. The MEF2 family of transcription factors is essential for the expression of many muscle-specific genes (*26*). There are four different mef2 genes (mef2a, -b,-c, and –d), all of them are capable of binding to the A/T rich consensus sequence YTA(A/T)$_4$TAR. MEF2 transcription factors are mainly expressed in all muscle types, but high levels of MEF2 are also found in the developing central nervous system. During the differentiation of skeletal muscle, MEF2d is the first family member to appear, followed by MEF2a and MEF2c (*27*). MEF2 is capable to interact with members of the MyoD family proteins to control the expression of many muscle-specific genes (*26*).

MEF2 is a common target for several hypertrophic pathways, although its precise function in cardiac remodeling and the

cardiac genes that are modulated by this factor are still under investigation by several groups. MEF2 proteins are responsive to calcium-controlled signaling pathways, such CaMK and CN (*26*; Fig. 1). In the adult heart, only basal MEF2 transcriptional activity is found. This basal activity is required for the continuous expression of genes involved in the maintenance of cardiomyocytes homeostasis (*28*). The effect of MEF-2 inhibition in CH and HF has been tested in physiological models of pressure overload by subjecting transgenic mice expressing a dominant negative form of MEF2 to transverse aortic constriction. In this model, echocardiographic data showed that the inhibition of MEF2 by the expression of the dominant negative form of MEF2 does not prevent CH development. However, MEF2 activity is upregulated by pro-hypertrophic Ca^{2+}-dependent pathways, such as CN and CaMKII, PKC, PKD, MAPK-1, and p38-MAPK. Also, expression of NADH dehydrogenase subunit 6 (ND6), part of Complex I of the oxidative phosphorylation system, and a main source of energy in cardiac muscle was decreased. The reduced expression of ND6 produces an increased cell death by the overproduction of reactive oxygen species followed by pressure overload (*28*). In contrast to these findings, previous reports show that a lower MEF2 activity in CN-transgenic mice was associated with attenuation of cardiac dysfunction (*29*). This could be explained because of the activation of CN pathways occurs along with the activation of many other signaling cascades, such as p38-MAPK and CaMKII, which produces a different effect in the setting of pressure overload (*28*).

CaMKs are involved in the regulation of many transcription factors such AP-1, SRF, CREB, and MEF2. The CaMK-dependent activation of MEF2 is not produced by direct phosphorylation of MEF2 by CaMKII, but via class II HDAC interaction (*30*). Class II HDACs expressed in heart have a MEF2 binding domain in the N-terminal, which is not present in other HDACs. This N-terminal domain is capable of binding to chaperone 14-3-3, then this complex is exported from the nucleus with the consequent relieving of MEF2 repression (*2*).

It was reported that whereas CaMKIIδ_B and CaMKIIδ_C have different effects in the phosphorylation of Ca^{2+} handling proteins, such RyR2 and PLB, both isoforms have effect in the HDAC-mediated MEF2 gene expression. Nuclear CaMKIIδ_B phosphorylates HDAC4 in the nucleus, then allowing the binding of the chaperone protein 14-3-3, with the consequent export of phospho-HDAC4/14-3-3 complex out of the nucleus. As consequence, MEF2 is relieved from the HDAC4 repression and his downstream genes are transcribed. In addition, cytoplasmic CaMKIIδ_C activation phosphorylates HDAC4 in the cytosol and prevents the import of HDAC4 from the cytosol to the nucleus. As well as HDAC4 is regulated by Ca^{2+}, HDAC5 is also partially regulated by $[Ca^{2+}]_c$, as it is imported to the nucleus by its dimerization with HDAC4 in a Ca^{2+}-dependent manner (*31*). Once in the nucleus, HDAC5 interacts with MEF-2, and this interaction is inhibited by direct binding of Ca^{2+}/calmodulin to HDAC5 (*32*; Fig. 1). In other words, cytoplasmic CaMKIIδ_C is also capable of activate MEF2 and other transcription factors, in addition to its effects on RyR2 and PLB (*33*). In addition, it was found that a constitutively active form of CaMKIIδ_C reduces CN activity by phosphorylating it, resulting in a decreased nuclear NFAT translocation, whereas a double negative form of CaMKIIδ_C indirectly reduces NFAT phosphorylation with its consequent nuclear translocation. Because of both CN and CaMKIIδ_C that are activated in a Ca^{2+}-dependent way, these findings suggest that CaMKIIδ_C could act as a negative modulator of CN activity (*34*).

It has been shown that activated Ca^{2+}-calmodulin-dependent protein kinases-I and -IV (CaMKI and CaMKIV) also induce hypertrophic responses in cardiomyocytes *in vitro*, and that CaMKIV overexpressing mice develop CH with increased left ventricular end-diastolic diameter and decreased fractional shortening (*35*). It was demonstrated that CaMKIV activates the transcription factor MEF2 through a posttranslational mechanism in the hypertrophic heart *in vivo*. Activated CN is a less efficient activator of MEF2-dependent transcription, suggesting that the CN-NFAT and CaMK-MEF2 pathways act in parallel. These findings identify MEF2 as a downstream target for CaMK signaling in the hypertrophic heart and suggest that the CaMK and CN pathways preferentially target different transcription factors to induce CH (*35*). Thus, the evidence indicates that MEF2 activation is indispensable for triggering the hypertrophic response in physiologic and pathologic CH, suggesting a conserved molecular mechanism. The data support the hypothesis that cardiac adaptation may be dependent on activating multiple cardiac transcription factors such as NFAT, GATA4, Nkx2.5, and MEF2, which recapitulate the expression pattern observed for developmental cardiac morphogenesis (*36*).

The endoplasmic reticulum stress (ERS) is involved in various cardiovascular diseases and takes part in the development of CH; however, its molecular mechanism is still unclear. MEF2c has been reported to be involved in Ca^{2+} triggered ERS-induced CH (*37*). The role of the CN signaling pathway in CH induced by Tg in neonatal rat cardiomyocytes has been investigated. Tg is a specific irreversible inhibitor of the SERCA2a pump that also functions as ERS inductor. Tg induces a significant ERS response along with hypertrophy in cardiomyocytes. Furthermore, Tg induces a significant elevation of the intracellular Ca^{2+} level, CN activation, and MEF2c expression in a dose- and time-dependent manner in the cardiomyocyte. In a recent study, CsA-suppressed MEF2c nuclear translocation and inhibited Tg-induced hypertrophy. In this study, the results demonstrate that ERS induces CH and that the CN-MEF2c pathway is involved in ERS-induced hypertrophy in cardiomyocytes (*37*).

The exchange protein directly activated by cAMP (EPAC) is a sensor for cAMP and is a novel mechanism for transducing cAMP signaling. Recent data show that EPAC activates a pro-hypertrophic signaling pathway that involves PLC, H-Ras, CaMKII, and HDAC4 nuclear export. EPAC activation also

increases the prohypertrophic transcription factor MEF2 in a CaMKII dependent manner in primary cardiac myocytes (*38*).

Crosstalk Among Ca^{2+}-regulated Pathways

As described above, CN-NFAT and CAMK-MEF2 are the most important pathways controlling calcium-dependent cardiac gene expression; however, there are other signals, which can regulate these pathways at many levels (Fig. 1). Further, both pathways can regulate each other providing a more complex and efficient regulation of gene expression. For example, in a study using an *in vitro* mechanical stretch model of cultured neonatal cardiomyocytes to evaluate the proposed mechanisms of calcium-induced CN activation, it was shown that L-type Ca^{2+} channels, capacitative Ca^{2+} entry, Na^+/H^+ exchanger, and Na^+/Ca^{2+} exchanger are also involved in this response, and therefore, are potential therapeutic targets for the inhibition of CH (*39*).

MicroRNAs (miRNAs) are involved in hypertrophy and development, miR-195 overexpression was shown to evoke CH and HF, whereas constitutively active CN-mediated hypertrophy downregulates miR-133 (*40*). Recently miR-1, a cardiomyocytes-restricted miRNA, was found to post-transcriptionally inhibit expression of CaM and MEF2a directly, and indirectly that of GATA4, positioning this miRNA as a central regulator of calcium signaling in CH (*41*; Fig. 1). Furthermore, miR-199b (another hypertrophy-upregulated miRNA), was shown to be a NFAT target. Also miR-199b is able to inhibit the nuclear NFAT kinase dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase-1a (Dyrk1a), thus constituting a pathogenic feedback (*42*). Additionally, it has been reported that Dyrk1a and Dyrk3 phosphorylate and activate SIRT1 in HEK293T cells (*43*). SIRT1 is a known cardioprotective NAD-dependent histone deacetylase which also has been shown to upregulate genes that enhance heart function (*44*).

Microarray analysis of mRNA expression in failing and nonfailing human hearts were done to find genes containing conserved MEF2, NFAT, NKX2, GATA, and FOX DNA binding motifs within the first 1,000 bp of their promoter region, to investigate correlation between these transcription factors. When differential expression of genes is modeled as a function of transcription factors combinations present in their promoter regions, it is possible to predict several combinations of differential gene expression in the failing heart, finding the highest odds ratios for genes containing both NFAT and MEF2 binding sites in the same promoter region. The above finding provides evidence for coregulation of myocardial gene expression by NFAT and MEF2 in human HF (*45*). Recently, it was reported, using a CN-induced animal model of HF, which MEF2 activates a genetic program that promotes chamber dilation, mechanical dysfunction, and dilated cardiomyopathy, but minimally affects cardiac growth, demonstrating that cardiac MEF2 transcription factors are activated in an NFAT-dependent manner by CN (*29*).

These and other evidences (some of them discussed in previous sections) highlight the importance of an integrated view of calcium-regulated expression in heart. Each pathway regulating cardiac gene expression has specific target genes, and final output is a combination of all converging signals. Furthermore, feedback loops between specific genes, crosstalk, combinatorial, and synergistic transcriptional regulation has to be considered to better understand the complex network which implies the regulation of cardiac gene expression by calcium.

TRANSCRIPTIONAL REGULATION OF THE SERCA2 AND CASQ2 GENES BY CALCIUM

Resting $[Ca^{2+}]_c$ can be raised in cardiac myocytes by partially inhibiting SERCA2a activity with Tg. In a recent report, the treatment of cardiomyocytes with low Tg concentrations, resulted in CN activation, as this treatment increased expression of a reporter gene under the control of the NFAT promoter and increases NFAT nuclear translocation (*4*). Exposure of cardiac myocytes to Tg was followed by SERCA2 expression increase, and was further increased when CN inactivation by CaMKII was prevented with KN93 (a CaMKII inhibitor). In addition, SERCA2 expression is reduced by CN inhibition with CsA (Fig. 1). These results demonstrate that Tg activates the CN-NFAT pathway by a rise of resting cytosolic $[Ca^{2+}]_c$, suggesting that the increased expression of SERCA2, may be mediated at the transcriptional level, providing a homeostatic mechanism for long-term control of cytosolic Ca^{2+}.

It has been shown that ERS caused by Ca^{2+} depletion is followed by ATF6 translocation to the nucleus (*46, 47*). ATF6 is a transcription factor involved in the transcription of endoplasmic reticulum stress-related proteins like grp78 and Bip but also proteins involved in calcium homeostasis such as SERCA2, CaM, Na^+/K^+-ATPase, α-MHC, plasma membrane Ca^{2+}-ATPases (PMCAs), and S100 calcium-binding protein; as well as transcription factors involved in cardiac gene expression such as Egr-1 and klf15 (*46, 48, 49*). The ATF6-mediated transcriptional activation is driven by the endoplasmic reticulum stress element (ERSE) in target genes and requires a high-affinity NF-Y binding site to be selective among ERSEs (*47*). The SERCA2 gene proximal promoter has a consensus ERSE that was shown to be responsible for ATF6 activation, although no for all the transcriptional activation mediated by Tg, suggesting that other calcium-mediated signaling pathways participate in transcriptional activation of the SERCA2 gene (*48*). Another important ATF6-inducible gene is MCIP-1 (also known as regulator of calcineurin-1), as ERS can be transduced by this way to the regulation of many pathways involving NFAT function, for example, growth and development (*49*).

The effects of calcium-dependent signaling by 2,3-butanedione monoxime (BDM), a drug that maintains calcium fluxes but abolishes contraction, or treatment with verapamil, a drug that inhibits both calcium fluxes and contraction, show that BDM increases SERCA2a mRNA level, which can be abolished

by Verapamil and reduced by CsA and KN-93 (*50*). In functional studies by transient transfection in neonatal cardiomyocytes, the transcriptional activity of the SERCA2 promoter was stimulated by MEF2c and NFATc4, but only when both factors were cotransfected. This upregulation might result of synergistic stimulation of SERCA2 promoter activity by NFATc4 and MEF2c, although a physical direct interaction between both factors has not been demonstrated. It becomes evident that contractile activity opposes the SERCA2 upregulation through distinct and independent pathways. These results suggest that in BDM-contractile arrested cardiomyocytes, CN and CaMKII mediate the upregulation of SERCA2a mRNA expression.

Although downregulation of SERCA2a expression in CH and HF is well documented, there are some reports regarding calcium-dependent upregulation of SERCA2 (*4, 50*). Intriguingly, while NFAT hypertrophic expression is mediated by high cytoplasmic calcium, it is also able to promote SERCA2 gene transcription. This apparent contradiction raises the need to describe the precise molecular mechanisms regulating the calcium dependence of SERCA2 expression and also other independent mechanisms that participate in development of hypertrophy, which ultimately lead to downregulation of SERCA2 expression in the hypertrophic and failing heart. Currently, ongoing studies in our laboratory are directed to understand the precise transcriptional mechanisms responsible for calcium regulation of SERCA2 gene expression.

Calsequestrin (Casq2) is the major calcium storage protein that links ECC in the cardiac SR. In complex with the proteins Triadin and Junctin, Casq2 is able to regulate the activity of RyR2 channels (*51*). Recently, it was demonstrated that MEF2- regulates transcriptional activity of the hCasq2 gene (*52*). It was also found in H9c2 cells that treatment with the CN inhibitor CsA reduced the expression of Casq2 (*53*). These data support the involvement of Ca^{2+}-dependent pathways in the control of Casq2 expression in cardiac muscle. However, contrary to these findings, in some hypertrophic conditions, which are characterized by abnormal calcium handling, there is no evidence of altered Casq2 expression; suggesting that human Casq2 promoter could be used to stably direct calcium handling proteins expression in gene therapy (*52*).

THE USE OF SERCA2 GENE THERAPY FOR HEART FAILURE

The existing reports have focused in the correction of the observed defects in HF mainly trying to increase SERCA2a expression. The SERCA2a calcium pump has a fundamental role in the normal myocardium function during muscular relaxation, recapturing the majority of the released Ca^{2+} from the SR for contraction and reloading the SR, maintaining an amount of Ca^{2+} that is sufficient to achieve an optimal contraction. Because of this role, it is a central point of the molecular basis of HF; where the overall SERCA2 expression is regulated in a negative manner, diminishing the capacity to reload the SR with Ca^{2+} after each contraction and resulting in a deficient contraction due to lack of Ca^{2+} inside the SR (*13*).

In the last years, SERCA2a expression became one of the most explored pharmacological targets in basic research and to a lesser extent in clinical investigation, although without conclusive positive results obtained (*54, 55*). The use of gene therapy to overexpress SERCA2a, has the advantage of eliminating the associated effects of drugs used to increase SERCA2a expression (*56*). In a similar way that the induction of SERCA2a expression has been extensively explored by pharmacological means, the overexpression by transference of genetic material also has been analyzed *in vitro* and *in vivo*. SERCA2a overexpression, improves the contractile function and the energy consumption in animal models with HF, improving the thickness of the anterior wall, and reducing ventricular arrhythmias. Other studies have also shown a decrease of inflammatory mediators and proapoptotic markers, after transference of viral vectors overexpressing SERCA2a in pressure overload induced HF; inhibition of ventricular remodeling in ischemic and volume overload induced HF (*57–60*).

Recent studies suggest that the myocardium of the failing heart is not refractory to treatment. However, different studies have demonstrated that the intra-arterial route is of little effectiveness unless the permeability of the endothelial lining is increased, or a high-pressure gradient is used; in these cases, a problem to consider is the increased tissue distribution and ectopic expression of the transferred gene (*61*). An option to avoid the ectopic expression of the transferred gene is to put the expression of the transferred gene under the control of a specific cardiac promoter, as is the case of the human cardiac calsequestrin gene (hCasq2) (*52*). At the moment, in our laboratory, we are developing adenoviral vectors that contain the SERCA2 gene under the control of hCasq2 promoter in order to achieve cardiac myocytes-specific expression (unpublished results).

It is important to mention that the ability of SERCA2a overexpression to reduce NFAT activity represents a potential novel therapeutic effect of SERCA2a that should be further considered in the development of cardiac gene therapy strategies. Recently, in the United States, a phase 2 clinical trial named Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) of intracoronary delivery of an adeno-associated viral vector AAV1/SERCA2a (MYDICAR®) to 39 patients with HF that started in 2007 was completed (*62, 63*). This trial was designed to evaluate the safety profile and biological effects of SERCA2a gene transfer, after 12 months follow-up, the patients showed an acceptable safety profile and improvement in heart function. Currently, there are two other ongoing gene therapy clinical trials to express SERCA2a in the hearts of patients with HF using AAV6/SERCA2a; one in England and one in France. Therefore, the transfer of SERCA2a cDNA into cardiomyocytes is a promising approach to treat HF by gene therapy.

In transgenic mice overexpressing SERCA2a subjected to ascending aortic constriction, the down-regulation of SERCA2a

in hypertrophic hearts was prevented; however, the hearts showed no increase in inotropic response compared to the wild-type mice hypertrophic hearts, suggesting that energy production may be compromised and a limiting factor for the beneficial effect of SERCA2a overexpression in hypertrophic and failing hearts (*64*). As mentioned before, there is a NADH dehydrogenase subunit-6 (ND6)-reduced expression in CH, which is a main source of energy in the cardiomyocyte (*28*). Therefore, in the future, there should be considered novel strategies combining energetic support with increasing SERCA2 activity to improve the therapeutic effectiveness for HF.

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REFERENCES

1. Bers, D. M. (2002) Cardiac excitation-contraction coupling. *Nature* **415**, 198–205.
2. Bers, D. M. (2008) Calcium cycling and signaling in cardiac myocytes. *Annu. Rev. Physiol.* **70**, 23–49.
3. Yano, K. and Zarain-Herzberg, A. (1994) Sarcoplasmic reticulum calsequestrins: structural and functional properties. *Mol. Cell. Biochem.* **135**, 61–70.
4. Prasad, A. M. and Inesi, G. (2011) Silencing calcineurin A subunit reduces SERCA2 expression in cardiac myocytes. *Am. J. Physiol. Heart Circ. Physiol.* **300**, H173–H180.
5. Reyes-Juarez, J. L. and Zarain-Herzberg, A. (2006) [Function and role of the sarcoplasmic reticulum in heart disease]. *Arch. Cardiol. Mex.* **76**(Suppl 4), S18–S32.
6. Zarain-Herzberg, A. (2006) Regulation of the sarcoplasmic reticulum Ca2+-ATPase expression in the hypertrophic and failing heart. *Can. J. Physiol. Pharmacol.* **84**, 509–521.
7. Alpert, N. R., Brosseau, C., Federico, A., Krenz, M., Robbins, J., et al. (2002) Molecular mechanics of mouse cardiac myosin isoforms. *Am. J. Physiol. Heart Circ. Physiol.* **283**, H1446–H1454.
8. Vannier, C., Chevassus, H., and Vassort, G. (1996) Ca-dependence of isometric force kinetics in single skinned ventricular cardiomyocytes from rats. *Cardiovasc. Res.* **32**, 580–586.
9. Baker, A. J., Figueredo, V. M., Keung, E. C., and Camacho, S. A. (1998) Ca2+ regulates the kinetics of tension development in intact cardiac muscle. *Am. J. Physiol.* **275**, H744–H750.
10. Palmer, S. and Kentish, J. C. (1998) Roles of Ca2+ and crossbridge kinetics in determining the maximum rates of Ca2+ activation and relaxation in rat and guinea pig skinned trabeculae. *Circ. Res.* **83**, 179–186.
11. Teucher, N., Prestle, J., Seidler, T., Currie, S., Elliott, E. B., et al. (2004) Excessive sarcoplasmic/endoplasmic reticulum Ca2+-ATPase expression causes increased sarcoplasmic reticulum Ca2+ uptake but decreases myocyte shortening. *Circulation* **110**, 3553–3559.
12. Bassani, J. W., Qi, M., Samarel, A. M., and Bers, D. M. (1994) Contractile arrest increases sarcoplasmic reticulum calcium uptake and SERCA2 gene expression in cultured neonatal rat heart cells. *Circ. Res.* **74**, 991–997.
13. Kaprielian, R., del Monte, F., and Hajjar, R. J. (2002) Targeting Ca2+ cycling proteins and the action potential in heart failure by gene transfer. *Basic Res. Cardiol.* **97**(Suppl 1), I136–I145.
14. Soltis, A. R., and Saucerman, J. J. (2010) Synergy between CaMKII substrates and beta-adrenergic signaling in regulation of cardiac myocyte Ca(2+) handling. *Biophys. J.* **99**, 2038–2047.
15. Hoshijima, M., Knoll, R., Pashmforoush, M., and Chien, K. (2006) Reversal of calcium cycling defects in advanced heart failure toward molecular therapy. *J. Am. Coll. Cardiol.* **48**, A15–A23.
16. Braunwald, E. and Bristow, M. R. (2000) Congestive heart failure: fifty years of progress. *Circulation* **102**, IV14–IV23.
17. Ai, X., Curran, J. W., Shannon, T. R., Bers, D. M., and Pogwizd, S. M. (2005) Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure. *Circ. Res.* **97**, 1314–1322.
18. van Oort, R. J., McCauley, M. D., Dixit, S. S., Pereira, L., Yang, Y., et al. (2010) Ryanodine receptor phosphorylation by calcium/calmodulin-dependent protein kinase II promotes life-threatening ventricular arrhythmias in mice with heart failure. *Circulation* **122**, 2669–2679.
19. Belevych, A. E., Terentyev, D., Terentyeva, R., Nishijima, Y., Sridhar, A., et al. (2011) The relationship between arrhythmogenesis and impaired contractility in heart failure: role of altered ryanodine receptor function. *Cardiovasc. Res.* **90**, 493–502.
20. Undrovinas, N. A., Maltsev, V. A., Belardinelli, L., Sabbah, H. N., and Undrovinas, A. (2010) Late sodium current contributes to diastolic cell Ca2+ accumulation in chronic heart failure. *J. Physiol. Sci.* **60**, 245–257.
21. Sossalla, S., Wagner, S., Rasenack, E. C., Ruff, H., Weber, S. L., et al. (2008) Ranolazine improves diastolic dysfunction in isolated myocardium from failing human hearts--role of late sodium current and intracellular ion accumulation. *J. Mol. Cell. Cardiol.* **45**, 32–43.
22. Diedrichs, H., Hagemeister, J., Chi, M., Boelck, B., Muller-Ehmsen, J., et al. (2007) Activation of the calcineurin/NFAT signalling cascade starts early in human hypertrophic myocardium. *J. Int. Med. Res.* **35**, 803–818.
23. Schaeffer, P. J., Desantiago, J., Yang, J., Flagg, T. P., Kovacs, A., et al. (2009) Impaired contractile function and calcium handling in hearts of cardiac-specific calcineurin b1-deficient mice. *Am. J. Physiol. Heart Circ. Physiol.* **297**, H744–H750.
24. Sanna, B., Bueno, O. F., Dai, Y. S., Wilkins, B. J., and Molkentin, J. D. (2005) Direct and indirect interactions between calcineurin-NFAT and MEK1-extracellular signal-regulated kinase 1/2 signaling pathways regulate cardiac gene expression and cellular growth. *Mol. Cell. Biol.* **25**, 865–878.
25. Colella, M., Grisan, F., Robert, V., Turner, J. D., Thomas, A. P., et al. (2008) Ca2+ oscillation frequency decoding in cardiac cell hypertrophy: role of calcineurin/NFAT as Ca2+ signal integrators. *Proc. Natl. Acad. Sci. USA* **105**, 2859–2864.
26. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2002) MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends Biochem. Sci.* **27**, 40–47.
27. Black, B. L. and Olson, E. N. (1998) Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu. Rev. Cell. Dev. Biol.* **14**, 167–196.
28. el Azzouzi, H., van Oort, R. J., van der Nagel, R., Sluiter, W., Bergmann, M. W., et al. (2010) MEF2 transcriptional activity maintains mitochondrial adaptation in cardiac pressure overload. *Eur. J. Heart Fail.* **12**, 4–12.
29. van Oort, R. J., van Rooij, E., Bourajjaj, M., Schimmel, J., Jansen, M. A., et al. (2006) MEF2 activates a genetic program promoting chamber dilation and contractile dysfunction in calcineurin-induced heart failure. *Circulation* **114**, 298–308.
30. Gordon, J. W., Pagiatakis, C., Salma, J., Du, M., Andreucci, J. J., et al. (2009) Protein kinase A-regulated assembly of a MEF2{middle dot}HDAC4 repressor complex controls c-Jun expression in vascular smooth muscle cells. *J. Biol. Chem.* **284**, 19027–19042.
31. Backs, J., Backs, T., Bezprozvannaya, S., McKinsey, T. A., and Olson, E. N. (2008) Histone deacetylase 5 acquires calcium/calmodulin-dependent kinase II responsiveness by oligomerization with histone deacetylase 4. *Mol. Cell. Biol.* **28**, 3437–3445.

