

Sigma1 receptors inhibit store-operated Ca^{2+} entry by attenuating coupling of STIM1 to Orai1

Shyam Srivats,¹ Dilshan Balasuriya,¹ Mathias Pasche,² Gerard Vistal,¹ J. Michael Edwardson,¹ Colin W. Taylor,¹ and Ruth D. Murrell-Lagnado^{1,3}

¹Department of Pharmacology, University of Cambridge, Cambridge CB2 1PD, England, UK

²MRC Laboratory for Molecular Biology, Cambridge CB2 0QH, England, UK

³Sussex Neuroscience, School of Life Sciences, University of Sussex, Brighton BN1 9QG, England, UK

Sigma1 receptors (σ 1Rs) are expressed widely; they bind diverse ligands, including psychotropic drugs and steroids, regulate many ion channels, and are implicated in cancer and addiction. It is not known how σ 1Rs exert such varied effects. We demonstrate that σ 1Rs inhibit store-operated Ca^{2+} entry (SOCE), a major Ca^{2+} influx pathway, and reduce the Ca^{2+} content of the intracellular stores. SOCE was inhibited by expression of σ 1R or an agonist of σ 1R and enhanced by loss of σ 1R or an antagonist. Within the endoplasmic reticulum (ER), σ 1R associated with STIM1, the ER Ca^{2+} sensor that regulates SOCE. This interaction was modulated by σ 1R ligands. After depletion of Ca^{2+} stores, σ 1R accompanied STIM1 to ER–plasma membrane (PM) junctions where STIM1 stimulated opening of the Ca^{2+} channel, Orai1. The association of STIM1 with σ 1R slowed the recruitment of STIM1 to ER–PM junctions and reduced binding of STIM1 to PM Orai1. We conclude that σ 1R attenuates STIM1 coupling to Orai1 and thereby inhibits SOCE.

Introduction

Sigma1 receptors (σ 1Rs) are widely distributed in the brain and peripheral tissues, including lung, kidney, liver, and spleen, and highly expressed in some tumor cells (Walker et al., 1990; Vilner et al., 1995; Monnet, 2005; Stone et al., 2006; Cobos et al., 2008; Wu and Bowen, 2008; Su et al., 2010; Brune et al., 2013). They are regulated by an unusually diverse array of ligands, including endogenous steroids, drugs of abuse such as cocaine and methamphetamine, and drugs used to treat depression, anxiety, psychosis, pain, and neurodegenerative diseases (Maurice et al., 1999; Waterhouse et al., 2007; Maurice and Su, 2009; Su et al., 2010; Navarro et al., 2012; Robson et al., 2012; Wünsch, 2012; Kourrich et al., 2013; Tsai et al., 2014). Changes in expression and polymorphisms of σ 1Rs are associated with heart failure (Ito et al., 2012, 2013), addiction (Maurice et al., 2002; Kourrich et al., 2013), neurodegenerative and psychiatric disorders (Miki et al., 2014; Tsai et al., 2014), and cancer (Spruce et al., 2004; Wang et al., 2004; Aydar et al., 2006; Maurice and Su, 2009; Crottès et al., 2013). These associations have provoked interest in σ 1Rs as both therapeutic targets and diagnostic tools (van Waarde et al., 2015).

The σ 1R is an integral membrane protein with two trans-membrane domains. It is expressed in the ER, where it is

concentrated in cholesterol-rich mitochondrion-associated ER membrane (MAM) domains and bound to the ER luminal chaperone, BiP (Fig. S1; Hayashi and Su, 2003, 2007; Palmer et al., 2007). Agonists of σ 1R cause it to dissociate from BiP and MAM, allowing σ 1Rs to move within ER membranes and interact with signaling proteins in the plasma membrane (PM), most notably ion channels, thereby regulating their activity (Su et al., 2010; Balasuriya et al., 2012; Pabba, 2013). Antagonists block this effect (Fig. S1 and Table S1). Loss of Ca^{2+} from the ER can also release σ 1Rs from their interaction with BiP, freeing them to interact with other proteins (Hayashi and Su, 2007). In addition to regulating the activity of these proteins, σ 1Rs can also act as chaperones, stabilizing signaling proteins as they traffic along the secretory pathway (Tsai et al., 2014). σ 1Rs may also be expressed in the nuclear envelope (Hayashi and Su, 2005a,b; Brune et al., 2013; Mori et al., 2013) and PM (Lupardus et al., 2000; Aydar et al., 2002; Brune et al., 2013; Balasuriya et al., 2014a) and may even be secreted into the extracellular space (Hayashi and Su, 2003; Su et al., 2010). The interactions between σ 1Rs and ion channels may therefore occur within the plane of a membrane (ER or PM) or across ER–PM junctions (Hayashi and Su, 2007; Kourrich et al., 2013; Balasuriya et al., 2014a). Clearly, σ 1Rs are important links between diverse ligands, physiological stimuli, and many key signaling molecules (Hayashi and Su, 2007; Su et al., 2010; Kourrich et al., 2013).

Correspondence to Ruth D. Murrell-Lagnado: R.Murrell-Lagnado@sussex.ac.uk

Abbreviations used in this paper: AFM, atomic force microscopy; ANOVA, analysis of variance; CAD, channel-activating domain; CPA, cyclopiazonic acid; HBS, Hepes-buffered saline; HEK, human embryonic kidney; IP_3 R, IP_3 receptor; MAM, mitochondrion-associated ER membrane; NFAT, nuclear factor of activated T cells; PM, plasma membrane; POST, partner of STIM1; SARAF, SOCE-associated regulatory factor; σ 1R, sigma1 receptor; SOCE, store-operated Ca^{2+} entry; STIM, stromal interaction molecule; TIRF, total internal reflection fluorescence; TS, Triton solution.

© 2016 Srivats et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see <http://www.rupress.org/terms>). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at <http://creativecommons.org/licenses/by-nc-sa/3.0/>).



Receptors that stimulate PLC and formation of inositol 1,4,5-trisphosphate (IP₃) evoke both Ca²⁺ release from the ER through IP₃ receptors (IP₃Rs) and Ca²⁺ entry across the PM. At MAMs, Ca²⁺ released by IP₃Rs can be rapidly accumulated by mitochondria, thereby stimulating oxidative phosphorylation (Rizzuto et al., 2012) and promoting cell survival (Cárdenas et al., 2010), whereas excessive mitochondrial Ca²⁺ uptake triggers apoptosis (Mallilankaraman et al., 2012). The association of IP₃Rs with σ 1Rs at MAMs supports the transfer of Ca²⁺ from the ER to mitochondria by curtailing the degradation of active IP₃Rs (Hayashi and Su, 2007). The increase in mitochondrial Ca²⁺ concentration and resultant boost in oxidative phosphorylation are thought to contribute to the prosurvival effects of σ 1Rs in the central nervous system and cancer cells (Lewis et al., 2014). One effect of σ 1Rs may therefore be to support transfer of Ca²⁺ from the ER to mitochondria, but this transfer also depends on the Ca²⁺ content of the ER.

The Ca²⁺ entry evoked by receptors that stimulate PLC is usually mediated by store-operated Ca²⁺ entry (SOCE), which is stimulated by loss of Ca²⁺ from the ER (Parekh and Putney, 2005; Hogan and Rao, 2015). The reduction in Ca²⁺ concentration within the ER is detected by the luminal EF hands of stromal interaction molecule 1 (STIM1), an integral ER membrane protein. This causes STIM1 to cluster and accumulate at ER–PM junctions. STIM1 then binds to Orai1, a Ca²⁺-permeable channel in the PM, and activates it (Lewis, 2007; Soboloff et al., 2012; Wu et al., 2014). The contributions of related proteins (Orai2, Orai3, and STIM2) to SOCE are not fully resolved (Hoth and Niemeyer, 2013), although STIM2 is usually more important than STIM1 for refilling of Ca²⁺ stores (Brandman et al., 2007). Additional proteins, including juncate, CRACR2, and SOCE-associated regulatory factor (SARAF), also interact with STIM1–Orai1 signaling complexes and regulate both activation and deactivation of SOCE (Srikanth et al., 2010, 2012, 2013; Palty et al., 2012; Srikanth and Gwack, 2012, 2013).

We show that σ 1Rs constitutively inhibit SOCE and reduce the Ca²⁺ content of the ER and that σ 1R ligands modulate this inhibition. The σ 1R associates with STIM1 in the ER and is conveyed with STIM1 to ER–PM junctions after store depletion. This association slows the recruitment of STIM1 to the junctions and reduces binding of STIM1 to Orai1. Our results establish that σ 1Rs inhibit a ubiquitous Ca²⁺ entry pathway and suggest a general model for directed translocation of σ 1R to its PM targets.

Results

σ 1R inhibits SOCE

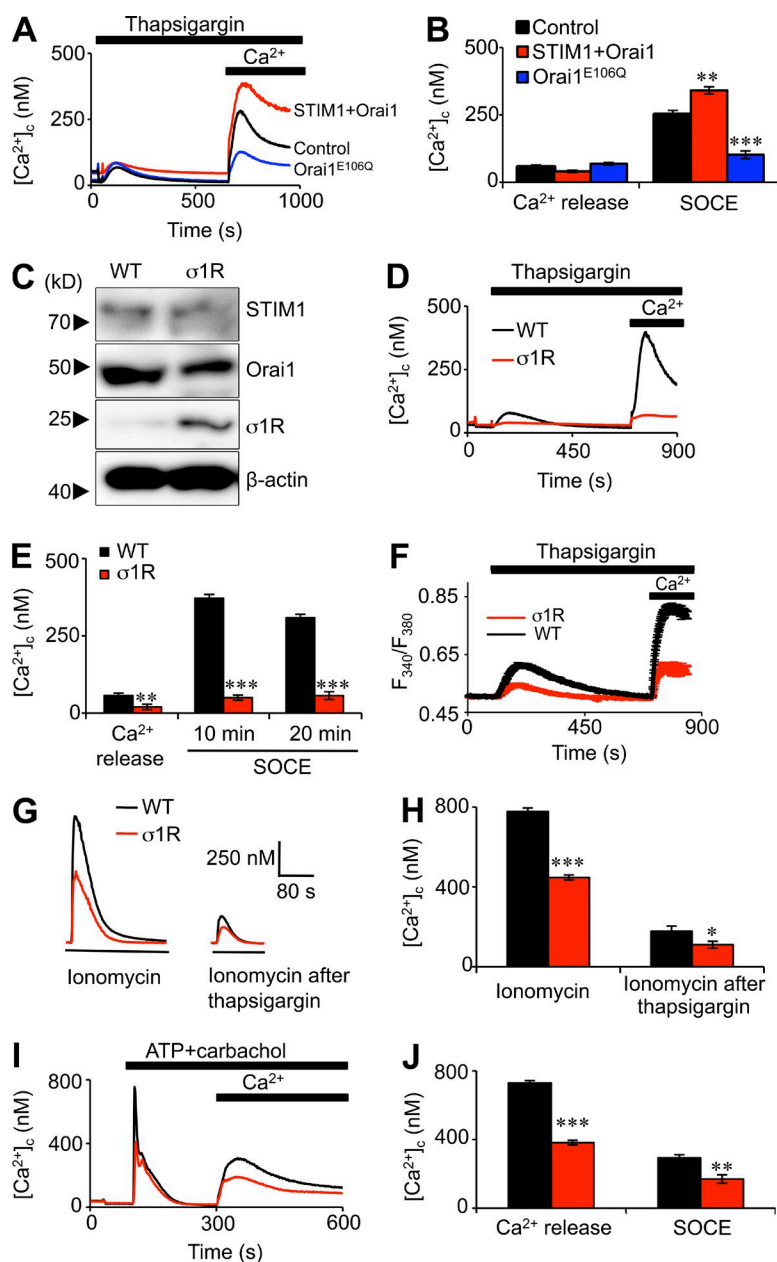
SOCE in human embryonic kidney (HEK) cells can be activated by depletion of intracellular Ca²⁺ stores using thapsigargin to inhibit the ER Ca²⁺ pump or by stimuli of endogenous receptors (e.g., ATP or carbachol) that activate PLC. The contributions of Orai1 and STIM1 to SOCE (Parekh and Putney, 2005; DeHaven et al., 2009; Soboloff et al., 2012) are clear from the inhibition of thapsigargin-evoked Ca²⁺ entry in HEK cells expressing a dominant-negative form of Orai1 (Orai1^{E106Q}; Prakriya et al., 2006) and the enhancement of SOCE after overexpression of Orai1 with STIM1 (Fig. 1, A and B). The initial Ca²⁺ release evoked by thapsigargin was unaffected by these effects of Orai1 and STIM on SOCE. Stable expression of a V5-tagged σ 1R in HEK cells (HEK- σ 1R cells) attenuated the Ca²⁺ signals

evoked by thapsigargin without affecting expression of Orai1 or STIM1 ($103 \pm 5\%$ and $91 \pm 8\%$ of wild-type cells, respectively; Fig. 1 C) or the basal cytosolic free Ca²⁺ concentration ([Ca²⁺]_i; 45 ± 7 nM and 50 ± 3 nM in wild-type and HEK- σ 1R cells, respectively). The increase in [Ca²⁺]_i after addition of thapsigargin in Ca²⁺-free medium was reduced by $65 \pm 9\%$, and the SOCE detected after restoration of extracellular Ca²⁺ was reduced by $86 \pm 4\%$ in HEK- σ 1R cells (Fig. 1, D and E). The rate of increase of [Ca²⁺]_i during SOCE decreased from 8.8 ± 0.3 nM·s⁻¹ in wild-type HEK cells to 2.8 ± 0.3 nM·s⁻¹ in HEK- σ 1R cells. SOCE in HEK- σ 1R cells was similarly reduced across a range of extracellular Ca²⁺ concentrations (Fig. S2 A). The inhibition of both thapsigargin-evoked Ca²⁺ release and SOCE in HEK- σ 1R cells was also observed at 37°C (Fig. S2 B) and in single-cell measurements (Fig. 1 F). The diminished SOCE did not result from ineffective store emptying because it was unaffected by prolonging the incubation with thapsigargin from 10 to 20 min (Fig. 1 E). Indeed, both the initial Ca²⁺ content of the stores (determined by addition of ionomycin in Ca²⁺-free medium, the effects of which are not restricted to the ER) and the residual content after thapsigargin treatment were reduced in HEK- σ 1R cells (Fig. 1, G and H). When ATP and carbachol were used to deplete Ca²⁺ stores via endogenous pathways, the Ca²⁺ release and Ca²⁺ entry were also attenuated in HEK- σ 1R cells (Fig. 1, I and J). The lesser Ca²⁺ release evoked by ATP and carbachol in HEK- σ 1R cells ($52 \pm 8\%$ of wild-type cells) matched the reduced Ca²⁺ content of the stores ($59 \pm 9\%$), suggesting that this was responsible for the diminished response to PLC-coupled receptors.

