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

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Sigma1 receptors inhibit store-operated Ca\(^{2+}\) entry and reduce the Ca\(^{2+}\) content of the intracellular stores. These effects are regulated by Sigma1 receptor ligands. A ligand-dependent interaction between Sigma1R and STIM1 reduces STIM1 association with Orai1 channels.
**Abbreviations:** AFM, atomic force microscopy; [Ca^{2+}]_{c}, cytosolic free Ca^{2+} concentration; CAD, channel-activating domain of STIM1; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; HBS, Hepes-buffered saline; HEK-σ1R, human embryonic kidney cell stably expressing V5-tagged σ1R; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; MAM, mitochondrion-associated membrane; PBS, phosphate-buffered saline; PLC, phospholipase C; PM, plasma membrane; POST, partner of STIM1; SARAF, SOCE-associated regulatory factor; σ1R, sigma1 receptor; SOCE, store-operated Ca^{2+} entry; STIM1, stromal interaction molecule 1; TS, Triton solution.
ABSTRACT

Sigma1 receptors (σ1Rs) 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 σ1Rs exert such varied effects. We demonstrate that σ1Rs inhibit store-operated Ca\textsuperscript{2+} entry (SOCE), a major Ca\textsuperscript{2+} influx pathway, and reduce the Ca\textsuperscript{2+} content of the intracellular stores. SOCE was inhibited by expression of σ1R or an agonist of σ1R, and enhanced by loss of σ1R or an antagonist. Within the endoplasmic reticulum (ER), σ1R associated with STIM1, the ER Ca\textsuperscript{2+}-sensor that regulates SOCE. This interaction was modulated by σ1R ligands. After depletion of Ca\textsuperscript{2+} stores, σ1R accompanied STIM1 to ER-plasma membrane (PM) junctions where STIM1 stimulated opening of the Ca\textsuperscript{2+} channel, Orai1. The association of STIM1 with σ1R slowed the recruitment of STIM1 to ER-PM junctions and reduced binding of STIM1 to PM Orai1. We conclude that σ1R attenuates STIM1 coupling to Orai1, and thereby inhibits SOCE.
Introduction

Sigma1 receptors (σ1Rs) are widely distributed in the brain and peripheral tissues, including lung, kidney, liver and spleen, and highly expressed in some tumor cells (Brune et al., 2013; Cobos et al., 2008; Monnet, 2005; Su et al., 2010; Vilner et al., 1995; Walker et al., 1990; Wu and Bowen, 2008; Stone et al., 2006). 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 (Kourrich et al., 2013; Maurice and Su, 2009; Maurice et al., 1999; Navarro et al., 2012; Robson et al., 2012; Su et al., 2010; Tsai et al., 2014; Waterhouse et al., 2007; Wünsch, 2012). Changes in expression and polymorphisms of σ1Rs are associated with heart failure (Ito et al., 2012, 2013), addiction (Kourrich et al., 2013; Maurice et al., 2002), neurodegenerative and psychiatric disorders (Miki et al., 2014; Tsai et al., 2014), and cancer (Aydar et al., 2006; Crottès et al., 2013; Maurice and Su, 2009; Spruce et al., 2004; Wang et al., 2004). These associations have provoked interest in σ1Rs as both therapeutic targets and diagnostic tools (Van Waarde et al., 2014).