32. Berger, I., Bieniossek, C., Schaffitzel, C., Hassler, M., Santelli, E., et al. (2003) Direct interaction of Ca2+/calmodulin inhibits histone deacetylase 5 repressor core binding to myocyte enhancer factor 2. *J. Biol. Chem.* **278**, 17625–17635.
33. Zhang, T., Kohlhaas, M., Backs, J., Mishra, S., Phillips, W., et al. (2007) CaMKIIdelta isoforms differentially affect calcium handling but similarly regulate HDAC/MEF2 transcriptional responses. *J. Biol. Chem.* **282**, 35078–35087.
34. MacDonnell, S. M., Weisser-Thomas, J., Kubo, H., Hanscome, M., Liu, Q., et al. (2009) CaMKII negatively regulates calcineurin-NFAT signaling in cardiac myocytes. *Circ. Res.* **105**, 316–325.
35. Passier, R., Zeng, H., Frey, N., Naya, F. J., Nicol, R. L., et al. (2000) CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. *J. Clin. Investig.* **105**, 1395–1406.
36. Kolodziejczyk, S. M., Wang, L., Balazsi, K., DeRepentigny, Y., Kothary, R., et al. (1999) MEF2 is upregulated during cardiac hypertrophy and is required for normal post-natal growth of the myocardium. *Curr. Biol.* **9**, 1203–1206.
37. Zhang, Z. Y., Liu, X. H., Hu, W. C., Rong, F., and Wu, X. D. (2010) The calcineurin-myocyte enhancer factor 2c pathway mediates cardiac hypertrophy induced by endoplasmic reticulum stress in neonatal rat cardiomyocytes. *Am. J. Physiol. Heart Circ. Physiol.* **298**, H744–H750.
38. Metrich, M., Laurent, A. C., Breckler, M., Duquesnes, N., Hmitou, I., et al. (2010) Epac activation induces histone deacetylase nuclear export via a Ras-dependent signalling pathway. *Cell. Signal.* **22**, 1459–1468.
39. Zobel, C., Rana, O. R., Saygili, E., Bolck, B., Diedrichs, H., et al. (2007) Mechanisms of Ca2+-dependent calcineurin activation in mechanical stretch-induced hypertrophy. *Cardiology* **107**, 281–290.
40. van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., McAnally, J., et al. (2006) A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc. Natl. Acad. Sci. USA* **103**, 18255–18260.
41. Ikeda, S., He, A., Kong, S. W., Lu, J., Bejar, R., et al. (2009) MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. *Mol. Cell. Biol.* **29**, 2193–2204.
42. da Costa Martins, P. A., Salic, K., Gladka, M. M., Armand, A. S., Leptidis, S., et al. (2010) MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signalling. *Nat. Cell Biol.* **12**, 1220–1227.
43. Guo, X., Williams, J. G., Schug, T. T., and Li, X. (2010) DYRK1A and DYRK3 promote cell survival through phosphorylation and activation of SIRT1. *J. Biol. Chem.* **285**, 13223–13232.
44. Sundaresan, N. R., Pillai, V. B., and Gupta, M. P. (2011) Emerging roles of SIRT1 deacetylase in regulating cardiomyocyte survival and hypertrophy. *J. Mol. Cell. Cardiol.*
45. Putt, M. E., Hannenhalli, S., Lu, Y., Haines, P., Chandrupatla, H. R., et al. (2009) Evidence for coregulation of myocardial gene expression by MEF2 and NFAT in human heart failure. *Circ. Cardiovasc. Genet* **2**, 212–219.
46. Adachi, Y., Yamamoto, K., Okada, T., Yoshida, H., Harada, A., et al. (2008) ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. *Cell Struct. Funct.* **33**, 75–89.
47. Li, M., Baumeister, P., Roy, B., Phan, T., Foti, D., et al. (2000) ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. *Mol. Cell. Biol.* **20**, 5096–5106.
48. Thuerauf, D. J., Hoover, H., Meller, J., Hernandez, J., Su, L., et al. (2001) Sarco/endoplasmic reticulum calcium ATPase-2 expression is regulated by ATF6 during the endoplasmic reticulum stress response: intracellular signaling of calcium stress in a cardiac myocyte model system. *J. Biol. Chem.* **276**, 48309–48317.
49. Belmont, P. J., Tadimalla, A., Chen, W. J., Martindale, J. J., Thuerauf, D. J., et al. (2008) Coordination of growth and endoplasmic reticulum stress signaling by regulator of calcineurin 1 (RCAN1), a novel ATF6-inducible gene. *J. Biol. Chem.* **283**, 14012–14021.
50. Vlasblom, R., Muller, A., Musters, R. J., Zuidwijk, M. J., Van Hardeveld, C., et al. (2004) Contractile arrest reveals calcium-dependent stimulation of SERCA2a mRNA expression in cultured ventricular cardiomyocytes. *Cardiovasc. Res.* **63**, 537–544.
51. Beard, N. A., Wei, L., and Dulhunty, A. F. (2009) Control of muscler-yanodine receptor calcium release channels by proteins in the sarcoplasmic reticulum lumen. *Clin. Exp. Pharmacol. Physiol.* **36**, 340–345.
52. Reyes-Juarez, J. L., Juarez-Rubi, R., Rodriguez, G., and Zarain-Herzberg, A. (2007) Transcriptional analysis of the human cardiac calsequestrin gene in cardiac and skeletal myocytes. *J. Biol. Chem.* **282**, 35554–35563.
53. Kasneci, A., Kemeny-Suss, N. M., Komarova, S. V., and Chalifour, L. E. (2009) Egr-1 negatively regulates calsequestrin expression and calcium dynamics in ventricular cells. *Cardiovasc. Res.* **81**, 695–702.
54. Zarain-Herzberg, A., Rupp, H., Elimban, V., and Dhalla, N. S. (1996) Modification of sarcoplasmic reticulum gene expression in pressure overload cardiac hypertrophy by etomoxir. *FASEB, J.* **10**, 1303–1309.
55. Zarain-Herzberg, A. and Rupp, H. (2002) Therapeutic potential of CPT I inhibitors: cardiac gene transcription as a target. *Expert Opin. Investig. Drugs* **11**, 345–356.
56. Gianni, D., Chan, J., Gwathmey, J., del Monte, F., and Hajjar, R. (2005) SERCA2a in heart failure: role and therapeutic prospects. *J. Bioenerg. Biomembr.* **37**, 375–380.
57. Miyamoto, M. I., del Monte, F., Schmidt, U., DiSalvo, T. S., Kang, Z. B., et al. (2000) Adenoviral gene transfer of SERCA2a improves left-ventricular function in aortic-banded rats in transition to heart failure. *Proc. Natl. Acad. Sci. USA* **97**, 793–798.
58. Beeri, R., Chaput, M., Guerrero, J. L., Kawase, Y., Yosefy, C., et al. (2010) Gene delivery of sarcoplasmic reticulum calcium ATPase inhibits ventricular remodeling in ischemic mitral regurgitation. *Circulation: Heart Fail.* **3**, 627–634.
59. Molina, E. J., Gupta, D., Palma, J., Gaughan, J. P., and Macha, M. (2010) Right ventricular beneficial effects of intracoronary SERCA2a gene transfer in an experimental model of heart failure. *Folia Biol. (Praha)* **56**, 1–8.
60. Mariani, J. A., Smolic, A., Preovolos, A., Byrne, M. J., Power, J. M., et al. (2011) Augmentation of left ventricular mechanics by recirculation-mediated AAV2/1-SERCA2a gene delivery in experimental heart failure. *Eur. J. Heart Fail.* **13**, 247–253.
61. Wright, M. J., Wightman, L. M., Latchman, D. S., and Marber, M. S. (2001) In vivo myocardial gene transfer: optimization and evaluation of intracoronary gene delivery in vivo. *Gene Ther.* **8**, 1833–1839.
62. Hajjar, R. J., Zsebo, K., Deckelbaum, L., Thompson, C., Rudy, J., et al. (2008) Design of a phase 1/2 trial of intracoronary administration of AAV1/SERCA2a in patients with heart failure. *J. Card. Fail.* **14**, 355–367.
63. Jaski, B. E., Jessup, M. L., Mancini, D. M., Cappola, T. P., Pauly, D. F., et al. (2009) Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID Trial), a first-in-human phase 1/2 clinical trial. *J. Card. Fail.* **15**, 171–181.
64. Pinz, I., Tian, R., Belke, D., Swanson, E., Dillmann, W., et al. (2011) Compromised myocardial energetics in hypertrophied mouse hearts diminish the beneficial effect of overexpressing SERCA2a. *J. Biol. Chem.* **286**, 10163–10168.

REVIEW / SYNTHÈSE

Regulation of sarco(endo)plasmic reticulum Ca^{2+}-ATPase and calsequestrin gene expression in the heart

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Abstract: The precise control of Ca^{2+} levels during the contraction–relaxation cycle in cardiac myocytes is extremely important for normal beat-to-beat contractile activity. The sarcoplasmic reticulum (SR) plays a key role controlling calcium concentration in the cytosol. The SR Ca^{2+}-ATPase (SERCA2) transports Ca^{2+} inside the SR lumen during relaxation of the cardiac myocyte. Calsequestrin (Casq2) is the main protein in the SR lumen, functioning as a Ca^{2+} buffer and participating in Ca^{2+} release by interacting with the ryanodine receptor 2 (RyR2) Ca^{2+}-release channel. Alterations in normal Ca^{2+} handling significantly contribute to the contractile dysfunction observed in cardiac hypertrophy and in heart failure. Transcriptional regulation of the *SERCA2* gene has been extensively studied and some of the mechanisms regulating its expression have been elucidated. Overexpression of Sp1 factor in cardiac hypertrophy downregulates *SERCA2* gene expression and increased levels of thyroid hormone up-regulates its transcription. Other hormones such norepinephrine, angiotensin II, endothelin-1, parathyroid hormone, prostaglandin-F2α, as well the cytokines tumor necrosis factor-α and interleukin-6 also downregulate *SERCA2* expression. Calcium acting through the calcineurin–NFAT (nuclear factor of activated T cells) pathway has been suggested to regulate *SERCA2* and *CASQ2* gene expression. This review focuses on the current knowledge regarding transcriptional regulation of *SERCA2* and *CASQ2* genes in the normal and pathologic heart.

Key words: SERCA2, calsequestrin, heart, transcription, gene expression, sarcoplasmic reticulum.

Résumé : Le contrôle précis des niveaux de Ca^{2+} lors du cycle de contraction–relaxation des myocytes cardiaques est extrêmement important afin de maintenir une activité contractile de battement à battement normale. Le réticulum sarcoplasmique joue un rôle clé en contrôlant la concentration de calcium du cytosol. La Ca^{2+}-ATPase du réticulum sarcoplasmique (RS) SERCA2 transporte le Ca^{2+} à l'intérieur de la lumière du RS lors de la relaxation du myocyte cardiaque. La calséquestrine (Casq2) est la principale protéine de la lumière du RS qui agit comme tampon du Ca^{2+} et qui participe à la libération de Ca^{2+} en interagissant avec le canal calcique de récepteurs à la ryanodine 2 (RyR2). Des modifications de la prise en charge normale du Ca^{2+} contribuent de façon significative à la dysfonction contractile observée dans l'hypertrophie cardiaque et l'insuffisance cardiaque. La régulation transcriptionnelle du gène *SERCA2* a été étudiée dans le détail et certains des mécanismes qui régulent son expression ont été élucidés. La surexpression du facteur SP1 dans les cas d'hypertrophie cardiaque régule à la baisse l'expression du gène *SERCA2* alors que des niveaux accrus d'hormone thyroïdienne stimulent sa transcription. D'autres hormones comme la norépinephrine, l'angiotensine II, l'endothéline-1, la parathormone, la prostaglandine-F2α ainsi que des cytokines comme le facteur nécrosant des tumeurs-α et l'interleukine-6 régulent l'expression de *SERCA2* à la baisse. Le calcium agissant par l'intermédiaire du sentier de la calcineurine–NFAT (les facteurs nucléaires des lyphocytes T) a été suggéré pour réguler l'expression des gènes *SERCA2* et *CASQ2*. Cet article de revue se concentre sur les connaissances actuelles de la régulation transcriptionnelle des gènes *SERCA2* et *CASQ2* dans le cœur normal et pathologique.

Mots-clés : SERCA2, calséquestrine, cœur, transcription, expression génique, réticulum sarcoplasmique.

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Introduction

The excitation–contraction coupling process (ECC) in the cardiac myocyte starts when a depolarizing stimulus reaches the sarcolemmal voltage dependent L-type calcium (Ca^{2+}) channels, also known as dihydropyridine receptors (DHPR), allowing the entrance of extracellular Ca^{2+} into the cell sarcoplasm at the triad junction, triggering opening of the sarcoplasmic reticulum (SR) Ca^{2+} release channels named ryanodine receptor 2 (RyR2), which results in a rapid and massive Ca^{2+} release from the SR lumen to the sarcoplasm. The rise in cytosolic calcium concentration ($[Ca^{2+}]_c$) is from 20–50 nmol/L to ~1 μmol/L. The free Ca^{2+} binds to troponin C (TnC), triggering a conformational change, then exposing the actin-binding site for myosin allowing the cross bridging of actin and myosin. Upon the cross-bridge formation there is a power stroke that moves the actin filament relative to myosin filament and ADP release. Immediately ATP binds to α- and β-myosin heavy chains, leading to detachment of myosin from actin. The Ca^{2+} released during EC coupling is actively transported from the sarcoplasm into the SR lumen by the sarco(endo)plasmic reticulum Ca^{2+}-ATPase pump (SERCA2a) (60%–80% of total), the remaining Ca^{2+} is extruded to the extracellular space by the Na^+–Ca^{2+} exchanger (NCX) (20%–40% of total) to preserve cardiomyocyte Ca^{2+} homeostasis (Bers 2002, 2008) (Fig. 1).

Inside the SR, Ca^{2+} binds to the main protein present, calsequestrin (Casq2), which has a high Ca^{2+} binding capacity (40 mol Ca^{2+}/mol protein) and moderate affinity for Ca^{2+} (K_d ~1 mmol/L); and is responsible for the storage of Ca^{2+}, but also participates in Ca^{2+}-release by RyR2 during contraction (Yano and Zarain-Herzberg 1994; Beard et al. 2004, 2009; Györke et al. 2009; MacLennan and Chen 2009; Lee et al. 2012). Thus, the precise control of Ca^{2+} levels during the contraction–relaxation cycle plays a key role for normal contractile beat-to-beat activity of the cardiac myocyte. Alterations in normal Ca^{2+} handling significantly contribute to the contractile dysfunction observed in cardiac hypertrophy (CH) and in heart failure (HF).

The Ca^{2+} ion serves as an intracellular signaling mechanism that couples membrane excitation to intracellular functions in most tissues. In the myocardial cell, variations of $[Ca^{2+}]_c$ are involved in several signaling functions, including contraction and activation of gene transcription. Therefore, it is meaningful to understand the regulation of expression of the proteins that participate in Ca^{2+} transport, binding, and release by the SR in the cardiomyocyte. In this paper, we review the current status of literature regarding transcriptional regulation of the *SERCA2* and calsequestrin-2 genes (*CASQ2*) in the normal and pathologic heart.

Alterations in calcium handling in the hypertrophic and failing heart

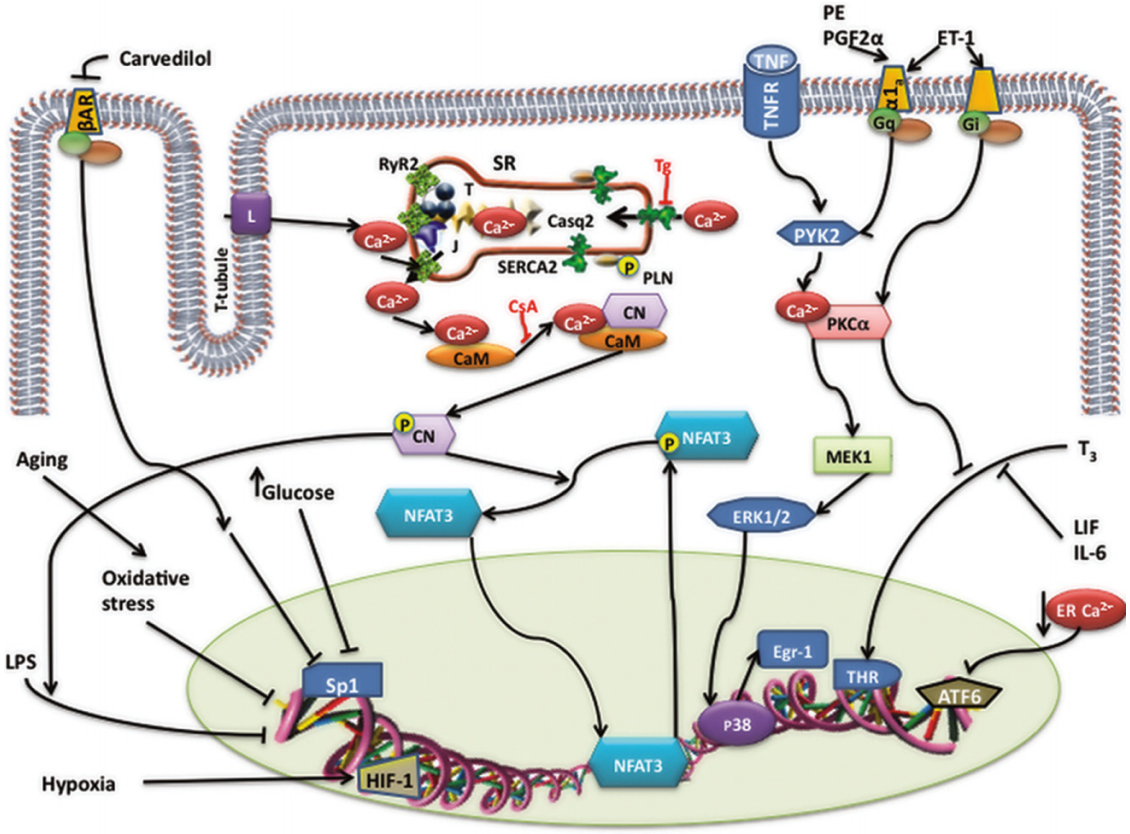
Physiological contraction and relaxation parameters are altered in cardiomyocytes of hearts in animal models with CH and in patients with HF (Bers 2002; Reyes-Juárez and Zarain-Herzberg 2006). It has been shown that a decrease in shortening velocity of the cardiomyocyte during contraction correlates with a reduced myofibrillar MHC-ATPase activity (Alpert et al. 2002), as well with a diminished $[Ca^{2+}]_c$ (Vannier et al. 1996; Baker et al. 1998; Palmer and Kentish 1998; Bers 2002). A decreased rate of Ca^{2+} removal from the sarcoplasm by the failing cardiomyocyte during myocardial relaxation leads to increased diastolic $[Ca^{2+}]_c$ levels, contributing to diastolic dysfunction development that is a distinctive feature of HF, which is also characterized by increased end-diastolic pressure and ventricular filling abnormalities.

The Ca^{2+} transients of cardiomyocytes from hearts in animal models with CH and from patients with end-stage HF, have decreased peak systolic $[Ca^{2+}]_c$ and a reduced velocity of Ca^{2+} transport by the SERCA2a pump into the SR (Reyes-Juárez and Zarain-Herzberg 2006). Because of the reduced peak $[Ca^{2+}]_c$ derived from reduced Ca^{2+} load to the SR there is a reduction of force development by the heart. Because low SR Ca^{2+} content has been observed in failing cardiomyocytes (Bassani et al. 1994; Teucher et al. 2004), altered SR Ca^{2+} handling is thought to be a key component contributing to the decreased contractility and force generation during cardiomyopathy. Cardiac ECC is a highly coordinated process controlled by several signaling pathways, including protein kinase A (PKA) and Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII). Among the alterations involved in CH and HF are changes in the expression levels of these regulatory proteins that act as sensors of intracellular Ca^{2+} (Kaprielian et al. 2002). An increased expression and activity of CaMKII has been demonstrated in HF, which plays a critical role in the decreased contractile properties of the heart, and which uncouples ECC and may lead to sudden death (Soltis and Saucerman 2010). These alterations also include decreased levels of SERCA2a with a subsequent decrease of the recapture of Ca^{2+} to the SR during diastole, resulting in a smaller amount of Ca^{2+} available to be released in the following contraction cycle.

Closely related to SERCA2a is phospholamban (PLN), a 52 amino acid peptide, which is the primary regulator of SERCA2a activity. PLN inhibits the pump in its native state, and dissociates from the pump when it is phosphorylated by CaMKII or protein kinase A (PKA) in response to β-adrenergic stimulation, in turn increasing the Ca^{2+} transport velocity by SERCA2a. In the failing heart, the SERCA2a:PLN ratio is decreased to a great extent by the reduction of SERCA2a pumps present in the SR membrane. This reduction causes a decrease in the amount of SERCA2a that is dissociated from PLN, and thus, reduces its activity and capacity to recapture the released Ca^{2+} during contraction (Hoshijima et al. 2006).

The decrease in phosphorylated PLN functional levels causes the SERCA2a activity to be decreased during HF; thus, reduced SERCA2a activity is not only due to a smaller expression of the SERCA2a protein, but also to alteration in the PLN phosphorylation level. Because of increased protein kinase C-α (PKC-α) activity, the inhibitor (I-1) of the protein phophatase-1 (PP1) is phosphorylated, relieving the PP1 inactivation, in turn causing PLN dephosphorylation by PP1 with the consequent PLN activation. The SR Ca^{2+} release channel (RyR2) has been reported in an unstable state, dissociated from the protein calstabin, which results in a higher channel opening probability. As a result, spontaneous Ca^{2+} liberations appear during the diastole (diastolic leak), which are associated with ventricular arrhythmias and, more importantly, with a decrease of the SR Ca^{2+} reserve available for the next contraction (Braunwald and Bristow 2000; Bers 2008).

Fig. 1. Proposed pathways involved in the transcriptional regulation of the *SERCA2* gene in the heart. Calcineurin (CN) silencing, or its inhibition with cyclosporine A (CsA) reduce *SERCA2* transcription, thus, NFAT3 is proposed as a positive regulator of *SERCA2* transcription. Thyroid hormone (T3) through thyroid hormone receptor (THR), endoplasmic reticulum (ER) stress through ATF6 transcription factor, and hypoxia through HIF-1 and Sp1 transcription factor up-regulate *SERCA2* gene transcription. Phenylephrine (PE) and prostaglandin-F2α (PGF2α) repress *SERCA2* transcription through the MAP kinases MEK1, ERK1/2, and p38; this process is mediated by the scaffolding protein PYK2 and PKC, since PKC inhibition prevents PE-mediated *SERCA2* downregulation. Gq and Gi proteins are necessary for ET-1-mediated *SERCA2* downregulation. PE and the cytokines LIF and IL-6 prevent T3-mediated *SERCA2* upregulation. Tumor necrosis factor-α (TNF-α) downregulates *SERCA2* transcription, probably through PYK2, which represses *SERCA2* expression via p38 protein. Egr-1 transcription factor represses *SERCA2* transcription, and p38 activity is required for this effect. Aging provokes oxidative stress that in turn diminishes *SERCA2* transcription; carvedilol, a β-adrenergic receptor blocker, avoids oxidative stress by promoting Sp1 binding to *SERCA2* promoter. LPS represses *SERCA2* transcription, and this effect requires CN activity. High glucose levels produce Sp1 transcription factor *O*-glucose-*N*-acylation diminishing its ability to bind and promote *SERCA2* transcription. NFAT, nuclear factor of activated T cells; ATF6, activating transcription factor-6; HIF-1, hypoxia inducible factor-1; MAP, mitogen-activated protein; MEK1, MAPK/extracellular signal-regulated kinase 1; ERK1/2, extracellular signal-regulated kinases 1 and 2; PYK2, protein tyrosine kinase 2; PKC, protein kinase C; ET-1, endothelin 1; LIF, leukemia inhibitory factor; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α; Egr-1, early growth response protein; LPS, lipopolysaccharide.



CaMKII shares common functional targets with PKA regulating ECC. Both PLN and RyR2 are phosphorylated by PKA and CaMKII, resulting in an increased SR Ca^{2+} release. The increased CaMKII activity in HF contributes to reduced SR calcium content and systolic function, and also causes diastolic SR Ca^{2+} leak and Ca^{2+} current changes that may be arrhythmogenic. One of the principal causes of sudden death in patients with HF is ventricular arrhythmia. Although abnormal Ca^{2+} release from the SR through RyR2 has been linked to arrhythmogenesis, the exact molecular mechanisms are unknown. Recent studies suggest that CaMKII-dependent phosphorylation of RyR2 is involved in enhanced SR diastolic Ca^{2+} leak and reduced SR Ca^{2+} load in HF, and that it may contribute to arrhythmias and contractile dysfunction in HF (Ai et al. 2005). RyR2 phosphorylation by CaMKII at S2814 plays a dominant role in arrhythmogenesis and sudden cardiac death in mice with HF. This fact suggests that destabilization of RyR2 activity due to excessive CaMKII-mediated phosphorylation results in reduced post-release refractoriness, which is a common mechanism involved in arrhythmogenesis and contractile dysfunction in the failing heart. These findings could lead to the development of more specific drug therapies that could modify the level of RyR2 phosphorylation by CaMKII, thus reducing diastolic Ca^{2+} leak and death from arrhythmias (van Oort et al. 2010; Belevych et al. 2011).

Cardiac pressure overload, as is seen in hypertensive patients, is the major pathologic stimulus for CH and is the main risk factor for HF development. Initially, hypertrophic growth is an adaptive response of the heart to pathological

stimuli that is characterized by cardiomyocyte enlargement, sarcomere assembly, and activation of a fetal cardiac gene expression program (Passier et al. 2000).

Two calcium-dependent signaling pathways have been identified in cardiomyocytes: the calcineurin–nuclear factor of activated T cell (CN–NFAT) pathway and the CaMKII–myocyte enhancer factor-2 (MEF2) pathway; the latter involving histone deacetylases (HDACs) nuclear export (Passier et al. 2000; Bers 2008). Both pathways participate in normal heart development and are altered in CH, but whether these pathways are independent or interdependent, and whether there is specificity among them is still unclear and under investigation. One of the features of CH and HF is downregulation of SERCA2a expression, leading to alterations in Ca^{2+} signaling pathways. Ca^{2+} signaling fluctuations and contractile function alterations have been demonstrated following specific inhibition of SERCA2a transport activity with thapsigargin (Tg), reduction of *SERCA2* gene expression by a gene null mutation, and *SERCA2* gene silencing with short interference RNA (siRNA) (Prasad and Inesi 2011).

Transcriptional regulation of the *SERCA2* gene

The *SERCA2* genes of several species have been cloned. The human *SERCA2* gene is located in chromosome 12q24.1 and contains 23 exons that by post-transcriptional alternative splicing mRNA processing produces 3 isoforms named SERCA2a, SERCA2b, and SERCA2c. Only the SERCA2a isoform is expressed in cardiomyocytes. The proximal regulatory region of the *SERCA2* gene (225 bp) is 80% G + C-rich and is conserved among human, rabbit, rat, and mouse species. It contains a TATA-like-box, an E-box/USF sequence, a CAAT-box, Egr-1, AP2, and several Sp1 binding sites, and 3 thyroid hormone responsive elements (TREs). There are 2 other conserved regulatory regions located between positions –410 to –661 bp and from –919 to –1410 bp. Among the DNA cis-elements present in these 2 regulatory regions there are potential binding sites for GATA-4, -5, -6, Nkx-2.5/Csx, OTF-1, USF, MEF2, serum response factor (SRF), PPAR/RXR, AP-2, and TREs. Upstream from position –1.5 kb, there is no significant homology among the *SERCA2* genes from different species, suggesting that the regulatory region is contained within the first 2 kb (Zarain-Herzberg and Alvarez-Fernandez 2002). Of particular relevance is the finding that Sp1 and Sp3 factors regulate *SERCA2* transcription in cardiomyocytes (Brady et al. 2003), and that in pressure overload induced CH, Sp1 levels are increased and are partially responsible for regulating decrease in *SERCA2* transcription (Takizawa et al. 2003).

Although several research groups have investigated the transcriptional regulation of the *SERCA2* gene, and there are some reviews about this topic and the diseases associated with the dysregulation of the protein (Vangheluwe et al. 2005; Zarain-Herzberg 2006; Brini and Carafoli 2009), recent reports have highlighted new views of previously unexplored pathways (Fig. 1). Different transcriptional mechanisms are involved in the regulation of *SERCA2* gene expression in the heart to maintain calcium homeostasis, in the following sections we review the knowledge of the known participating mechanisms.

Regulation by thyroid hormone

The effect of 3,3′,5-triiodo-L-thyronine or thyroid hormone (T_3) on heart function and *SERCA2* expression has been extensively investigated by several groups (Kahaly and Dillmann 2005). Thyroid hormone enhances *SERCA2* expression through its binding to the nuclear receptor THR. Thyroid hormone response elements (TREs) can be configured as elements consisting of 2 direct 6 bp repeats separated by a 4 bp tract, as palindromes or as inverted palindromes. Thyroid hormone receptor (THR) is coded by 2 different genes *THR-α* and *THR-β*, both of them produce different isoforms of the receptor by alternative splicing (Dillmann 2010).

Thyroid hormone increases the speed of diastolic relaxation in the heart, owing in part to the increase of *SERCA2* mRNA in normal rats and rabbits (Rohrer and Dillmann 1988; Nagai et al. 1989), thus there is an increased level of *SERCA2* mRNA in hyperthyroid hearts, but a decreased level in hypothyroid hearts. T_3 administration to hypothyroid rat hearts rescued *SERCA2* mRNA level. It is interesting to notice that T_3-mediated *SERCA2* mRNA and protein increase is gene and tissue specific, since *SERCA2* mRNA level in fast-twitch extensor digitorum longus muscle is decreased after T_3 administration to hypothyroid rats (in which *SERCA2* mRNA level is upregulated), and is also reduced under hyperthyroidism. Furthermore, in the soleus muscle, both hyperthyroidism and hypothyroidism lead to *SERCA2* decrease that is unaffected in the second case by T_3 administration (Sayen et al. 1992). These findings raised the question about the precise mechanisms controlling the T_3-mediated transcriptional regulation of *SERCA2* in the heart. In 1994, it was shown that the *SERCA2* response to T_3 was mediated by the binding of THR-α1 and (or) THR-β1 to a region containing 3 T_3 response elements (TREs) located in the proximal promoter of the rabbit and rat *SERCA2* genes (Hartong et al. 1994; Zarain-Herzberg et al. 1994). Site-directed mutagenesis of the 3 TREs of the *SERCA2* gene resulted in a loss of the thyroid hormone effect on the activation of *SERCA2* gene promoter. Additionally, T_3 is able to regulate phospholamban gene (*PLN*) expression in an opposite fashion to that observed for SERCA2a (Nagai et al. 1989). In hypothyroid hearts, the levels of phospholamban were increased with the consequent inhibition of SERCA2 activity (Dillmann 2010).

Norepinephrine, compared with thyroid hormone effect on *SERCA2* expression, is detrimental and depends on contractility, since *SERCA2* expression was unaffected in contraction-arrested cardiomyocytes exposed to norepinephrine, but was increased in those treated with T_3 (Muller et al. 1997). Regarding the role of T_3 under pathologic conditions, Wu et al. (1997) found that the repressive effect of phenylephrine (α_1-adrenergic agonist) on *SERCA2* expression inhibits the T_3-mediated SERCA2 increase. This effect was shown to be mediated directly by α_1-adrenergic stimulation since a constitutively active G protein (G_q) also downregulated *SERCA2* expression, and furthermore, was proposed to be transcriptionally mediated.

Even though T_3 has a positive effect on *SERCA2* expression, in end-stage heart failure there is an increase of the co-repressor protein FOG-2 (friend of GATA-2), and recently it was reported that this co-repressor disrupts the increased *SERCA2* expression driven by thyroid hormone, constituting

a novel mechanism of cardiac resistance to thyroid hormone (Rouf et al. 2008).

Regulation by other hormones

A family of critical hormones in the heart is the group of different vasoconstrictors including angiotensin II, endothelin-1 (ET-1), and prostaglandin-F2α. The suppressor effect of ET-1 on *SERCA2* expression has been shown to take place in a process that requires cross-talk between G_q and PTX-sensitive G_i pathways (Hilal-Dandan et al. 2009). Additionally, PKC signaling was also observed to be necessary for ET-1-mediated *SERCA2* repression (Uehara et al. 2012). It is important to note that PKC signaling is sufficient to induce *SERCA2* repression (Hartong et al. 1996), and that this repression is likely PKC isoenzyme dependent (Porter et al. 2003). Furthermore, the myocyte hypertrophy and downregulation of *SERCA2* elicited by phenylephrine, a well-known adrenergic agent promoting hypertrophy in neonatal cardiomyocytes, is prevented by PKC inhibition with Gö6983 (Prasad and Inesi 2012). Angiotensin II is another vasoconstrictor whose action on *SERCA2* transcription is detrimental in neonatal cardiomyocytes (Ju et al. 1996) but does not affect in adult counterparts. The same behavior occurs with phenylephrine, since adult rat cardiac myocytes treated with this adrenergic agent even showed an upregulation of *SERCA2* transcription (Anwar et al. 2005).