To investigate whether sustained depletion of Ca²⁺ stores might itself cause down-regulation of SOCE, HEK cells were treated with cyclopiazonic acid (CPA) to reversibly inhibit the ER Ca²⁺ pump for a 2-h period that later experiments (see Fig. 3) show is sufficient for σ 1R agonists to inhibit SOCE. This CPA treatment caused a more substantial depletion of the intracellular Ca²⁺ stores than was observed in HEK- σ 1R cells, but a much smaller inhibition of the SOCE evoked by subsequent addition of thapsigargin (Fig. 2, A and B). These results establish that loss of Ca²⁺ from the ER does not cause the reduced SOCE in HEK- σ 1R cells.

The smaller increase in [Ca²⁺]_i evoked by SOCE in HEK- σ 1R cells could result from decreased Ca²⁺ entry or enhanced Ca²⁺ extrusion. However, rates of recovery from Ca²⁺ signals evoked by carbachol and ATP in Ca²⁺-free medium (measured over matched [Ca²⁺]_i) were unaffected by expression of σ 1R (half-times, $t_{1/2} = 36 \pm 5$ s and 32 ± 6 s for wild-type and HEK- σ 1R cells, respectively). The smaller [Ca²⁺]_i increases in HEK- σ 1R cells were not, therefore, due to more effective buffering or Ca²⁺ extrusion. Because most Ca²⁺ extrusion pathways do not transport Mn²⁺, we used quenching of fura 2 fluorescence to measure unidirectional Mn²⁺ influx through the SOCE pathway (Fig. 2 C). Thapsigargin, or carbachol with ATP, stimulated Mn²⁺ entry in HEK cells, consistent with the activation of SOCE in response to store depletion. In HEK- σ 1R cells, there was no change in the rate of Mn²⁺ entry in response to either stimulus (Fig. 2 D). Together, these results establish that stable expression of σ 1R inhibits SOCE.

Selection of polyclonal HEK cells stably expressing σ 1R might have propagated cells with different Ca²⁺ signaling behaviors. However, the thapsigargin-evoked increase in [Ca²⁺]_i and SOCE and the Ca²⁺ content of the intracellular stores were also reduced in HEK cells transiently expressing σ 1Rs (Fig. S2,



C–E). The reduced SOCE correlated with the level of expression of σ 1R (Fig. S2, F and G). Translocation of GFP-tagged nuclear factor of activated T cells (NFAT) from the cytosol to the nucleus requires SOCE (Kar et al., 2011). SOCE stimulated NFAT translocation in HEK cells, and the response was attenuated to similar degrees in cells stably or transiently expressing σ 1R (Fig. 2, E and F).

We conclude that expression of σ 1R inhibits SOCE by reducing the coupling of empty stores to the activation of SOCE.

Agonists and antagonists of σ 1R regulate SOCE

The σ 1R agonist (+)SKF10047 (Su et al., 2010; Navarro et al., 2012) and the antagonist BD1047 (Fig. S1; Skuza and Rogó, 2006; Gromek et al., 2014) were used to investigate the acute effects of σ 1Rs in CHO cells and HEK- σ 1R cells. In CHO cells, σ 1Rs are endogenously expressed (Hayashi and Su, 2007). As in HEK cells, SOCE was inhibited by transient expression of

Orai1^{E106Q}, although in CHO cells, the thapsigargin-evoked Ca^{2+} release was also inhibited (Fig. S3 A). In both CHO and HEK- σ 1R cells, preincubation with BD1047 increased the amplitude of the Ca^{2+} signals evoked by SOCE, whereas the agonist (+)SKF10047 had the opposite effect (Fig. 3, A–D). Neither ligand affected SOCE in wild-type HEK cells (Fig. 3, E and F), confirming that the effects are mediated by σ 1Rs. The temperature dependence and slow equilibration of ligand binding to σ 1Rs (Yamamoto et al., 2001; Chu and Ruoho, 2016), together with the need to load cells with Ca^{2+} indicators at 20°C, limited opportunities to investigate the time course of the effects of σ 1R ligands. Nevertheless, it is clear that treatment with ligands for at least 1 h at 37°C before loading cells with Ca^{2+} indicator (1.5 h) in the continued presence of ligands was required to detect significant effects of the ligands on SOCE (Fig. S3, B–D).

In CHO cells, siRNA to σ 1R almost abolished expression of endogenous σ 1R, but this was accompanied by reduced expression of Orai1 and increased expression of STIM1 (Fig. 3, G

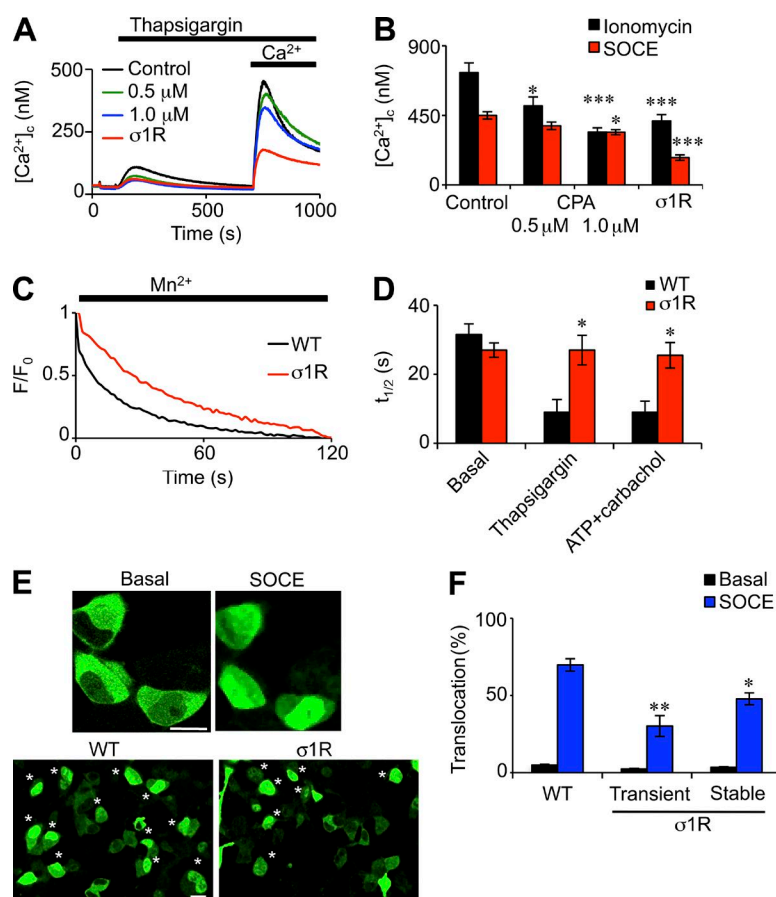


Figure 2. Stable and transient expression of σ 1R inhibits SOCE. (A) Ca^{2+} signals evoked by 1 μ M thapsigargin in Ca^{2+} -free HBS followed by restoration of 4 mM extracellular Ca^{2+} in HEK wild-type cells treated with CPA (0.5 μ M or 1 μ M for 2.5 h) or HEK- σ 1R cells. (B) Summary results show peak increases in $[Ca^{2+}]_i$ evoked by SOCE or by addition of ionomycin in Ca^{2+} -free HBS ($n = 3$). (C) Populations of fura 2-loaded cells were treated with thapsigargin (5 μ M for 10 min) in nominally Ca^{2+} -free HBS before addition of 5 mM $MnCl_2$. Results show normalized fluorescence intensity (F/F_0) for six replicates. WT, wild type. (D) Summary results ($n = 3$) show half-times ($t_{1/2}$) for fluorescence quenching from unstimulated cells (basal) and cells treated with thapsigargin (5 μ M for 10 min) or ATP and carbachol (100 μ M each for 3.5 min). (E) Typical images of HEK cells expressing NFAT-GFP before and 30 min after addition of 5 μ M thapsigargin in normal HBS (top). Bar, 10 μ m. Images of larger fields (bottom) show thapsigargin-treated HEK wild-type and HEK- σ 1R cells. Asterisks indicate cells used for analysis. Bar, 20 μ m. (F) Summary results show nuclear translocation of NFAT-GFP before and after treatment with thapsigargin (percentage of cells; six independent fields, with between 595 and 660 cells counted for each condition). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Student's t test (D) or ANOVA with Tukey's posthoc analysis (B and F). Results show mean \pm SEM.

and H). The loss of Orai1 could reflect a chaperone role for σ 1R similar to the stabilization of human ether-a-go-go-related gene (HERG) K^+ channels by σ 1R (Hayashi and Su, 2007; Crottès et al., 2011). Alternatively, Orai1 expression may be down-regulated through an adaptive feedback mechanism arising from the reduced inhibition of SOCE after loss of σ 1Rs. Overstimulation of SOCE by constitutively active STIM1 was shown previously to reduce Orai1 expression (Kilch et al., 2013). Despite the reduced expression of Orai1, SOCE was increased in CHO cells lacking σ 1Rs, and it was then unaffected by (+)SKF10047 or BD1047 (Fig. 3, I and J). The enhanced SOCE in CHO cells lacking σ 1Rs was abolished by expression of the dominant-negative form of Orai1 (Fig. S3 G), confirming that it was mediated by Orai1. SOCE monitored by unidirectional Mn^{2+} entry was also increased in CHO cells treated with siRNA to σ 1R (Fig. S3, E and F). In normal CHO cells, (+)SKF10047 reduced the Ca^{2+} content of the stores, whereas the σ 1R antagonist BD1047 increased their content to a level that matched that of cells without σ 1Rs. Neither ligand affected the Ca^{2+} stores in CHO cells lacking σ 1Rs (Fig. 3, K and L). Although comparison of SOCE-mediated Ca^{2+} signals in CHO cells with and without σ 1Rs is compromised by accompanying changes in STIM1 and Orai1 expression (Fig. 3, G and H), the analyses demonstrate that σ 1R ligands are effective only in cells expressing σ 1Rs, and they establish a constitutive inhibition of SOCE by endogenous σ 1Rs and an associated reduction in ER Ca^{2+} content in CHO cells (Fig. 3, I–L). Similar results were obtained in HEK- σ 1R cells: siRNA to σ 1R abolished the effects of σ 1R ligands on both SOCE and the Ca^{2+} content of the stores; it also increased the basal Ca^{2+} content of the stores and the rate of

Mn^{2+} entry evoked by either thapsigargin or by the more physiological stimuli, ATP and carbachol (Fig. S3, H and J).

Determining whether ligands of σ 1R are more effective before or after depletion of Ca^{2+} stores was frustrated by the need for prolonged preincubations at 37°C for optimal effects (Fig. S3, B–D). In a modified protocol, fluo 4-loaded HEK- σ 1R cells in Ca^{2+} -free Hepes-buffered saline (HBS) were incubated with (+)SKF10047 or BD1047 for 2 h at 20°C, with thapsigargin added either immediately before the ligands or after the 2-h incubation. Under these conditions, where the effects of the ligands were much reduced, (+)SKF10047 modestly inhibited SOCE, and BD1047 modestly enhanced SOCE, but only when added before store depletion (Fig. S3 K). These results suggest that σ 1R ligands affect an early step in the activation of SOCE.

Breast cancer cells express high levels of σ 1Rs (Spruce et al., 2004; Wang et al., 2004; Aydar et al., 2006). In MDA-MB-231 human breast cancer cells, which also express σ 1Rs, SOCE was enhanced by BD1047 and inhibited by (+)SKF10047 (Fig. S4). The Ca^{2+} content of the stores was also reduced by (+)SKF10047. Hence, in three cell types, HEK- σ 1R, CHO, and MDA-MB-231 cells, σ 1Rs both inhibit SOCE and decrease the Ca^{2+} content of the ER. The inverse agonist effect of BD1047 in CHO and MDA-MB-231 cells suggests a constitutive regulation of SOCE and ER Ca^{2+} content by endogenous σ 1Rs.

σ 1R and STIM1 associate and move to ER-PM junctions after store depletion

Interactions between σ 1R and STIM1 in unstimulated cells were investigated using HEK cells transiently expressing STIM1-Myc and σ 1R-FLAG. Anti-Myc beads pulled down σ 1R-FLAG