Parathyroid hormone (PTH) is increased in plasma from patients with heart disease, compared with healthy individuals. Furthermore, a synthetic form of PTH-related protein (PTHrP) is able to induce hypertrophy in cardiac myocytes, this process being abolished by a truncated form of the same synthetic PTHrP (Meyer et al. 2007). *SERCA2* is upregulated by synthetic PTHrP lacking the first N-terminal 6 amino acids, presenting the possibility of a dual function for PTHrP as seen in THR (Rouf et al. 2008).

Prostaglandin-F2α (PGF2α) is a vasoconstrictor also involved in inflammatory response that is produced in the myocardium subsequent to infarction or pressure overload (Berger et al. 1976; Chazov et al. 1979; Lai et al. 1996). It was shown that PGF2α stimulates hypertrophic growth of neonatal rat cardiac myocytes, including downregulation of SERCA2a. PGF2α increases Egr-1 expression and overexpression of Egr-1 decreases transcription of the proximal *SERCA2* gene promoter that contains a putative Egr-1 binding site. PGF2α was shown to exert repressive effects on *SERCA2* transcription through Egr-1 transcription factor in a process mediated by MAPK-p38, Ras, and Rac, but not ERK (Hara et al. 2008).

Regulation by cytokines

Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels are increased in plasma from patients with diastolic heart failure, and have been reported to downregulate *SERCA2* expression (Gloss et al. 2000; Wu et al. 2011). In neonatal cardiomyocytes, a reduction of the thyroid-hormone-induced increase in *SERCA2* mRNA level and transcriptional activity of the gene following co-treatment with the leukemia inhibitory factor (LIF) or IL-6 has been observed. These data suggest that cytokine-mediated inhibitory effects on the *SERCA2* gene promoter may be mediated by interfering with the thyroid hormone action (Gloss et al. 2000). Moreover, the mechanism by which TNF-α represses *SERCA2* expression was investigated, and an increased *SERCA2* promoter methylation due to TNF-α treatment in HL-1 cells was found (Kao et al. 2010). In this context, it was recently shown that H9c2 cells and rat embryonic heart tissue exposed to the solvent trichloroethylene exhibit *SERCA2* promoter hypermethylation and reduced concentration of *S*-adenosyl-methionine (Palbykin et al. 2011). One of the proteins involved in TNF-α signaling pathway is PYK2, a scaffolding protein that transduces signals from G protein-coupled receptors to MAPK downstream signaling (Blaukat et al. 1999; Pandey et al. 1999). Heidkamp et al. (2005) showed that PYK2 represses *SERCA2* expression through JNK1/2 and MAPK p38 phosphorylation, thus reinforcing other data showing the importance of TNF-α, MAPK p38, and even PKC signaling in *SERCA2* transcriptional regulation (Hartong et al. 1996; Hara et al. 2008; Kao et al. 2010).

Regulation by hypoxia and ischemia

Intracellular $[Ca^{2+}]_C$ is a key regulator of myocardial ischemia–reperfusion injury. It has been reported that during ischemic heart disease and in hypoxic myocardium, SERCA2a expression is decreased, inducing contractile dysfunction (Zhang and Zhu 2010). Recently, it was reported that under hypoxic conditions of cardiomyocytes in culture, *SERCA2* gene transcription is downregulated (Ronkainen et al. 2011). Activation of the hypoxia-inducible factor-1 (HIF-1) or overexpression of normoxia-stabile HIF-1α (HIF-1α/VP16) decreased endogenous *SERCA2a* mRNA expression and gene promoter activity by binding 2 functional HIF-1 elements. The above results may explain the diminished SERCA2 activity observed in the ischemic and hypoxic myocardium, and probably establish a link between oxygen supply and Ca^{2+} concentrations. In agreement with the above observation, it has been reported that exercise improves myocardial Ca^{2+}-handling and SERCA2a function (Kemi et al. 2008). Interestingly, it has been found that the mitochondrial transcription factors TFAM and TFB2M regulate transcription of the *SERCA2* gene in cardiomyocytes, providing a link between mitochondrial ATP production and spending by the SERCA2a pump (Watanabe et al. 2011).

Regulation by calcium signaling pathways

The CN and the CaMKII signaling pathways have been reported to play a key role in regulating *SERCA2* gene expression in cardiomyocytes (Vlasblom et al. 2004). Changes in cytoplasmic calcium concentration act as a trigger of the CN-NFAT pathway, which has been proposed as a primary driver of *SERCA2* expression, since cardiac myocytes treated with the specific SERCA inhibitor thapsigargin show increased *SERCA2* transcription. In addition, the calcium-mediated increase in *SERCA2* transcription is inhibited by blocking or silencing calcineurin activity, reinforcing the importance of CN-NFAT signaling in *SERCA2* gene transcriptional regulation (Prasad and Inesi 2011). Furthermore, recent findings show that competitive engagement of the CN-NFAT pathway by other signaling pathways also activated by calcium could explain the reduced SERCA2 expression during CH, even though high CN activity is observed in this pathology (Prasad and Inesi 2012).

Calcium sensing is crucial for proper cell function, for instance, inside the endoplasmic reticulum (ER) calcium level must be controlled within certain range, and if this level drops, the ER stress response is triggered (Yoshida 2007). Among all of the features involved in ER stress response, is the release of the transcriptional factor ATF6 from the ER and its translocation to the nucleus, where it promotes transcription of several genes, including *SERCA2* (Thuerauf et al. 2001). Moreover, ATF6 is able to induce both *SERCA2* and regulator of calcineurin-1 (*RCAN1*) transcription, thus, ATF6 controls the *SERCA2* transcription and one of its regulators at the same time (Belmont et al. 2008).

Owing to the key role of SERCA2 in cardiac function, it is not unusual to find multiple signals regulating its transcription. One of these examples is MAP kinases, whose action has been found to regulate, both directly and indirectly, CN-NFAT pathway that in turn regulates *SERCA2* transcription during CH (Molkentin 2004). Another less explored pathway involved in CH, and consequently, in SERCA2 expression, is the inflammatory response. For instance, the lipopolysaccharide (LPS) from gram negative bacteria has been shown to produce hypertrophic features in H9c2 cells, such features being prevented by CN blocking (Liu et al. 2008).

The transcription and (or) function of SERCA2 can be modified indirectly by many factors, such as the function and (or) expression of other calcium handling proteins interacting, or not, with SERCA2 (Vandecaetsbeek et al. 2009). Also, the aging process affects the function and (or) expression of SERCA2, either by downregulating it or diminishing its enzymatic activity (Assayag et al. 1997; Azhar et al. 2007; Kaplan et al. 2007), and this process is intimately related to oxidative stress. Since oxidative stress is known to repress SERCA2 transcription and function (Bigelow 2009), strategies for restoring the redox state have been proposed to attenuate declining heart function produced by oxidative stress (Dai et al. 2009). In fact, carvedilol, a β-adrenoreceptor blocker, elicits its beneficial effects on cardiac function through the restoration of SERCA2 expression in patients with heart failure. Furthermore, carvedilol prevents oxidative-stress-mediated *SERCA2* downregulation by promoting Sp1 and Sp3 binding to elements located in the proximal promoter, increasing transcription of the gene by this mechanism (Koitabashi et al. 2005).

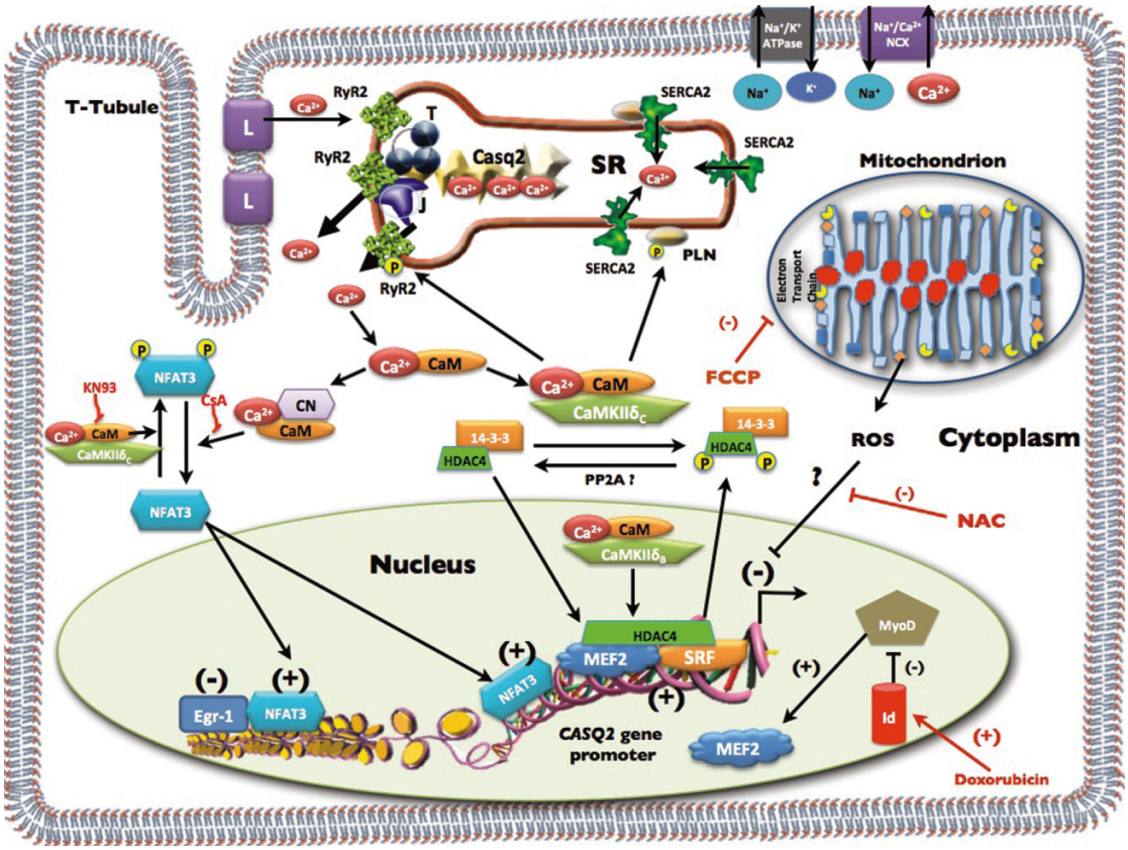
Among the causes provoking left ventricular dysfunction, the dysregulation of energetic balance plays a substantial role, owing to the highly energy-consuming process that contraction–relaxation cycling represents. For example, mice with cardiomyocyte-restricted knockout of the insulin receptor show increased mitochondrial dysfunction following myocardial infarction when compared with wild-type littermates, and this impairment is accompanied by a decrease in several proteins including SERCA2, PPAR-α, and GLUT4 (Sena et al. 2009). Actually, GLUT4 knockout mice exhibit deficient contractile performance, cardiomyocyte hypertrophy, fibrosis, and reduced expression of RyR2 and SERCA2 proteins (Domenighetti et al. 2010). Since diabetes is a highly prevalent cause of cardiomyopathy, several studies have addressed SERCA2 expression under diabetic conditions, and the reader is directed to other reviews for a deeper discussion (Hayat et al. 2004; Boudina and Abel 2007). Here, we only highlight some data regarding the SERCA2 repression caused by *O*-GlcNAcylation of the transcription factor Sp1 in cardiomyocytes exposed to high glucose (Clark et al. 2003). Since Sp1 is a key transcriptional regulator of SERCA2 expression in the normal and hypertrophic heart (Brady et al. 2003; Takizawa et al. 2003), Sp1 modification constitutes a relatively novel mechanism of SERCA2 regulation. Moreover, high glucose treatment in cardiomyocytes was recently shown to promote PLN *O*-GlcNAcylation, and it was proposed that this modification could improve PLN-mediated SERCA2a inhibition, thus providing a mechanism by which SERCA2a function would be reduced under diabetic cardiomyopathy (Yokoe et al. 2010).

Transcriptional regulation of the cardiac calsequestrin gene

As mentioned before, calsequestrin is the principal Ca^{2+}-binding protein inside the terminal cistern of the SR in cardiac and skeletal muscle cells. Being a highly acidic protein, especially in its C-terminal portion, calsequestrin is able to bind high quantities of Ca^{2+} (approximately 30–50 mol of Ca^{2+}/mol of Casq) (Beard et al. 2004). The primary role proposed for Casq inside the SR is to be a Ca^{2+} buffer; however, in association with the proteins triadin (T) and junctin (J), Casq has the capacity to regulate the activity of the SR Ca^{2+}-release channel RyR2 (Beard et al. 2009; Györke et al. 2009; MacLennan and Chen 2009; Lee et al. 2012). In absence of Ca^{2+}, it is thought that Casq has a random structure, but above 1.0 mmol/L luminal SR Ca^{2+}, Casq protein monomers fold into thiorredoxin-like domains. In this polymeric form, Casq is able to associate with T and J transmembranal proteins then form a RyR2 channel regulatory complex (Beard et al. 2009; Lee et al. 2012). In the heart, the Casq2–T–J complex buffers the SR free Ca^{2+} levels to 1 mmol/L, even though the total Ca^{2+} level varies significantly between cardiac muscle contraction cycles (Beard et al. 2009). The SR Ca^{2+} levels are lowered during Ca^{2+}-induced Ca^{2+} release (CICR) during excitation–contraction coupling (EC) in cardiac muscle, so the Casq2–T–J complex inhibits the RyR2 channel. When the SR Ca^{2+} load is restored by the action of the SERCA2a pump, Casq2 is dissociated from T and J then relieving the inhibitory action exerted on RyR2 by Casq2 (Györke et al. 2009).

In mammals, there are 2 Casq isoforms coded by 2 different genes. Both isoforms are exclusively expressed in muscle cells (Frank et al. 2001). In humans, the Casq1 isoform is coded by the *CASQ1* gene located in chromosome 1q21 and is expressed in fast- and slow-twitch skeletal muscles. The Casq2 isoform is encoded by the *CASQ2* gene located in 1p23 and is mainly expressed in cardiac muscle, and to a lesser extent in slow-twitch skeletal muscle, counting for approximately 25% of total Casq protein in the latter. The remaining 75% Casq protein content in slow-twitch skeletal muscle corresponds to the Casq1 isoform. During fetal development, Casq2 is the only isoform expressed in cardiomyocytes, and this pattern remains unchanged during the post-natal period as well as adulthood. In skeletal muscle, *CASQ2* gene is expressed during fast- and slow-twitch muscle development; however, during the post-natal period there is an isoform switch to the Casq1 isoform, making this one the only isoform expressed in fast-twitch adult skeletal muscle. However, as mentioned above, Casq2 is also ex-

Fig. 2. Physiological role of calsequestrin and proposed transcriptional regulation of the *CASQ2* gene in cardiomyocytes. Casq2 is the main intraluminal sarcoplasmic reticulum (SR) Ca^{2+}-binding protein. In association with the proteins triadin (T) and junctin (J), Casq2 is able to regulate the activity of the SR Ca^{2+}-release channel ryanodine receptor 2 (RyR2). Ca^{2+}–calmodulin-dependent kinases (CaMKIIδb and CaMKIIδc) phosphorylate histone deacetylase 4 (HDAC4), allowing its interaction with the chaperone protein 14-3-3, resulting either in HDAC4 export from the nucleus to the cytoplasm (CaMKIIδb), or avoiding nuclear import (CaMKIIδc). The location of HDAC4 in the cytoplasm permits myocyte enhancer factor-2 (MEF2)-dependent and SRF-dependent activation of the *CASQ2* gene. Also, CaMKIIδc is able to phosphorylate RyR2 channel and phospholamban (PLN), resulting in an enhanced SR Ca^{2+}-release. Treatment of H9c2 cells with Ca^{2+}–CaM–calcineurin inhibitor cyclosporine A (CsA) inhibits nuclear factor of activated T cells (NFAT) activation and down-regulates *CASQ2* gene expression. These data support the idea of NFAT as a positive regulator of the *CASQ2* gene. In Egr-1 overexpressing H9c2 cells, Egr-1 transcription factor has been suggested as a negative regulator of *CASQ2* gene expression. Doxorubicin inhibits MyoD-dependent transcription up-regulating the Id-protein. Because MEF-2 is a target of MyoD, this mechanism could be responsible of the *CASQ2* gene down-regulation observed in doxorubicin-induced cardiomyopathy models. Finally, the mitochondrial uncoupling agent carbonyl-cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) down-regulates *CASQ2* gene expression by an unknown mechanism. Oxidative-stress-activated pathways must be involved because *CASQ2* gene down-regulation could be prevented by the reactive oxygen species (ROS) scavenger *N*-acetyl-cysteine (NAC). MEF2, myocyte enhancer factor 2; HDAC4, histone deacetylase class II-4; SRF, serum response factor. PP2A, protein phosphatase-2A.



pressed, but to a lesser extent, in adult slow-twitch skeletal muscle (Frank et al. 2001; Reyes-Juárez et al. 2007).

The *CASQ2* gene mRNA levels have been measured in pathological states such as heart failure, CH, and dilated cardiomyopathy, although no alterations have been found (Lehnart et al. 1998). Despite this fact, in Casq2-overexpressing mice, CH, induction of fetal gene expression program, lowered shortening fraction, increased SR-Ca^{2+} storage capacity, and decreased SR Ca^{2+} release with the consequent contractile impairment of muscle contraction were found (Sato et al. 1998). In the opposite situation, gene ablation of *CASQ2* produces an arrhythmic phenotype without contractile function alterations. When Casq2 is absent, there is an increased probability of spontaneous SR Ca^{2+} release (Knollmann 2009). These data could provide a pathophysiological mechanism for the catecholaminergic polymorphic ventricular tachycardia (CPVT) disorder in which several mutations in the *CASQ2* gene are found. CPVT is a rare inherited arrhythmia syndrome characterized by adrenergic-stress-induced ventricular tachyarrhythmias without obvious heart structural changes (Chopra and Knollmann 2009). Taken together, these data support the idea of Casq2 as a central protein in the SR–Ca^{2+} homeostasis control in cardiac muscle.

The thyroid hormones have a deep influence in the expression of many Ca^{2+}-homeostasis control proteins such SERCA2, phospholamban, Na^{+}–Ca^{2+} exchanger (NCX), and Casq. In methimazole-induced hypothyroid Lewis rats, the levels of Casq1 are decreased in the fast-twitch skeletal extensor digitorum longus muscle (EDL). By comparison, treatment with 3,3′,5-triiodo-L-thyronine increased Casq1 levels

in EDL muscle. These changes in Casq1 expression in skeletal muscle are mainly due to the increased proportion of the fastest 2B muscle fibers in hyperthyroid status. Also, the methimazole-induced decreased level of Casq1 can result in decreased proportion of these fibers. Additional studies must be done to explain the role of thyroid hormones in the regulation of the Casq protein expression (Novák and Soukup 2011).

Although the *CASQ2* gene has been reported to be exclusively expressed in cardiac muscle, there have been recent reports on the role of autoimmunity against Casq2 in patients with Graves disease. Gene-expression analysis of thyroid tissue from patients with Grave's ophthalmopathy revealed a 2-fold increase in *CASQ2* mRNA levels. Although Casq2 has no known function in the thyroid gland and is primarily located in SR, it is also found in other membranes during the micro-tubular development, and could be exposed to the immune system during this stage. The above data support the idea of an up-regulation of *CASQ2* gene induced by TSH-R antibodies (Wescombe et al. 2010). However, a correlation between thyroid hormone and Casq2 expression has not been found before (Arai et al. 1991). The data mentioned above underline the role of *CASQ2* gene expression in different pathologic situations involving the cardiovascular and endocrine systems.

In spite of the importance of Casq2 in many different physiologic and pathologic situations, the transcriptional control of *CASQ2* gene expression is still poorly understood. In-silico DNA sequence analysis of the 5′-regulatory region of human *CASQ2* gene, revealed high sequence homology between –130 to –30 bp relative to the transcription start site. Within this region there is an MEF-2 and a SRF transcription factor binding site. Using functional assays, it was demonstrated that these sites confer high transcriptional activity in cardiomyocytes. Also, site-directed mutagenesis of these binding sites resulted in a loss of transcriptional activity in cultured rat cardiomyocytes (Reyes-Juárez et al. 2007). A reduction in Casq2 expression has been reported in Egr-1 overexpressing H9c2 cells, so Egr-1 transcription factor could be a negative regulator of *CASQ2* gene expression. Additionally, the treatment of H9c2 cells with cyclosporine A (an inhibitor of the Ca^{2+}–CaM–CN–NFAT pathway) resulted in a reduced Casq2 expression (Kasneci et al. 2009). It should be noted that there are several putative NFAT transcription factor binding sites in both rat (Kasneci et al. 2009) and human *CASQ2* genes (unpublished observation). Therefore, taken together, these data suggest a role of the Ca^{2+}–CaM–CN–NFAT pathway in *CASQ2* gene regulation (Fig. 2).

It is relevant to mention that Ca^{2+}-controlled pathways (such calcineurin and CaMKII) activate MEF-2, NFAT, and SRF transcription factors. Interestingly, Casq2 expression changes have been not found in cardiac pathologies with altered Ca^{2+} handling, such as in CH and HF (Lehnart et al. 1998). To complicate the scenario, it has been shown that a hyperthyroid state results in a reduced level of CaMKII. However, this is not associated with a reduction of Casq2 protein (Jiang et al. 2006). These data may be disturbing because the activation of MEF2-dependent transcription relies on CaMKII activity, and MEF2 was shown to be critical in *CASQ2* gene transcriptional regulation (Reyes-Juárez et al. 2007).

Contrary to previous reports in which Casq2 was unchanged in cardiac pathologies (Lehnart et al. 1998), Casq2 expression was lowered in a rabbit model of doxorubicin-induced cardiomyopathy. Rabbits treated with doxorubicin had a significant decrement in CASQ2 mRNA levels as well as in other proteins involved in cardiac Ca^{2+}-homeostasis control such as RyR2 channel, SERCA2, and its regulatory protein PLN (Arai et al. 1998). Doxorubicin is able to inhibit the expression of MyoD-controlled genes up-regulating the expression of the Id factor, which results in a MyoD-dependent transcription impairment (Kurabayashi et al. 1994). These results are interesting because MEF-2 relies on MyoD activity for its expression. Considering the role of MEF-2 in *CASQ2* gene expression, this could provide a molecular basis for the reduced *CASQ2* gene mRNA levels induced by doxorubicin. Because of sequence analysis of the *CASQ2* gene 5′-regulatory region revealed several putative MyoD-binding sites (Reyes-Juárez et al. 2007), the doxorubicin-induced up-regulation of Id factor could also directly impact *CASQ2* gene expression.

Finally, a recent report suggested that oxidative stress could have a role in the *CASQ2* gene expression. Cultured rat cardiomyocytes treated with the mitochondrial uncoupling agent carbonyl-cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) resulted in a concentration-dependent *CASQ2* mRNA downregulation. The treatment of cardiomyocytes with the reactive oxygen species (ROS) scavenger *N*-acetyl-cysteine prevented the *CASQ2* gene downregulation (Hänninen et al. 2010). However, the molecular mechanism for this effect remains unknown.

Taking together all the facts discussed above, it is obvious that *CASQ2* gene transcriptional control is more complex than was reported earlier (Reyes-Juárez et al. 2007), and many Ca^{2+}-dependent and Ca^{2+}-independent pathways must be involved in its regulation. Considering the central role of Casq2 in the SR–Ca^{2+} release in cardiac muscle, and its involvement in cardiac pathologies such CPVT and doxorubicin-induced cardiomyopathy, this suggests that it is important that the molecular mechanisms involved in the *CASQ2* gene expression are studied further.

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References

Ai, X., Curran, J.W., Shannon, T.R., Bers, D.M., and Pogwizd, S.M. 2005. Ca^{2+}/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca^{2+} leak in heart failure. Circ. Res. **97**(12): 1314–1322. doi:10.1161/01.RES.0000194329.41863.89. PMID:16269653.

Alpert, N.R., Brosseau, C., Federico, A., Krenz, M., Robbins, J., and Warshaw, D.M. 2002. Molecular mechanics of mouse cardiac myosin isoforms. Am. J. Physiol. Heart Circ. Physiol. **283**(4): H1446–H1454. PMID:12234796.

Anwar, A., Taimor, G., Korkusuz, H., Schreckenberg, R., Berndt, T.,

Abdallah, Y., et al. 2005. PKC-independent signal transduction pathways increase SERCA2 expression in adult rat cardiomyocytes. J. Mol. Cell. Cardiol. **39**(6): 911–919. doi:10.1016/j.yjmcc.2005.08.001. PMID:16236312.

Arai, M., Otsu, K., MacLennan, D.H., Alpert, N.R., and Periasamy, M. 1991. Effect of thyroid hormone on the expression of mRNA encoding sarcoplasmic reticulum proteins. Circ. Res. **69**(2): 266–276. PMID:1830516.

Arai, M., Tomaru, K., Takizawa, T., Sekiguchi, K., Yokoyama, T., Suzuki, T., and Nagai, R. 1998. Sarcoplasmic reticulum genes are selectively down-regulated in cardiomyopathy produced by doxorubicin in rabbits. J. Mol. Cell. Cardiol. **30**(2): 243–254. doi:10.1006/jmcc.1997.0588. PMID:9515001.

Assayag, P., Charlemagne, D., de Leiris, J., Boucher, F., Valere, P.E., Lortet, S., et al. 1997. Senescent heart compared with pressure overload-induced hypertrophy. Hypertension, **29**(1): 15–21. PMID:9039074.

Azhar, G., Zhang, X., Wang, S., Zhong, Y., Quick, C.M., and Wei, J.Y. 2007. Maintaining serum response factor activity in the older heart equal to that of the young adult is associated with better cardiac response to isoproterenol stress. Basic Res. Cardiol. **102**(3): 233–244. doi:10.1007/s00395-006-0634-z. PMID:17122890.

Baker, A.J., Figueredo, V.M., Keung, E.C., and Camacho, S.A. 1998. Ca^{2+} regulates the kinetics of tension development in intact cardiac muscle. Am. J. Physiol. **275**(3): H744–H750. PMID:9724275.

Bassani, J.W., Qi, M., Samarel, A.M., and Bers, D.M. 1994. Contractile arrest increases sarcoplasmic reticulum calcium uptake and *SERCA2* gene expression in cultured neonatal rat heart cells. Circ. Res. **74**(5): 991–997. PMID:8156646.

Beard, N.A., Laver, D.R., and Dulhunty, A.F. 2004. Calsequestrin and the calcium release channel of skeletal and cardiac muscle. Prog. Biophys. Mol. Biol. **85**(1): 33–69. doi:10.1016/j.pbiomolbio.2003.07.001. PMID:15050380.

Beard, N.A., Wei, L., and Dulhunty, A.F. 2009. Control of muscle ryanodine receptor calcium release channels by proteins in the sarcoplasmic reticulum lumen. Clin. Exp. Pharmacol. Physiol. **36**(3): 340–345. doi:10.1111/j.1440-1681.2008.05094.x. PMID:19278523.

Belevych, A.E., Terentyev, D., Terentyeva, R., Nishijima, Y., Sridhar, A., Hamlin, R.L., et al. 2011. The relationship between arrhythmogenesis and impaired contractility in heart failure: role of altered ryanodine receptor function. Cardiovasc. Res. **90**(3): 493–502. doi:10.1093/cvr/cvr025. PMID:21273243.

Belmont, P.J., Tadimalla, A., Chen, W.J., Martindale, J.J., Thuerauf, D.J., Marcinko, M., et al. 2008. Coordination of growth and endoplasmic reticulum stress signaling by regulator of calcineurin 1 (RCAN1), a novel ATF6-inducible gene. J. Biol. Chem. **283**(20): 14012–14021. doi:10.1074/jbc.M709776200. PMID:18319259.

Berger, H.J., Zaret, B.L., Speroff, L., Cohen, L.S., and Wolfson, S. 1976. Regional cardiac prostaglandin release during myocardial ischemia in anesthetized dogs. Circ. Res. **38**(6): 566–571. PMID:1269107.

Bers, D.M. 2002. Cardiac excitation-contraction coupling. Nature, **415**(6868): 198–205. doi:10.1038/415198a. PMID:11805843.

Bers, D.M. 2008. Calcium cycling and signaling in cardiac myocytes. Annu. Rev. Physiol. **70**(1): 23–49. doi:10.1146/annurev.physiol.70.113006.100455. PMID:17988210.

Bigelow, D.J. 2009. Nitrotyrosine-modified SERCA2: a cellular sensor of reactive nitrogen species. Pflugers Arch. **457**(3): 701–710. doi:10.1007/s00424-007-0429-6. PMID:18172601.

Blaukat, A., Ivankovic-Dikic, I., Gronroos, E., Dolfi, F., Tokiwa, G., Vuori, K., and Dikic, I. 1999. Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. J. Biol. Chem. **274**(21): 14893–14901. doi:10.1074/jbc.274.21.14893. PMID:10329689.

Boudina, S., and Abel, E.D. 2007. Diabetic cardiomyopathy revisited. Circulation, **115**(25): 3213–3223. doi:10.1161/CIRCULATIONAHA.106.679597. PMID:17592090.

Brady, M., Koban, M.U., Dellow, K.A., Yacoub, M., Boheler, K.R., and Fuller, S.J. 2003. Sp1 and Sp3 transcription factors are required for trans-activation of the human SERCA2 promoter in cardiomyocytes. Cardiovasc. Res. **60**(2): 347–354. doi:10.1016/S0008-6363(03)00529-7. PMID:14613864.

Braunwald, E., and Bristow, M.R. 2000. Congestive heart failure: fifty years of progress. Circulation, **102**(20 Suppl. 4): IV14–IV23. PMID:11080127.

Brini, M., and Carafoli, E. 2009. Calcium pumps in health and disease. Physiol. Rev. **89**(4): 1341–1378. doi:10.1152/physrev.00032.2008. PMID:19789383.

Chazov, E.I., Pomoinetsky, V.D., Geling, N.G., Orlova, T.R., Nekrasova, A.A., and Smirnov, V.N. 1979. Heart adaptation to acute pressure overload: an involvement of endogenous prostaglandins. Circ. Res. **45**(2): 205–211. PMID:445704.

Chopra, N., and Knollmann, B.C. 2009. Cardiac calsequestrin: the new kid on the block in arrhythmias. J. Cardiovasc. Electrophysiol. **20**(10): 1179–1185. doi:10.1111/j.1540-8167.2009.01531.x. PMID:19563351.

Clark, R.J., McDonough, P.M., Swanson, E., Trost, S.U., Suzuki, M., Fukuda, M., and Dillmann, W.H. 2003. Diabetes and the accompanying hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear *O*-GlcNAcylation. J. Biol. Chem. **278**(45): 44230–44237. doi:10.1074/jbc.M303810200. PMID:12941958.

Dai, D.F., Santana, L.F., Vermulst, M., Tomazela, D.M., Emond, M.J., MacCoss, M.J., et al. 2009. Overexpression of catalase targeted to mitochondria attenuates murine cardiac aging. Circulation, **119**(21): 2789–2797. doi:10.1161/CIRCULATIONAHA.108.822403. PMID:19451351.

Dillmann, W. 2010. Cardiac hypertrophy and thyroid hormone signaling. Heart Fail. Rev. **15**(2): 125–132. doi:10.1007/s10741-008-9125-7. PMID:19125327.

Domenighetti, A.A., Danes, V.R., Curl, C.L., Favaloro, J.M., Proietto, J., and Delbridge, L.M. 2010. Targeted GLUT-4 deficiency in the heart induces cardiomyocyte hypertrophy and impaired contractility linked with Ca(2+) and proton flux dysregulation. J. Mol. Cell. Cardiol. **48**(4): 663–672. doi:10.1016/j.yjmcc.2009.11.017. PMID:19962383.

Frank, K.F., Mesnard-Rouiller, L., Chu, G., Young, K.B., Zhao, W., Haghighi, K., et al. 2001. Structure and expression of the mouse cardiac calsequestrin gene. Basic Res. Cardiol. **96**(6): 636–644. doi:10.1007/s003950170016. PMID:11770083.

Gloss, B., Villegas, S., Villarreal, F.J., Moriscot, A., and Dillmann, W.H. 2000. Thyroid hormone-induced stimulation of the sarcoplasmic reticulum Ca^{2+} ATPase gene is inhibited by LIF and IL-6. Am. J. Physiol. Endocrinol. Metab. **278**(4): E738–E743. PMID:10751209.

Györke, S., Stevens, S.C.W., and Terentyev, D. 2009. Cardiac calsequestrin: quest inside the SR. J. Physiol. **587**(13): 3091–3094. doi:10.1113/jphysiol.2009.172049. PMID:19567748.

Hänninen, S.L., Ronkainen, J.J., Leskinen, H., and Tavi, P. 2010. Mitochondrial uncoupling downregulates calsequestrin expression and reduces SR Ca^{2+} stores in cardiomyocytes. Cardiovasc. Res. **88**(1): 75–82. doi:10.1093/cvr/cvq180. PMID:20525644.

Hara, S., Arai, M., Tomaru, K., Doi, H., Koitabashi, N., Iso, T., et al. 2008. Prostaglandin F2α inhibits *SERCA2* gene transcription through an induction of Egr-1 in cultured neonatal rat cardiac myocytes. Int. Heart J. **49**(3): 329–342. doi:10.1536/ihj.49.329. PMID:18612190.

Hartong, R., Wang, N., Kurokawa, R., Lazar, M.A., Glass, C.K.,

Apriletti, J.W., and Dillmann, W.H. 1994. Delineation of three different thyroid hormone-response elements in promoter of rat sarcoplasmic reticulum Ca^{2+}ATPase gene. Demonstration that retinoid X receptor binds 5′ to thyroid hormone receptor in response element 1. J. Biol. Chem. **269**(17): 13021–13029. PMID: 8175722.

Hartong, R., Villarreal, F.J., Giordano, F., Hilal-Dandan, R., McDonough, P.M., and Dillmann, W.H. 1996. Phorbol myristate acetate-induced hypertrophy of neonatal rat cardiac myocytes is associated with decreased sarcoplasmic reticulum Ca^{2+} ATPase (*SERCA2*) gene expression and calcium reuptake. J. Mol. Cell. Cardiol. **28**(12): 2467–2477. doi:10.1006/jmcc.1996.0239. PMID: 9004163.

Hayat, S.A., Patel, B., Khattar, R.S., and Malik, R.A. 2004. Diabetic cardiomyopathy: mechanisms, diagnosis and treatment. Clin. Sci. **107**(6): 539–557. doi:10.1042/CS20040057. PMID:15341511.

Heidkamp, M.C., Scully, B.T., Vijayan, K., Engman, S.J., Szotek, E.L., and Samarel, A.M. 2005. PYK2 regulates *SERCA2* gene expression in neonatal rat ventricular myocytes. Am. J. Physiol. Cell Physiol. **289**(2): C471–C482. doi:10.1152/ajpcell.00130. 2005. PMID:15829561.

Hilal-Dandan, R., He, H., Martin, J.L., Brunton, L.L., and Dillmann, W.H. 2009. Endothelin downregulates SERCA2 gene and protein expression in adult rat ventricular myocytes: regulation by pertussis toxin-sensitive Gi protein and cAMP. Am. J. Physiol. Heart Circ. Physiol. **296**(3): H728–H734. doi:10.1152/ajpheart. 00584.2008. PMID:19151257.

Hoshijima, M., Knoll, R., Pashmforoush, M., and Chien, K. 2006. Reversal of calcium cycling defects in advanced heart failure toward molecular therapy. J. Am. Coll. Cardiol. **48**(9 Suppl. 1): A15–A23. doi:10.1016/j.jacc.2006.06.070. PMID:17084280.

Jiang, M., Xu, A., and Narayanan, N. 2006. Thyroid hormone downregulates the expression and function of sarcoplasmic reticulum-associated CaM kinase II in the rabbit heart. Am. J. Physiol. Heart. Circ. Physiol. **291**: H1384–H1394. doi:10.1152/ ajpheart.00875.2005. PMID:16617128.

Ju, H., Scammel-La Fleur, T., and Dixon, I.M. 1996. Altered mRNA abundance of calcium transport genes in cardiac myocytes induced by angiotensin II. J. Mol. Cell. Cardiol. **28**(5): 1119–1128. doi:10. 1006/jmcc.1996.0103. PMID:8762048.

Kahaly, G.J., and Dillmann, W.H. 2005. Thyroid hormone action in the heart. Endocr. Rev. **26**(5): 704–728. doi:10.1210/er.2003-0033. PMID:15632316.

Kao, Y.H., Chen, Y.C., Cheng, C.C., Lee, T.I., Chen, Y.J., and Chen, S.A. 2010. Tumor necrosis factor-α decreases sarcoplasmic reticulum Ca^{2+}-ATPase expressions via the promoter methylation in cardiomyocytes. Crit. Care Med. **38**(1): 217–222. doi:10.1097/ CCM.0b013e3181b4a854. PMID:19730253.

Kaplan, P., Jurkovicova, D., Babusikova, E., Hudecova, S., Racay, P., Sirova, M., et al. 2007. Effect of aging on the expression of intracellular Ca(2+) transport proteins in a rat heart. Mol. Cell. Biochem. **301**(1–2): 219–226. doi:10.1007/s11010-007-9414-9. PMID:17549608.

Kaprielian, R., del Monte, F., and Hajjar, R.J. 2002. Targeting Ca^{2+} cycling proteins and the action potential in heart failure by gene transfer. Basic Res. Cardiol. **97**(7 Suppl. 1): I136–I145. doi:10. 1007/s003950200042. PMID:12479247.

Kasneci, A., Kemeny-Suss, N.M., Komarova, S.V., and Chalifour, L.E. 2009. Egr-1 negatively regulates calsequestrin expression and calcium dynamics in ventricular cells. Cardiovasc. Res. **81**(4): 695–702. doi:10.1093/cvr/cvn357. PMID:19103607.

Kemi, O.J., Ceci, M., Condorelli, G., Smith, G.L., and Wisloff, U. 2008. Myocardial sarcoplasmic reticulum Ca^{2+} ATPase function is increased by aerobic interval training. Eur. J. Cardiovasc. Prev. Rehabil. **15**(2): 145–148. doi:10.1097/HJR.0b013e3282efd4e0. PMID:18391639.

Knollmann, B.C. 2009. New roles of calsequestrin and triadin in cardiac muscle. J. Physiol. **587**(13): 3081–3087. doi:10.1113/ jphysiol.2009.172098. PMID:19451205.

Koitabashi, N., Arai, M., Tomaru, K., Takizawa, T., Watanabe, A., Niwano, K., et al. 2005. Carvedilol effectively blocks oxidative stress-mediated downregulation of sarcoplasmic reticulum Ca^{2+}-ATPase 2 gene transcription through modification of Sp1 binding. Biochem. Biophys. Res. Commun. **328**(1): 116–124. doi:10. 1016/j.bbrc.2004.12.139. PMID:15670758.

Kurabayashi, M., Jeyaseelan, R., and Kedes, L. 1994. Doxorubicin represses the function of the myogenic helix–loop–helix transcription factor MyoD. Involvement of *Id* gene induction. J. Biol. Chem. **269**(8): 6031–6039. PMID:8119948.

Lai, J., Jin, H., Yang, R., Winer, J., Li, W., Yen, R., et al. 1996. Prostaglandin F2 alpha induces cardiac myocyte hypertrophy in vitro and cardiac growth in vivo. Am. J. Physiol. **271**(6): H2197–H2208. PMID:8997275.

Lee, K.W., Maeng, J.S., Choi, J.Y., Lee, Y.R., Hwang, C.Y., Park, S.S., et al. 2012. Role of junctin protein interactions in cellular dynamics of calsequestrin polymer upon calcium perturbation. J. Biol. Chem. **287**(3): 1679–1687. doi:10.1074/jbc.M111.254045. PMID:22123818.

Lehnart, S.E., Schillinger, W., Pieske, B., Prestle, J., Just, H., and Hasenfuss, G. 1998. Sarcoplasmic reticulum proteins in heart failure. Ann. N. Y. Acad. Sci. **853**(1 CARDIAC SARCO): 220–230. doi:10.1111/j.1749-6632.1998.tb08270.x. PMID:10603950.

Liu, C.J., Cheng, Y.C., Lee, K.W., Hsu, H.H., Chu, C.H., Tsai, F.J., et al. 2008. Lipopolysaccharide induces cellular hypertrophy through calcineurin/NFAT-3 signaling pathway in H9c2 myocardiac cells. Mol. Cell. Biochem. **313**(1–2): 167–178. doi:10.1007/s11010-008-9754-0. PMID:18398669.

MacLennan, D.H., and Chen, S.R. 2009. Store overload-induced Ca^{2+} release as a triggering mechanism for CPVT and MH episodes caused by mutations in *RYR* and *CASQ* genes. J. Physiol. **587** (13): 3113–3115. doi:10.1113/jphysiol.2009.172155. PMID: 19567749.

Meyer, R., Schreckenberg, R., Kretschmer, F., Bittig, A., Conzelmann, C., Grohe, C., and Schluter, K. 2007. Parathyroid hormone-related protein (PTHrP) signal cascade modulates myocardial dysfunction in the pressure overloaded heart. Eur. J. Heart Fail. **9**(12): 1156–1162. doi:10.1016/j.ejheart.2007.10.007. PMID:17997355.

Molkentin, J.D. 2004. Calcineurin-NFAT signaling regulates the cardiac hypertrophic response in coordination with the MAPKs. Cardiovasc. Res. **63**(3): 467–475. doi:10.1016/j.cardiores.2004.01. 021. PMID:15276472.

Muller, A., Zuidwijk, M.J., Simonides, W.S., and van Hardeveld, C. 1997. Modulation of SERCA2 expression by thyroid hormone and norepinephrine in cardiocytes: role of contractility. Am. J. Physiol. **272**(4): H1876–H1885. PMID:9139974.

Nagai, R., Zarain-Herzberg, A., Brandl, C.J., Fujii, J., Tada, M., MacLennan, D.H., et al. 1989. Regulation of myocardial Ca^{2+}-ATPase and phospholamban mRNA expression in response to pressure overload and thyroid hormone. Proc. Natl. Acad. Sci. U.S.A. **86** (8): 2966–2970. doi:10.1073/pnas.86.8.2966. PMID:2523077.

Novák, P., and Soukup, T. 2011. Calsequestrin distribution, structure and function, its role in normal and pathological situations and the effect of thyroid hormones. Physiol. Res. **60**(3): 439–452. PMID: 21401301.

Palbykin, B., Borg, J., Caldwell, P.T., Rowles, J., Papoutsis, A.J., Romagnolo, D.F., and Selmin, O.I. 2011. Trichloroethylene induces methylation of the Serca2 promoter in H9c2 cells and

embryonic heart. Cardiovasc. Toxicol. **11**(3): 204–214. doi:10.1007/s12012-011-9113-3. PMID:21479763.

Palmer, S., and Kentish, J.C. 1998. Roles of Ca^{2+} and crossbridge kinetics in determining the maximum rates of Ca^{2+} activation and relaxation in rat and guinea pig skinned trabeculae. Circ. Res. **83** (2): 179–186. PMID:9686757.

Pandey, P., Avraham, S., Kumar, S., Nakazawa, A., Place, A., Ghanem, L., et al. 1999. Activation of p38 mitogen-activated protein kinase by PYK2/related adhesion focal tyrosine kinase-dependent mechanism. J. Biol. Chem. **274**(15): 10140–10144. doi:10.1074/jbc.274.15.10140. PMID:10187797.

Passier, R., Zeng, H., Frey, N., Naya, F.J., Nicol, R.L., McKinsey, T.A., et al. 2000. CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. J. Clin. Invest. **105**(10): 1395–1406. doi:10.1172/JCI8551. PMID: 10811847.

Porter, M.J., Heidkamp, M.C., Scully, B.T., Patel, N., Martin, J.L., and Samarel, A.M. 2003. Isoenzyme-selective regulation of *SERCA2* gene expression by protein kinase C in neonatal rat ventricular myocytes. Am. J. Physiol. Cell Physiol. **285**(1): C39–C47. PMID:12606313.

Prasad, A.M., and Inesi, G. 2011. Silencing calcineurin A subunit reduces SERCA2 expression in cardiac myocytes. Am. J. Physiol. Heart Circ. Physiol. **300**(1): H173–H180. doi:10.1152/ajpheart.00841.2010. PMID:21057045.

Prasad, A.M., and Inesi, G. 2012. Regulation and rate limiting mechanisms of Ca^{2+} ATPase (SERCA2) expression in cardiac myocytes. Mol. Cell. Biochem. **361**(1–2): 85–96. doi:10.1007/s11010-011-1092-y. PMID:21964539.

Reyes-Juárez, J.L., and Zarain-Herzberg, A. 2006. Función del retículo sarcoplásmico y su papel en las enfermedades cardíacas [Function and role of the sarcoplasmic reticulum in heart disease]. Arch. Cardiol. Mex. **76**(Suppl. 4): S18–S32. PMID:17469332.

Reyes-Juárez, J.L., Juárez-Rubi, R., Rodríguez, G., and Zarain-Herzberg, A. 2007. Transcriptional analysis of the human cardiac calsequestrin gene in cardiac and skeletal myocytes. J. Biol. Chem. **282**(49): 35554–35563. doi:10.1074/jbc.M707788200. PMID: 17938175.

Rohrer, D., and Dillmann, W.H. 1988. Thyroid hormone markedly increases the mRNA coding for sarcoplasmic reticulum Ca^{2+}-ATPase in the rat heart. J. Biol. Chem. **263**(15): 6941–6944. PMID: 2966798.

Ronkainen, V.P., Skoumal, R., and Tavi, P. 2011. Hypoxia and HIF-1 suppress SERCA2a expression in embryonic cardiac myocytes through two interdependent hypoxia response elements. J. Mol. Cell. Cardiol. **50**(6): 1008–1016. doi:10.1016/j.yjmcc.2011.02.017. PMID:21382378.

Rouf, R., Greytak, S., Wooten, E.C., Wu, J., Boltax, J., Picard, M., et al. 2008. Increased FOG-2 in failing myocardium disrupts thyroid hormone-dependent *SERCA2* gene transcription. Circ. Res. **103** (5): 493–501. doi:10.1161/CIRCRESAHA.108.181487. PMID: 18658259.

Sato, Y., Ferguson, D.G., Sako, H., Dorn, G.W., II, Kadambi, V.J., Yatani, A., et al. 1998. Cardiac-specific overexpression of mouse cardiac calsequestrin is associated with depressed cardiovascular function and hypertrophy in transgenic mice. J. Biol. Chem. **273** (43): 28470–28477. doi:10.1074/jbc.273.43.28470. PMID: 9774476.

Sayen, M.R., Rohrer, D.K., and Dillmann, W.H. 1992. Thyroid hormone response of slow and fast sarcoplasmic reticulum Ca^{2+} ATPase mRNA in striated muscle. Mol. Cell. Endocrinol. **87**(1–3): 87–93. doi:10.1016/0303-7207(92)90236-Y. PMID:1446789.

Sena, S., Hu, P., Zhang, D., Wang, X., Wayment, B., Olsen, C., et al. 2009. Impaired insulin signaling accelerates cardiac mitochondrial dysfunction after myocardial infarction. J. Mol. Cell. Cardiol. **46** (6): 910–918. doi:10.1016/j.yjmcc.2009.02.014. PMID:19249310.

Soltis, A.R., and Saucerman, J.J. 2010. Synergy between CaMKII substrates and beta-adrenergic signaling in regulation of cardiac myocyte Ca(2+) handling. Biophys. J. **99**(7): 2038–2047. doi:10.1016/j.bpj.2010.08.016. PMID:20923637.

Takizawa, T., Arai, M., Tomaru, K., Koitabashi, N., Baker, D.L., Periasamy, M., and Kurabayashi, M. 2003. Transcription factor Sp1 regulates *SERCA2* gene expression in pressure-overloaded hearts: a study using in vivo direct gene transfer into living myocardium. J. Mol. Cell. Cardiol. **35**(7): 777–783. doi:10.1016/S0022-2828(03)00122-6. PMID:12818568.

Teucher, N., Prestle, J., Seidler, T., Currie, S., Elliott, E.B., Reynolds, D.F., et al. 2004. Excessive sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase expression causes increased sarcoplasmic reticulum Ca^{2+} uptake but decreases myocyte shortening. Circulation, **110** (23): 3553–3559. doi:10.1161/01.CIR.0000145161.48545.B3. PMID:15505097.

Thuerauf, D.J., Hoover, H., Meller, J., Hernandez, J., Su, L., Andrews, C., et al. 2001. Sarco/endoplasmic reticulum calcium ATPase-2 expression is regulated by ATF6 during the endoplasmic reticulum stress response: intracellular signaling of calcium stress in a cardiac myocyte model system. J. Biol. Chem. **276**(51): 48309–48317. PMID:11595740.

Uehara, Y., Azuma, Y., Minai, K., Yoshida, H., Yoshimura, M., and Shimizu, M. 2012. Endothelin-1 prolongs intracellular calcium transient decay in neonatal rat cardiac myocytes. Heart Vessels, **27** (1): 98–105. doi:10.1007/s00380-011-0133-5. PMID:21445599.

van Oort, R.J., McCauley, M.D., Dixit, S.S., Pereira, L., Yang, Y., Respress, J.L., et al. 2010. Ryanodine receptor phosphorylation by calcium/calmodulin-dependent protein kinase II promotes life-threatening ventricular arrhythmias in mice with heart failure. Circulation, **122**(25): 2669–2679. doi:10.1161/CIRCULATIONAHA.110.982298. PMID:21098440.

Vandecaetsbeek, I., Raeymaekers, L., Wuytack, F., and Vangheluwe, P. 2009. Factors controlling the activity of the SERCA2a pump in the normal and failing heart. Biofactors, **35**(6): 484–499. doi:10.1002/biof.63. PMID:19904717.

Vangheluwe, P., Raeymaekers, L., Dode, L., and Wuytack, F. 2005. Modulating sarco(endo)plasmic reticulum Ca^{2+} ATPase 2 (SERCA2) activity: cell biological implications. Cell Calcium, **38**(3–4): 291–302. doi:10.1016/j.ceca.2005.06.033. PMID: 16105684.

Vannier, C., Chevassus, H., and Vassort, G. 1996. Ca-dependence of isometric force kinetics in single skinned ventricular cardiomyocytes from rats. Cardiovasc. Res. **32**(3): 580–586. PMID:8881518.

Vlasblom, R., Muller, A., Musters, R.J., Zuidwijk, M.J., Van Hardeveld, C., Paulus, W.J., and Simonides, W.S. 2004. Contractile arrest reveals calcium-dependent stimulation of SERCA2a mRNA expression in cultured ventricular cardiomyocytes. Cardiovasc. Res. **63**(3): 537–544. doi:10.1016/j.cardiores.2004.04.005. PMID:15276479.

Watanabe, A., Arai, M., Koitabashi, N., Niwano, K., Ohyama, Y., Yamada, Y., et al. 2011. Mitochondrial transcription factors TFAM and TFB2M regulate *SERCA2* gene transcription. Cardiovasc. Res. **90**(1): 57–67. doi:10.1093/cvr/cvq374. PMID:21113058.

Wescombe, L., Lahooti, H., Gopinath, B., and Wall, J.R. 2010. The cardiac calsequestrin gene (*CASQ2*) is up-regulated in the thyroid in patients with Graves' ophthalmopathy — support for a role of autoimmunity against calsequestrin as the triggering event. Clin. Endocrinol. (Oxf.), **73**(4): 522–528. PMID:20039900.

Wu, P.S., Moriscot, A.S., Knowlton, K.U., Hilal-Dandan, R., He, H., and Dillmann, W.H. 1997. Alpha 1-adrenergic stimulation inhibits 3,5,3′-triiodothyronine-induced expression of the rat heart sarco-

plasmic reticulum Ca^{2+} adenosine triphosphatase gene. Endocrinology, **138**(1): 114–120. doi:10.1210/en.138.1.114. PMID: 8977393.

Wu, C.K., Lee, J.K., Chiang, F.T., Yang, C.H., Huang, S.W., Hwang, J.J., et al. 2011. Plasma levels of tumor necrosis factor-alpha and interleukin-6 are associated with diastolic heart failure through downregulation of sarcoplasmic reticulum Ca^{2+} ATPase. Crit. Care Med. **39**(5): 984–992. doi:10.1097/CCM.0b013e31820a91b9. PMID:21263314.

Yano, K., and Zarain-Herzberg, A. 1994. Sarcoplasmic reticulum calsequestrins: structural and functional properties. Mol. Cell. Biochem. **135**(1): 61–70. doi:10.1007/BF00925961. PMID: 7816057.

Yokoe, S., Asahi, M., Takeda, T., Otsu, K., Taniguchi, N., Miyoshi, E., and Suzuki, K. 2010. Inhibition of phospholamban phosphorylation by *O*-GlcNAcylation: implications for diabetic cardiomyopathy. Glycobiology, **20**(10): 1217–1226. doi:10.1093/glycob/cwq071. PMID:20484118.

Yoshida, H. 2007. ER stress and diseases. FEBS J. **274**(3): 630–658. doi:10.1111/j.1742-4658.2007.05639.x. PMID:17288551.

Zarain-Herzberg, A. 2006. Regulation of the sarcoplasmic reticulum Ca^{2+}-ATPase expression in the hypertrophic and failing heart. Can. J. Physiol. Pharmacol. **84**(5): 509–521. doi:10.1139/y06-023. PMID:16902596.

Zarain-Herzberg, A., and Alvarez-Fernandez, G. 2002. Sarco(endo) plasmic reticulum Ca^{2+}-ATPase-2 gene: structure and transcriptional regulation of the human gene. ScientificWorldJournal, **2**: 1469–1483. doi:10.1100/tsw.2002.228. PMID:12805933.

Zarain-Herzberg, A., Marques, J., Sukovich, D., and Periasamy, M. 1994. Thyroid hormone receptor modulates the expression of the rabbit cardiac sarco (endo) plasmic reticulum Ca(2+)-ATPase gene. J. Biol. Chem. **269**(2): 1460–1467. PMID:8166809.

Zhang, N., and Zhu, B. 2010. The role of the rat sarcoplasmic reticulum Ca^{2+}-ATPase promoter in myocardial ischemia-preconditioning. Mol. Cell. Biochem. **333**(1–2): 311–321. doi:10.1007/s11010-009-0232-0. PMID:19714449.

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REVIEW

SERCA2a: its role in the development of heart failure and as a potential therapeutic target

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Abstract: The complexity of heart physiology has delayed the implementation of efficient, feasible, and safe therapies to fight against heart diseases for many years. As knowledge of the precise mechanisms governing cardiac hypertrophy and heart failure development increases, the availability of new therapeutic alternatives also grows. Since the cardiomyocyte physiology deeply depends on the correct calcium handling, many efforts to describe accurately the excitation–contraction coupling process in the heart and the proteins involved have been made. Among the proteins participating in calcium handling, sarco/endoplasmic reticulum Ca^{2+} adenosine triphosphatase-2a (SERCA2a), whose expression and function is decreased in heart failure, stands out because of its critical role regulating Ca^{2+} concentration in the cardiomyocyte. The importance of SERCA2a has been reflected in numerous studies aimed to describe its expression and function. Recently, gene therapy to deliver SERCA2a has shown promising results in human clinical trials. This paper reviews the current literature knowledge exploring diverse approaches to rescue SERCA2a expression in heart failure. It also discusses some data suggesting other possible therapies that could improve SERCA2a expression and function in cardiac diseases.

Keywords: SERCA2a, cardiac hypertrophy, heart failure, gene therapy, calcium

Introduction

Heart contractile function depends on the correct calcium ion (Ca^{2+}) cycling in the cardiomyocyte accomplished by a tightly synchronized process called excitation–contraction coupling (ECC). In the cardiomyocyte the ECC process starts when a depolarizing stimulus reaches the sarcolemmal voltage-dependent L-type Ca^{2+} channels also known as dihydropyridine receptors. This allows the entrance of extracellular Ca^{2+} into the cell sarcoplasm at the triad junction, triggering the opening of the sarcoplasmic reticulum (SR) Ca^{2+} release channels named ryanodine receptors type-2 (RyR2), which results in a rapid and massive Ca^{2+} release from the SR lumen to the sarcoplasm and causes cytosolic free calcium concentration ($[Ca^{2+}]_c$) to rise from around 100 nM to 1 μM.[1] Then, free Ca^{2+} binds to the troponin complex, which triggers a conformational change that exposes the actin-binding site for myosin, allowing the cross-bridge between actin and myosin. Upon cross-bridge formation there is a power stroke that moves the actin filament relative to myosin filament and adenosine diphosphate is released, resulting in cardiomyocyte contraction. During relaxation, adenosine triphosphate (ATP) binds to α- and β-myosin heavy chain (MHC) leading to detachment of myosin from actin, and the Ca^{2+} released from the SR contraction is actively transported from the sarcoplasm into the SR lumen by the cardiac sarco/endoplasmic reticulum Ca^{2+} ATPase-2a

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(SERCA2a) pump, varying from around 60% in humans to 90% in mice. The remainder Ca^{2+} is extruded to the extracellular space mainly by the sodium (Na^+)/Ca^{2+} exchanger, but also by the mitochondrial Ca^{2+} uniporter and the sarcolemmal Ca^{2+} ATPase (plasma membrane Ca^{2+} ATPase) to maintain cardiomyocyte Ca^{2+} homeostasis.[2,3] Inside the SR, Ca^{2+} binds to the main present protein calsequestrin (CASQ2), which has high Ca^{2+}-binding capacity (40 mol Ca^{2+}/mol protein), moderate affinity for Ca^{2+} (Kd ~1 mM), and is responsible for storage of Ca^{2+} but also participates in Ca^{2+} release by RyR2 during contraction.[4–6] The precise control of Ca^{2+} levels during the contraction–relaxation cycle plays a key role in the normal contractile function of the cardiac myocyte. Altered Ca^{2+} handling is the main hallmark of the contractile dysfunction observed in heart failure (HF).

This review analyzes the evidence demonstrating alterations in the function of the components regulating Ca^{2+} handling in cardiac hypertrophy (CH) and HF. It also reviews the current knowledge about the different strategies directed to normalize cardiac SERCA2a activity and/or expression, including pharmacological and gene therapy approaches.