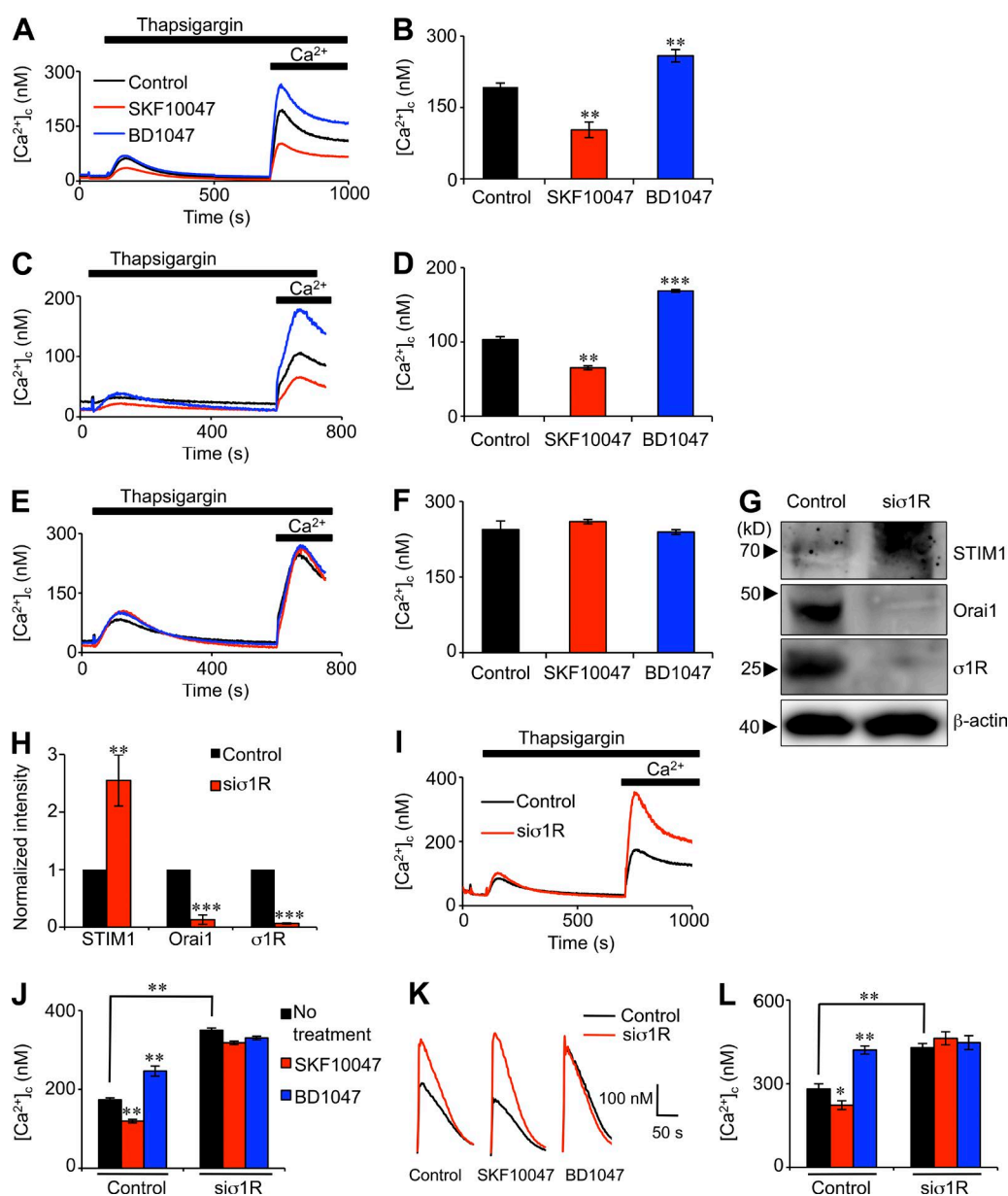


Figure 3. Ligands of $\sigma 1R$ modulate SOCE. (A–F) Populations of cells were treated with 25 μM (+)SKF10047 or 10 μM BD1047 before removal of extracellular Ca^{2+} , addition of 5 μM thapsigargin, and then restoration of extracellular 4 mM Ca^{2+} to CHO (A and B), HEK- $\sigma 1R$ (C and D), or wild-type HEK cells (E and F). Representative results (B, D, and F) show peak increases in $[Ca^{2+}]_i$ after restoration of extracellular Ca^{2+} . The color codes in A apply to all panels (A–F). (G) Representative immunoblot from CHO cells transfected with control plasmid or plasmid encoding siRNA for $\sigma 1R$ (si $\sigma 1R$). (H) Summary results show band intensities for the indicated proteins normalized to those from cells treated with control plasmid. (I) Ca^{2+} signals evoked by addition of thapsigargin in Ca^{2+} -free HBS and then restoration of extracellular Ca^{2+} in CHO cells treated with si $\sigma 1R$ or control plasmid. (J) Summary shows peak $[Ca^{2+}]_i$ after restoration of extracellular Ca^{2+} to thapsigargin-treated CHO cells treated with si $\sigma 1R$ or control plasmid. Cells were pretreated with 25 μM (+) SKF10047 or 10 μM BD1047, as indicated. (K and L) Effects of si $\sigma 1R$ or control plasmid and pretreatment with $\sigma 1R$ ligands on the Ca^{2+} signals evoked by 5 μM ionomycin in Ca^{2+} -free HBS. Typical traces (K) and summary results (L) are shown. Legends for L are the same as J. All summary results show mean \pm SEM. $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ANOVA with Tukey's posthoc analysis (B, D, F, J, and L) or Student's t test (H and comparison of no treatment conditions in J and L).

from solubilized cell extracts, but only in cells expressing STIM1-Myc. Conversely, anti-FLAG beads pulled down STIM1-Myc, but only in cells expressing $\sigma 1R$ -FLAG (Fig. 4, A and B). Coimmunoprecipitation of $\sigma 1R$ -FLAG with STIM1-Myc was enhanced by (+)SKF10047 and reduced by BD1047 (Fig. 4, C–E). These results show that STIM1 and $\sigma 1R$ are associated in unstimulated cells and that their interaction is regulated by $\sigma 1R$ ligands. Furthermore, the increased association

of $\sigma 1R$ with STIM1 evoked by the $\sigma 1R$ agonist (Fig. 4) correlates with the inhibition of SOCE (Fig. 3).

To investigate the intracellular dynamics of $\sigma 1R$ and STIM1, we used HeLa cells because they are better suited than HEK cells for optical analyses of ER proteins while still lacking detectable endogenous $\sigma 1R$ s (Fig. 5 A). In cells expressing $\sigma 1R$ -EGFP with mCh-STIM1, $\sigma 1R$ -EGFP and mCh-STIM1 colocalized within the ER (Mander's correlation coefficient

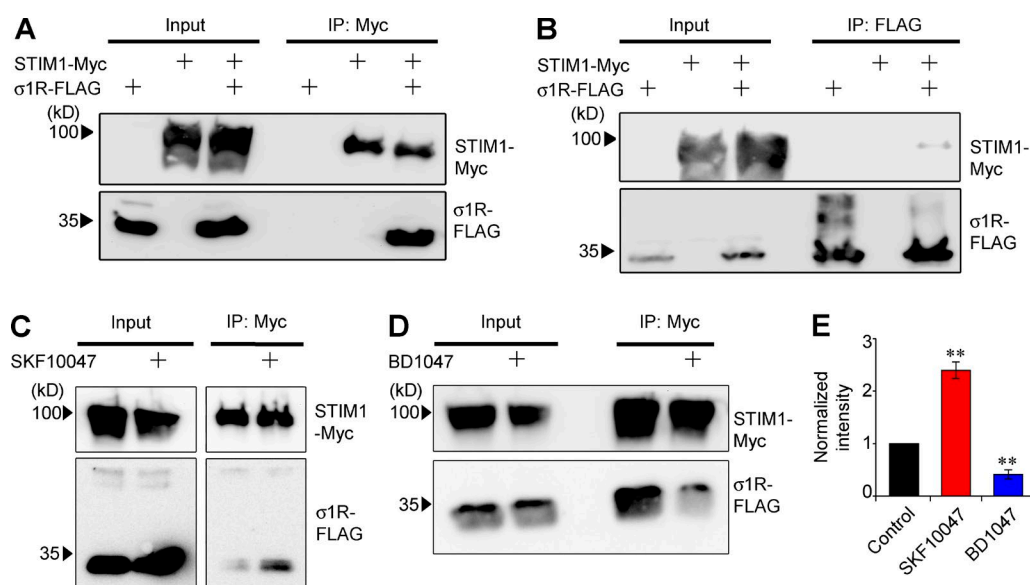


Figure 4. Ligands regulate association of σ 1R with STIM1. (A and B) Solubilized HEK cells expressing STIM1-Myc, σ 1R-FLAG, or both were immunoprecipitated (IP) with anti-Myc (A) or anti-FLAG (B) antibodies before immunoblotting. Input lanes were loaded with 10 μ l of the 500- μ l sample and immunoprecipitation lanes with 20 μ l of the 50- μ l eluate. (C and D) Similar immunoprecipitation analyses from cells expressing STIM1-Myc and σ 1R-FLAG after pretreatment with 25 μ M (+)SKF10047 or 10 μ M BD1047. (E) Summary results (normalized to control cells; mean \pm SEM; $n = 3$) show amounts of σ 1R-FLAG immunoprecipitated by anti-Myc antibody in cells transiently expressing STIM1-Myc and σ 1R-FLAG. **, $P < 0.01$. ANOVA with Tukey's posthoc analysis.

was 0.77 ± 0.03 ; $n = 8$; Fig. 5 B). We used total internal reflection fluorescence (TIRF) microscopy to visualize translocation of mCh-STIM1 and σ 1R-EGFP in response to thapsigargin. In cells expressing mCh-STIM1, thapsigargin stimulated an accumulation of mCh-STIM1 in puncta immediately beneath the PM (Fig. 5 C, top). This is consistent with evidence that store depletion causes STIM1 to aggregate into sub-PM clusters, where they interact with Orai1 to activate SOCE (Liou et al., 2007; Wu et al., 2014). In contrast, thapsigargin had no detectable effect on the sub-PM distribution of σ 1R-EGFP expressed alone (Fig. 5 C, bottom). However, when mCh-STIM1 and σ 1R-EGFP were coexpressed, thapsigargin caused both proteins to accumulate in sub-PM puncta, within which the proteins colocalized (Mander's correlation coefficient was 0.77 ± 0.04 ; $n = 8$; Fig. 5 D), but expression of σ 1R slowed the rate of formation of the mCh-STIM1 puncta (Fig. 5 E). Rates of formation of mCh-STIM1 puncta were unaffected by expression of another ER membrane protein, IP₃R1 (times to 50% accumulation were 325 ± 14 s and 342 ± 11 s, with and without IP₃R1, respectively), confirming that the effects of σ 1R were not caused by nonspecific accumulation of ER proteins. Furthermore, in cells coexpressing Orai1-EGFP, σ 1R-mKate, and HA-STIM1, Orai1-EGFP and σ 1R-mKate accumulated into colocalized puncta after thapsigargin treatment, but neither formed puncta in the absence of STIM1 (Fig. 5, F and G). These results demonstrate that after store depletion, σ 1R accompanies STIM1 to ER-PM junctions, but σ 1R slows the accumulation of STIM1.

In related experiments, HeLa cells expressing different combinations of σ 1R-EGFP, mCh-STIM1, and Orai1-Myc were fixed for immunolabeling, and confocal images were analyzed to assess colocalization of the proteins before and after treatment with thapsigargin. As expected, in cells coexpressing STIM1 and Orai1, thapsigargin caused their colocalization to increase, consistent with evidence that clustered STIM1 at ER-PM junctions captures Orai1 as it diffuses within the PM (Wu et al., 2014). In contrast, when σ 1R and Orai1 were coexpressed,

their colocalization was enhanced by store depletion only in the presence of STIM1, and overlapping puncta of all three proteins were then apparent at the cell periphery (Fig. 5, H and I). These results agree with those obtained using TIRF microscopy and demonstrate the importance of STIM1 in recruiting both Orai1 and σ 1R to the same junctions.

σ 1R reduces the association of STIM1 with PM Orai1

The requirement for STIM1 in recruiting σ 1R to ER-PM junctions containing Orai1 was investigated further by expressing σ 1R-FLAG and Orai1-Myc with and without HA-STIM1. After treatment with thapsigargin and cell surface biotinylation, PM protein complexes were purified using avidin. Immunoblotting showed that the amount of σ 1R within the biotinylated sample was significantly increased in cells overexpressing STIM1 and Orai1, but not when only Orai1 was overexpressed (Fig. 6, A and B). This indicates that STIM1 either promotes trafficking of σ 1R to the PM, where it is directly biotinylated, or it promotes association of σ 1R with a biotinylated PM protein. Similar analyses established that expression of σ 1R-FLAG reduced the amount of STIM1 in the biotinylated sample to $47 \pm 12\%$ ($n = 3$) of that measured without σ 1R (Fig. 6, C and D). The β -actin control showed no evidence of cell permeabilization or biotinylation of intracellular proteins. The biotinylated PM sample was subjected to a further round of purification using anti-Myc beads. Immunoblotting confirmed that when all three proteins were expressed, they were each captured in the final extract, suggesting that both STIM1 and σ 1R are associated with the PM Orai1 channel complex (and that there is no need to invoke cell surface expression of σ 1R to account for its presence in the biotinylated sample). The amount of STIM1 within this complex was again reduced by σ 1R to $51 \pm 7\%$ of that measured without σ 1R (Fig. 6, C and D). These results indicate that σ 1R reduces the amount of STIM1 bound to PM Orai1. This was confirmed by purifying HA-STIM1 with anti-HA beads:

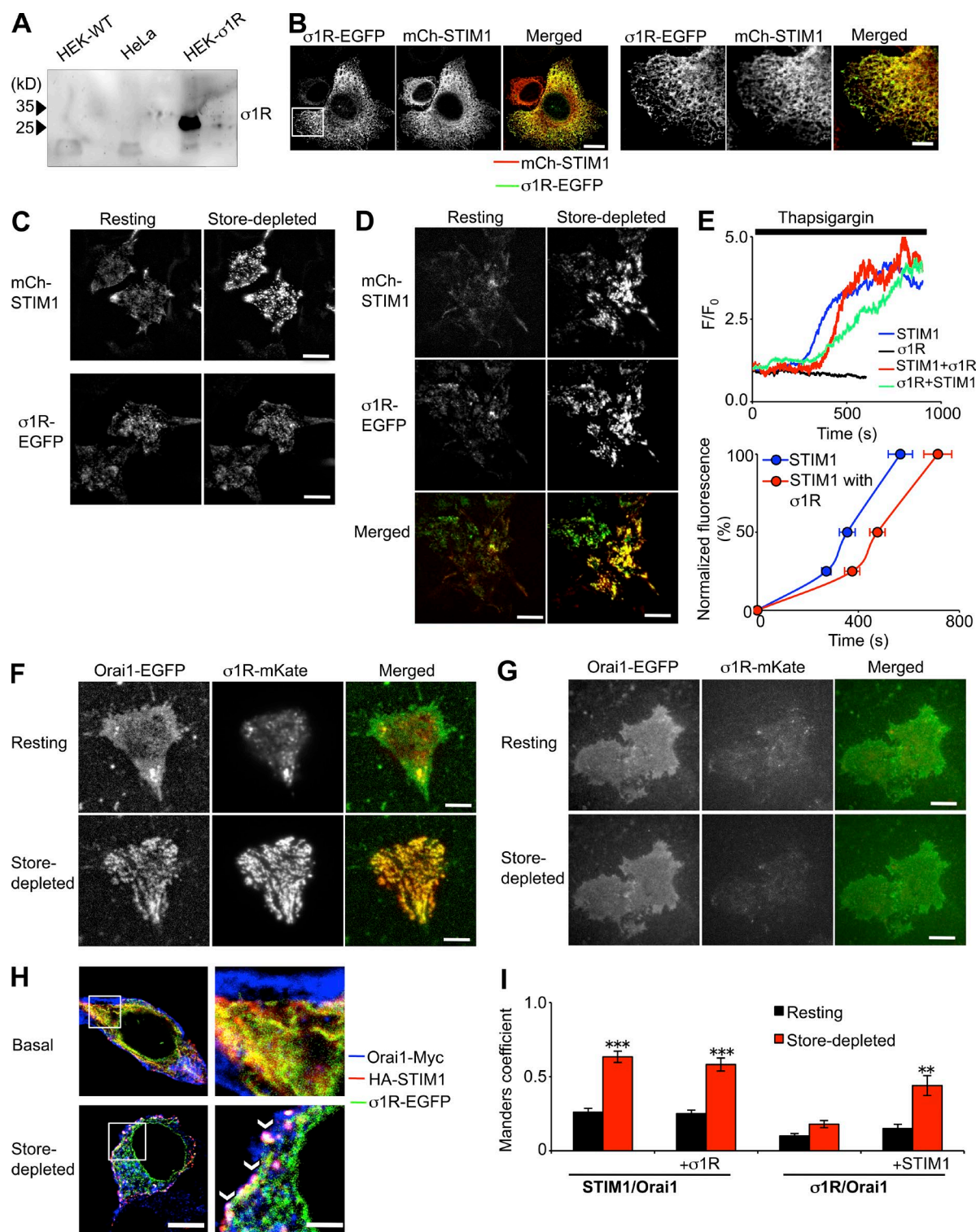


Figure 5. σ 1R accompanies STIM1 to ER-PM junctions after store depletion. (A) Immunoblot of lysates from wild-type (WT) HEK, HEK- σ 1R, or HeLa cells. The same amount of protein was loaded in each lane. (B) Confocal images of unstimulated HeLa cells transiently transfected with σ 1R-EGFP and mCh-STIM1. Bar, 10 μ m. (Right) Enlargement of the boxed area. Bar, 2.5 μ m. (C and D) TIRF images of HeLa cells expressing mCh-STIM1 (C, top), σ 1R-EGFP (C, bottom), or both (D) before and 10 min after addition of 5 μ M thapsigargin in Ca^{2+} -free HBS. (E, top) Traces show time courses of the fluorescence changes (F/F_0) within the TIRF field after addition of thapsigargin (mean values for 30 puncta for each or size-matched regions of interest for σ 1R alone). (E, bottom) Summary results show changes in mCh fluorescence (normalized to maximal intensity) after store depletion in cells with and without σ 1R ($n = 87$). (F and G) TIRF images of HeLa cells expressing Orai1-EGFP and σ 1R-mKate either with (F) or without HA-STIM1 (G). Bars (C, D, F, and G), 10 μ m. (H) Confocal images of HeLa cells expressing σ 1R-EGFP, HA-STIM1, and Orai1-Myc, immunostained after treatment with 5 μ M thapsigargin. Boxed areas in the left panels (bar, 5 μ m) are enlarged on the right (bar, 2 μ m). Arrowheads show colocalization of all three proteins as white puncta at the PM. (I) Summary results ($n = 8$) show Mander's overlap coefficient for colocalization of the indicated pairs of proteins in cells expressing only those tagged proteins or with σ 1R-EGFP or HA-STIM1, as indicated, with and without thapsigargin treatment. **, $P < 0.01$; ***, $P < 0.001$, relative to resting cells. Student's t test. Results show mean \pm SEM.