Alterations in calcium handling in the hypertrophic and failing heart

Physiological CH occurs as a result of an adaptation to increased workload, like the one observed in athletes, and is a benign modification of the myocardium. Some of the changes observed are increased heart size, better cardiac performance, enhanced energy utilization, and increased (or unchanged) expression of SERCA2a and α-MHC. In contrast, in pathological CH the increased heart size is accompanied by poor cardiac performance, poor fatty acid utilization, decreased expression of SERCA2a and α-MHC, and activation of a fetal cardiac gene expression program.[7] Although adrenergic stimulation has been shown to increase SERCA2a expression,[8] a time-course follow-up of hypertrophy has shown that this upregulation occurs in the first stages, but in severe CH the *SERCA2* gene is downregulated as occurs in HF.[9]

Physiological contraction and relaxation parameters are altered in cardiomyocytes of hearts in animal models with CH and in patients with HF.[2,10] It has been shown that a decrease in shortening velocity of the cardiomyocyte during contraction correlates with a reduced myofibrillar MHC/ATPase activity,[11] as well as with a diminished $[Ca^{2+}]_c$.[2,12–14] A decreased rate of Ca^{2+} removal from the sarcoplasm by the failing cardiomyocyte during myocardial relaxation leads to increased diastolic $[Ca^{2+}]_c$ levels, contributing to diastolic dysfunction development present in HF. The observed dysfunction is characterized by increased end-diastolic pressure and ventricular filling abnormalities. The Ca^{2+} transients of cardiomyocytes from hearts in animal models with CH and from patients with end-stage HF have decreased peak systolic $[Ca^{2+}]_c$ and a reduced velocity of Ca^{2+} transport by the SERCA2a pump into the SR.[10] Because of the reduced peak $[Ca^{2+}]_c$ derived from reduced Ca^{2+} load to the SR, there is a reduction of force development by the heart. Due to a low SR Ca^{2+} content in failing cardiomyocytes,[15,16] altered SR Ca^{2+} handling is thought to be a key component contributing to the decreased contractility and force generation during cardiomyopathy.

Cardiac ECC is controlled by several signaling pathways, including protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Among the alterations involved in CH and HF are changes in the expression levels of these regulatory proteins that act as sensors of intracellular Ca^{2+}.[17] In HF, increased activity and expression of CaMKII has been demonstrated, which plays a role in decreased contractile properties of the myocardium, uncoupling ECC and possibly leading to sudden death.[18] Several studies have shown dilated cardiomyopathy, CH and HF development in models of CaMKII overexpression,[5,19] and protective effects against HF development in CaMKII knockout animals.[7,20] The alterations include decreased levels of SERCA2a with a subsequent decrease of the recapture of Ca^{2+} to the SR during diastole, resulting in a smaller amount of Ca^{2+} available to be released in the following heartbeat.

Closely related to SERCA2a is phospholamban (PLN), a 52 amino acid peptide that is the primary regulator of SERCA2a activity. PLN inhibits the pump associating with SERCA2a, and dissociates from the pump when it is phosphorylated by CaMKII or PKA in response to β-adrenergic stimulation, resulting in increased Ca^{2+} transport velocity by SERCA2a.[21,22] In the failing heart, the SERCA2a/PLN ratio is decreased to a great extent by the reduction of SERCA2a pumps present in the SR membrane. This reduction causes the amount of active SERCA2a to decrease, and therefore decreases its activity and capacity to recapture the Ca^{2+} released during contraction.[23]

PLN assembling in pentamers is necessary for fully active SERCA2a inhibition, and equilibrium between phosphorylated (unbound) and dephosphorylated (bound) PLN forms is classically thought to determine SERCA2a activity.[24] Although, recent findings[25,26] suggest that SERCA activation can occur without dissociation of the SERCA/PLN complex, the decrease in phosphorylated PLN levels causes the SERCA2a activity to decrease. During HF, PLN is

hypophosphorylated, which causes a chronic decrease in SERCA2a activity.[27] Therefore, reduced SERCA2a activity is not only due to a smaller expression of SERCA2a protein, but also to alterations in the PLN phosphorylation levels as well. Because of increased PKA activity, the inhibitor-1 of the protein phosphatase-1 (PP1) is phosphorylated, releasing PP1 inactivation and resulting in PLN dephosphorylation by PP1 with the consequent activation of PLN.[28,29] The SR Ca^{2+} release channel of the SR (RyR2) has been reported in an unstable state when it is dissociated from the protein calstabin, resulting in an increased probability of channel opening. This results in spontaneous Ca^{2+} liberations that appear during diastole (diastolic leak) and are associated with ventricular arrhythmias and a decrease in the SR Ca^{2+} reserve available for the next contraction–relaxation cycle.[3,30]

CaMKII shares common functional targets with PKA regulating ECC. PLN and RyR2 are phosphorylated by PKA and CaMKII, resulting in an increased SR Ca^{2+} release. The increased CaMKII activity in HF contributes to the reduced SR calcium content and systolic function, and also causes diastolic SR Ca^{2+} leak and Ca^{2+} current changes that may be arrhythmogenic. One of the main causes of sudden death in patients with HF is ventricular arrhythmia. Abnormal RyR2 Ca^{2+} release from the SR has been linked to arrhythmogenesis; in fact, there is evidence for RyR2 hyperphosphorylation due to PKA and the involvement of phosphodiesterase D in this process.[31,32] Recent reports suggest that CaMKII-dependent phosphorylation of RyR2 is involved in enhanced SR diastolic Ca^{2+} leak and also reduced SR Ca^{2+} load in HF, contributing to arrhythmias and contractile dysfunction.[33] RyR2 phosphorylation by CaMKII at serine 2814 plays a dominant role in arrhythmogenesis and sudden cardiac death in mice with HF. This fact suggests that a destabilization of RyR2 activity due to increased CaMKII phosphorylation results in reduced post-release refractoriness, which is a mechanism involved in arrhythmogenesis and dysfunction in contractility of the failing heart.

Cardiac pressure overload, as is seen in hypertensive patients, is the major pathologic stimulus for CH and is the main risk factor for HF development. There are several reports demonstrating that in human pressure overload hypertrophic and failing hearts, as well in CH animal models, there is a marked decrease in SERCA2a content (~50%) of cardiomyocytes.[3,34] Since SERCA2a activity is essential for Ca^{2+} handling and this is altered as a consequence of CH and HF development, efforts to investigate calcium-dependent signaling pathways in this context have been conducted. The best-known two calcium-dependent signaling pathways in cardiomyocytes are the calcineurin/nuclear factor of activated T-cells (NFAT) pathway and the CaMK/myocyte enhancer factor-2 pathway. Both pathways participate in normal heart development and are altered in CH, but the cross-talk between these pathways is still not fully understood.[35] One of the common features of CH and HF is downregulation of *SERCA2* gene expression, leading to alterations in Ca^{2+} signaling pathways. Ca^{2+} signaling fluctuations and contractile function alterations have been demonstrated following specific inhibition of SERCA2a transport activity with thapsigargin, reduction of *SERCA2* gene expression by a gene null mutation, and *SERCA2* gene silencing with small interfering RNA.[36] *SERCA2* gene direct transcriptional regulation by NFAT activity has been proposed,[37] although the precise molecular mechanisms have not been yet described. Whether NFAT and/or myocyte enhancer factor-2 are involved directly in *SERCA2* gene transcriptional regulation is currently under investigation in the authors' laboratory. The above data highlight the importance of SERCA2 gene expression in the physiopathology of cardiac diseases.

SERCA2a as a therapeutic target

Pharmacological therapies

The current most widely used drugs to treat HF patients include angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, adrenergic β-blockers, digoxin or digitalis, diuretics, aldosterone antagonists, and inotropes. As a multifactorial clinical syndrome, HF still represents an epidemic threat; therefore, diverse strategies and molecular targets are being investigated. SERCA2a, as a key calcium handling protein, has received special attention in the last 20 years. The following section reviews current literature knowledge positioning SERCA2a as a therapeutic target in the treatment of cardiac diseases. Several drugs that have been recently investigated for their effect on SERCA2a activity and expression are shown in Table 1.

Istaroxime is a luso-inotropic compound that activates SERCA2a and inhibits the sodium/potassium ATPase.[38,39] Due to its double action, istaroxime has been proved to treat acute HF syndrome in guinea pigs and dogs, improving systolic and diastolic function, central hemodynamics, systolic blood pressure, and reducing heart rate.[40–42] Istaroxime has also been used in human patients; the Hemodynamic, Echocardiographic, and Neurohormonal Effects of Istaroxime, a Novel Intravenous Inotropic and Lusitropic Agent: a Randomized Controlled Trial in Patients Hospitalized with Heart Failure (HORIZON-HF) trial showed that in addition to its beneficial effects on heart

Table 1 Potential drug therapies targeting SERCA2a for treatment of heart failure

Post-translational modification, protein, or drug	Activator or inhibitor	Sites in the protein or mechanism of action	References
Istaroxime	Activator	Favoring E1 SERCA2a conformation	38–47
Carvedilol	Activator	Increased Sp1 binding to SERCA2 promoter	48
Oxymatrine	Activator	Possibly through TNF-α inhibition or Sp1 induction	49–56
Acetylation	Unknown	K464, K510, and K533; possibly through SIRT1 or other deacetylases	78,81,83–85
Nitration	Inhibitor	Tyr294, Tyr295, and Tyr753	93–97
S-glutathiolation	Activator	Cys674	98
Egr-1	Inhibitor	Transcriptional repression	102,103,105
O-GlcNAcylation	Inhibitor	Sp1 and PLN modification	119,120,122,123

Abbreviations: Cys, cysteine; Egr-1, early growth response protein-1; PLN, phospholamban; SERCA2a, sarco/endoplasmic reticulum Ca^{2+} adenosine triphosphatase-2a; SIRT1, sirtuin-1; Sp1, specificity protein-1; TNF-α, tumor-necrosis factor-α; Tyr, tyrosine; O-GlcNAcylation, β-O-linkage of N-acetylglucosamine.

function, istaroxime produces few side effects since there were no neurohormonal or renal changes and no troponin I release, and adverse effects were not life-threatening and only observed at the highest dose.[43] Istaroxime effect on SERCA2a is isoform specific since no effect of the drug was observed on SERCA1 from skeletal muscle.[40] Recently, the mechanism of istaroxime-driven SERCA2a activation has been addressed and it was observed that this effect is dependent on PLN presence. The SERCA2a/PLN complex was found to dissociate with increasing nanomolar istaroxime concentrations in a similar way to that observed with increasing Ca^{2+} concentrations. Furthermore, SERCA2a activation was shown to be cyclic adenosine monophosphate (cAMP)/PKA independent since the stimulation was persistent using the PKA inhibitor staurosporine.[44] As istaroxime is the only small molecule able to activate SERCA2a activity, and there are some concerns about its specificity, studies have been carried out to design new lusitropic/inotropic compounds with higher effectiveness and lower toxicity. Searching for these molecules has yielded istaroxime analogs with better activity, and even structure–activity relationships have been found.[45,46] Further, a high-throughput fluorescence resonance energy transfer assay has been used to find allosteric SERCA2a activators capable of decreasing SERCA2a/PLN-derived fluorescence resonance energy transfer.[47] In this study, several compounds previously reported to correct aberrant Ca^{2+} regulation in HF were shown to activate SERCA2a activity, thus validating the mechanism of action for these drugs.

Carvedilol is a β-adrenergic receptor blocker that elicits its beneficial effects on cardiac function through the restoration of SERCA2a expression in patients with HF. Furthermore, carvedilol prevents oxidative stress-mediated *SERCA2* gene downregulation by promoting specificity protein-1 (Sp1) and Sp3 binding to elements located in the proximal promoter, increasing transcription of the gene by this mechanism.[48]

Among the current therapies to prevent heart disease, traditional medicine has played an especially important role in the People's Republic of China where herbal medicine is very diverse. Oxymatrine (OMT) is an alkaloid obtained from the roots of the Chinese herb *Sophora japonica (Sophora falvescens* Ait), also known as ku shen. It has anti-inflammatory, antiviral, immune reaction inhibiting, and anti-hepatic fibrosis protective activities. A few years ago the properties of OMT in treating myocardial infarction in rats were studied, and it was found that a dose of 20 mg/kg reduced infarct size and inhibited lipid peroxidation. Beneficial effects possibly were due to increased endogenous superoxide dismutase activity, increased B-cell lymphoma-2 expression, and a reduction of the apoptotic mediator Fas and $[Ca^{2+}]_c$ overload.[49] OMT has also been reported to exert antiarrhythmic effects in a rat model of arrhythmia induced by coronary ligation.[50] In this study, OMT reduced the action potential duration of cardiomyocytes through reduction of L-type calcium current, enhancement of transient outward potassium current, and inhibition of inward rectifier potassium current. Also, in rats with acute myocardial infarction, this alkaloid was shown to improve hemodynamic parameters and the left ventricle weight/body weight ratio, and to decrease expression of transforming growth factor-β receptor-1 and Smad3 messenger RNAs.[51] OMT downregulated the angiotensin-converting enzyme messenger RNA expression and extracellular signal-regulated kinase-1/2, c-Jun N-terminal kinase, and p38 phosphorylation in spontaneous hypertensive rats to levels comparable to those obtained with captopril, a widely used drug to treat HF.[52] OMT has been shown to inhibit Janus kinase/signal transducer and activator of transcription protein signaling

pathway activation and expression of interleukin-1β and tumor necrosis factor-α;[53] the latter being a cytokine that has been associated with SERCA2a downregulation.[54] Since the reported effects for OMT include regulation of ionic currents, calcium handling was assessed in a rat model of chronic HF. The Ca^{2+} transients and SR Ca^{2+} content were improved by 50 and 100 mg/kg of OMT along with the upregulation of SERCA2a and dihydropyridine receptor expression.[55] Furthermore, OMT was shown to induce the Sp1 transcription factor expression in human embryonic kidney 293 cells.[56] Taken together, the OMT beneficial effects on cardiac tissue seem to be related to the activation of SERCA2a expression through tumor necrosis factor-α inhibition or Sp1 increased expression. These findings encourage further investigation about OMT and other natural products that could serve as SERCA2a direct or indirect activators.

Gene therapy to increase SERCA2a levels

The goal of cardiac gene therapy is to reverse some of the molecular alterations in the failing heart. An obvious approach to restore the SERCA2a protein deficiency caused by HF is to provide more SERCA2a protein. Despite barriers and risks associated with gene therapy, SERCA2a overexpression use for HF shows promising results.[66] The first attempts to restore SERCA2a through gene therapy were done using adenoviruses,[57,58] since previous studies had shown feasibility for SERCA2a in vivo overexpression.[59,60] Lentiviral vectors are also an alternative for gene delivery because they integrate their DNA into host chromosomes, providing a long-term expression of the delivered gene. SERCA2a complementary DNA delivery by a lentiviral vector improved heart function in rat hearts subjected to myocardial infarction.[61] Recombinant adeno-associated viruses (AAV) were used in the first gene therapy clinical trial for HF called Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID).[62,63,64] The second phase of this trial used antegrade percutaneous intracoronary artery infusion of the AAV vector AAV1/SERCA2a (MYDICAR®, Celladon Corporation, San Diego, CA, USA), in 39 patients. It began in 2007 and was designed to evaluate the safety profile and biological effects of SERCA2a gene transfer.[64–66] After a 3-year follow-up, patients from the CUPID trial showed an acceptable safety profile and improvement in heart function.[66] In the trial it was not found to increase adverse effects, laboratory abnormalities, disease-related events, or arrhythmias. The study concluded that after a single intracoronary infusion of AAV1/SERCA2a in patients with advanced HF, the beneficial cardiovascular effects last for several years.

Since the CUPID clinical trial showed promising good results, a clinical trial called CUPID2,[67] enrolling 250 patients with HF, has just started in the Cardiovascular Biomedical Research Unit at the Royal Brompton Hospital of England and results will be available in 2015. There are two ongoing gene therapy clinical trials to express SERCA2a in the hearts of patients with HF using AAV1/SERCA2a – one in England and one in France.[68] Lastly, another recent trial, named Investigation of the Safety and Feasibility of SERCA Gene Transfer in the Human Failing Heart Using an Adeno-associated Viral Vector, (SERCA/LVAD), started recruitment of patients in the UK in the summer of 2013.[69] This trial will test *SERCA2* gene therapy in 16 HF patients already fitted with mechanical heart pumps, also known as left ventricular assist devices. These trials will collect information about the effectiveness of the therapy by measuring the amount of the *SERCA2* gene and protein that has been introduced into heart muscle (Table 2). Although the above mentioned trials are promising, boosting SERCA2a function has been shown to be counterproductive in the setting of dysregulated RyR2 function in mice.[70]

Table 2 *SERCA2* gene therapy clinical trials for heart failure

Trial	Diseases	Route/vector	Stage	Number of patients
CUPID[62–66,*]	Heart failure, congestive dilated cardiomyopathy	Intracoronary/AAV1-SERCA2a (MYDICAR®)	Phase II, completed	51
AGENT-HF[68,*]	Heart failure, ischemic, and nonischemic	Intracoronary/AAV1-SERCA2a	Phase II, enrolling	44
SERCA2 Gene Therapy in LVAD patients[69,*]	Advanced heart failure with LVAD, ischemic, and nonischemic	Intracoronary/AAV6-SERCA2a	Phase II, enrolling	16
CUPID2[67,*]	Heart failure, ischemic, and nonischemic	Intracoronary/AAV1-SERCA2a (MYDICAR®)	Phase IIb, enrolling	250

Note: *From ClinicalTrials.gov. MYDICAR®, Celladon Corporation, San Diego, CA, USA.

Abbreviations: AAV, adeno-associated virus; AGENT-HF, AAV1–cytomegalovirus–SERCA2a Gene Therapy Trial in Heart Failure; CUPID, Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease; LVAD, left ventricular assist device; SERCA2a, sarco/endoplasmic reticulum Ca^{2+} adenosine triphosphatase-2a.

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder characterized by mutations in the dystrophin gene. The vast majority of dystrophin gene mutations lead to loss of dystrophin protein and as a consequence smooth, skeletal, and cardiac muscles of DMD patients show myofiber damage and membrane leakage. Although DMD patients usually die from respiratory failure before their 20s, many men show abnormal electrocardiograms by the age of 18 years. This fact, and the absence of an effective therapy to correct the absence of dystrophin, raises the need for a therapy to correct performance in smooth and cardiac muscle.[71] SERCA2a overexpression has been explored to treat DMD heart disease.[72] SERCA2a was overexpressed in old female mdx mice, a murine model of DMD cardiomyopathy, finding that the characteristic tachycardia was corrected by the overexpression of SERCA2a. SERCA2a overexpression has also been used to treat skeletal muscle dystrophy. The δ-sarcoglycan-null mice and dystrophin mutant mdx mice subjected to skeletal muscle-specific overexpression of SERCA1 showed dramatically reduced myofiber central nucleation, fibrosis, and serum creatine kinase levels compared with non-treated animals. The loss of exercise capacity and dystrophic phenotype in the gastrocnemius muscle of δ-sarcoglycan-null mice were also rescued. Furthermore, SERCA1 overexpression reduced total cytosolic Ca^{2+}, reversed mitochondria swelling, and reduced calpain activation, suggesting protection from Ca^{2+}-driven necrosis.[73]

Post-translational SERCA2a modifications as possible therapeutic targets

SERCA2a function in the normal heart is modulated by different means, such as PLN, S100A1, and sarcoglycan interaction, Small ubiquitin-like modifier-1 addition to serine or threonine residues (SUMOylation), glutathiolation, and nitration.[74,75] Some of these modifications are presented in Table 3 as possible unexplored therapeutic targets that are under investigation. Increasing SERCA2a activity in cardiomyocytes can be achieved by diminishing the SERCA2a/PLN interaction. Using fluorescent fusion proteins in human embryonic kidney cells and measuring SERCA/PLN interaction by fluorescence resonance energy transfer, PLN mutants with no SERCA inhibitory function competed with wild type for SERCA binding and led to increased SERCA activity.[76] Furthermore, it was shown that phosphorylation of PLN induced by forskolin increased SERCA2a/PLN interaction, suggesting that SERCA2 inhibition can be relieved without dissociation of the complex. This strategy raises the possibility of using PLN mutants for gene therapy in heart diseases.

Table 3 Possible gene therapies to modify SERCA2a activity or expression

Gene delivered	Mechanism of action	References
SERCA2a	Direct SERCA2a upregulation	57–69
Mutant PLN	Interference with wild type PLN-mediated inhibition	76
SUMO-1	Increased SERCA2a activity	78
miR-1	ET-1 repression and MAPK inhibition	125–126
S100A1	Increased SERCA2a activity	130–132

Abbreviations: ET-1, endothelin-1; MAPK, mitogen-activated protein kinase; PLN, phospholamban; SERCA2a, sarco/endoplasmic reticulum Ca^{2+} adenosine triphosphatase-2a; SUMO, Small ubiquitin-like modifier-1; miR-1, micro RNA-1.

Since transgenic mice overexpressing CaMKIIδ have increased RyR2 phosphorylation, enhanced SR Ca^{2+} leak, and lowered SR Ca^{2+} load, PLN ablation was explored as an alternative to rescue SERCA2 function, ie, SR Ca^{2+} handling in this animal model.[77] Although double mutant mice showed normalized SR Ca^{2+} loading, SR Ca^{2+} leak and mitochondrial Ca^{2+} were enhanced, compromising mice survival. Similarly, in the study by Kalyanasundaram et al[70] mentioned in the previous section, the overexpression of SERCA1a in a transgenic model that interferes with RyR2 function was counterproductive. These two studies suggest that increasing SERCA2a function for HF treatment should be used with caution in the setting of dysregulated RyR2 function.

As stated before, a series of post-translational modifications can alter SERCA2a function. SUMOylation is a modification occurring at lysine (Lys) residues of target proteins. It was shown that SERCA2a SUMOylation occurring at Lys480 and Lys585 is essential for the correct ATPase activity and stability of the pump in mouse and human cells.[78] Small ubiquitin-like modifier-1 (SUMO-1) and SUMOylated SERCA2a levels are reduced in mouse failing hearts and AAV-mediated gene delivery of SUMO-1 was able to restore cardiac function and SERCA2a protein abundance. Further, SUMO-1 downregulation led to deterioration of cardiac function and decreased SERCA2a function. Although in this study SERCA2a knockdown was not rescued by SUMO-1 overexpression, SERCA2a SUMOylation could serve as a basis for the design of new therapies for HF.

Nkx2.5 is a homeobox domain transcription factor essential for heart specification and morphogenesis that has also been shown to be SUMOylated.[79] SUMO-1 overexpression in the spontaneously beating HL-1 cell line strongly enhanced Nkx2.5 transcriptional activity and promoted the SUMOylation of undetermined Nkx2.5 cofactors. These data support the role of SUMO-1 as a potential therapy, not only as a SERCA2a activator but also as a complex transcriptional

remodeling factor for HF. Lys residues in proteins are as important for SUMOylation as they are for acetylation. Acetylation has been widely reported in histones as an epigenetic mechanism for gene expression control. Recently, acetylation has been investigated as a more extensive post-translational modification in the whole proteome and its role in cancer is a currently addressed topic.[80] In this context, acetylation of cardiac proteins has already been reported in the guinea pig acetylome.[81] Particularly, SERCA2a can be acetylated in Lys464, Lys510, and Lys533. These three sites lie on the surface of the ATP binding domain, and whether their acetylation affects ATP binding to the pump has not been reported. Sirtuin-1 is a cardioprotective nicotinamide adenine dinucleotide-dependent histone deacetylase[82] that has been related to the beneficial effects of resveratrol on cardiac function.[83,84] Resveratrol has been shown to rescue SERCA2a expression in the setting of diabetic cardiomyopathy[83] and histone deacetylases have been found in sarcomeres.[85] Thus, these findings raise the question of whether SERCA2a acetylation may be a prominent modification that would compete or interfere with SERCA2a SUMOylation as proposed by Kho et al,[78] and therefore could be of therapeutic interest.

Obesity, diabetes, and hypertension are health problems that have alarmingly grown over the last two decades in the developing world and are major predisposing factors to the development of cardiovascular disease.[86] High-fat-fed rats develop obesity that mimics human Western health problems. It has been found that a decreased level of phosphorylated PLN is clearly linked with cardiovascular problems present in this animal model.[87,88] Exercise training and diet-induced weight loss have been shown to exert beneficial effects on cardiac performance for both animal HF models and human HF patients.[89–92] However, rescued expression of SERCA2a and other calcium handling proteins is not complete, and hence more effective therapies are needed.

A characteristic feature of aging, obesity, and diabetes is oxidative and nitrative stress that alters many protein functions, including SERCA2a activity.[93–95] Increased SERCA2a nitration has been shown in human failing hearts.[96] This modification has been correlated with increased time to half relaxation in myocytes isolated from control and idiopathic dilated cardiomyopathy hearts. This study also showed in vesicles from porcine hearts that SERCA2a was inactivated by peroxynitrite ($ONOO^-$) exposure and that this inactivation was prevented by PKA pre-treatment. A 60% decrease in SERCA2a activity in the senescent Fischer-344 rat heart relative to that of young adult rat hearts has been reported.[97] Although 18% of this decrease was attributed to lower SERCA2a protein abundance, the rest was shown to be due to 3-nitrotyrosine modification of the pump. It was found that there was a more than two-fold increase in age-dependent nitration of SERCA2a and that this nitration was distributed over tyrosine 122 (Tyr122), Tyr130, Tyr497, Tyr586, and Tyr990 in young rat hearts in contrast with senescent rat hearts, where Tyr294, Tyr 295, and Tyr753 were additionally found to be nitrated. Skeletal muscle was also analyzed in this study and full site (1 mol/mol) of Tyr753 nitration was found in young hearts versus additional nitration of Tyr294 and Tyr295 in senescent muscle. The appearance of these latter two nitrotyrosines correlated with diminished ATP utilization by the SERCA2a pump in both skeletal and cardiac muscle under nitrative stress.

Nitric oxide (NO) is one of the signals leading to relaxation in cardiac, skeletal, and smooth muscle, and it performs this function through SERCA2a activation. Using purified protein reconstituted in phospholipid vesicles, it was shown that NO-derived $ONOO^-$ directly activated SERCA2a by S-glutathiolation and that this modification was blocked by irreversible oxidation of relevant cysteine thiols. As mutation of SERCA cysteine 674 to serine abolished the pump activity dependence on glutathione and superoxide scavengers decreased S-glutathiolation of SERCA and relaxation by NO, $ONOO^-$ was proposed as the mediator. Additionally, atherosclerosis was found to decrease NO-dependent relaxation as well as the S-glutathiolation of SERCA with its consequent activation. Furthermore, a high content of SERCA2a cysteine 674 irreversibly oxidized to sulfonic acid in atherosclerotic aorta was found, confirming a key role for this modification in the diseased tissue.[98]

Prostaglandin-F2 (PGF2) is a vasoconstrictor involved in inflammatory response that is produced in the myocardium subsequent to infarction or pressure overload.[99–101] It was shown that PGF2 stimulates hypertrophic growth of neonatal rat cardiac myocytes, including downregulation of SERCA2a. Interestingly, PGF2 increased early growth response protein 1 (Egr-1) expression and overexpression of Egr-1 decreased transcription of the proximal SERCA2 gene promoter. PGF2 was shown to exert repressive effects on SERCA2 transcription through Egr-1 transcription factor in a process mediated by mitogen-activated protein kinase (MAPK)/p38, Ras, and Rac but not extracellular signal-regulated kinase.[102] The MAPK-activated protein kinases 2 and 3 (MK2/3) are known regulators of SERCA2a expression,[103] and important players in the cardiac hypertrophic response.[104] Recently, it was reported that SERCA2 gene expression was upregulated in MK2/3 double-knockout

mice and that this regulation was due to a decreased ratio of transcription factor Egr-1/Sp1.[105] It was found that SERCA2 gene promoter activity was directly dependent on MK2 catalytic activity. Also, the MK2/3 double-knockout mice showed enhanced expression of the peroxisome proliferator-activated receptor-γ co-activator-1α gene, which has been shown to be decreased in HF[106] and restored after losartan treatment accompanied by exercise training.[107] The above data show that Egr-1 transcription factor and its trigger MAPK proteins are important SERCA2a regulators that could potentially be used as therapeutic targets in cardiac diseases alone or combined with other approaches.

O-GlcNAcylation is a modification that has been recently found to play an important role in CH. The β-O-linkage of N-acetylglucosamine (O-GlcNAc) is controlled by two enzymes – the uridine diphospho-N-acetylglucosamine: peptide β-N-acetylglucosaminyltransferase (O-GlcNAc transferase [OGT]) and the O-β-N-acetylglucosaminidase (O-GlcNAcase) in mammalian cells.[108] Increased O-GlcNAcylation has been reported in many cardiac dysfunctions,[109] but it has also been shown that in vivo ischemic preconditioning increases O-GlcNAc.[110] In the latter study, pharmacological augmentation of O-GlcNAc levels was sufficient to reduce myocardial infarct size in mice subjected to acute myocardial infarct. On the other hand, OGT overexpression was reported to reduce post-hypoxic damage in mouse cardiac myocytes and OGT silencing or inhibition to sensitize the formation of mitochondrial permeability transition pore induced by calcium.[111,112] O-GlcNAcylation has also been investigated in vascular smooth muscle cells and it was found that increased O-GlcNAc levels mediated by endothelin-1 were abolished by OGT silencing.[113] It was reported that pressure overload-induced CH (via transverse aortic constriction) in mice or treatment of neonatal rat cardiac myocytes with phenylephrine increased total O-GlcNAc levels that mirrored the enhanced activated NFAT signaling.[114]

Among the causes provoking left ventricular dysfunction, the dysregulation of energetic balance plays a substantial role due to the highly energy-consuming process that contraction–relaxation cycling represents. For example, mice with cardiomyocyte-restricted knockout of the insulin receptor show increased mitochondrial dysfunction following myocardial infarction when compared with wild type littermates and this impairment is accompanied by a decrease in several proteins including SERCA2a, peroxisome proliferator-activated receptor-α, and glucose transporter type-4.[115] Glucose transporter type-4 knockout mice exhibit deficient contractile performance, CH, fibrosis, and reduced expression of RyR2 and SERCA2a proteins.[116] Sp1 is a key transcriptional regulator of SERCA2 gene expression in the normal and hypertrophic heart;[117,118] its transcriptional activity is decreased by O-GlcNAcylation.[119] High O-GlcNAcylation levels in streptozotocin-mediated diabetic rats have been related to deleterious effects of diabetic cardiomyopathy. In this model, the overexpression of O-GlcNAcase diminished global O-GlcNAcylation level and also diminished SERCA2a and PLN expression, but increased phosphorylated PLN.[120] Since one of the main therapies in the setting of diabetes is exercise training, its effects on O-GlcNAcylation interactions with transcriptional co-repressors were studied in a type-2 diabetes mouse model.[121] Paradoxically, it was shown that exercise increased O-GlcNAc level. Diabetic hearts showed higher levels of histone deacetylase-1 and -2 activity and lower levels of mammalian switch-independent-3A. In sedentary diabetic hearts, mammalian switch-independent-3A and OGT were shown to be less associated with histone deacetylase-1 and -2, respectively, compared with controls, but exercise removed these differences. In fact, derived from Sp1 O-GlcNAcylation, SERCA2 gene transcriptional repression was also reported in cardiomyocytes exposed to high glucose.[122] Moreover, high glucose treatment in cardiomyocytes was recently shown to promote PLN O-GlcNAcylation and it was proposed that this modification could improve PLN-mediated SERCA2a inhibition.[123] The Nkx2.5 transcription factor is also downregulated in the heart tissue of streptozotocin-induced diabetic mice.[124] Thus, the above data provide a possible mechanism that explains decreased SERCA2a function and expression in diabetic cardiomyopathy.

Other gene therapies regulating SERCA2a function and expression

As the cardiomyocyte physiology and the SERCA2a interactions are complex, other targets for gene therapy have been tested to override deleterious effects produced by CH (Table 3). Some of them ultimately impact SERCA2a function or expression, highlighting the importance of correct calcium handling in the cardiomyocyte.

Recently, miR-1, a cardiomyocyte-restricted micro RNA, was found to post-transcriptionally directly inhibit the expression of calmodulin and myocyte enhancer factor-2a, and indirectly the expression of GATA4, suggesting that miR-1 is a central regulator of calcium signaling in CH.[125] As miR-1 has been shown to inhibit endothelin-1 expression,[126] low levels of miR-1 would result in low SERCA2a levels and hence in a poor cardiac performance. A recent publication

showed that AAV-mediated restoration of miR-1 expression in male Sprague Dawley rats subjected to ascending aortic stenosis ameliorated adverse effects derived from CH such as fibrosis, MAPK activation, and SERCA2a downregulation.[127] The above results show a regulation of SERCA2a expression by miR-1.

As stated above, the phosphorylated/dephosphorylated ratio of PLN derived from phosphorylation by CaMKII or PKA or dephosphorylation by PP1a delimits SERCA2a pump activity. Hajjar et al showed that not only PLN phosphorylation status modifies SERCA2a activity since recombinant adenovirus-mediated PLN overexpression (Ad.RSV.PL) led to a significant prolongation of the relaxation phase, a decrease in peak $[Ca^{2+}]_c$, and an elevation in resting $[Ca^{2+}]_c$ in membrane preparations from cardiomyocytes. Ad.RSV.PL also prolonged the time course of shortening in myocytes, but simultaneous SERCA2a overexpression partially reverted these effects.[128]

S100A1 is an EF-hand calcium-binding sensor protein that has been shown to interact with SERCA2a and activate it, among other functions like regulating RyR2, titin, and mitochondrial ATPase function.[129] Due to these functions, S100A1 gene therapy has recently been explored in small and large animal models for HF.[130,131] In both rat and domestic pig models, S100A1 transgene expression normalized cardiomyocyte Ca^{2+} cycling and SR calcium handling, and energy homeostasis resulted from cryoinfarction in rats or balloon occlusion of the left circumflex coronary artery in domestic pigs.[132]

Conclusion

Cardiovascular diseases are the main cause of morbidity and mortality in the world. Decreased SERCA2a content in the SR of patients with failing hearts is responsible for its decreased contractility. The current goal of the cardiovascular research is to fight back against HF by targeting and reversing some of the critical molecular changes arising in the heart when it fails. During the last decade, several researchers have focused their efforts on trying to correct SERCA2a expression in the hypertrophic and failing heart. Various pharmacologic and genetic approaches aimed to correct SERCA2a expression have been recently explored, shedding new light on the molecular mechanisms responsible for SERCA2a expression and activity. A growing number of approved gene therapy clinical trials have been designed to increase SERCA2a expression in the myocardium of patients with HF. The results published show long lasting results improving cardiac function after a single intracoronary administration of AAV carrying the SERCA2a complementary DNA. The biosafety and efficacy of AAV/SERCA2a vectors has been demonstrated for the treatment of patients with HF, opening the field for testing new targets to modulate cardiac gene expression with more advanced vector systems for cardiovascular gene therapy in the near future.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Bers DM. *Excitation – Contraction Coupling and Cardiac Contractile Force*. 2nd ed. Boston, MA: Kluwer Academic Publishers; 2001.
2. Bers DM. Cardiac excitation – contraction coupling. *Nature*. 2002; 415(6868):198–205.
3. Bers DM. Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol*. 2008;70:23–49.
4. Yano K, Zarain-Herzberg A. Sarcoplasmic reticulum calsequestrins: structural and functional properties. *Mol Cell Biochem*. 1994;135(1): 61–70.
5. Beard NA, Laver DR, Dulhunty AF. Calsequestrin and the calcium release channel of skeletal and cardiac muscle. *Prog Biophys Mol Biol*. 2004;85(1):33–69.
6. Beard NA, Wei L, Dulhunty AF. Control of muscle ryanodine receptor calcium release channels by proteins in the sarcoplasmic reticulum lumen. *Clin Exp Pharmacol Physiol*. 2009;36(3):340–345.
7. Bernardo BC, Weeks KL, Pretorius L, McMullen JR. Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. *Pharmacol Ther*. 2010;128(1):191–227.
8. Anwar A, Taimor G, Korkusuz H, et al. PKC-independent signal transduction pathways increase SERCA2 expression in adult rat cardiomyocytes. *J Mol Cell Cardiol*. 2005;39(6):911–919.
9. Arai M, Suzuki T, Nagai R. Sarcoplasmic reticulum genes are upregulated in mild cardiac hypertrophy but downregulated in severe cardiac hypertrophy induced by pressure overload. *J Mol Cell Cardiol*. 1996;28(8):1583–1590.
10. Reyes-Juarez JL, Zarain-Herzberg A. El retículo sarcoplásmico, su función y su papel en las enfermedades cardiacas. [Function and role of the sarcoplasmic reticulum in heart disease]. *Arch Cardiol Mex*. 2006; 76(Suppl 4):S18–S32. Spanish.
11. Alpert NR, Brosseau C, Federico A, Krenz M, Robbins J, Warshaw DM. Molecular mechanics of mouse cardiac myosin isoforms. *Am J Physiol*. 2002;283(4):H1446–H1454.
12. Vannier C, Chevassus H, Vassort G. Ca-dependence of isometric force kinetics in single skinned ventricular cardiomyocytes from rats. *Cardiovasc Res*. 1996;32(3):580–586.
13. Baker AJ, Figueredo VM, Keung EC, Camacho SA. Ca2+ regulates the kinetics of tension development in intact cardiac muscle. *Am J Physiol*. 1998;275(3 Pt 2):H744–H750.
14. Palmer S, Kentish JC. Roles of Ca2+ and crossbridge kinetics in determining the maximum rates of Ca2+ activation and relaxation in rat and guinea pig skinned trabeculae. *Circ Res*. 1998;83(2): 179–186.
15. Teucher N, Prestle J, Seidler T, et al. Excessive sarcoplasmic/endoplasmic reticulum Ca2+-ATPase expression causes increased sarcoplasmic reticulum Ca2+ uptake but decreases myocyte shortening. *Circulation*. 2004;110(23):3553–3559.

16. Bassani JW, Qi M, Samarel AM, Bers DM. Contractile arrest increases sarcoplasmic reticulum calcium uptake and SERCA2 gene expression in cultured neonatal rat heart cells. *Circ Res*. 1994;74(5): 991–997.
17. Kaprielian R, del Monte F, Hajjar RJ. Targeting Ca2+ cycling proteins and the action potential in heart failure by gene transfer. *Basic Res Cardiol*. 2002;97(Suppl 1):I136–I145.
18. Soltis AR, Saucerman JJ. Synergy between CaMKII substrates and β-adrenergic signaling in regulation of cardiac myocyte Ca(2+) handling. *Biophys J*. 2010;99(7):2038–2047.
19. Zhang T, Johnson EN, Gu Y, et al. The cardiac-specific nuclear delta(B) isoform of Ca2+/calmodulin-dependent protein kinase II induces hypertrophy and dilated cardiomyopathy associated with increased protein phosphatase 2A activity. *J Biol Chem*. 2002;277(2):1261–1267.
20. Backs J, Backs T, Neef S, et al. The delta isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload. *Proc Natl Acad Sci U S A*. 2009;106(7): 2342–2347.
21. Tada M, Kirchberger MA, Repke DI, Katz AM. The stimulation of calcium transport in cardiac sarcoplasmic reticulum by adenosine 3′:5′-monophosphate-dependent protein kinase. *J Biol Chem*. 1974;249(19): 6174–6180.
22. Katz S, Richter B, Eibschutz B. Characterization of calmodulin-dependent and cyclic-AMP-dependent protein kinase stimulation of cardiac sarcoplasmic reticulum calcium transport. *Adv Myocardiol*. 1985;6:233–247.
23. Hoshijima M, Knoll R, Pashmforoush M, Chien K. Reversal of calcium cycling defects in advanced heart failure toward molecular therapy. *J Am Coll Cardiol*. 2006;48(9 Suppl 1):A15–A23.
24. Chu G, Li L, Sato Y, et al. Pentameric assembly of phospholamban facilitates inhibition of cardiac function in vivo. *J Biol Chem*. 1998;273(50):33674–33680.
25. Negash S, Yao Q, Sun H, Li J, Bigelow DJ, Squier TC. Phospholamban remains associated with the Ca2+- and Mg2+-dependent ATPase following phosphorylation by cAMP-dependent protein kinase. *Biochem J*. 2000;351(Pt 1):195–205.
26. Bidwell P, Blackwell DJ, Hou Z, Zima AV, Robia SL. Phospholamban binds with differential affinity to calcium pump conformers. *J Biol Chem*. 2011;286(40):35044–35050.
27. Reiken S, Gaburjakova M, Guatimosim S, et al. Protein kinase A phosphorylation of the cardiac calcium release channel (ryanodine receptor) in normal and failing hearts. Role of phosphatases and response to isoproterenol. *J Biol Chem*. 2003;278(1):444–453.
28. Huang FL, Glinsmann WH. Separation and characterization of two phosphorylase phosphatase inhibitors from rabbit skeletal muscle. *Eur J Biochem*. 1976;70(2):419–426.
29. Nimmo GA, Cohen P. The regulation of glycogen metabolism. Purification and characterisation of protein phosphatase inhibitor-1 from rabbit skeletal muscle. *Eur J Biochem*. 1978;87(2):341–351.
30. Braunwald E, Bristow MR. Congestive heart failure: 50 years of progress. *Circulation*. 2000;102(Suppl 4):IV14–IV23.
31. Lehnart SE, Wehrens XH, Reiken S, et al. Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. *Cell*. 2005;123(1):25–35.
32. Marx SO, Reiken S, Hisamatsu Y, et al. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell*. 2000;101(4):365–376.
33. Ai X, Curran JW, Shannon TR, Bers DM, Pogwizd SM. Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure. *Circ Res*. 2005;97(12):1314–1322.
34. Nagai R, Zarain-Herzberg A, Brandl CJ, et al. Regulation of myocardial Ca2+-ATPase and phospholamban mRNA expression in response to pressure overload and thyroid hormone. *Proc Natl Acad Sci U S A*. 1989;86(8):2966–2970.
35. Zarain-Herzberg A, Fragoso-Medina J, Estrada-Aviles R. Calcium-regulated transcriptional pathways in the normal and pathologic heart. *IUBMB Life*. 2011;63(10):847–855.
36. Prasad AM, Inesi G. Silencing calcineurin A subunit reduces SERCA2 expression in cardiac myocytes. *Am J Physiol Heart Circ Physiol*. 2011;300(1):H173–H180.
37. Prasad AM, Inesi G. Regulation and rate limiting mechanisms of Ca2+ ATPase (SERCA2) expression in cardiac myocytes. *Mol Cell Biochem*. 2012;361(1–2):85–96.
38. Rocchetti M, Besana A, Mostacciuolo G, et al. Modulation of sarcoplasmic reticulum function by Na+/K+ pump inhibitors with different toxicity: digoxin and PST2744 ([E,Z]-3-[[2-aminoethoxy] imino]androstane-6,17-dione hydrochloride). *J Pharmacol Exp Ther*. 2005;313(1):207–215.
39. Micheletti R, Mattera GG, Rocchetti M, et al. Pharmacological profile of the novel inotropic agent (E,Z)-3-([2-aminoethoxy]imino) androstane-6,17-dione hydrochloride (PST2744). *J Pharmacol Exp Ther*. 2002;303(2):592–600.
40. Micheletti R, Palazzo F, Barassi P, et al. Istaroxime, a stimulator of sarcoplasmic reticulum calcium adenosine triphosphatase isoform 2a activity, as a novel therapeutic approach to heart failure. *Am J Cardiol*. 2007;99(2A):24A–32A.
41. Mattera GG, Lo Giudice P, Loi FM, et al. Istaroxime: a new luso-inotropic agent for heart failure. *Am J Cardiol*. 2007;99(2A):33A–40A.
42. Sabbah HN, Imai M, Cowart D, Amato A, Carminati P, Gheorghiade M. Hemodynamic properties of a new-generation positive luso-inotropic agent for the acute treatment of advanced heart failure. *Am J Cardiol*. 2007;99(2A):41A–46A.
43. Shah SJ, Blair JE, Filippatos GS, et al. Effects of istaroxime on diastolic stiffness in acute heart failure syndromes: results from the Hemodynamic, Echocardiographic, and Neurohormonal Effects of Istaroxime, a Novel Intravenous Inotropic and Lusitropic Agent: a Randomized Controlled Trial in Patients Hospitalized with Heart Failure (HORIZON-HF) trial. *Am Heart J*. 2009;157(6):1035–1041.
44. Ferrandi M, Barassi P, Tadini-Buoninsegni F, et al. Istaroxime stimulates SERCA2a and accelerates calcium cycling in heart failure by relieving phospholamban inhibition. *Br J Pharmacol*. 2013;169(8): 1849–1861.
45. Gobbini M, Armaroli S, Banfi L, et al. Novel analogues of istaroxime, a potent inhibitor of Na+, K+-ATPase: synthesis and structure–activity relationship. *J Med Chem*. 2008;51(15):4601–4608.
46. Gobbini M, Armaroli S, Banfi L, et al. Novel analogues of istaroxime, a potent inhibitor of Na(+), K(+)-ATPase: synthesis, structure–activity relationship and 3D-quantitative structure-activity relationship of derivatives at position 6 on the androstane scaffold. *Bioorg Med Chem*. 2010;18(12):4275–4299.
47. Cornea RL, Gruber SJ, Lockamy EL, et al. High-throughput FRET assay yields allosteric SERCA activators. *J Biomol Screen*. 2013;18(1): 97–107.
48. Koitabashi N, Arai M, Tomaru K, et al. Carvedilol effectively blocks oxidative stress-mediated downregulation of sarcoplasmic reticulum Ca2+-ATPase 2 gene transcription through modification of Sp1 binding. *Biochem Biophys Res Commun*. 2005;328(1):116–124.
49. Hong-Li S, Lei L, Lei S, et al. Cardioprotective effects and underlying mechanisms of oxymatrine against ischemic myocardial injuries of rats. *Phytother Res*. 2008;22(7):985–989.
50. Cao YG, Jing S, Li L, et al. Antiarrhythmic effects and ionic mechanisms of oxymatrine from Sophora flavescens. *Phytother Res*. 2010;24(12):1844–1849.
51. Shen XC, Yang YP, Xiao TT, Peng J, Liu XD. Protective effect of oxymatrine on myocardial fibrosis induced by acute myocardial infarction in rats involved in TGF-β_1-Smads signal pathway. *J Asian Nat Prod Res*. 2011;13(3):215–224.
52. Huang XY, Chen CX. Effect of oxymatrine, the active component from Radix Sophorae flavescentis (Kushen), on ventricular remodeling in spontaneously hypertensive rats. *Phytomedicine*. 2013;20(3–4):202–212.

53. Zhang M, Wang X, Wang X, et al. Oxymatrine protects against myocardial injury via inhibition of JAK2/STAT3 signaling in rat septic shock. *Mol Med Rep*. 2013;7(4):1293–1299.
54. Wu CK, Lee JK, Chiang FT, et al. Plasma levels of tumor necrosis factor-α and interleukin-6 are associated with diastolic heart failure through downregulation of sarcoplasmic reticulum Ca2+ ATPase. *Crit Care Med*. 2011;39(5):984–992.
55. Hu ST, Tang Y, Shen YF, et al. Protective effect of oxymatrine on chronic rat heart failure. *J Physiol Sci*. 2011;61(5):363–372.
56. Zhang Y, Dong Z, Jin L, et al. Arsenic trioxide-induced hERG K(+) channel deficiency can be rescued by matrine and oxymatrine through up-regulating transcription factor Sp1 expression. *Biochem Pharmacol*. 2013;85(1):59–68.
57. Davia K, Bernobich E, Ranu HK, et al. SERCA2A overexpression decreases the incidence of aftercontractions in adult rabbit ventricular myocytes. *J Mol Cell Cardiol*. 2001;33(5):1005–1015.
58. del Monte F, Harding SE, Schmidt U, et al. Restoration of contractile function in isolated cardiomyocytes from failing human hearts by gene transfer of SERCA2a. *Circulation*. 1999;100(23):2308–2311.
59. Hajjar RJ, Kang JX, Gwathmey JK, Rosenzweig A. Physiological effects of adenoviral gene transfer of sarcoplasmic reticulum calcium ATPase in isolated rat myocytes. *Circulation*. 1997;95(2):423–429.
60. He H, Giordano FJ, Hilal-Dandan R, et al. Overexpression of the rat sarcoplasmic reticulum Ca2+ ATPase gene in the heart of transgenic mice accelerates calcium transients and cardiac relaxation. *J Clin Invest*. 1997;100(2):380–389.
61. Niwano K, Arai M, Koitabashi N, et al. Lentiviral vector-mediated SERCA2 gene transfer protects against heart failure and left ventricular remodeling after myocardial infarction in rats. *Mol Ther*. 2008;16(6):1026–1032.
62. Celladon Corporation. Efficacy and Safety Study of Genetically Targeted Enzyme Replacement Therapy for Advanced Heart Failure. Available from http://clinicaltrials.gov/ct2/show/study/NCT00454818. Accessed January 9, 2014.
63. Jaski BE, Jessup ML, Mancini DM, et al. Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID trial), a first-in-human Phase I/II clinical trial. *J Card Fail*. 2009;15(3):171–181.
64. Hajjar RJ, Zsebo K, Deckelbaum L, et al. Design of a Phase I/II trial of intracoronary administration of AAV1/SERCA2a in patients with heart failure. *J Card Fail*. 2008;14(5):355–367.
65. Jessup M, Greenberg B, Mancini D, et al. Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID): a Phase II trial of intracoronary gene therapy of sarcoplasmic reticulum Ca2+-ATPase in patients with advanced heart failure. *Circulation*. 2011;124(3):304–313.
66. Zsebo KM, Yaroshinsky A, Rudy JJ, et al. Long term effects of AAV1/SERCA2a gene transfer in patients with severe heart failure: analysis of recurrent cardiovascular events and mortality. *Circ Res*. Epub September 24, 2013.
67. Celladon Corporation. A Study of Genetically Targeted Enzyme Replacement Therapy for Advanced Heart Failure (CUPID-2b). Available from http://clinicaltrials.gov/ct2/show/record/NCT01643330. Accessed January 9, 2014.
68. Assistance Publique - Hôpitaux de Paris. AAV1-CMV-Serca2a GENe Therapy Trial in Heart Failure (AGENT-HF). Available from: http://clinicaltrials.gov/ct2/show/NCT01966887. Accessed January 9, 2014.
69. Imperial College London. The recruitment status of this study is unknown because the information has not been verified recently. Available from: http://clinicaltrials.gov/show/NCT00534703. Accessed January 9, 2014.
70. Kalyanasundaram A, Lacombe VA, Belevych AE, et al. Up-regulation of sarcoplasmic reticulum Ca(2+) uptake leads to cardiac hypertrophy, contractile dysfunction and early mortality in mice deficient in CASQ2. *Cardiovasc Res*. 2013;98(2):297–306.
71. Nowak KJ, Davies KE. Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. *EMBO Rep*. 2004;5(9):872–876.
72. Shin JH, Bostick B, Yue Y, Hajjar R, Duan D. SERCA2a gene transfer improves electrocardiographic performance in aged mdx mice. *J Transl Med*. 2011;9:132.
73. Goonasekera SA, Lam CK, Millay DP, et al. Mitigation of muscular dystrophy in mice by SERCA overexpression in skeletal muscle. *J Clin Invest*. 2011;121(3):1044–1052.
74. Vandecaetsbeek I, Raeymaekers L, Wuytack F, Vangheluwe P. Factors controlling the activity of the SERCA2a pump in the normal and failing heart. *Biofactors*. 2009;35(6):484–499.
75. Brini M, Carafoli E. Calcium pumps in health and disease. *Physiol Rev*. 2009;89(4):1341–1378.
76. Gruber SJ, Haydon S, Thomas DD. Phospholamban mutants compete with wild type for SERCA binding in living cells. *Biochem Biophys Res Commun*. 2012;420(2):236–240.
77. Zhang T, Guo T, Mishra S, et al. Phospholamban ablation rescues sarcoplasmic reticulum Ca(2+) handling but exacerbates cardiac dysfunction in CaMKIIδ(C) transgenic mice. *Circ Res*. 2010;106(2): 354–362.
78. Kho C, Lee A, Jeong D, et al. SUMO1-dependent modulation of SERCA2a in heart failure. *Nature*. 2011;477(7366):601–605.
79. Costa MW, Lee S, Furtado MB, et al. Complex SUMO-1 regulation of cardiac transcription factor Nkx2-5. *PLoS One*. 2011;6(9):e24812.
80. Choudhary C, Kumar C, Gnad F, et al. Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science*. 2009;325(5942):834–840.
81. Foster DB, Liu T, Rucker J, et al. The cardiac acetyl-lysine proteome. *PLoS One*. 2013;8(7):e67513.
82. Sundaresan NR, Pillai VB, Gupta MP. Emerging roles of SIRT1 deacetylase in regulating cardiomyocyte survival and hypertrophy. *J Mol Cell Cardiol*. 2011;51(4):614–618.
83. Sulaiman M, Matta MJ, Sunderesan NR, Gupta MP, Periasamy M, Gupta M. Resveratrol, an activator of SIRT1, upregulates sarcoplasmic calcium ATPase and improves cardiac function in diabetic cardiomyopathy. *Am J Physiol*. 2010;298(3):H833–H843.
84. Rimbaud S, Ruiz M, Piquereau J, et al. Resveratrol improves survival, hemodynamics and energetics in a rat model of hypertension leading to heart failure. *PLoS One*. 2011;6(10):e26391.
85. Gupta MP, Samant SA, Smith SH, Shroff SG. HDAC4 and PCAF bind to cardiac sarcomeres and play a role in regulating myofilament contractile activity. *J Biol Chem*. 2008;283(15):10135–10146.
86. Hossain P, Kawar B, El Nahas M. Obesity and diabetes in the developing world – a growing challenge. *N Engl J Med*. 2007;356(3):213–215.
87. Relling DP, Esberg LB, Fang CX, et al. High-fat diet-induced juvenile obesity leads to cardiomyocyte dysfunction and upregulation of Foxo3a transcription factor independent of lipotoxicity and apoptosis. *J Hypertens*. 2006;24(3):549–561.
88. Ouwens DM, Boer C, Fodor M, et al. Cardiac dysfunction induced by high-fat diet is associated with altered myocardial insulin signalling in rats. *Diabetologia*. 2005;48(6):1229–1237.
89. Alves AJ, Ribeiro F, Goldhammer E, et al. Exercise training improves diastolic function in heart failure patients. *Med Sci Sports Exerc*. 2012;44(5):776–785.
90. de las Fuentes L, Waggoner AD, Mohammed BS, et al. Effect of moderate diet-induced weight loss and weight regain on cardiovascular structure and function. *J Am Coll Cardiol*. 2009;54(25):2376–2381.
91. Rolim NP, Medeiros A, Rosa KT, et al. Exercise training improves the net balance of cardiac Ca2+ handling protein expression in heart failure. *Physiol Genomics*. 2007;29(3):246–252.
92. Paulino EC, Ferreira JC, Bechara LR, et al. Exercise training and caloric restriction prevent reduction in cardiac Ca2+-handling protein profile in obese rats. *Hypertension*. 2010;56(4):629–635.
93. Assayag P, Charlemagne D, de Leiris J, et al. Senescent heart compared with pressure overload-induced hypertrophy. *Hypertension*. 1997;29(1 Pt 1):15–21.

94. Azhar G, Zhang X, Wang S, Zhong Y, Quick CM, Wei JY. Maintaining serum response factor activity in the older heart equal to that of the young adult is associated with better cardiac response to isoproterenol stress. *Basic Res Cardiol*. 2007;102(3):233–244.
95. Kaplan P, Jurkovicova D, Babusikova E, et al. Effect of aging on the expression of intracellular Ca(2+) transport proteins in a rat heart. *Mol Cell Biochem*. 2007;301(1–2):219–226.
96. Lokuta AJ, Maertz NA, Meethal SV, et al. Increased nitration of sarcoplasmic reticulum Ca2+-ATPase in human heart failure. *Circulation*. 2005;111(8):988–995.
97. Knyushko TV, Sharov VS, Williams TD, Schoneich C, Bigelow DJ. 3-Nitrotyrosine modification of SERCA2a in the aging heart: a distinct signature of the cellular redox environment. *Biochemistry*. 2005;44(39):13071–13081.
98. Adachi T, Weisbrod RM, Pimentel DR, et al. S-glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med*. 2004;10(11):1200–1207.
99. Berger HJ, Zaret BL, Speroff L, Cohen LS, Wolfson S. Regional cardiac prostaglandin release during myocardial ischemia in anesthetized dogs. *Circ Res*. 1976;38(6):566–571.
100. Lai J, Jin H, Yang R, et al. Prostaglandin F2α induces cardiac myocyte hypertrophy in vitro and cardiac growth in vivo. *Am J Physiol*. 1996;271(6 Pt 2):H2197–H2208.
101. Chazov EI, Pomoinetsky VD, Geling NG, Orlova TR, Nekrasova AA, Smirnov VN. Heart adaptation to acute pressure overload: an involvement of endogenous prostaglandins. *Circ Res*. 1979;45(2):205–211.
102. Hara S, Arai M, Tomaru K, et al. Prostaglandin F2α inhibits SERCA2 gene transcription through an induction of Egr-1 in cultured neonatal rat cardiac myocytes. *Int Heart J*. 2008;49(3):329–342.
103. Andrews C, Ho PD, Dillmann WH, Glembotski CC, McDonough PM. The MKK6–p38 MAPK pathway prolongs the cardiac contractile calcium transient, downregulates SERCA2, and activates NF-AT. *Cardiovasc Res*. 2003;59(1):46–56.
104. Molkentin JD. Calcineurin–NFAT signaling regulates the cardiac hypertrophic response in coordination with the MAPKs. *Cardiovasc Res*. 2004;63(3):467–475.
105. Scharf M, Neef S, Freund R, et al. Mitogen-activated protein kinase-activated protein kinases 2 and 3 regulate SERCA2a expression and fiber type composition to modulate skeletal muscle and cardiomyocyte function. *Mol Cell Biol*. 2013;33(13):2586–2602.
106. Rowe GC, Jiang A, Arany Z. PGC-1 coactivators in cardiac development and disease. *Circ Res*. 2010;107(7):825–838.
107. Kemi OJ, Hoydal MA, Haram PM, et al. Exercise training restores aerobic capacity and energy transfer systems in heart failure treated with losartan. *Cardiovasc Res*. 2007;76(1):91–99.
108. Ngoh GA, Facundo HT, Zafir A, Jones SP. O-GlcNAc signaling in the cardiovascular system. *Circ Res*. 2010;107(2):171–185.
109. Lunde IG, Aronsen JM, Kvaloy H, et al. Cardiac O-GlcNAc signaling is increased in hypertrophy and heart failure. *Physiol Genomics*. 2012;44(2):162–172.
110. Jones SP, Zachara NE, Ngoh GA, et al. Cardioprotection by N-acetylglucosamine linkage to cellular proteins. *Circulation*. 2008;117(9):1172–1182.
111. Ngoh GA, Watson LJ, Facundo HT, Dillmann W, Jones SP. Non-canonical glycosyltransferase modulates post-hypoxic cardiac myocyte death and mitochondrial permeability transition. *J Mol Cell Cardiol*. 2008;45(2):313–325.
112. Hirose K, Tsutsumi YM, Tsutsumi R, et al. Role of the O-linked β-N-acetylglucosamine in the cardioprotection induced by isoflurane. *Anesthesiology*. 2011;115(5):955–962.
113. Lima VV, Giachini FR, Carneiro FS, et al. O-GlcNAcylation contributes to the vascular effects of ET-1 via activation of the RhoA/Rho-kinase pathway. *Cardiovasc Res*. 2011;89(3):614–622.
114. Facundo HT, Brainard RE, Watson LJ, et al. O-GlcNAc signaling is essential for NFAT-mediated transcriptional reprogramming during cardiomyocyte hypertrophy. *Am J Physiol Heart Circ Physiol*. 2012;302(10):H2122–H2130.
115. Sena S, Hu P, Zhang D, et al. Impaired insulin signaling accelerates cardiac mitochondrial dysfunction after myocardial infarction. *J Mol Cell Cardiol*. 2009;46(6):910–918.
116. Domenighetti AA, Danes VR, Curl CL, Favaloro JM, Proietto J, Delbridge LM. Targeted GLUT-4 deficiency in the heart induces cardiomyocyte hypertrophy and impaired contractility linked with Ca(2+) and proton flux dysregulation. *J Mol Cell Cardiol*. 2010;48(4):663–672.
117. Brady M, Koban MU, Dellow KA, Yacoub M, Boheler KR, Fuller SJ. Sp1 and Sp3 transcription factors are required for trans-activation of the human SERCA2 promoter in cardiomyocytes. *Cardiovasc Res*. 2003;60(2):347–354.
118. Takizawa T, Arai M, Tomaru K, et al. Transcription factor Sp1 regulates SERCA2 gene expression in pressure-overloaded hearts: a study using in vivo direct gene transfer into living myocardium. *J Mol Cell Cardiol*. 2003;35(7):777–783.
119. Yang X, Su K, Roos MD, Chang Q, Paterson AJ, Kudlow JE. O-linkage of N-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability. *Proc Natl Acad Sci U S A*. 2001;98(12):6611–6616.
120. Hu Y, Belke D, Suarez J, et al. Adenovirus-mediated overexpression of O-GlcNAcase improves contractile function in the diabetic heart. *Circ Res*. 2005;96(9):1006–1013.
121. Cox EJ, Marsh SA. Exercise and diabetes have opposite effects on the assembly and O-GlcNAc modification of the mSin3A/HDAC1/2 complex in the heart. *Cardiovasc Diabetol*. 2013;12(1):101.
122. Clark RJ, McDonough PM, Swanson E, et al. Diabetes and the accompanying hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear O-GlcNAcylation. *J Biol Chem*. 2003;278(45):44230–44237.
123. Yokoe S, Asahi M, Takeda T, et al. Inhibition of phospholamban phosphorylation by O-GlcNAcylation: implications for diabetic cardiomyopathy. *Glycobiology*. 2010;20(10):1217–1226.
124. Kim HS, Woo JS, Joo HJ, Moon WK. Cardiac transcription factor Nkx2.5 is downregulated under excessive O-GlcNAcylation condition. *PLoS One*. 2012;7(6):e38053.
125. Ikeda S, He A, Kong SW, et al. MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. *Mol Cell Biol*. 2009;29(8):2193–2204.
126. Li D, He B, Zhang H, et al. The inhibitory effect of miRNA-1 on ET-1 gene expression. *FEBS Lett*. 2012;586(7):1014–1021.
127. Karakikes I, Chaanine AH, Kang S, et al. Therapeutic cardiac-targeted delivery of miR-1 reverses pressure overload-induced cardiac hypertrophy and attenuates pathological remodeling. *J Am Heart Assoc*. 2013;2(2):e000078.
128. Hajjar RJ, Schmidt U, Kang JX, Matsui T, Rosenzweig A. Adenoviral gene transfer of phospholamban in isolated rat cardiomyocytes. Rescue effects by concomitant gene transfer of sarcoplasmic reticulum Ca(2+)-ATPase. *Circ Res*. 1997;81(2):145–153.
129. Most P, Remppis A, Pleger ST, Katus HA, Koch WJ. S100A1: a novel inotropic regulator of cardiac performance. Transition from molecular physiology to pathophysiological relevance. *Am J Physiol Regul Integr Comp Physiol*. 2007;293(2):R568–R577.
130. Pleger ST, Shan C, Ksienzyk J, et al. Cardiac AAV9-S100A1 gene therapy rescues post-ischemic heart failure in a preclinical large animal model. *Sci Transl Med*. 2011;3(92):92ra64.
131. Pleger ST, Most P, Boucher M, et al. Stable myocardial-specific AAV6-S100A1 gene therapy results in chronic functional heart failure rescue. *Circulation*. 2007;115(19):2506–2515.
132. Ritterhoff J, Most P. Targeting S100A1 in heart failure. *Gene Ther*. 2012;19(6):613–621.