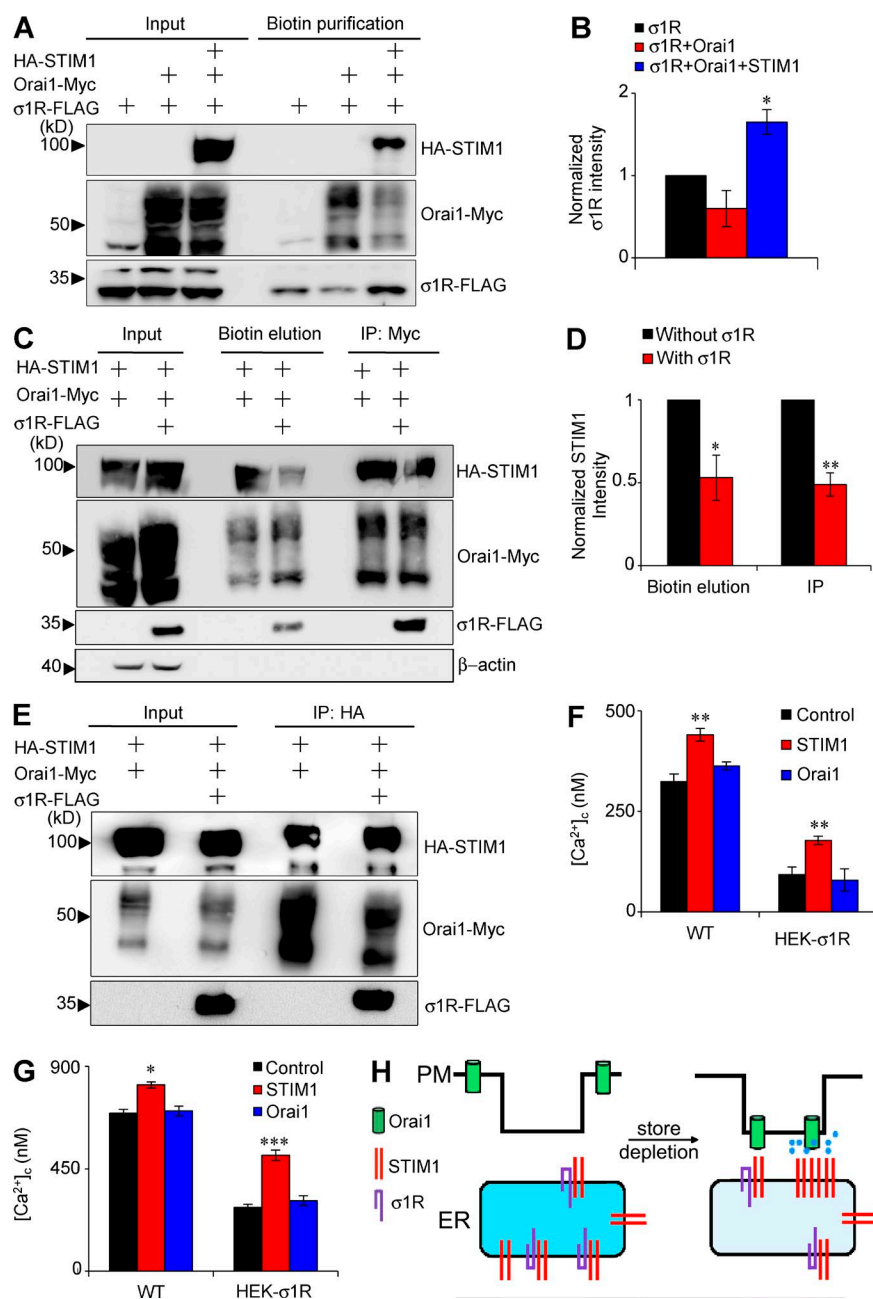


Figure 6. STIM1, Orai1, and σ1R interact within a macromolecular complex at the PM. (A) HEK cells expressing σ1R-FLAG alone or with Orai1-Myc or Orai1-Myc and HA-STIM1 were treated with thapsigargin (5 μM for 30 min in Ca²⁺-free HBS), and then the cell surface was biotinylated. The representative immunoblot shows the inputs and the proteins detected after purification with avidin beads. Input lanes were loaded with 10 μl of the 500-μl sample, and surface biotinylation lanes were loaded with 10 μl of the 50-μl eluate. (B) Summary shows the amounts of σ1R-FLAG detected in the avidin pull-downs (normalized to cells expressing only σ1R-FLAG). (C) HEK cells expressing Orai1-Myc and HA-STIM1 with or without σ1R-FLAG were cell surface biotinylated before sequential purification by elution from avidin-agarose with biotin and then from anti-Myc-agarose with Myc peptide. The immunoblot (anti-HA, anti-FLAG, anti-Myc, and anti-β-actin) shows the input and the two eluates. Input lanes were loaded with 10 μl of the 500-μl sample and elution lanes with 10 μl of the 50-μl eluate. (D) Summary shows the amounts of HA-STIM1 detected in the avidin (biotin elution) and anti-Myc pull-downs (normalized to Orai1-Myc pull-down in each condition). (E) HEK cells expressing Orai1-Myc and HA-STIM1 with or without σ1R-FLAG were immunoprecipitated (IP) with anti-HA antibody. (F) Peak [Ca²⁺]_i signals evoked by SOCE were recorded from HEK or HEK-σ1R cells after treatment with thapsigargin (5 μM in Ca²⁺-free HBS for 10 min) and then restoration of 4 mM extracellular Ca²⁺. The effects of transiently overexpressing STIM1 or Orai1 are shown. WT, wild type. (G) The Ca²⁺ contents of the intracellular stores of the same cells were measured by recording peak increases in [Ca²⁺]_i from cells exposed to ionomycin (5 μM in Ca²⁺-free HBS). Results (B, D, F, and G) are mean ± SEM. *n* = 3. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001, relative to control. ANOVA with Tukey's posthoc analysis (B, F, and G) and Student's *t* test (D). (H) The results suggest that STIM1 and σ1R within the ER are associated. When STIM1 is activated by depletion of the ER Ca²⁺ stores, STIM1 conveys σ1R to the PM, where STIM1 and Orai1 associate, trapping them within ER-PM junctions. The interaction between STIM1 and Orai1 is weakened by σ1R.

the amount of Orai1 that copurified with STIM1 was reduced in the presence of σ1R (Fig. 6 E).

If the reduction in STIM1 binding to Orai1 contributes to inhibition of SOCE by σ1R, we might expect increased expression of STIM1 to relieve the inhibition. We therefore tested the effects of overexpressing STIM1 on the amplitude of SOCE in wild-type HEK and HEK-σ1R cells. Expression of STIM1 produced a similar increase in the amplitude of SOCE in wild-type and HEK-σ1R cells, but the percent increase was greater in the HEK-σ1R cells (36 ± 5% in wild-type and 81 ± 8% in HEK-σ1R cells; Fig. 6 F). This suggests that activation of SOCE is more limited by STIM1 in HEK-σ1R cells than in wild-type cells. The effects of STIM1 on SOCE were matched by its effects on Ca²⁺ stores: overexpression of STIM1 increased the Ca²⁺ content of the stores, and the effect was greater in HEK-σ1R relative to wild-type cells (83 ± 7% and 18 ± 5% increases, respectively; Fig. 6 G). Furthermore, the effects of

σ1R ligands on SOCE were much reduced in HEK-σ1R cells overexpressing STIM1 and Orai1 (Fig. S5). These results support the idea that σ1Rs inhibit the association of STIM1 with PM Orai1, thereby reducing SOCE (Fig. 6 H). Coincident with this inhibition of SOCE by σ1Rs, we invariably detected a decrease in the Ca²⁺ content of the ER.

σ1R inhibits binding of STIM1 to PM Orai1 channel complexes

To examine the structure of the PM Orai1 channel complex in the presence and absence of σ1R, we used atomic force microscopy (AFM). Previous AFM images of complexes purified from cells overexpressing Orai1 and STIM1 showed a hexameric arrangement of STIM1 around a central Orai1 complex and a few strings of STIM1 molecules associated with Orai1, consistent with the oligomerization of STIM1 after depletion of Ca²⁺ stores (Balasuriya et al., 2014b). We examined extracts from

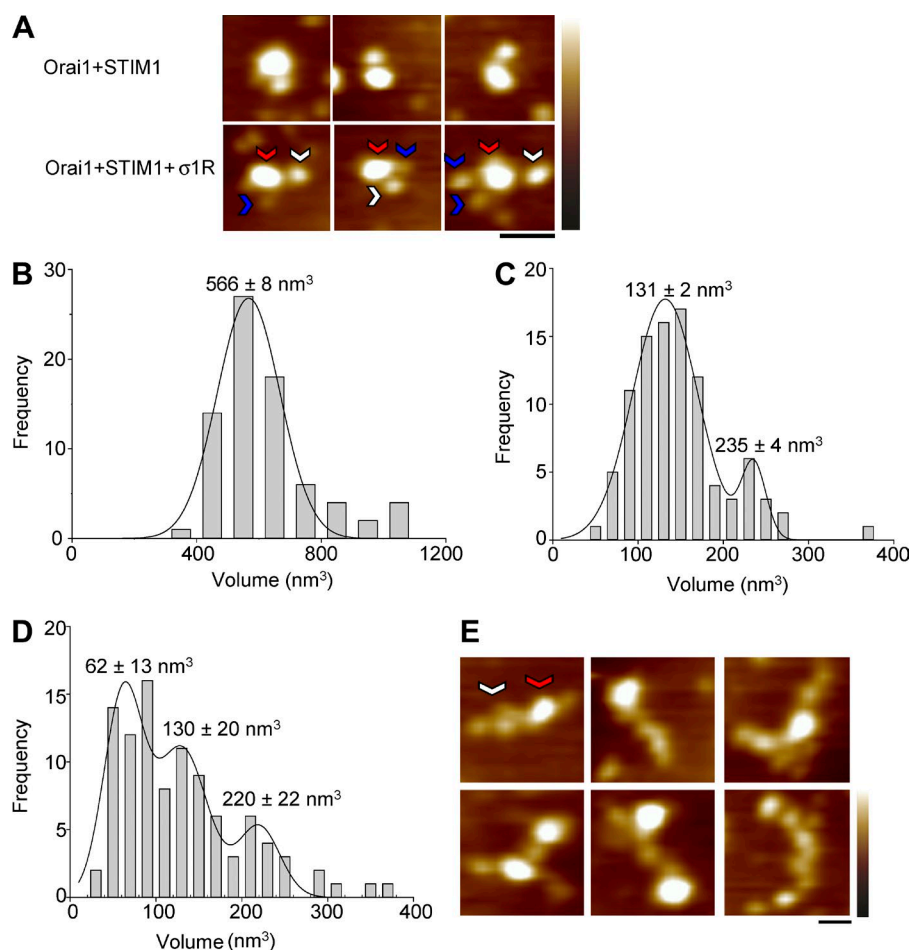


Figure 7. AFM analyses of interactions between $\sigma 1R$, STIM1, and Orai1 at the PM. (A) AFM images of Orai1 isolated with $\sigma 1R$ and STIM1 showing the central Orai1 (red arrowheads) channel decorated by either STIM1 (white arrowheads) or both STIM1 and $\sigma 1R$ (blue arrowheads). (B–D) Frequency distributions of the volumes of the decorated central particles (Orai1; B), bound peripheral particles (STIM1) from cells expressing Orai1 and STIM1 (C), and bound peripheral particles ($\sigma 1R$ and STIM1) from cells expressing Orai1, STIM1, and $\sigma 1R$ (D). For these analyses, volume ranges of 30–100 nm³ and 120–300 nm³ were set for $\sigma 1R$ and STIM1 particles, respectively; the intermediate volumes (100–120 nm³) were disregarded. (E) AFM images showing strings of STIM1 (white arrowhead) connecting several Orai1 channels (red arrowhead). These infrequent structures were observed only in the absence of $\sigma 1R$ s. Bars, 20 nm; height scales, 0–3 nm (dark to light).

thapsigargin-treated HEK cells expressing Orai1-Myc/His and HA-STIM1, with or without $\sigma 1R$ -FLAG, in which cell surface proteins had been biotinylated and complexes were isolated by sequential purification using avidin and anti-Myc beads. AFM images showed large particles decorated by smaller peripheral particles (Fig. 7 A). The large central particle had the volume expected of hexameric Orai1 (566 ± 8 nm³; Fig. 7 B). A volume distribution of bound peripheral particles for the Orai1-Myc/HA-STIM1 sample had two peaks at 131 ± 2 and 235 ± 4 nm³ (Fig. 7 C), consistent with the expected volumes of STIM1 monomers and dimers. For the Orai1-Myc/HA-STIM1/ $\sigma 1R$ -FLAG sample, the volume distribution of the peripheral particles had three peaks (62 ± 13, 130 ± 20, and 220 ± 22 nm³) corresponding to STIM1 monomers and dimers and a smaller peak consistent with the expected volume of $\sigma 1R$ monomers (~63 nm³; Fig. 7 D). Of the 300 Orai1 complexes analyzed when expressed with STIM1 alone, 73 had bound particles and were either singly or doubly decorated. The total number of bound STIM1 was 96. From the 300 Orai1 complexes analyzed when coexpressed with STIM1 and $\sigma 1R$, 76 had bound particles; there were 59 bound STIM1 and 46 bound $\sigma 1R$. So the total number of bound STIM1 was reduced by 39% in the presence of $\sigma 1R$. AFM images of Orai1 isolated from cells expressing Orai1 and STIM1 revealed, albeit with low frequency, that Orai1 bound to strings of STIM1 (Fig. 7 E). These assemblies were never seen in images from cells coexpressing $\sigma 1R$. These results provide evidence for a PM complex of Orai1, STIM1, and $\sigma 1R$ and for reduced binding of STIM1 to Orai1 in the presence of $\sigma 1R$.