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The CCAAT box in the proximal SERCA2 gene promoter regulates basal and stress-induced transcription in cardiomyocytes

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Abstract The cardiac sarco/endoplasmic reticulum Ca^{2+}-ATPase-2a (SERCA2a) is vital for the correct handling of calcium concentration in cardiomyocytes. Recent studies showed that the induction of endoplasmic reticulum (ER) stress (ERS) with the SERCA2 inhibitor Thapsigargin (Tg) increases the mRNA and protein levels of SERCA2a. The *SERCA2* gene promoter contains an ERS response element (ERSE) at position −78 bp that is conserved among species and might transcriptionally regulate *SERCA2* gene expression. However, its involvement in *SERCA2* basal and calcium-mediated transcriptional activation has not been elucidated. In this work, we show that in cellular cultures of neonatal rat ventricular myocytes, the treatment with Tg or the calcium ionophore A23187 increases the SERCA2a mRNA and protein abundance, as well as the transcriptional activity of two chimeric human *SERCA2* gene constructs, containing −254 and −2579 bp of 5′-regulatory region cloned in the pGL3-basic vector and transiently transfected in cultured cardiomyocytes. We found that the ERSE present in the *SERCA2* proximal promoter contains a CCAAT box that is involved in basal and ERS-mediated *hSERCA2* transcriptional activation. The EMSA results showed that the CCAAT box present in the ERSE recruits the NF-Y transcription factor. Additionally, by ChIP assays, we confirmed in vivo binding of NF-Y and C/EBPβ transcription factors to the *SERCA2* gene proximal promoter.

Keywords *SERCA2* · Stress · Calcium · Transcriptional regulation · NF-Y · C/EBPβ

Introduction

The calcium ion (Ca^{2+}) is an important second messenger involved in signal transduction of a variety of processes such as excitability, muscle contraction, and gene transcription [1]. In cardiomyocytes, the Ca^{2+}-handling is a tightly regulated process, controlled by several Ca^{2+} handling proteins such as the calcium release channel RyR2, calsequestrin, and the sarco/endoplasmic reticulum Ca^{2+}-ATPase calcium pump (SERCA2a). The joint action of these proteins plays a crucial role in excitation–contraction coupling in cardiomyocytes [2]. The SERCA2a inhibition with Thapsigargin (Tg) has multiple effects, such as sarcoplasmic reticulum (SR) Ca^{2+} depletion, increased cytoplasmic Ca^{2+} concentration, and increased SERCA2a expression [3–5]. Additionally, the SR Ca^{2+} depletion induces sarco/endoplasmic reticulum stress (ERS), triggers the unfolded protein response (UPR), and induces expression of several Ca^{2+} handling proteins like SERCA2, GRP78, GRP94, ERP72, ATF-6, and calreticulin [3, 6–10]. The ER stress response element (ERSE) is present in the promoter of many genes upregulated by ERS. The *SERCA2* gene (*ATP2A2*) promoter contains an ERSE site that is conserved among species and shares high homology with the ERSEs in other genes such as *grp78, grp94*, and calreticulin [11, 12]. The 19 bp sequence (−78 bp 5′-**CCAAT**GA**GCGGCGT**C**CACA**-3′ −60 bp), contains three regions: (1) a CCAAT motif that could bind C/EBP and NF-Y; (2) a 6-nucleotide GC-rich domain that

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could bind TF-II-I and ERSF; and (3) a 3′-nucleotide sequence that has been shown to bind YY1 and ATF6 [11–15].

Nuclear Factor-Y (NF-Y) is a transcription factor that consists of three subunits NF-YA, NF-YB, and NF-YC. The NF-YA subunit binds specifically to DNA at the 5′-CCAAT-3′ sequence, whereas the B and C subunits interact with DNA as core histones in non-specific manner [16]. NF-Y can interact with a vast number of proteins including general transcription factors, cofactors, and histones providing it with a wide range of functions [17]. The C/EBP (CCAAT/Enhancer-Binding Protein) family of transcription factors constitutes of 6 members (C/EBPα, -β, -γ, -δ, -ε, and -ζ) that are able to bind CCAAT motif with the exception of C/EBPζ, which is also known as CHOP (CCAAT/Enhancer-Binding Protein Homologous Protein) and acts as a negative regulator of other C/EBPs and stress-induced genes [18]. Among the members of the C/EBP family, C/EBPβ is expressed in heart and has been shown to regulate different cardiac functions [19–21].

It has been reported that the SR Ca^{2+} depletion upregulated rat *SERCA2* gene expression through a process involving the ATF6 transcription factor [11]. In the same study it was shown that the YY1 binding sequence of the ERSE site in the *SERCA2* promoter is necessary for the *SERCA2* induction elicited by ATF6 overexpression. However, this site was only partially responsible for increased *SERCA2* transcription induced by SR Ca^{2+} depletion. Therefore, the authors concluded that *SERCA2* upregulation due to SR Ca^{2+} depletion cannot be completely attributed to the action of ATF6 and that other transcription factors might be involved.

In this work, we show that the CCAAT sequence that is part of the ERSE in the *SERCA2* promoter region binds NF-Y and C/EBPβ transcription factors and is necessary for the basal transcriptional activity of the gene in neonatal rat ventricular cardiomyocytes. We also show that the SR Ca^{2+} depletion induced by Tg increases *SERCA2* transcription and this increment is mediated through the CCAAT box present in the *SERCA2* gene promoter.

Materials and methods

Cell culture

Primary cultures of ventricular cardiomyocytes were prepared from neonatal Wistar rat hearts (1–2 days-old) according to the method previously described and approved by the ethics and animal care committee of the School of Medicine, National Autonomous University of Mexico [22]. Cells were plated in DMEM culture medium (Sigma-Aldrich, MO, USA) supplemented with fetal bovine serum (FBS) (10%) (Gibco, MA, USA), kanamycin (70 mg/mL) (Sigma-Aldrich, MO, USA), penicillin (10 U/mL) (Gibco, MA, USA), streptomycin (10 mg/mL) (Gibco, MA, USA), amphotericin B (0.025 mg/mL) (Gibco, MA, USA), nystatin (10 U/mL) (Sigma-Aldrich, MO, USA), and 5-bromodeoxyuridine (0.1 μM final concentration) in order to minimize fibroblast proliferation. After 24 h the culture medium was switched to Opti-MEM (Life Technologies, CA, USA) supplemented with 5-bromodeoxyuridine and 2% FBS. For the mRNA experiments, 48 h after plating, cardiac myocytes were exposed for 12 h to pharmacological agents and RNA, and proteins were extracted. For the transfections assays, 48 h after plating, cells were transfected and incubated for 12 h with or without pharmacological agents as indicated above for luciferase activity measurements. Cells used for ChIP experiments were fixed 48 h after plating.

Real-time quantitative RT-PCR

Total RNA was extracted from cardiomyocytes primary cultures using TRIsure reagent (Bioline Inc, MA, USA) following manufacturer's protocol. One μg total RNA was used to synthesize cDNA by reverse transcription using M-MLV retro-transcriptase (Life Technologies, CA, USA) according to manufacturer's protocol with minor modifications: final concentration of reagents was as follows: 150 ng/μL for random primers, 7.5 ng/μL for dT20 oligonucleotide, 10 mM for dNTPs, 0.1 M for DTT, and 100 units of M-MLV enzyme in 20 μL total volume per reaction. PCR was performed using the Rotor-gene Real-Time PCR System (Qiagen, CA, USA), the Maxima SYBR Green qPCR Master Mix (Thermo Scientific, MA, USA), 200 nM final concentration of each primer, and 1 μL of cDNA reaction in a final volume of 10 μL per reaction. The relative SERCA2a mRNA abundance was calculated using GAPDH mRNA as the normalizing gene by the method previously described [23]. Oligonucleotides used were purchased from Sigma-Aldrich, MO, USA, and are listed in ESM 1.

Western blot analysis

Total protein was extracted from organic phase derived from TRIsure-RNA extracts following the method described by Chomczynski [24]. Protein lysates (25 μg) were separated on 12% polyacrylamide gels and transferred to a PVDF membrane (Bio-Rad CA, USA). Membranes were blocked with 5% non-fat dry milk (Bio-Rad CA, USA) dissolved in Tris-buffered saline with 0.1% Tween (TBST 0.1%). Membranes were incubated overnight at 4 °C with primary antibody against SERCA2 (1:10,000 dilution; ab3625, Abcam, MA, USA) using β-actin antibody (1:5000

dilution; a kindly gift from Dr. Martha Robles-Flores, Dept. Biochemistry, School of Medicine, UNAM, México) as the normalizing control. Membranes were incubated with the goat anti-rabbit (H + L) secondary antibody (1:5000) (Thermo Fisher 32460 MA, USA) for 1 h at room temperature. Signals were detected with SuperSignal West Dura reagent (34075, Thermo Fisher MA, USA) in a C-Digit Blot Scanner (LI-COR, NE, USA) and analyzed using the Image-Studio Lite 5.2.5 software (LI-COR, NE, USA).

Functional assays of human *SERCA2* promoter

Primary cultures of neonatal rat cardiomyocytes were transfected with two *SERCA2* promoter constructs containing Luciferase as the reporter gene (pGL3-*hSERCA2*p-254 bp and pGL3-*hSERCA2*p-2579 bp) previously cloned by our group [25]. Transient transfections were done in 24-well plates using Lipofectamine 2000 reagent (Invitrogen, CA, USA); 12 h later, the cells were harvested, lysed using the Passive Lysis Buffer (Promega, WI, USA), and the luciferase activity was determined using the Luciferase Reporter Assay System (Promega, WI, USA) in a Wallac Victor^2 1420 Luminometer (Perkin Elmer, MA, USA), using protein concentration to normalize data.

Site-directed mutagenesis

The site-directed mutagenesis of the putative binding sites was done by a mutagenic PCR reaction using *Pfu* X DNA polymerase (Jena Biosciences, Germany). Briefly, for the amplification reaction, 200 ng of the pGL3-*hSERCA2*p-254 bp construct as a template was mixed with 125 ng of specific primers designed to mutate the −78 bp CCAAT box from 5′-CCAAT-3 to 5′-CCCCT-3 (Fwd: 5′-GGGCG CTCTCGGCCCCTGAGCGGCG-3 and Rev: 5′-CGCCGC TCAGGGGCCGAGAGCGCCC-3), the ERSF site from 5′-GAGCTTCGT-3 to 5′-GAGCGGCGT-3′ (Fwd: 5′-CTCGGCCAATGAGCTTCGTCCACATGCCGCGG-3 and Rev: 5′-CCGCGGCATGTGGACGCCGCTCATTGGCCGA G-3), and the −63 bp YY1 site from 5′-CCACA-3 to 5′-TA CAA-3 (Fwd: 5′-GAGCGGCGTTACAATGCCGCGGCG G-3 and Rev: 5′-CCGCCGCGGCATTGTAACGCCGCT C-3). The mutagenic PCR reaction conditions were as follows: 95 °C for 5 min, 18 cycles (95 °C for 50 s, 75 °C for 50 s, 68 °C for 5 min), and 68 °C for 7 min. The final reaction volume was 50 µL. The reaction product was digested with 10 U of methylation-sensitive enzyme *DpnI* at 37 °C for 2 h (New England Biolabs, MA, USA). *E. coli* DH5-α competent cells were transformed with the amplified products. Posteriorly, the plasmids were purified using the PureLink plasmid DNA purification kit (Catalog K2100-04, Invitrogen, CA, USA). The mutated plasmids were sequenced and used in functional assays.

Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides of the human *SERCA2* −78 bp ERSE site or the rat *grp78* at position-98 bp ERSE site (ESM 2) were labeled with ^{32}[P]-ATP and polynucleotide kinase as described previously and incubated with cardiomyocytes nuclear extracts [22]. Specific complexes between probes and NF-Y or C/EBPβ transcription factors were demonstrated by using molar excess of non-labeled oligonucleotides and by super-shift assays using NF-YA (H-209, sc10779, Santa Cruz Biotechnology, TX, USA), NF-YB (FL-207, sc13045, Santa Cruz Biotechnology, TX, USA), NF-YC (C-19, sc7714, Santa Cruz Biotechnology, TX, USA), C/EBPβ (C-19, sc-150, Santa Cruz Biotechnology, TX, USA), or phosphorylated C/EBPβ (ab52194, Abcam, MA, USA) antibodies. The gels were dried, exposed to X-ray films, and photographed.

Chromatin immunoprecipitation (ChIP) assay

Chromatin obtained from primary cultures of two-day-old ventricular cardiomyocytes was fixed with 1% formaldehyde for 15 min, and the reaction was stopped with glycine (Sigma-Aldrich, MO, USA) at a final concentration of 0.125 M. Culture medium was removed, and the cells were washed with PBS 1× with PMSF 1 mM. The cells were then lysed with lysis buffer [Tris–HCl 50 mM pH 8.0, EDTA 10 mM, SDS 1%, and Sigma Fast Protease Inhibitor Cocktail (Sigma-Aldrich, MO, USA)]. Lysed cells were subjected to 5 sonication cycles of 60 s ON with 60 s OFF in a Bioruptor Pico sonication device (Diagenode, NJ, USA). Immunoprecipitation was done with One-day ChIP kit (Catalog number C01010081, Diagenode, NJ, USA) following the manufacturer instructions. Sonicated chromatin was incubated with 4 µg of antibody against NF-YB (FL-207, sc13045, Santa Cruz Biotechnology, TX, USA), C/EBPβ (C-19, sc-150, Santa Cruz Biotechnology, TX, USA), or Sp1 (PEP2, sc-59×, Santa Cruz Biotechnology, TX, USA). Rabbit IgG (kch 504-250, Diagenode, NJ, USA) was used as negative control. Five microliters per sample of the obtained DNA were subjected to PCR using 2 µl of each 10 µM of the rat *SERCA2* gene promoter primers (Fwd: −268 F 5′-CAGCGAGCACAGCGAGGAC-3′ −260 bp and Rev: +60 5′-GACAGCGGCGGAG-GAAACTG-3′ +41 bp; product 328 bp) and 10 µL of ZymoTaq PreMix (Zymo Research, USA) in a final volume of 20 µL per reaction with a final 5% DMSO concentration. Cycling conditions were as follows: activation at 94 °C for 4 min, 40 cycles of amplification at 94 °C for 30 s followed by annealing at 68 °C for 1 min, and final amplification at

72 °C for 5 min. DNA was subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed.

Statistical analysis

Results of at least three experiments are shown as the mean ± standard error. The statistical analysis was done using ANOVA (Holm–Sidak test) for group comparison; a level of significance of $p \leq 0.05$ was considered as significant.

Results

Thapsigargin and Ca^{2+} ionophore A23187 increase transcription of the *SERCA2* gene

To upregulate *SERCA2* expression, cardiomyocytes were exposed to Tg (200 nM) or the Ca^{2+} ionophore A23187 (0.5 μM) for 12 h. SERCA2 protein level was increased about 1.5-fold as shown in Fig. 1a and previously reported [4]. Additionally, we measured SERCA2 mRNA levels in response to the above-mentioned treatments, and mRNA levels showed a similar increase (∼1.6-fold) to those of protein (Fig. 1b).

To investigate if the increase in SERCA2 mRNA was due to transcriptional regulation of the *SERCA2* promoter, we used two pGL3-luciferase reporters under the control of the *hSERCA2* 5′-regulatory region previously cloned by our group in pGL3-basic (Promega, USA) [25]. The *hSERCA2* gene promoter constructs used contain +318 bp of the 5′-untranslated sequence, and −254 or −2579 bp (pGL3-*hSERCA2*p-254 bp or pGL3-*hSERCA2*p-2579 bp) of the regulatory region. These constructs were transiently transfected into neonatal rat cardiomyocytes; the cells were treated with Tg; and Ca^{2+} ionophore A23187 and the luciferase activity due to pharmacological treatments was measured. Although pGL3-*hSERCA2*p-2579 bp construct showed a higher luciferase activity due to Tg and ionophore A23187 treatments indicating the presence of regulatory elements additional to those present in the short construct pGL3-*hSERCA2*p-254 bp (Fig. 2), the activation due to the first 254 bp of 5′-regulatory region was considered strong enough to contain the main sequences responsible for the induction. Thus, we focused on the short construct (pGL3-*hSERCA2*p-254 bp) for further experiments.

The CCAAT box at −78 bp is essential for basal and Tg-induced transcription of h*SERCA2* gene constructs

The in silico DNA sequence analysis of the *SERCA2* 5′-regulatory region reveals the presence of an endoplasmic

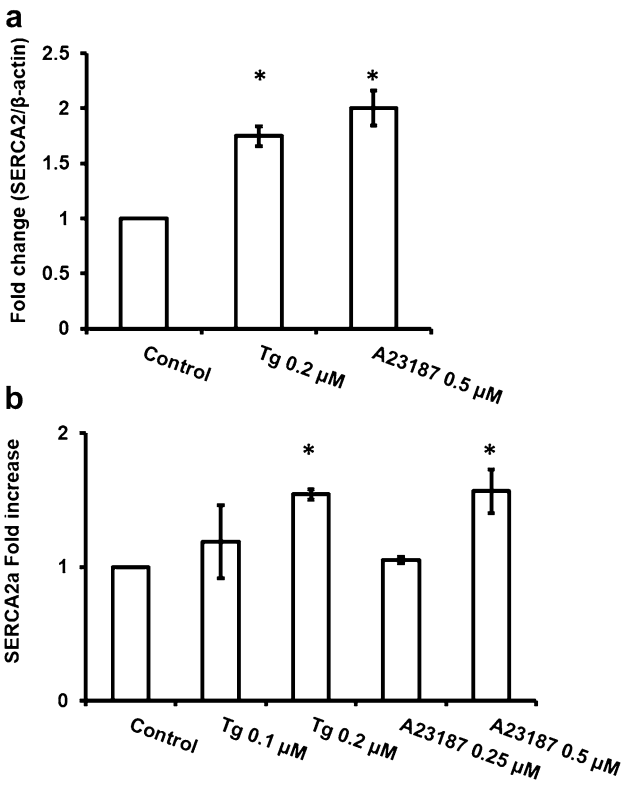

Fig. 1 Thapsigargin and the calcium ionophore A23187 upregulate SERCA2 expression in cardiomyocytes. Neonatal rat ventricular cardiomyocytes were treated with the drugs 48 h after seeding. The cells were harvested after 12 h of treatment using TRIsure reagent, and total RNA and protein were isolated. **a** Densitometry analysis of Western blots: β-actin was used as loading control. **b** SERCA2a mRNA abundance using GAPDH to normalize data. Data are mean ± standard error, $n \geq 3$. Statistical significance was determined using ANOVA and Holm–Sidak test ($p \leq 0.05$), and comparisons between control and treatments are indicated by an asterisk

reticulum stress response element (ERSE) site at position −78 bp of the promoter (−78 bp 5′-CCAAT-GAGCGGCGTCCACA-3′ −60 bp) which is conserved among species and with different genes such as *grp78* (−98 bp 5′-CCAATCGGAGGCCTCCACG-3′ −80 bp). Therefore, we decided to investigate whether the ERSE at −78 bp could be responsible for Tg-mediated *SERCA2* gene upregulation. The ERSE elements comprise three regions: a CCAAT motif, an ERSF binding site, and a YY-1 binding site. We mutated the CCAAT box (from 5′-CCAAT-3 to 5′-CCCCT-3, named as mCAT), the ERSF site (5′-CGGAGGCCT-3′ to 5′-CGGATTCCT-3, named as mERSF), and the YY1 site (from CCACA to TACAA, named as mYY1) in the pGL3-*hSERCA2*p-254 bp construct. To assess the influence of the above-mentioned sites on Tg-mediated *SERCA2* upregulation, neonatal ventricular rat cardiomyocytes were transiently transfected with the wild-type or mutated pGL3-*hSERCA2* promoter constructs and treated with pharmacological stressors. ERSF site

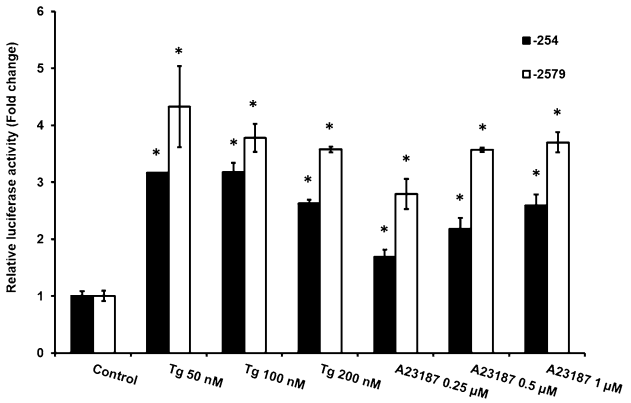

Fig. 2 Thapsigargin and calcium ionophore A23187 increase human *SERCA2* gene transcription in cardiomyocytes. Neonatal rat ventricular cardiomyocytes were transfected with the pGL3-*hSERCA2*p-254 bp and pGL3-*hSERCA2*p-2579 bp wild-type constructs 48 h after seeding, and the drugs were administered 2 h after transfection. Cells were harvested 12 h later, and Luciferase activity and protein concentration were measured. Data are mean ± standard error, $n \geq 3$. Statistical significance was determined using ANOVA and Holm–Sidak test ($p \leq 0.05$), and comparisons between control and treatments are indicated by an asterisk

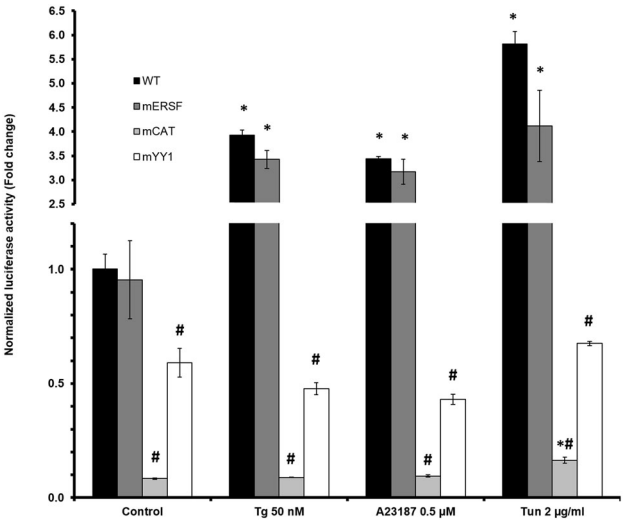

Fig. 3 Transcriptional activities of the −254 bp human *SERCA2* wild-type and mutant constructs. The wild-type construct and the construct mutated at the ERSF site were induced by Thapsigargin, A23187, and Tunicamycin. The constructs where the CCAAT box or the YY1 site was mutated do not exhibit the increase with the drugs. Moreover, the basal transcriptional activity of the mCAT and mYY1 mutants is decreased compared with wild-type construct. Neonatal rat ventricular cardiomyocytes were transfected with the pGL3-*hSERCA2*p-254 bp wild-type (WT), or the three mutant constructs 48 h after seeding, the drugs were administered 2 h after transfection. Cells were harvested 12 h later, and Luciferase activity and protein concentration were measured. Data are mean ± standard error, $n \geq 3$. Statistical significance was determined using ANOVA and Holm–Sidak test ($p \leq 0.05$). Statistically significant differences with respect to the control without pharmacological treatment are marked by asterisks and differences between WT and mutants are indicated by a hash symbol

mutation showed no influence on the basal or the Tg- or A23187-activated *SERCA2* upregulation. YY1 mutant (mYY1) showed 40% reduction of basal activity compared with WT construct (pGL3-*hSERCA2*p-254 bp) and CCAAT box mutant (mCAT) 90% reduction (Fig. 3). YY1 and CCAAT box mutations blunted pGL3-*hSERCA2*p-254 bp upregulation induced by Tg or A23187. As a control of ER/SR stress induction, the N-linked protein glycosylation inhibitor Tunicamycin was used at a concentration of 2 μg/mL. While in the WT pGL3-*hSERCA2*p-254 bp promoter there was a 6-fold increase by Tunicamycin, mutation of the YY1 site at position −63 bp prevented the upregulation, and in the CCAAT box-mutated reporter, there was a remaining two-fold activation (Fig. 3). When the ERSF site was mutated, there was no change in pGL3-*hSERCA2*p-254 bp upregulation induced by Tunicamycin.

The *SERCA2* gene CCAAT box at −78 bp is essential for the ERSE complex formation

To further understand the participation of the CCAAT box in *SERCA2* transcriptional regulation, EMSA experiments were performed using ^{32}P-labeled oligonucleotides for the human *SERCA2* −78 bp ERSE site. EMSAs showed the existence of a specific complex between nuclear extracts of rat cardiomyocytes and human *SERCA2* −78 ERSE site (Fig. 4). Molar excesses of unlabeled (cold probes) and the mutated versions of human *SERCA2* −78 ERSE were used to investigate the importance of the three regions described above. Even 200× molar excesses of cold probes containing the mutated CCAAT box failed to compete ERSE complex formation. We showed that molar excess (50×) of unlabeled *hSERCA2* −78 ERSE site can effectively compete for the formation of the ERSE complex driven by the ERSE site at position −98 bp in the rat *grp78* gene, considered a classical ERS marker (Fig. 5).

NF-Y and C-EBPβ transcription factors bind to the *SERCA2* promoter

The CCAAT sequence has been reported to bind NF-Y and C/EBPβ transcription factors. Therefore, we investigated the participation of NF-Y and C/EBPβ transcription factors in the formation of ERSE complex; antibodies against the three NF-Y transcription factors (NF-YA, NF-YB, and NF-YC), C/EBPβ, and phosphorylated C/EBPβ (C/EBPβ-P) were used. Adding NF-Y antibodies cause disappearance (super-shift) of the ERSE complex formed with nuclear extracts. As Fig. 6 shows, when the rat *grp78* ERSE site at

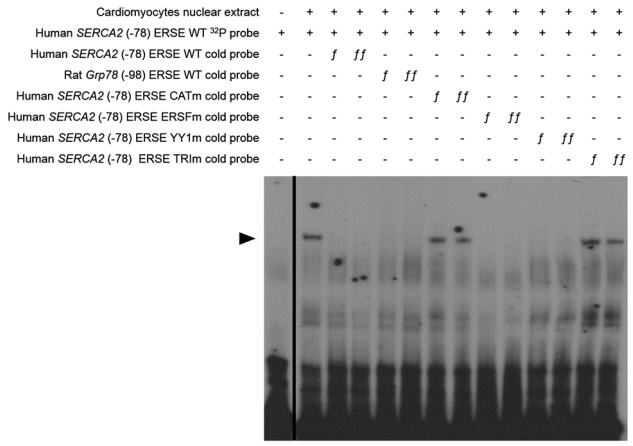

Fig. 4 The CCAAT box present in the ERSE in the human *SERCA2* gene is necessary to form a specific DNA–protein complex. EMSAs were conducted on nuclear extracts from neonatal rat ventricular cardiomyocytes using a ^{32}P probe for *hSERCA2* wild-type (WT) ERSE and the indicated molar excess of unlabeled (cold) probes (*f*, 100-fold; *ff*, 200-fold) as follows: *hSERCA2* wild-type (WT) ERSE (lanes 3–4); rat *grp78* wild-type (WT) ERSE (lanes 5–6); *hSERCA2* ERSE with the mutated −78 CCAAT box (lanes 7–8); *hSERCA2* ERSE with the mutated −72 ERSF site (lanes 9–10); *hSERCA2* ERSE with the mutated −63 YY1 site (lanes 11–12); and *hSERCA2* ERSE with the three sites mutated (TRIm) (lanes 13–14). The specific ERSE complex is indicated by an arrow. Representative image of three independent experiments. All lanes are from the same representative film

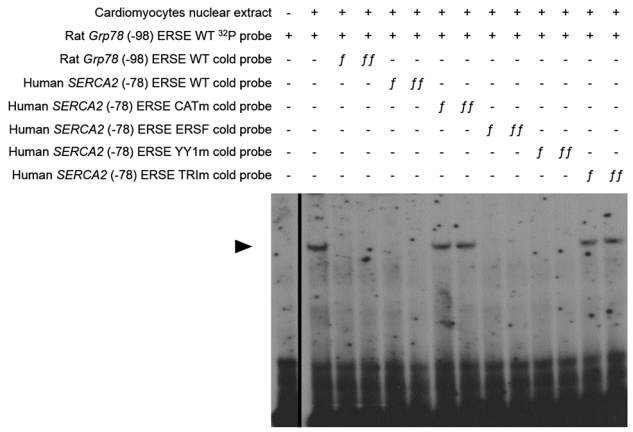

Fig. 5 The CCAAT box from the ERSE in the human *SERCA2* gene site efficiently competes for the formation of rat *grp78* ERSE complex. EMSAs were conducted on nuclear extracts from neonatal rat ventricular cardiomyocytes using a ^{32}P probe for rat *grp78* wild-type (WT) ERSE and indicated molar excess of unlabeled probe (*f*, 50-fold; *ff*, 100-fold) as follows: rat *grp78* wild-type (WT) ERSE (lanes 3–4); *hSERCA2* wild-type (WT) ERSE (lanes 5–6); *hSERCA2* ERSE with the mutated CCAAT box (lanes 7–8); *hSERCA2* ERSE with the mutated ERSF site (lanes 9–10); *hSERCA2* ERSE with the mutated −63 YY1 site (lanes 11–12); and *hSERCA2* ERSE with the three sites mutated (TRIm) (lanes 13–14). The ERSE-specific complex is indicated by an arrow. Representative image of three independent experiments. All lanes are from the same film

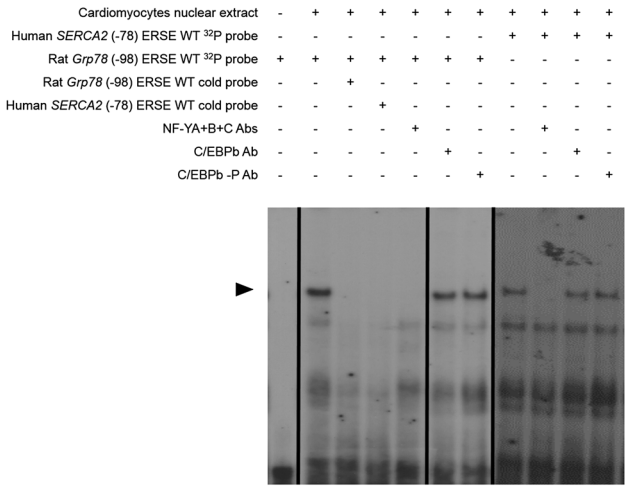

Fig. 6 The CCAAT box in the ERSE of *SERCA2* and *grp78* genes binds NF-Y. Nuclear extracts from neonatal rat ventricular cardiomyocytes were incubated with ^{32}P-labeled *grp78* wild-type ERSE probe (lanes 2–7), and competed with 200× molar excess of unlabeled rat *grp78* wild-type ERSE (lane 3); 200× molar excess of unlabeled human *SERCA2* wild-type ERSE (lane 4); 6 μg of NF-Y antibodies (2 μg of NF-YA + 2 μg of NF-YB + 2 μg of NF-YC) (lane 5); 2 μg of C/EBPβ antibody (lane 6); 2 μg of phosphorylated C/EBPβ antibody (lane 7); ^{32}P-labeled *hSERCA2* wild-type ERSE probe (lane 8–11); 6 μg of NF-Y antibodies (2 μg of NF-YA + 2 μg of NF-YB + 2 μg of NF-YC) (lane 9); 2 μg of C/EBPβ antibody (lane 10); 2 μg of phosphorylated C/EBPβ antibody (lane 11). The specific ERSE complex is indicated by an arrow. Representative image of four independent experiments. Lanes 8–11 are from a different film than lanes 1–7

−98 bp was used for EMSA to determine if NF-Y or C/EBPβ were able to super-shift ERSE complex, results mirrored those obtained with the ERSE in the *hSERCA2* promoter at −78 bp, confirming the participation of NF-Y in the formation of the ERSE complex. The presence of the C/EBPβ antibodies did not affect the formation of ERSE complex in a visible manner (Fig. 6).

An additional EMSA was carried out to investigate if the complex formation changed upon Tg treatment, and results in Fig. 7a show that Tg diminished the formation of the complex during the first 4 h but increased again after 6 h. Also, in Fig. 7b it is shown that Tg diminishes ERSE complex formation but does not compete for the interaction when using antibodies against C/EBPβ, but NF-Y antibodies compete for the formation of the complex.

To confirm if NF-Y binds in vivo to the *SERCA2* promoter, chromatin from cultures of neonatal rat ventricular cardiomyocytes was used to perform ChIP assays. In these experiments, Sp1 antibody was used as a positive control due to the presence of several Sp1 binding sites on the proximal *SERCA2* promoter [26]. The ChIP results shown in Fig. 8 demonstrate that NF-YB and C-EBPβ binding are

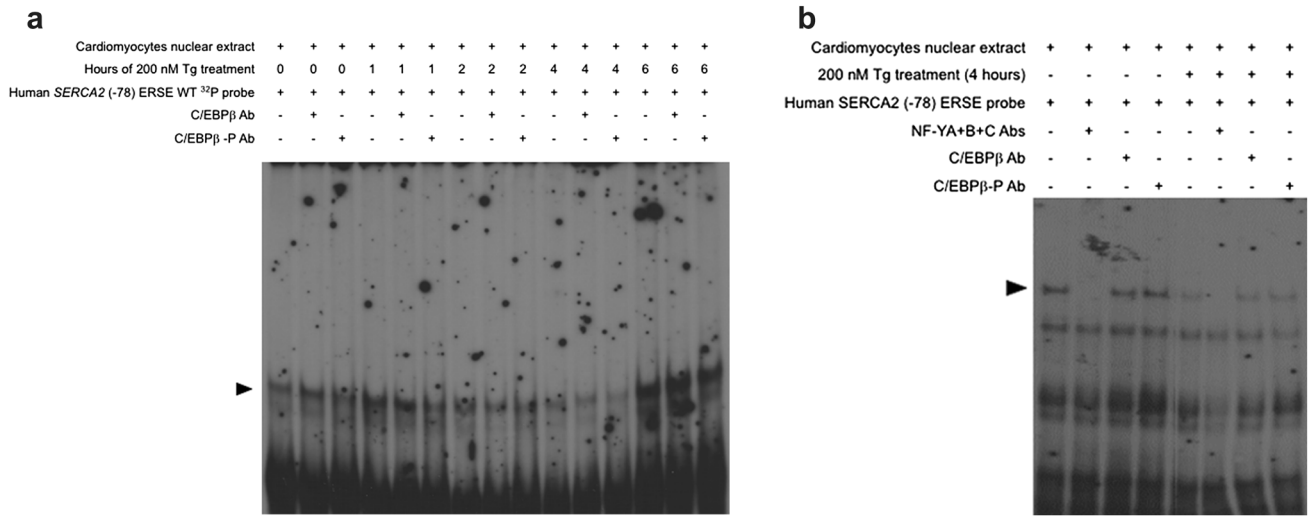

Fig. 7 Thapsigargin treatment modifies formation of the SERCA2-ERSE complex. **a** EMSAs were conducted using nuclear extracts from neonatal rat cardiomyocytes treated with Tg (200 nM) for the indicated times, and detected using a probe for human SERCA2 wild-type ERSE. **b** Nuclear extracts from untreated (lanes 1–4) or 200 nM Tg-treated neonatal rat cardiomyocytes for 4 h (lanes 5–8), were incubated with 6 μg of NF-Y antibodies (2 μg of anti NF-Y-A + 2 μg of anti NF-Y-B + 2 μg of anti NF-Y-C; lanes 2 and 6), 2 μg of anti C/EBPβ (lanes 3 and 7), or 2 μg of anti-phosphorylated C/EBPβ (lanes 4 and 8). The specific ERSE complex is indicated by an arrow. Representative image of four independent experiments

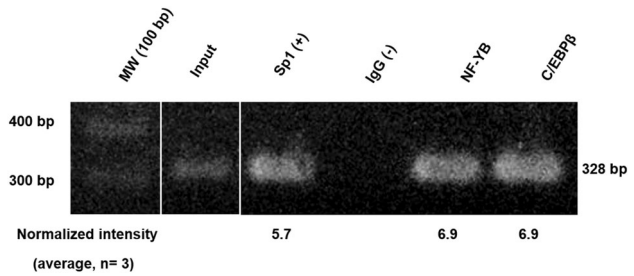

Fig. 8 NF-YB and C/EBPβ bind in vivo to the *SERCA2* proximal promoter. Representative image of ChIP assay where a region of 328 bp containing −268 bp of the rat *SERCA2* proximal promoter and 60 bp of 5′-non-translated was amplified. Fragmented chromatin obtained from neonatal rat ventricular cardiomyocytes was immunoprecipitated with NF-YB or C/EBPβ antibodies. Sp1 antibody and IgG were used as positive and negative controls, respectively. ChIPs were performed in three independent experiments, and each PCR was done twice. Average increase of the PCR product is shown below the image. All bands showed are from the same representative gel

enriched at region comprising positions between −268 and +60 of the *SERCA2* proximal promoter. These results corroborated EMSA experiments showing binding of NF-Y factors to the *SERCA2* promoter, demonstrating a strong participation of the CCAAT box in ERSE site at −78 bp in transcriptional regulation of basal and stress-mediated *SERCA2* increased expression.

Discussion

The participation of ATF6 in ER stress-induced *SERCA2* transcription was previously shown [11]. In that study, a constitutively active form of ATF6 was used to increase transcriptional activity of a construct containing 324 bp of rat *SERCA2* promoter. By depleting calcium from the SR of rat cardiomyocytes, ER stress was produced, and ATF6 participation in this process was shown. The ERSE site at −78 bp of the rat *SERCA2* promoter was described as the responsiveness motif to ATF6 and calcium depletion. Further, mutation of the YY1 site at −63 bp was shown to completely prevent responsiveness to constitutively active ATF6 but only partially prevented increase due to SR calcium depletion.

In this work, we show that mutation of the CCAAT box at −78 bp of the *hSERCA2* promoter completely abrogated the response to increased cytosolic calcium-induced by the SERCA2 inhibitor Tg and the calcium ionophore A23187. Furthermore, transient transfections and DNA–protein interaction studies remarked the importance of NF-Y transcription factor and the CCAAT box binding site in basal and Tg-induced *SERCA2* transcription since the presence of the CCAAT sequence is essential to promote the formation of a transcriptional complex in the *SERCA2* proximal ERSE. Functional assays showed that Tg was unable to increase pGL3-hSERCA2p-254 bp

transcriptional activity when the CCAAT box or YY1 sites were mutated (Fig. 2).

As shown in Figs. 4, 5, and 6, the ERSE at −98 bp of the *grp78* promoter highly resembles the −78 bp ERSE in the *SERCA2* gene. This similarity might help to extrapolate some previous observations about *grp78* transcriptional regulation to that of *SERCA2* since the *grp78* promoter is an extensively used model for transcriptional activation through ERSE sites.

NF-Y is a known ubiquitous transcription factor that controls transcription of several stress-inducible genes [17], and other gene promoters that bind NF-Y to the CCAAT box have been shown to be transcriptionally repressed in basal conditions [27, 28]. For example, in HepG2 cells, NF-Y specific binding to a CCAAT box present in the human Proline-rich Nuclear Receptor Co-activator (PNRC) promoter was demonstrated. Further, NF-Y overexpression repressed PNRC promoter transcriptional activity when CCAAT box was intact, but it did not when CCAAT box was mutated [27]. In cardiomyocytes, an isolated CCAAT box present at the Protein Disulfide Isomerase-associated 6 (PDI-6) promoter was shown to be bound by NF-Y and mediate PDI-6 basal repression [28]. The ability of NF-Y (A, B, and C) to bind CCAAT box has been well documented in many systems [17]; however, its direct influence on *SERCA2* transcription has not been described. Nevertheless, the CCAAT box is not the preferred binding site for C/EBP, its reported association with NF-Y led us to investigate if it could be involved in SERCA2 regulation [29–31]. As shown in Fig. 6, antibodies against NF-Y were capable of disrupting the interaction between cardiomyocyte nuclear extracts and human *SERCA2* −78 bp ERSE site. NF-Y binding to the human acetylcholinesterase promoter was shown in response to HeLa cells treatment with A23187 or Tg [32]. Further, in a second study, a decreased intensity for the NF-Y complex when cells were treated with A23187 was found [33]. On the other hand, intracellular calcium concentration reduction by the first-generation calcium channel blocker verapamil was shown to reduce cardiac expression of the pro-apoptotic thioredoxin-interacting protein (TXNIP) through NF-Y [34]. Further, that study showed that both verapamil and calcineurin inhibitor Cyclosporine A treatments induced NF-Y binding to TXNIP promoter suggesting that NF-Y binding to its targets in the heart is enhanced by decreased intracellular calcium and vice versa. Then, NF-Y binding to the *SERCA2* promoter confirms the importance of CCAAT box in *SERCA2* regulation while it raises the possibility of a repressing role for the ERSE complex in basal conditions. Regarding the effect of intracellular calcium concentration on NF-Y and/or C/EBP interaction with human *SERCA2* −78 bp ERSE, Fig. 7 showed that Tg diminished ERSE complex formation when administered for a maximum of 4 h but increased it when treatment was applied for 6 h. This behavior, even unexpected, is in accordance with the previous findings of transcriptional activation of the rat grp78 gene [14]. Figure 7b showed that Tg only diminished the intensity of bands due to ERSE complex, but it did not alter the interactions of the NF-Y antibody with the complex.

The ERSE complex formation decreased after the first 4 h of Tg treatment; it might be related to the previous observation that in the first hours of Tg treatment total ATF6 protein diminished [35, 36] and that ATF6 overexpression was shown to activate ERSE activity in the absence of stress stimuli [14]. It might be possible that in unstressed cells, Tg treatment initially produces a decrease in ERSE complex formation due to the reorganization of the transcription factors involved, but after stress-induced proteins have reached optimal levels and localizations, total content is increased reflecting the observed increase in *SERCA2* transcription.

Intriguingly, C/EBPβ antibody did not affect ERSE complex formation as shown in EMSA experiments (Figs. 6, 7), but ChIP assays showed that C/EBPβ is recruited to the proximal *SERCA2* gene promoter in vivo as effectively as NF-YB (Fig. 8). Both transcription factors have been associated with basal transcriptional machinery and stress response. Some studies have found coordination between these two transcription factors, showing that they can act synergistically in HepG2, mouse adrenal, and ameloblast-like cell lines [29–31]. Specifically, the mentioned cooperation was reported in murine macrophage-like J774.2 cells to activate the *mdr1b* gene, in HepG2 cells to activate microsomal epoxide hydrolase gene, and in the ameloblast-like cell line LS8 to increase amelogenin gene expression. In the last two studies, a mechanism for the synergistic activity of the transcription factors was proposed suggesting that C/EBPα would not activate gene expression directly through its interaction with DNA but rather through its association with DNA-bound NF-Y.

SERCA2 transcriptional regulation by stress is complex, and data presented in this study are focused on the ERSE located at −78 bp using a chimeric reporter that contains 254 bp upstream the transcription initiation site. However, it is important to mention that other cis-elements along the full length *SERCA2* regulatory region might also be important for stress response. One limitation of this study is the identification of other transcription factors that may participate along with NF-Y and C/EBPβ in the ERSE complex that regulates *SERCA2* transcription and requires future research that would clarify the mechanism investigated in our study. The specific differences and similarities between transcriptional control through NF-Y, C/EBPβ, or other transcription factors remain to be fully investigated,

and certainly, their elucidation will help to better understand *SERCA2* transcriptional regulation in the normal heart and different pathologies where ERS is involved.

The *SERCA2* 5′-regulatory region reveals the presence of potential binding sites for SP1, ATF, CREB, NFAT, MEF2, and GATA transcription factors that could participate in *SERCA2* transcription activated by calcium and pressure-overload in the heart [25, 37, 38]. Distal cis-acting elements have been found to regulate *SERCA2* expression. For instance, the MCAT element located at −1394 bp of the rabbit *SERCA2* gene interacts with TEF1, and enhances *SERCA2* transcription along with E box/AT-rich and CArG elements in Sol8 myotubes [39]. Hypoxic conditions have also been reported to regulate *SERCA2* transcription through hypoxia response elements at −1075 and −709 bp of the mouse *SERCA2* promoter [40].

In conclusion, in this work, we showed that the CCAAT box at −78 bp of *SERCA2* is an essential transcriptional regulatory site responsible for the basal and ERS-mediated *SERCA2* transcriptional regulation. Furthermore, binding of NF-YB and C/EBPβ to the *SERCA2* promoter, strongly suggests that *SERCA2* gene basal repression due to an ERSE complex might be a previously unexplored mechanism involved in normal and pathological cardiac function.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Clapham DE (2007) Calcium signaling. Cell 131(6):1047–1058. doi:10.1016/j.cell.2007.11.028
2. Bers DM (2002) Cardiac excitation-contraction coupling. Nature 415(6868):198–205. doi:10.1038/415198a
3. Caspersen C, Pedersen PS, Treiman M (2000) The sarco/endoplasmic reticulum calcium-ATPase 2b is an endoplasmic reticulum stress-inducible protein. J Biol Chem 275(29):22363–22372. doi:10.1074/jbc.M001569200
4. Prasad AM, Inesi G (2009) Effects of thapsigargin and phenylephrine on calcineurin and protein kinase C signaling functions in cardiac myocytes. Am J Physiol Cell Physiol 296(5):C992–C1002. doi:10.1152/ajpcell.00594.2008
5. Wu KD, Bungard D, Lytton J (2001) Regulation of SERCA Ca2 + pump expression by cytoplasmic Ca2 + in vascular smooth muscle cells. Am J Physiol Cell Physiol 280(4):C843–C851
6. Kaufman RJ (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 13(10):1211–1233
7. Marcus N, Green M (1997) NF-Y, a CCAAT box-binding protein, is one of the trans-acting factors necessary for the response of the murine ERp72 gene to protein traffic. DNA Cell Biol 16 (9):1123–1131. doi:10.1089/dna.1997.16.1123
8. Janetzki S, Palla D, Rosenhauer V, Lochs H, Lewis JJ, Srivastava PK (2000) Immunization of cancer patients with autologous cancer-derived heat shock protein gp96 preparations: a pilot study. Int J Cancer 88(2):232–238. doi:10.1002/1097-0215(20001015)88:2<232:AID-IJC14>3.0.CO
9. Ferrari DM, Soling HD (1999) The protein disulphide-isomerase family: unravelling a string of folds. Biochem J 339(Pt 1):1–10
10. High S, Lecomte FJ, Russell SJ, Abell BM, Oliver JD (2000) Glycoprotein folding in the endoplasmic reticulum: a tale of three chaperones? FEBS Lett 476(1–2):38–41
11. Thuerauf DJ, Hoover H, Meller J, Hernandez J, Su L, Andrews C, Dillmann WH, McDonough PM, Glembotski CC (2001) Sarco/endoplasmic reticulum calcium ATPase-2 expression is regulated by ATF6 during the endoplasmic reticulum stress response: intracellular signaling of calcium stress in a cardiac myocyte model system. J Biol Chem 276(51):48309–48317. doi:10.1074/jbc.M107146200
12. Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M, Mori K (2001) Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6alpha and 6beta that activates the mammalian unfolded protein response. Mol Cell Biol 21 (4):1239–1248. doi:10.1128/MCB.21.4.1239-1248.2001
13. Baumeister P, Luo S, Skarnes WC, Sui G, Seto E, Shi Y, Lee AS (2005) Endoplasmic reticulum stress induction of the Grp78/BiP promoter: activating mechanisms mediated by YY1 and its interactive chromatin modifiers. Mol Cellular Biol 25(11):4529–4540. doi:10.1128/MCB.25.11.4529-4540.2005
14. Li M, Baumeister P, Roy B, Phan T, Foti D, Luo S, Lee AS (2000) ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. Mol Cell Biol 20 (14):5096–5106
15. Roy B, Lee AS (1999) The mammalian endoplasmic reticulum stress response element consists of an evolutionarily conserved tripartite structure and interacts with a novel stress-inducible complex. Nucl Acids Res 27(6):1437–1443
16. Nardini M, Gnesutta N, Donati G, Gatta R, Forni C, Fossati A, Vonrhein C, Moras D, Romier C, Bolognesi M, Mantovani R (2013) Sequence-specific transcription factor NF-Y displays histone-like DNA binding and H2B-like ubiquitination. Cell 152 (1–2):132–143. doi:10.1016/j.cell.2012.11.047
17. Dolfini D, Gatta R, Mantovani R (2012) NF-Y and the transcriptional activation of CCAAT promoters. Crit Rev Biochem Mol Biol 47(1):29–49. doi:10.3109/10409238.2011.628970
18. Tsukada J, Yoshida Y, Kominato Y, Auron PE (2011) The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. Cytokine 54(1):6–19. doi:10.1016/j.cyto.2010.12.019
19. Ambrosino C, Iwata T, Scafoglio C, Mallardo M, Klein R, Nebreda AR (2006) TEF-1 and C/EBPbeta are major p38alpha

MAPK-regulated transcription factors in proliferating cardiomyocytes. Biochem J 396(1):163–172. doi:10.1042/BJ20051502
20. Bostrom P, Mann N, Wu J, Quintero PA, Plovie ER, Panakova D, Gupta RK, Xiao C, MacRae CA, Rosenzweig A, Spiegelman BM (2010) C/EBPbeta controls exercise-induced cardiac growth and protects against pathological cardiac remodeling. Cell 143 (7):1072–1083. doi:10.1016/j.cell.2010.11.036
21. Huang GN, Thatcher JE, McAnally J, Kong Y, Qi X, Tan W, DiMaio JM, Amatruda JF, Gerard RD, Hill JA, Bassel-Duby R, Olson EN (2012) C/EBP transcription factors mediate epicardial activation during heart development and injury. Science 338 (6114):1599–1603. doi:10.1126/science.1229765
22. Reyes-Juarez JL, Juarez-Rubi R, Rodriguez G, Zarain-Herzberg A (2007) Transcriptional analysis of the human cardiac calsequestrin gene in cardiac and skeletal myocytes. J Biol Chem 282 (49):35554–35563. doi:10.1074/jbc.M707788200
23. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucl Acids Res 29(9):e45
24. Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 15(3):532–534, 536–537
25. Zarain-Herzberg A, Alvarez-Fernandez G (2002) Sarco(endo)-plasmic reticulum Ca2 + -ATPase-2 gene: structure and transcriptional regulation of the human gene. Sci World J 2:1469–1483. doi:10.1100/tsw.2002.228
26. Brady M, Koban MU, Dellow KA, Yacoub M, Boheler KR, Fuller SJ (2003) Sp1 and Sp3 transcription factors are required for trans-activation of the human SERCA2 promoter in cardiomyocytes. Cardiovasc Res 60(2):347–354
27. Zhang Y, Chen B, Li Y, Chen J, Lou G, Chen M, Zhou D (2008) Transcriptional regulation of the human PNRC promoter by NFY in HepG2 cells. J Biochem 143(5):675–683. doi:10.1093/jb/mvn019
28. Vekich JA, Belmont PJ, Thuerauf DJ, Glembotski CC (2012) Protein disulfide isomerase-associated 6 is an ATF6-inducible ER stress response protein that protects cardiac myocytes from ischemia/reperfusion-mediated cell death. J Mol Cell Cardiol 53 (2):259–267. doi:10.1016/j.yjmcc.2012.05.005
29. Yu L, Wu Q, Yang CP, Horwitz SB (1995) Coordination of transcription factors, NF-Y and C/EBP beta, in the regulation of the mdr1b promoter. Cell Growth Differ 6(12):1505–1512
30. Xu Y, Zhou YL, Luo W, Zhu QS, Levy D, MacDougald OA, Snead ML (2006) NF-Y and CCAAT/enhancer-binding protein alpha synergistically activate the mouse amelogenin gene. J Biol Chem 281(23):16090–16098. doi:10.1074/jbc.M510514200
31. Zhu QS, Qian B, Levy D (2004) CCAAT/enhancer-binding protein alpha (C/EBPalpha) activates transcription of the human microsomal epoxide hydrolase gene (EPHX1) through the interaction with DNA-bound NF-Y. J Biol Chem 279(29):29902–29910. doi:10.1074/jbc.M400438200
32. Zhu H, Gao W, Jiang H, Jin QH, Shi YF, Tsim KW, Zhang XJ (2007) Regulation of acetylcholinesterase expression by calcium signaling during calcium ionophore A23187- and thapsigargin-induced apoptosis. Int J Biochem Cell Biol 39(1):93–108. doi:10.1016/j.biocel.2006.06.012
33. Zhu H, Gao W, Shi YF, Zhang XJ (2007) The CCAAT-binding factor CBF/NF-Y regulates the human acetylcholinesterase promoter activity during calcium ionophore A23187-induced cell apoptosis. Biochim Biophys Acta 1770(10):1475–1482. doi:10.1016/j.bbagen.2007.07.007
34. Cha-Molstad H, Xu G, Chen J, Jing G, Young ME, Chatham JC, Shalev A (2012) Calcium channel blockers act through nuclear factor Y to control transcription of key cardiac genes. Mol Pharmacol 82(3):541–549. doi:10.1124/mol.112.078253
35. Haze K, Yoshida H, Yanagi H, Yura T, Mori K (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 10(11):3787–3799
36. Yoshida H, Haze K, Yanagi H, Yura T, Mori K (1998) Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. J Biol Chem 273(50):33741–33749
37. Takizawa T, Arai M, Tomaru K, Koitabashi N, Baker DL, Periasamy M, Kurabayashi M (2003) Transcription factor Sp1 regulates SERCA2 gene expression in pressure-overloaded hearts: a study using in vivo direct gene transfer into living myocardium. J Mol Cell Cardiol 35(7):777–783
38. Takizawa T, Arai M, Yoguchi A, Tomaru K, Kurabayashi M, Nagai R (1999) Transcription of the SERCA2 gene is decreased in pressure-overloaded hearts: a study using in vivo direct gene transfer into living myocardium. J Mol Cell Cardiol 31(12):2167–2174. doi:10.1006/jmcc.1999.1045
39. Baker DL, Dave V, Reed T, Misra S, Periasamy M (1998) A novel E box/AT-rich element is required for muscle-specific expression of the sarcoplasmic reticulum Ca2 + -ATPase (SERCA2) gene. Nucl Acids Res 26(4):1092–1098
40. Ronkainen VP, Skoumal R, Tavi P (2011) Hypoxia and HIF-1 suppress SERCA2a expression in embryonic cardiac myocytes through two interdependent hypoxia response elements. J Mol Cell Cardiol 50(6):1008–1016. doi:10.1016/j.yjmcc.2011.02.017