$\sigma 1R$ inhibits SOCE via STIM1 rather than by direct effects on Orai1

Reduced binding of STIM1 to Orai1 caused by $\sigma 1R$ is expected to reduce SOCE, but $\sigma 1R$ might also directly inhibit gating of Orai1 channels. To address this possibility, we used the channel-activating domain (CAD) of STIM1, which directly activates Orai1 (Muik et al., 2009; Park et al., 2009; Yuan et al., 2009; Gudlur et al., 2014). mCh-CAD expressed alone in HeLa cells was diffusely distributed in the cytoplasm, but it was peripherally distributed when coexpressed with Orai1 (Fig. 8 A). Addition of extracellular Ca²⁺ to HEK or HEK- $\sigma 1R$ cells in Ca²⁺-free HBS had no significant effect on [Ca²⁺]_i, but there was a substantial increase in [Ca²⁺]_i in cells expressing CAD, consistent with constitutive activation of SOCE by CAD (Fig. 8, B and C). The response was indistinguishable in HEK and HEK- $\sigma 1R$ cells, suggesting that $\sigma 1R$ does not directly modulate PM expression of Orai1 nor its activity.

Discussion

We have shown that $\sigma 1R$ s inhibit SOCE by decreasing the effectiveness with which empty stores stimulate Orai1. The target for regulation of SOCE by $\sigma 1R$ appears to be STIM1 (Fig. 8 D). $\sigma 1R$ and STIM1 colocalize in the ER; they can be coimmunoprecipitated before and after depletion of Ca²⁺ stores, and their interaction is regulated by $\sigma 1R$ ligands. The agonist, (+) SKF10047, increases binding of STIM1 to $\sigma 1R$ and further

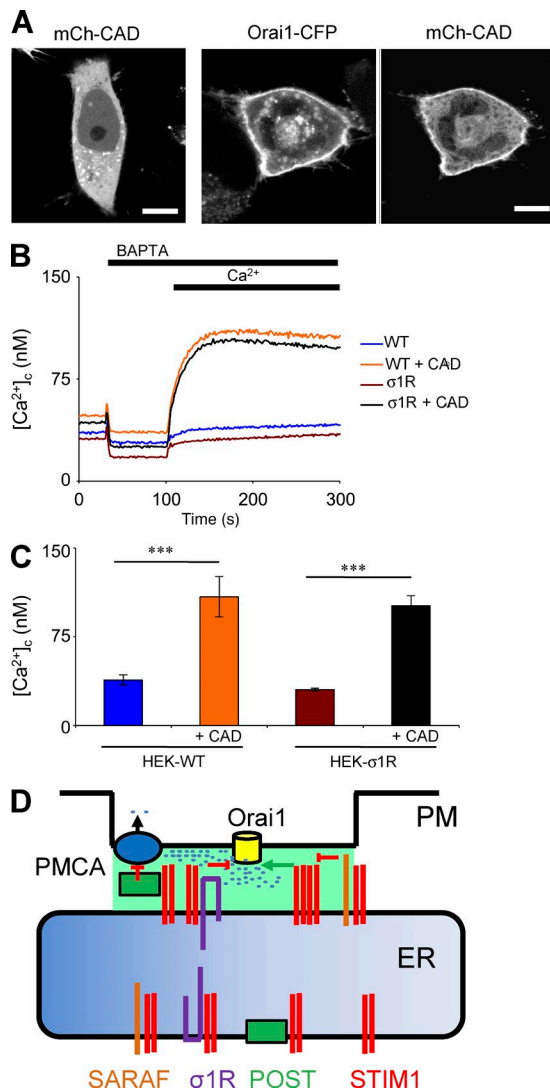


Figure 8. Translocation of Ca^{2+} -regulating proteins to ER-PM junctions by STIM1. (A) Confocal images of unstimulated HeLa cells transiently transfected with mCh-CAD alone (left) or with Orai1-CFP (right). Bars, 10 μm . (B) HEK or HEK- $\sigma 1\text{R}$ cells were mock transfected or transfected with CAD, and $[\text{Ca}^{2+}]_c$ was recorded after addition of 1 mM BAPTA and then restoration of 4 mM extracellular Ca^{2+} . WT, wild type. (C) Summary results show peak $[\text{Ca}^{2+}]_c$ signals evoked by restoration of extracellular Ca^{2+} (mean \pm SD from six replicates). ***, $P < 0.001$, relative to control. Student's t test. (D) Several proteins, including POST, $\sigma 1\text{R}$, and SARAF, associate with STIM1 in ER membranes (Krapivinsky et al., 2011; Palty et al., 2012). Loss of Ca^{2+} from the ER causes STIM1 molecules to oligomerize and become trapped, with their cargoes, in ER-PM junctions (green shading) as STIM1 binds to phosphatidylinositol 4,5-bisphosphate and Orai1. STIM1 activates Orai1 and thereby SOCE. The proteins associated with STIM1 also regulate SOCE. SARAF, by competing with STIM1, reduces STIM1 oligomerization and thereby contributes to the termination of SOCE (Palty et al., 2012). $\sigma 1\text{R}$ competes with STIM1 for binding to Orai1, thereby inhibiting SOCE. POST associates with and inhibits the PM Ca^{2+} pump (PMCA) and thereby reduces local Ca^{2+} extrusion (Krapivinsky et al., 2011).

inhibits SOCE, whereas the antagonist, BD1047, has the opposite effects. After store depletion, $\sigma 1\text{R}$ translocates with STIM1 to ER-PM junctions, but $\sigma 1\text{R}$ slows recruitment of STIM1 and reduces the amount of STIM1 bound to PM Orai1. This reduction in STIM1 binding to Orai1 suggests a likely mechanism for the inhibition of SOCE wherein $\sigma 1\text{R}$ accompanies STIM1 to ER-PM junctions, where it attenuates the interaction of STIM1

with Orai1. The gap between the ER and PM at the junctions where SOCE occurs is probably too large (>9 nm; Várnai et al., 2007) to be bridged by the short cytosolic loop of $\sigma 1\text{R}$ (Fig. S1; Hayashi and Su, 2007). The association of $\sigma 1\text{Rs}$ with PM Orai1 is therefore likely to be mediated by STIM1. Reduced binding of STIM1 to Orai1 in the presence of $\sigma 1\text{R}$ may be caused by $\sigma 1\text{R}$ inhibiting the oligomerization of STIM1 or directly reducing the affinity of STIM1 for Orai1.

There are interesting similarities between the behavior of $\sigma 1\text{Rs}$ and that of other ER membrane proteins, including SARAF (Palty et al., 2012) and POST (partner of STIM1; Krapivinsky et al., 2011). SARAF also translocates to ER-PM junctions in a STIM1-dependent manner, and it promotes deactivation of STIM1 by antagonizing interactions between STIM1 molecules (Palty et al., 2012). Translocation of POST modulates SOCE-evoked Ca^{2+} signals because it inhibits the PM Ca^{2+} pump that extrudes cytosolic Ca^{2+} (Krapivinsky et al., 2011). Hence, after loss of Ca^{2+} from the ER, STIM1 both activates SOCE and fine-tunes its activity by delivering additional Ca^{2+} -regulating proteins to ER-PM junctions (Fig. 8 D). For $\sigma 1\text{Rs}$, the effects of ER luminal Ca^{2+} on these delivery mechanisms may operate at two levels. Loss of ER Ca^{2+} (or a $\sigma 1\text{R}$ agonist) releases $\sigma 1\text{R}$ from its interaction with the ER luminal protein, BiP (Fig. S1; Hayashi and Su, 2007). Store depletion also causes STIM1 to oligomerize and thereby gain affinity for ER-PM junctions. Depletion of Ca^{2+} stores may therefore both release $\sigma 1\text{R}$ from its ER tethers and, via its association with oligomeric STIM1, allow it to accumulate at ER-PM junctions. We focused on SOCE, but recruitment of $\sigma 1\text{Rs}$ to ER-PM junctions by STIM1 might also be involved in regulation of other PM channels by $\sigma 1\text{Rs}$ (Maurice and Su, 2009; Su et al., 2010; Kourrich et al., 2013; Pabba, 2013). For example, the L-type Ca^{2+} channel is inhibited by $\sigma 1\text{R}$ (Tchedre et al., 2008) and by depletion of intracellular Ca^{2+} stores and STIM1 (Park et al., 2010; Wang et al., 2010). We suggest that STIM1-mediated translocation of $\sigma 1\text{R}$ to ER-PM junctions may inhibit voltage-gated Ca^{2+} entry and may also deliver $\sigma 1\text{Rs}$ to additional PM targets (Fig. 8 D).

Inhibition of SOCE by $\sigma 1\text{Rs}$ was invariably accompanied by a decrease in the Ca^{2+} content of the ER with no evident change in $[\text{Ca}^{2+}]_c$. In contrast, and consistent with a study by López et al. (2012), inhibition of SOCE by expression of Orai1^{E106Q} did not consistently affect ER Ca^{2+} content: it was normal in HEK cells but reduced in CHO cells. Inhibition of the STIM1-Orai1 interactions that mediate thapsigargin-evoked SOCE are not, therefore, sufficient to explain the effects of $\sigma 1\text{Rs}$ on ER Ca^{2+} content. It may be that $\sigma 1\text{Rs}$ also interact with STIM2, which plays a major role in maintaining the Ca^{2+} content of the stores (Brandman et al., 2007), or with other proteins, such as the sarco/ER Ca^{2+} ATPase, as was shown for orosomucoid-like 3 (Cantero-Recasens et al., 2010), or with Ca^{2+} channels that mediate Ca^{2+} uptake and release from the ER. For example, Sec61 mediates Ca^{2+} release from the ER, and it is inhibited by BiP (Schäuble et al., 2012). Expression of $\sigma 1\text{R}$ might sequester BiP (Fig. S1) and thereby enhance the Sec61-mediated Ca^{2+} leak. The decreased Ca^{2+} content of the ER might also arise from $\sigma 1\text{R}$ stabilizing IP₃R3 and thereby enhancing Ca^{2+} transport from the ER to mitochondria (Hayashi and Su, 2007).

The pathophysiological effects $\sigma 1\text{Rs}$ may, in part, result from inhibition of SOCE and the reduced Ca^{2+} content of the ER. The latter may affect protein folding (Hayashi and Su, 2007) and inhibit apoptosis by preventing excessive Ca^{2+} transfer to mitochondria (Maurice and Su, 2009; Giorgi et al., 2012).

The effects of σ 1Rs on mitochondrial Ca^{2+} uptake are probably finely balanced because σ 1Rs enhance delivery of Ca^{2+} to mitochondria at MAMs by stabilizing MAM-associated IP₃Rs (Hayashi and Su, 2007), whereas our results show that σ 1Rs reduce the ER Ca^{2+} content. The latter could explain the otherwise surprising antiapoptotic effects of σ 1Rs (Wang et al., 2005; Maurice and Su, 2009; Decuypere et al., 2011; Crottès et al., 2013). The σ 1R agonist, cocaine, was recently shown to attenuate SOCE in rat brain microvascular endothelial cells (Brailoiu et al., 2016). The neuroprotective effects of σ 1R agonists after ischemic injury (Katnik et al., 2006) and in patients with amyotrophic lateral sclerosis arising from loss-of-function mutations in σ 1R (Al-Saif et al., 2011; Ono et al., 2014) may also, at least in part, be due to inhibition of SOCE. Hyperactive SOCE may contribute to the motor deficiencies in σ 1R-knock-out mice (Maurice and Su, 2009; Sabino et al., 2009; Mavlyutov et al., 2010) and to neurodegeneration in Alzheimer's (Mishina et al., 2008; Ishikawa and Hashimoto, 2009; Hyrskyluoto et al., 2013) and Parkinson's diseases (Mishina et al., 2005; Hyrskyluoto et al., 2013; Francardo et al., 2014), where expression of σ 1R is reduced. These suggestions prompt consideration of whether σ 1R also interacts with STIM2 because it appears to play the major role in regulating SOCE in central neurons (Berna-Erro et al., 2009).

We conclude that σ 1Rs inhibit SOCE because they associate with STIM1, slow STIM1 recruitment to ER-PM junctions, and reduce its binding to Orai1 after depletion of Ca^{2+} stores. Our study highlights a role for STIM1 in translocating σ 1Rs to the PM and establishes σ 1Rs and their ligands as important regulators of SOCE, a ubiquitously expressed Ca^{2+} entry pathway (Fig. 8 D).

Materials and methods

Materials

(+)-SKF10047 and BD1047 were from Tocris Bioscience. Ionomycin was from MerckEurolab. Thapsigargin was from Alomone Labs. Anti-Myc monoclonal antibody (1:500 dilution for immunoblots; 46-0603), fura 2-AM, and fluo 4-AM were from Thermo Fisher Scientific. Anti-HA (1:500; 16B12) and anti-FLAG (1:500; F3165) monoclonal antibodies were from Covance and Sigma-Aldrich, respectively. The anti- σ 1R antibody (1:200; Ab53852), which recognizes a sequence conserved in human and mouse σ 1R, was from Abcam. Custom-made rabbit polyclonal antipeptide antisera to STIM1 (1:100; CDPQHGHSQRDLTR; the Cys used for conjugation is underlined) and Orai1 (1:200; CEFAWLQDQLDHRGD) were prepared by Sigma-Aldrich. Anti-actin (1:500; A5441) antibody was from Sigma-Aldrich. Anti-mouse (1:1,000) and anti-rabbit (1:1,000) HRP-conjugated secondary antibodies were from Thermo Fisher Scientific and Bio-Rad Laboratories, respectively. Sources of additional materials are provided within the relevant methods.

Plasmids and siRNA

Plasmids encoding HA-STIM1 and Orai1-Myc/His₆ have been described previously (Willoughby et al., 2012; Balasuriya et al., 2014b). For mCh-STIM1, human STIM1 was subcloned into mCherry-C1 (Takara Bio Inc.) using XbaI and NotI. For σ 1R-FLAG, σ 1R was subcloned into pcDNA3.1/FLAG using HindIII and AgeI. For σ 1R-GFP, σ 1R was subcloned into GFP-N1 (Takara Bio Inc.) using HindIII and KpnI. For σ 1R-V5, σ 1R was subcloned into pcDNA3.1/V5-His-TOPO using HindIII and AgeI. For σ 1R-mKate, σ 1R was subcloned into

mKate2-N (Evrogen) using HindIII and KpnI. The coding sequences of all new constructs were verified. pDsRed2-Mito was from Takara Bio Inc. A pSIREN vector encoding siRNA for σ 1R (5'-GATCCACACGTGGATGGTGGAGTATTCAAGAGATACTCCACCATCCACGTGTTTTTTTGTCTAGCG-3') was used to inhibit expression of σ 1R. pSIREN encoding the luciferase gene was used as a negative control. Both pSIREN constructs were gifts from T.-P. Su (National Institutes of Health, Bethesda, MD; Hayashi and Su, 2004). An expression plasmid (MO70) encoding a dominant-negative form of Orai1 in which Glu-106 is replaced by Gln (Orai1^{E106Q}) was a gift from Y. Gwack and S. Srikanth (University of California, Los Angeles, Los Angeles, CA; Srikanth et al., 2012). The expression plasmid for mouse GFP-NFAT1 was a gift from A. Parekh (University of Oxford, Oxford, England, UK; Kar et al., 2011). The mCh-STIM1 CAD expression plasmid was a gift from P. Hogan (La Jolla Institute for Allergy and Immunology, La Jolla, CA; Gudlur et al., 2014), and Orai1-CFP was from D.M.F. Cooper (University of Cambridge, Cambridge, England, UK).

Cell culture and transfection

All cells were maintained in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C in humidified air with 5% CO₂. tsA 201 cells were grown to 70% confluence in a 162-cm² flask and transfected using calcium phosphate. 50 μ g of plasmid DNA was mixed with 5 ml of 250 mM CaCl₂ and diluted with 5 ml of medium comprising 275 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 15 mM glucose, and 42 mM Hepes, pH 7.07. The mixture was added to the cells bathed in 25 ml of fresh growth medium. After 8 h, the medium was replaced with fresh growth medium. Cells were incubated for a further 48 h before being used for experiments.

HEK 293 cells were transfected using polyethylenimine. For cells grown to 70% confluence in 1 well of a 6-well plate, 1 μ g of plasmid DNA was mixed with 2 μ l of 7.5 mM polyethylenimine (Polysciences, Inc.) and then diluted with 150 μ l of serum-free DMEM. The mixture was incubated for 10 min at 20°C and then added to wells containing 2 ml of fresh growth medium for 48 h. The generation of a polyclonal HEK cell line stably expressing mouse σ 1R-V5 (HEK- σ 1R cells) was performed as described previously (Xu et al., 2012). These cells were maintained in medium supplemented with 0.8 mg/ml G418 (Thermo Fisher Scientific).

HeLa cells were grown on poly-L-lysine-coated 25-mm glass coverslips and transfected using Lipofectamine 2000 (Thermo Fisher Scientific). For 1 well of a 6-well plate, 2 μ g of plasmid DNA was diluted in 200 μ l Opti-MEM and incubated at 20°C for 5 min. This was combined with 200 μ l Opti-MEM containing 4 μ l Lipofectamine 2000 and left for a further 20 min at 20°C. The mixture was then added to cells in 2 ml of fresh medium. Cells were incubated for 48 h at 37°C and then used for experiments.

Measurements of $[\text{Ca}^{2+}]_i$

For measurements of $[\text{Ca}^{2+}]_i$ in populations of cells, HEK cells were seeded into poly-L-lysine-coated 96-well plates. After 24 h, cells were incubated with 2 μ M fluo 4-AM in HBS for 60 min at 20°C. HBS had the following composition: 150 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM Hepes, pH 7.3. In Ca^{2+} -free HBS, Ca^{2+} was omitted, and 1 mM BAPTA was added. For treatments with (+)-SKF10047 and BD1047, 10 mM stock solutions were prepared in DMSO and water, respectively. Cells were pretreated with 25 μ M (+)-SKF10047 or 10 μ M BD1047 in serum-free DMEM for 1 h at 37°C before loading cells with fluorescent Ca^{2+} indicators. Drug treatments were continued during loading and throughout $[\text{Ca}^{2+}]_i$ measurements. After loading, cells were washed and incubated in HBS for a further 30 min at 20°C. Fluorescence (excitation at 490 nm and emission at

520 nm) was measured at 20°C using a plate reader that allows on-line additions (FlexStation 3; Molecular Devices). Fluorescence was calibrated to $[Ca^{2+}]_c$ from

$$[Ca^{2+}]_c = K_D \left(\frac{F - F_{\min}}{F_{\max} - F} \right),$$

where $K_D = 345$ nM ($K_D = 190$ nM at 37°C; Fig. S2 B), F is the measured fluorescence, and F_{\max} and F_{\min} are the fluorescence values determined after addition of 0.1% Triton X-100 in HBS with 10 mM Ca^{2+} or 10 mM BAPTA, respectively.

Measurements of Mn^{2+} entry

Confluent cultures of HEK cells in 96-well plates were loaded with 2 μ M fura 2-AM using the method described for fluo 4. Fluorescence (excitation at 360 nm and emission at 510 nm) was measured using a plate reader (FlexStation 3) at 1.5-s intervals at 20°C. Quenching of fura 2 fluorescence (which reports unidirectional entry of Mn^{2+}) is reported as F/F_0 , where F is the fluorescence intensity recorded at each time and F_0 is the mean fluorescence intensity measured in the 5 s before addition of $MnCl_2$. Monoexponential curve fits to the time course of the changes in F/F_0 were used to compute half-times ($t_{1/2}$) for Mn^{2+} -evoked fluorescence quenching.

NFAT translocation assay

HEK cells were seeded onto poly-L-lysine-coated 25-mm coverslips, transfected with GFP-NFAT plasmid using polyethylenimine, and used after 48 h. The distribution of GFP fluorescence was measured before and 40 min after addition of 5 μ M thapsigargin to cells at 37°C in HBS. Fluorescence (excitation at 488 nm and emission at 510–540 nm) was collected using a confocal microscope (SP5; Leica Biosystems) with an oil-immersion 40 \times objective (NA 1.25). Analyses of nuclear translocation of GFP-NFAT were performed with coded images, which were decoded only when the analysis was complete.

Analyses of protein expression

Cells were grown in 162-cm² flasks. Where appropriate, cells were transfected with 50 μ g of plasmid DNA using polyethylenimine. Cells were extracted in ice-cold medium (138 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4 , 7.5 mM glucose, 21 mM Hepes, and 2 mM EDTA, pH 7.4) and centrifuged at 1,000 g for 5 min. Pelleted cells were solubilized at 4°C for 60 min in Triton solution (TS) containing 25 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, and 1 mg/ml protease inhibitor cocktail solution (Roche), pH 7.4, and samples were analyzed by SDS-PAGE followed by immunoblotting.

Immunoprecipitation analyses

tsA 201 cells, which are SV40-transformed HEK 293 cells, were used because they express heterologous proteins at high levels. Cells were grown in 162-cm² flasks and transfected using calcium phosphate. Pretreatments with (+)SKF10047 and BD1047 were for 2 h at 37°C, and stimulation with 5 μ M thapsigargin was for 30 min at 20°C. Cells were extracted in 25 ml of ice-cold medium, and all subsequent steps were performed at 4°C. The suspension was centrifuged at 1,000 g for 5 min, and pelleted cells were solubilized for 60 min in 500 μ l TS. After centrifugation (50,000 g for 60 min), 50 μ l of the supernatant was removed for analysis of total expression (input), and 450 μ l was incubated with 30 μ l anti-Myc (EZ View Red) or anti-FLAG beads (Sigma-Aldrich) for 3 h with rotation. Protein-bead complexes were isolated (20,800 g for 10 min) and washed three times in TS, and proteins were eluted either with 50 μ l of the peptides (1 mg/ml; Sigma-Aldrich), to which the anti-Myc or anti-FLAG antibodies had been raised, or with 50 μ l Laemmli buffer. The eluted samples were analyzed by SDS-PAGE followed by immunoblotting.

For immunoblots, lanes were loaded with 10 μ l of the 500- μ l sample (2% of the entire sample) for the measurement of input and with 10 or 20 μ l of the 50- μ l eluate for measurements of immunoprecipitation.

Isolation of surface biotinylated proteins

tsA 201 cells were grown in 162-cm² flasks and transfected using calcium phosphate. After appropriate stimulation, the medium was removed and replaced with 12.5 ml of ice-cold HBS containing 0.2 mg/ml biotin-sulfo-NHS (Thermo Fisher Scientific). After 60 min on ice, cells were washed three times with 15 ml Tris-buffered saline (25 mM Tris-HCl, 150 mM NaCl, and 10 mM EDTA, pH 7.4) and centrifuged at 1,000 g for 5 min, and the pellet was solubilized in 500 μ l TS for 60 min at 4°C. After centrifugation at 50,000 g for 60 min, the supernatant was incubated with 50 μ l monomeric avidin-coated agarose beads (Thermo Fisher Scientific) at 4°C for 2 h. Protein-bead complexes were collected at 20,800 g for 10 min, washed three times in TS, and eluted with either 50 μ l Laemmli buffer for immunoblots or biotin (1 mg/ml in 1 ml TS) for further immunopurification using anti-Myc beads as described in the previous paragraph. For analyses of avidin pull-downs of biotinylated proteins (Fig. 5, C–E), 2% of the total sample was loaded as input, and 40% of the Laemmli sample was loaded in the surface biotinylation lanes.

Immunostaining

HeLa cells were seeded on poly-L-lysine-coated glass coverslips, transfected, and used after 48 h. After stimulation, cells were washed with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 2 mM KH_2PO_4), fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.5 mg/ml saponin for 60 min (Sigma-Aldrich) in blocking solution (5% goat serum and 3% BSA in PBS). Cells were stained with primary antibody in blocking solution (PBS containing 3% BSA and 5% goat serum) for 60 min at 20°C, washed twice with PBS, and then incubated in the dark with secondary antibody in blocking solution for 60 min at 20°C, washed with PBS, dried, mounted onto a glass microscope slide, and stored at 4°C. Cells were imaged using an oil-immersion 60 \times objective (NA 1.40) using a confocal microscope (SP5; Leica Biosystems). For both Pearson's and Mander's coefficient measurements, images were analyzed with ImageJ (National Institutes of Health) using the JACoP plugin. For Mander's coefficient, only pixels in which HA-STIM1 (or σ 1R-EGFP) was detected were considered, and the fraction of those pixels in which Orai1-Myc was also detected was then computed to provide the colocalization coefficient.

TIRF microscopy

Coverslips were mounted on a TIRF microscope (IX51 inverted microscope [Olympus] with a 100 \times oil-immersion objective [NA 1.49] coupled to an electron-multiplying charged-coupled device camera [iXon; Andor Technology] and 488-nm argon ion and 561-nm diode lasers). Cells were incubated with HBS at 20°C and imaged (1 image/s) by exciting σ 1R-GFP at 488 nm (emission at 510–540 nm) and mCh-STIM1 at 561 nm (emission at 610–650 nm). For each experiment, there were suitable controls, with cells expressing the EGFP-tagged protein alone and the mCherry/mKate-tagged protein alone to ensure there was no bleed through. For depletion of stores, cells were incubated with 1 μ M thapsigargin in Ca^{2+} -free HBS. Fluorescence intensities were quantified using the time series analyzer plugin V2.0 in ImageJ. Individual regions of interest within the cell were selected, and the data were analyzed as F/F_0 , where F and F_0 are the fluorescence intensities at each time and at the start of the experiment, respectively.

AFM

tsA 201 cells expressing appropriate combinations of Orai1-Myc-His, σ 1R-FLAG, and HA-STIM1 were treated with thapsigargin, followed

by biotin-sulfo-NHS, and then purified using sequential avidin and anti-Myc affinity chromatography, as described in the Isolation of surface biotinylated proteins section. About 45 μ l of proteins was added to a 1-cm² mica disk, incubated at 20°C for 10 min, gently washed with water, and dried under nitrogen. Samples were imaged in air using an atomic force microscope (Multimode; Bruker). The silicon cantilever (OTESPA; Bruker) was set at a drive frequency of 271–321 kHz and spring constant of 12–103 N/m. The scan rate was 3 Hz, and the applied imaging force was kept as low as possible (target amplitude of 1.0 V and amplitude set point of 0.7–1.0 V). Molecular volumes for individual particles were determined using an image processor (version 5; Scanning Probe). For particles within complexes, particle heights (h) and radii (r) were measured manually using Nanoscope software. Particle volumes (V_m) were then calculated from

$$V_m = \frac{\pi h(3r^2 + h^2)}{6}.$$

Molecular volume (V_c), based on a known molecular mass (M_0), was calculated from

$$V_c = \frac{M_0(V_1 + dV_2)}{N_0},$$

where N_0 is Avogadro's number, V_1 is the specific particle volume (0.74 cm³/g), V_2 is the water specific volume (1 cm³/g), and d is the extent of hydration (assumed to be 0.4 g H₂O/g protein).

Data analysis

Most results are presented as mean \pm SEM from n independent experiments. Statistical analysis used Student's t test or analysis of variance (ANOVA) followed by Tukey's posthoc test as appropriate.

Online supplemental material

Fig. S1 illustrates key features of σ 1R, and Table S1 describes ligands targeting σ 1R. Fig. S2 shows the effects of expressing σ 1Rs in HEK cells on SOCE and the Ca²⁺ content of the intracellular stores. Fig. S3 shows the effects of σ 1R ligands on SOCE in CHO and HEK cells. Fig. S4 shows the effects of σ 1R ligands on SOCE and the Ca²⁺ content of the intracellular stores in MDA-MB-231 human breast cancer cells. Fig. S5 shows the effects of σ 1R ligands on SOCE in HEK- σ 1R cells overexpressing STIM1 and Orai1. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201506022/DC1>.

Acknowledgments

We thank T.-P. Su for σ 1R siRNA and control plasmids, S. Srikanth and Y. Gwack for the Orai1^{E106Q} construct, A. Parekh for the GFP-NFAT plasmid, P. Hogan for the CAD construct, and D.M.F. Cooper for the Orai1-CFP construct.

S. Srivats is supported by the Cambridge International and European Trust, D. Balasuriya is supported by a David James bursary from the Department of Pharmacology, University of Cambridge, and G. Vistal is supported by the Jardines Matheson student bursary. This work was supported by grants to J.M. Edwardson and R.D. Murrell-Lagnado from the Biotechnology and Biological Sciences Research Council (BB/J018236/1 and BB/F001320/1) and by a grant from the Wellcome Trust (101844) to C.W. Taylor.

The authors declare no competing financial interests.

Submitted: 4 June 2015

Accepted: 24 February 2016

References

- Al-Saif, A., F. Al-Mohanna, and S. Bohlega. 2011. A mutation in sigma-1 receptor causes juvenile amyotrophic lateral sclerosis. *Ann. Neurol.* 70:913–919. <http://dx.doi.org/10.1002/ana.22534>
- Aydar, E., C.P. Palmer, V.A. Klyachko, and M.B. Jackson. 2002. The sigma receptor as a ligand-regulated auxiliary potassium channel subunit. *Neuron* 34:399–410. [http://dx.doi.org/10.1016/S0896-6273\(02\)00677-3](http://dx.doi.org/10.1016/S0896-6273(02)00677-3)
- Aydar, E., P. Onganer, R. Perrett, M.B. Djamgoz, and C.P. Palmer. 2006. The expression and functional characterization of sigma (σ) 1 receptors in breast cancer cell lines. *Cancer Lett.* 242:245–257. <http://dx.doi.org/10.1016/j.canlet.2005.11.011>
- Balasuriya, D., A.P. Stewart, D. Crottès, F. Borgese, O. Soriani, and J.M. Edwardson. 2012. The sigma-1 receptor binds to the Nav1.5 voltage-gated Na⁺ channel with 4-fold symmetry. *J. Biol. Chem.* 287:37021–37029. <http://dx.doi.org/10.1074/jbc.M112.382077>
- Balasuriya, D., L. D'Sa, R. Talker, E. Dupuis, F. Maurin, P. Martin, F. Borgese, O. Soriani, and J.M. Edwardson. 2014a. A direct interaction between the sigma-1 receptor and the hERG voltage-gated K⁺ channel revealed by atomic force microscopy and homogeneous time-resolved fluorescence (HTRF®). *J. Biol. Chem.* 289:32353–32363. <http://dx.doi.org/10.1074/jbc.M114.603506>
- Balasuriya, D., S. Srivats, R.D. Murrell-Lagnado, and J.M. Edwardson. 2014b. Atomic force microscopy (AFM) imaging suggests that stromal interaction molecule 1 (STIM1) binds to Orai1 with sixfold symmetry. *FEBS Lett.* 588:2874–2880. <http://dx.doi.org/10.1016/j.febslet.2014.06.054>
- Berna-Erro, A., A. Braun, R. Kraft, C. Kleinschmitz, M.K. Schuhmann, D. Stegner, T. Wulsch, J. Eilers, S.G. Meuth, G. Stoll, and B. Nieswandt. 2009. STIM2 regulates capacitive Ca²⁺ entry in neurons and plays a key role in hypoxic neuronal cell death. *Sci. Signal.* 2:ra67. <http://dx.doi.org/10.1126/scisignal.2000522>
- Brailoiu, G.C., E. Deliu, L.M. Console-Bram, J. Soboloff, M.E. Abood, E.M. Unterwald, and E. Brailoiu. 2016. Cocaine inhibits store-operated Ca²⁺ entry in brain microvascular endothelial cells: critical role for sigma-1 receptors. *Biochem. J.* 473:1–5. <http://dx.doi.org/10.1042/BJ2015093426467159>
- Brandman, O., J. Liou, W.S. Park, and T. Meyer. 2007. STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca²⁺ levels. *Cell* 131:1327–1339. <http://dx.doi.org/10.1016/j.cell.2007.11.039>
- Brune, S., S. Pricl, and B. Wünsch. 2013. Structure of the σ 1 receptor and its ligand binding site. *J. Med. Chem.* 56:9809–9819. <http://dx.doi.org/10.1021/jm400660u>
- Cantero-Recasens, G., C. Fandos, F. Rubio-Moscardo, M.A. Valverde, and R. Vicente. 2010. The asthma-associated ORMDL3 gene product regulates endoplasmic reticulum-mediated calcium signaling and cellular stress. *Hum. Mol. Genet.* 19:111–121. <http://dx.doi.org/10.1093/hmg/ddp471>
- Cárdenas, C., R.A. Miller, I. Smith, T. Bui, J. Molgó, M. Müller, H. Vais, K.H. Cheung, J. Yang, I. Parker, et al. 2010. Essential regulation of cell bioenergetics by constitutive InsP₃ receptor Ca²⁺ transfer to mitochondria. *Cell* 142:270–283. <http://dx.doi.org/10.1016/j.cell.2010.06.007>
- Chu, U.B., and A.E. Ruoho. 2016. Biochemical pharmacology of the Sigma-1 receptor. *Mol. Pharmacol.* 89:142–153. <http://dx.doi.org/10.1124/mol.115.101170>
- Cobos, E.J., J.M. Entrena, F.R. Nieto, C.M. Cendán, and E. Del Pozo. 2008. Pharmacology and therapeutic potential of sigma1 receptor ligands. *Curr. Neuropharmacol.* 6:344–366. <http://dx.doi.org/10.2174/157015908787386113>
- Crottès, D., S. Martial, R. Rapetti-Mauss, D.F. Pisani, C. Lioriol, B. Pellissier, P. Martin, E. Chevet, F. Borgese, and O. Soriani. 2011. Sig1R protein regulates hERG channel expression through a post-translational mechanism in leukemic cells. *J. Biol. Chem.* 286:27947–27958. <http://dx.doi.org/10.1074/jbc.M111.226738>
- Crottès, D., H. Guizouarn, P. Martin, F. Borgese, and O. Soriani. 2013. The sigma-1 receptor: a regulator of cancer cell electrical plasticity? *Front. Physiol.* 4:175. <http://dx.doi.org/10.3389/fphys.2013.00175>
- Decuyper, J.P., G. Monaco, G. Bultynck, L. Missiaen, H. De Smedt, and J.B. Parys. 2011. The IP₃ receptor-mitochondria connection in apoptosis and autophagy. *Biochim. Biophys. Acta.* 1813:1003–1013. <http://dx.doi.org/10.1016/j.bbamer.2010.11.023>
- DeHaven, W.I., B.F. Jones, J.G. Petranka, J.T. Smyth, T. Tomita, G.S. Bird, and J.W. Putney Jr. 2009. TRPC channels function independently of STIM1 and Orai1. *J. Physiol.* 587:2275–2298. <http://dx.doi.org/10.1113/jphysiol.2009.170431>
- Francardo, V., F. Bez, T. Wieloch, H. Nissbrandt, K. Ruscher, and M.A. Cenci. 2014. Pharmacological stimulation of sigma-1 receptors has neurorestorative effects in experimental parkinsonism. *Brain* 137:1998–2014. <http://dx.doi.org/10.1093/brain/awu107>

- Giorgi, C., F. Baldassari, A. Bononi, M. Bonora, E. De Marchi, S. Marchi, S. Missiroli, S. Patergnani, A. Rimessi, J.M. Suski, et al. 2012. Mitochondrial Ca^{2+} and apoptosis. *Cell Calcium*. 52:36–43. <http://dx.doi.org/10.1016/j.ceca.2012.02.008>
- Gromek, K.A., F.P. Suchy, H.R. Meddaugh, R.L. Wrobel, L.M. LaPointe, U.B. Chu, J.G. Primm, A.E. Ruoho, A. Senes, and B.G. Fox. 2014. The oligomeric states of the purified sigma-1 receptor are stabilized by ligands. *J. Biol. Chem.* 289:20333–20344. <http://dx.doi.org/10.1074/jbc.M113.537993>
- Gudlur, A., A. Quintana, Y. Zhou, N. Hirve, S. Mahapatra, and P.G. Hogan. 2014. STIM1 triggers a gating rearrangement at the extracellular mouth of the ORAI1 channel. *Nat. Commun.* 5:5164. <http://dx.doi.org/10.1038/ncomms6164>
- Hayashi, T., and T.-P. Su. 2003. Intracellular dynamics of σ -1 receptors (σ_1 binding sites) in NG108-15 cells. *J. Pharmacol. Exp. Ther.* 306:726–733. <http://dx.doi.org/10.1124/jpet.103.051292>
- Hayashi, T., and T.-P. Su. 2004. Sigma-1 receptors at galactosylceramide-enriched lipid microdomains regulate oligodendrocyte differentiation. *Proc. Natl. Acad. Sci. USA*. 101:14949–14954. <http://dx.doi.org/10.1073/pnas.0402890101>
- Hayashi, T., and T.-P. Su. 2005a. The sigma receptor: evolution of the concept in neuropsychopharmacology. *Curr. Neuropharmacol.* 3:267–280. <http://dx.doi.org/10.2174/157015905774322516>
- Hayashi, T., and T.-P. Su. 2005b. The potential role of sigma-1 receptors in lipid transport and lipid raft reconstitution in the brain: implication for drug abuse. *Life Sci.* 77:1612–1624. <http://dx.doi.org/10.1016/j.lfs.2005.05.009>
- Hayashi, T., and T.-P. Su. 2007. Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca^{2+} signaling and cell survival. *Cell*. 131:596–610. <http://dx.doi.org/10.1016/j.cell.2007.08.036>
- Hogan, P.G., and A. Rao. 2015. Store-operated calcium entry: Mechanisms and modulation. *Biochem. Biophys. Res. Commun.* 460:40–49. <http://dx.doi.org/10.1016/j.bbrc.2015.02.110>
- Hoth, M., and B.A. Niemeyer. 2013. The neglected CRAC proteins: Orai2, Orai3, and STIM2. *Curr. Top. Membr.* 71:237–271. <http://dx.doi.org/10.1016/B978-0-12-407870-3.00010-X>
- Hyrskyluoto, A., I. Pulli, K. Törnqvist, T.H. Ho, L. Korhonen, and D. Lindholm. 2013. Sigma-1 receptor agonist PRE084 is protective against mutant huntingtin-induced cell degeneration: involvement of calpastatin and the NF- κ B pathway. *Cell Death Dis.* 4:e646. <http://dx.doi.org/10.1038/cddis.2013.170>
- Ishikawa, M., and K. Hashimoto. 2009. The role of sigma-1 receptors in the pathophysiology of neuropsychiatric diseases. *J. Receptor Ligand Channel Res.* 2010:25–36. <http://dx.doi.org/10.2147/JRLCR.S8453>
- Ito, K., Y. Hirooka, R. Matsukawa, M. Nakano, and K. Sunagawa. 2012. Decreased brain sigma-1 receptor contributes to the relationship between heart failure and depression. *Cardiovasc. Res.* 93:33–40. <http://dx.doi.org/10.1093/cvr/cvr255>
- Ito, K., Y. Hirooka, and K. Sunagawa. 2013. Brain sigma-1 receptor stimulation improves mental disorder and cardiac function in mice with myocardial infarction. *J. Cardiovasc. Pharmacol.* 62:222–228. <http://dx.doi.org/10.1097/FJC.0b013e3182970b15>
- Kar, P., C. Nelson, and A.B. Parekh. 2011. Selective activation of the transcription factor NFAT1 by calcium microdomains near Ca^{2+} release-activated Ca^{2+} (CRAC) channels. *J. Biol. Chem.* 286:14795–14803. <http://dx.doi.org/10.1074/jbc.M111.220582>
- Katnik, C., W.R. Guerrero, K.R. Pennypacker, Y. Herrera, and J. Cuevas. 2006. Sigma-1 receptor activation prevents intracellular calcium dysregulation in cortical neurons during in vitro ischemia. *J. Pharmacol. Exp. Ther.* 319:1355–1365. <http://dx.doi.org/10.1124/jpet.106.107557>
- Kilch, T., D. Alansary, M. Peglow, K. Dörr, G. Rychkov, H. Rieger, C. Peinelt, and B.A. Niemeyer. 2013. Mutations of the Ca^{2+} -sensing stromal interaction molecule STIM1 regulate Ca^{2+} influx by altered oligomerization of STIM1 and by destabilization of the Ca^{2+} channel Orai1. *J. Biol. Chem.* 288:1653–1664. <http://dx.doi.org/10.1074/jbc.M112.417246>
- Kourrich, S., T. Hayashi, J.-Y. Chuang, S.-Y. Tsai, T.-P. Su, and A. Bonci. 2013. Dynamic interaction between sigma-1 receptor and Kv1.2 shapes neuronal and behavioral responses to cocaine. *Cell*. 152:236–247. <http://dx.doi.org/10.1016/j.cell.2012.12.004>
- Krapivinsky, G., L. Krapivinsky, S.C. Stotz, Y. Manasian, and D.E. Clapham. 2011. POST, partner of stromal interaction molecule 1 (STIM1), targets STIM1 to multiple transporters. *Proc. Natl. Acad. Sci. USA*. 108:19234–19239. <http://dx.doi.org/10.1073/pnas.1117231108>
- Lewis, R.S. 2007. The molecular choreography of a store-operated calcium channel. *Nature*. 446:284–287. <http://dx.doi.org/10.1038/nature05637>
- Lewis, A., T. Hayashi, T.-P. Su, and M.J. Betenbaugh. 2014. Bcl-2 family in inter-organelle modulation of calcium signaling: roles in bioenergetics and cell survival. *J. Bioenerg. Biomembr.* 46:1–15. <http://dx.doi.org/10.1007/s10863-013-9527-7>
- Liou, J., M. Fivaz, T. Inoue, and T. Meyer. 2007. Live-cell imaging reveals sequential oligomerization and local plasma membrane targeting of stromal interaction molecule 1 after Ca^{2+} store depletion. *Proc. Natl. Acad. Sci. USA*. 104:9301–9306. <http://dx.doi.org/10.1073/pnas.0702866104>
- López, E., G.M. Salido, J.A. Rosado, and A. Bernal-Ero. 2012. Unraveling STIM2 function. *J. Physiol. Biochem.* 68:619–633. <http://dx.doi.org/10.1007/s13105-012-0163-1>
- Lupardus, P.J., R.A. Wilke, E. Aydar, C.P. Palmer, Y. Chen, A.E. Ruoho, and M.B. Jackson. 2000. Membrane-delimited coupling between sigma receptors and K^{+} channels in rat neurohypophyseal terminals requires neither G-protein nor ATP. *J. Physiol.* 526:527–539. <http://dx.doi.org/10.1111/j.1469-7793.2000.00527.x>
- Mallilankaraman, K., P. Doonan, C. Cárdenas, H.C. Chandramoorthy, M. Müller, R. Miller, N.E. Hoffman, R.K. Gandhirajan, J. Molgó, M.J. Birnbaum, et al. 2012. MICU1 is an essential gatekeeper for MCU-mediated mitochondrial Ca^{2+} uptake that regulates cell survival. *Cell*. 151:630–644. <http://dx.doi.org/10.1016/j.cell.2012.10.011>
- Maurice, T., and T.-P. Su. 2009. The pharmacology of sigma-1 receptors. *Pharmacol. Ther.* 124:195–206. <http://dx.doi.org/10.1016/j.pharmthera.2009.07.001>
- Maurice, T., V.L. Phan, A. Urani, H. Kamei, Y. Noda, and T. Nabeshima. 1999. Neuroactive neurosteroids as endogenous effectors for the sigma₁ (σ_1) receptor: pharmacological evidence and therapeutic opportunities. *Jpn. J. Pharmacol.* 81:125–155. <http://dx.doi.org/10.1254/jjp.81.125>
- Maurice, T., R. Martin-Fardon, P. Romieu, and R.R. Matsumoto. 2002. Sigma₁ (σ_1) receptor antagonists represent a new strategy against cocaine addiction and toxicity. *Neurosci. Biobehav. Rev.* 26:499–527. [http://dx.doi.org/10.1016/S0149-7634\(02\)00017-9](http://dx.doi.org/10.1016/S0149-7634(02)00017-9)
- Mavlyutov, T.A., M.L. Epstein, K.A. Andersen, L. Ziskind-Conhaim, and A.E. Ruoho. 2010. The sigma-1 receptor is enriched in postsynaptic sites of C-terminals in mouse motoneurons. An anatomical and behavioral study. *Neuroscience*. 167:247–255. <http://dx.doi.org/10.1016/j.neuroscience.2010.02.022>
- Miki, Y., F. Mori, T. Kon, K. Tanji, Y. Toyoshima, M. Yoshida, H. Sasaki, A. Kakita, H. Takahashi, and K. Wakabayashi. 2014. Accumulation of the sigma-1 receptor is common to neuronal nuclear inclusions in various neurodegenerative diseases. *Neuropathology*. 34:148–158. <http://dx.doi.org/10.1111/neup.12080>
- Mishina, M., K. Ishiwata, K. Ishii, S. Kitamura, Y. Kimura, K. Kawamura, K. Oda, T. Sasaki, O. Sakayori, M. Hamamoto, et al. 2005. Function of sigma₁ receptors in Parkinson's disease. *Acta Neurol. Scand.* 112:103–107. <http://dx.doi.org/10.1111/j.1600-0404.2005.00432.x>
- Mishina, M., M. Ohyama, K. Ishii, S. Kitamura, Y. Kimura, K. Oda, K. Kawamura, T. Sasaki, S. Kobayashi, Y. Katayama, and K. Ishiwata. 2008. Low density of sigma₁ receptors in early Alzheimer's disease. *Ann. Nucl. Med.* 22:151–156. <http://dx.doi.org/10.1007/s12149-007-0094-z>
- Monnet, F.P. 2005. Sigma-1 receptor as regulator of neuronal intracellular Ca^{2+} : clinical and therapeutic relevance. *Biol. Cell*. 97:873–883. <http://dx.doi.org/10.1042/BC20040149>
- Mori, T., T. Hayashi, E. Hayashi, and T.-P. Su. 2013. Sigma-1 receptor chaperone at the ER-mitochondrion interface mediates the mitochondrion-ER-nucleus signaling for cellular survival. *PLoS One*. 8:e76941. <http://dx.doi.org/10.1371/journal.pone.0076941>
- Muik, M., M. Fahrner, I. Derler, R. Schindl, J. Bergsmann, I. Frischauf, K. Groschner, and C. Romanin. 2009. A cytosolic homomerization and a modulatory domain within STIM1 C terminus determine coupling to ORAI1 channels. *J. Biol. Chem.* 284:8421–8426. <http://dx.doi.org/10.1074/jbc.C800229200>
- Navarro, J.F., D. Beltrán, and M. Cavas. 2012. Effects of (+) SKF 10,047, a sigma-1 receptor agonist, on anxiety, tested in two laboratory models in mice. *Psychopharmacol.* 24:427–430.
- Ono, Y., H. Tanaka, M. Takata, Y. Nagahara, Y. Noda, K. Tsuruma, M. Shimazawa, I. Hozumi, and H. Hara. 2014. SA4503, a sigma-1 receptor agonist, suppresses motor neuron damage in vitro and in vivo amyotrophic lateral sclerosis models. *Neurosci. Lett.* 559:174–178. <http://dx.doi.org/10.1016/j.neulet.2013.12.005>
- Pabba, M. 2013. The essential roles of protein-protein interaction in sigma-1 receptor functions. *Front. Cell. Neurosci.* 7:50. <http://dx.doi.org/10.3389/fncel.2013.00050>
- Palmer, C.P., R. Mahen, E. Schnell, M.B.A. Djamgoz, and E. Aydar. 2007. Sigma-1 receptors bind cholesterol and remodel lipid rafts in breast cancer cell lines. *Cancer Res.* 67:11166–11175. <http://dx.doi.org/10.1158/0008-5472.CAN-07-1771>
- Palty, R., A. Raveh, I. Kaminsky, R. Meller, and E. Reuveny. 2012. SARAF inactivates the store operated calcium entry machinery to prevent excess calcium refilling. *Cell*. 149:425–438. <http://dx.doi.org/10.1016/j.cell.2012.01.055>

- Parekh, A.B., and J.W. Putney Jr. 2005. Store-operated calcium channels. *Physiol. Rev.* 85:757–810. <http://dx.doi.org/10.1152/physrev.00057.2003>
- Park, C.Y., P.J. Hoover, F.M. Mullins, P. Bachhawat, E.D. Covington, S. Raunser, T. Walz, K.C. Garcia, R.E. Dolmetsch, and R.S. Lewis. 2009. STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. *Cell*. 136:876–890. <http://dx.doi.org/10.1016/j.cell.2009.02.014>
- Park, C.Y., A. Shcheglovitov, and R. Dolmetsch. 2010. The CRAC channel activator STIM1 binds and inhibits L-type voltage-gated calcium channels. *Science*. 330:101–105. <http://dx.doi.org/10.1126/science.1191027>
- Prakriya, M., S. Feske, Y. Gwack, S. Srikanth, A. Rao, and P.G. Hogan. 2006. Orai1 is an essential pore subunit of the CRAC channel. *Nature*. 443:230–233. <http://dx.doi.org/10.1038/nature05122>
- Rizzuto, R., D. De Stefani, A. Raffaello, and C. Mammucari. 2012. Mitochondria as sensors and regulators of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 13:566–578. <http://dx.doi.org/10.1038/nrm3412>
- Robson, M.J., B. Noorbakhsh, M.J. Seminerio, and R.R. Matsumoto. 2012. Sigma-1 receptors: Potential targets for the treatment of substance abuse. *Curr. Pharm. Des.* 18:902–919. <http://dx.doi.org/10.2174/138161212799436601>
- Sabino, V., P. Cottone, S.L. Parylak, L. Steardo, and E.P. Zorrilla. 2009. Sigma-1 receptor knockout mice display a depressive-like phenotype. *Behav. Brain Res.* 198:472–476. <http://dx.doi.org/10.1016/j.bbr.2008.11.036>
- Schäuble, N., S. Lang, M. Jung, S. Cappel, S. Schorr, Ö. Ulucan, J. Linxweiler, J. Dudek, R. Blum, V. Helms, et al. 2012. BiP-mediated closing of the Sec61 channel limits Ca²⁺ leakage from the ER. *EMBO J.* 31:3282–3296. <http://dx.doi.org/10.1038/emboj.2012.189>
- Skuza, G., and Z. Rogóz. 2006. Effect of BD 1047, a sigma₁ receptor antagonist, in the animal models predictive of antipsychotic activity. *Pharmacol. Rep.* 58:626–635.
- Soboloff, J., B.S. Rothberg, M. Madesh, and D.L. Gill. 2012. STIM proteins: dynamic calcium signal transducers. *Nat. Rev. Mol. Cell Biol.* 13:549–565. <http://dx.doi.org/10.1038/nrm3414>
- Spruce, B.A., L.A. Campbell, N. McTavish, M.A. Cooper, M.V.L. Appleyard, M. O'Neill, J. Howie, J. Samson, S. Watt, K. Murray, et al. 2004. Small molecule antagonists of the sigma-1 receptor cause selective release of the death program in tumor and self-reliant cells and inhibit tumor growth in vitro and in vivo. *Cancer Res.* 64:4875–4886. <http://dx.doi.org/10.1158/0008-5472.CAN-03-3180>
- Srikanth, S., and Y. Gwack. 2012. Orai1, STIM1, and their associating partners. *J. Physiol.* 590:4169–4177. <http://dx.doi.org/10.1113/jphysiol.2012.231522>
- Srikanth, S., and Y. Gwack. 2013. Molecular regulation of the pore component of CRAC channels, Orai1. *Curr. Top. Membr.* 71:181–207. <http://dx.doi.org/10.1016/B978-0-12-407870-3.00008-1>
- Srikanth, S., H.J. Jung, K.D. Kim, P. Souda, J. Whitelegge, and Y. Gwack. 2010. A novel EF-hand protein, TRACR2A, is a cytosolic Ca²⁺ sensor that stabilizes CRAC channels in T cells. *Nat. Cell Biol.* 12:436–446. <http://dx.doi.org/10.1038/ncb2045>
- Srikanth, S., M. Jew, K.D. Kim, M.K. Yee, J. Abramson, and Y. Gwack. 2012. Juncate is a Ca²⁺-sensing structural component of Orai1 and stromal interaction molecule 1 (STIM1). *Proc. Natl. Acad. Sci. USA*. 109:8682–8687. <http://dx.doi.org/10.1073/pnas.1200667109>
- Srikanth, S., B. Ribalet, and Y. Gwack. 2013. Regulation of CRAC channels by protein interactions and post-translational modification. *Channels (Austin)*. 7:354–363. <http://dx.doi.org/10.4161/chan.23801>
- Stone, J.M., E. Årstad, K. Erlandsson, R.N. Waterhouse, P.J. Ell, and L.S. Pilowsky. 2006. [¹²³I]TPCNE—a novel SPET tracer for the sigma-1 receptor: first human studies and in vivo haloperidol challenge. *Synapse*. 60:109–117. <http://dx.doi.org/10.1002/syn.2028116715498>
- Su, T.-P., T. Hayashi, T. Maurice, S. Buch, and A.E. Ruoho. 2010. The sigma-1 receptor chaperone as an inter-organelle signaling modulator. *Trends Pharmacol. Sci.* 31:557–566. <http://dx.doi.org/10.1016/j.tips.2010.08.007>
- Tchedre, K.T., R.-Q. Huang, A. Dibas, R.R. Krishnamoorthy, G.H. Dillon, and T. Yorio. 2008. Sigma-1 receptor regulation of voltage-gated calcium channels involves a direct interaction. *Invest. Ophthalmol. Vis. Sci.* 49:4993–5002. <http://dx.doi.org/10.1167/iovs.08-1867>
- Tsai, S.Y.A., M.J. Pokrass, N.R. Klauer, N.E. De Credico, and T.-P. Su. 2014. Sigma-1 receptor chaperones in neurodegenerative and psychiatric disorders. *Expert Opin. Ther. Targets*. 18:1461–1476. <http://dx.doi.org/10.1517/14728222.2014.972939>
- van Waarde, A., A.A. Rybczynska, N.K. Ramakrishnan, K. Ishiwata, P.H. Elsinga, and R.A.J.O. Dierckx. 2015. Potential applications for sigma receptor ligands in cancer diagnosis and therapy. *Biochim. Biophys. Acta*. 1848:2703–2714. <http://dx.doi.org/10.1016/j.bbame.2014.08.022>
- Várnai, P., B. Tóth, D.J. Tóth, L. Hunyady, and T. Balla. 2007. Visualization and manipulation of plasma membrane-endoplasmic reticulum contact sites indicates the presence of additional molecular components within the STIM1-Orai1 complex. *J. Biol. Chem.* 282:29678–29690. <http://dx.doi.org/10.1074/jbc.M704339200>
- Vilner, B.J., C.S. John, and W.D. Bowen. 1995. Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines. *Cancer Res.* 55:408–413.
- Walker, J.M., W.D. Bowen, F.O. Walker, R.R. Matsumoto, B. De Costa, and K.C. Rice. 1990. Sigma receptors: biology and function. *Pharmacol. Rev.* 42:355–402.
- Wang, B., R. Rouzier, C.T. Albarracin, A. Sahin, P. Wagner, Y. Yang, T.L. Smith, F. Meric-Bernstam, C. Marcelo Aldaz, G.N. Hortobagyi, and L. Pusztai. 2004. Expression of sigma 1 receptor in human breast cancer. *Breast Cancer Res. Treat.* 87:205–214. <http://dx.doi.org/10.1007/s10549-004-6590-0>
- Wang, L., A.R. Prescott, B.A. Spruce, J. Sanderson, and G. Duncan. 2005. Sigma receptor antagonists inhibit human lens cell growth and induce pigmentation. *Invest. Ophthalmol. Vis. Sci.* 46:1403–1408. <http://dx.doi.org/10.1167/iovs.04-1209>
- Wang, Y., X. Deng, S. Mancarella, E. Hendron, S. Eguchi, J. Soboloff, X.D. Tang, and D.L. Gill. 2010. The calcium store sensor, STIM1, reciprocally controls Orai and CaV1.2 channels. *Science*. 330:105–109. <http://dx.doi.org/10.1126/science.1191086>
- Waterhouse, R.N., R.C. Chang, N. Atuehene, and T.L. Collier. 2007. In vitro and in vivo binding of neuroactive steroids to the sigma-1 receptor as measured with the positron emission tomography radioligand [¹⁸F]FPS. *Synapse*. 61:540–546. <http://dx.doi.org/10.1002/syn.2036917447254>
- Willoughby, D., K.L. Everett, M.L. Halls, J. Pacheco, P. Skroblin, L. Vaca, E. Klussmann, and D.M.F. Cooper. 2012. Direct binding between Orai1 and AC8 mediates dynamic interplay between Ca²⁺ and cAMP signaling. *Sci. Signal.* 5:ra29. <http://dx.doi.org/10.1126/scisignal.2002299>
- Wu, Z., and W.D. Bowen. 2008. Role of sigma-1 receptor C-terminal segment in inositol 1,4,5-trisphosphate receptor activation: constitutive enhancement of calcium signaling in MCF-7 tumor cells. *J. Biol. Chem.* 283:28198–28215. <http://dx.doi.org/10.1074/jbc.M802099200>
- Wu, M.M., E.D. Covington, and R.S. Lewis. 2014. Single-molecule analysis of diffusion and trapping of STIM1 and Orai1 at endoplasmic reticulum-plasma membrane junctions. *Mol. Biol. Cell*. 25:3672–3685. <http://dx.doi.org/10.1091/mbc.E14-06-1107>
- Wünsch, B. 2012. The σ₁ receptor antagonist S1RA is a promising candidate for the treatment of neurogenic pain. *J. Med. Chem.* 55:8209–8210. <http://dx.doi.org/10.1021/jm3011993>
- Xu, X.J., M. Boumechache, L.E. Robinson, V. Marschall, D.C. Gorecki, M. Masin, and R.D. Murrell-Lagnado. 2012. Splice variants of the P2X7 receptor reveal differential agonist dependence and functional coupling with pannexin-1. *J. Cell Sci.* 125:3776–3789. <http://dx.doi.org/10.1242/jcs.099374>
- Yamamoto, H., J. Karasawa, N. Sagi, S. Takahashi, K. Horikomi, S. Okuyama, T. Nukada, I. Sora, and T. Yamamoto. 2001. Multiple pathways of σ₁ receptor ligand uptakes into primary cultured neuronal cells. *Eur. J. Pharmacol.* 425:1–9. [http://dx.doi.org/10.1016/S0014-2999\(01\)01143-8](http://dx.doi.org/10.1016/S0014-2999(01)01143-8)
- Yuan, J.P., W. Zeng, M.R. Dorwart, Y.J. Choi, P.F. Worley, and S. Muallem. 2009. SOAR and the polybasic STIM1 domains gate and regulate Orai channels. *Nat. Cell Biol.* 11:337–343. <http://dx.doi.org/10.1038/ncb1842>