The σ1R is an integral membrane protein with two transmembrane domains. It is expressed in the endoplasmic reticulum (ER), where it is concentrated in cholesterol-rich mitochondrion-associated ER membrane (MAM) domains and bound to the ER luminal chaperone, BiP (Hayashi and Su, 2003, 2007; Palmer et al., 2007) (Fig. S1). Agonists of σ1R cause it to dissociate from BiP and MAM, allowing σ1Rs to move within ER membranes and interact with signalling proteins in the plasma membrane (PM), most notably ion channels, thereby regulating their activity (Balasuriya et al., 2012; Pabba, 2013; Su et al., 2010). Antagonists block this effect (Fig. S1). Loss of Ca2+ from the ER can also release σ1Rs 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, σ1Rs can
also act as chaperones, stabilizing signalling proteins as they traffic along the secretory pathway (Tsai et al., 2014). σ1Rs may also be expressed in the nuclear envelope (Brune et al., 2013; Hayashi and Su, 2005; Mori et al., 2013) and PM (Aydar et al., 2002; Balasuriya et al., 2014a; Brune et al., 2013; Lupardus et al., 2000), and may even be secreted into the extracellular space (Hayashi and Su, 2003; Su et al., 2010). The interactions between σ1Rs and ion channels may therefore occur within the plane of a membrane (ER or PM) or across ER-PM junctions (Balasuriya et al., 2014a; Hayashi and Su, 2007; Kourrich et al., 2013). Clearly, σ1Rs are important links between diverse ligands, physiological stimuli and many key signalling molecules (Hayashi and Su, 2007; Kourrich et al., 2013; Su et al., 2010).

Receptors that stimulate phospholipase C (PLC), and formation of inositol 1,4,5-trisphosphate (IP$_3$), evoke both Ca$^{2+}$ release from the ER through IP$_3$ receptors (IP$_3$Rs) and Ca$^{2+}$ entry across the PM. At MAMs, Ca$^{2+}$ released by IP$_3$Rs can be rapidly accumulated by mitochondria, thereby stimulating oxidative phosphorylation (Rizzuto et al., 2012) and promoting cell survival (Cárdenas et al., 2010), while excessive mitochondrial Ca$^{2+}$ uptake triggers apoptosis (Mallilankaraman et al., 2012). The association of IP$_3$R3s with σ1Rs at MAMs supports the transfer of Ca$^{2+}$ from ER to mitochondria by curtailing the degradation of active IP$_3$R3s (Hayashi and Su, 2007). The increase in mitochondrial Ca$^{2+}$ concentration and resultant boost in oxidative phosphorylation are thought to contribute to the pro-survival effects of σ1Rs in the central nervous system and cancer cells (Lewis et al., 2014). One effect of σ1Rs may, therefore, be to support transfer of Ca$^{2+}$ from ER to mitochondria, but this transfer also depends on the Ca$^{2+}$ content of the ER.

The Ca$^{2+}$ entry evoked by receptors that stimulate PLC is usually mediated by store-operated Ca$^{2+}$ entry (SOCE), which is stimulated by loss of Ca$^{2+}$ from the ER (Hogan and Rao, 2015; Parekh and Putney, 2005). The reduction in Ca$^{2+}$ concentration within the ER is
detected by the luminal EF-hands of STIM1 (stromal interaction molecule 1), an integral ER membrane protein. This causes STIM1 to cluster and accumulate at ER-PM junctions. STIM1 then binds to Orai1, a Ca\(^{2+}\)-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 Ca\(^{2+}\) stores (Brandman et al., 2007). Additional proteins, including junctate, CRACR2 and SARAF, also interact with STIM1-Orai1 signalling complexes and regulate both activation and de-activation of SOCE (Palty et al., 2012; Srikanth and Gwack, 2012, 2013; Srikanth et al., 2010, 2012, 2013).

We show that σ1Rs constitutively inhibit SOCE and reduce the Ca\(^{2+}\) content of the ER, and that σ1R ligands modulate this inhibition. The σ1R 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 σ1Rs inhibit a ubiquitous Ca\(^{2+}\) entry pathway and suggest a general model for directed translocation of σ1R to its PM targets.
Results

σ1R inhibits SOCE

SOCE in HEK cells can be activated by depletion of intracellular Ca\(^{2+}\) stores using thapsigargin to inhibit the ER Ca\(^{2+}\) pump or by stimuli of endogenous receptors (e.g. ATP or carbachol) that activate PLC. The contributions of Orai1 and STIM1 to SOCE (DeHaven et al., 2009; Parekh and Putney, 2005; Soboloff et al., 2012) are clear from the inhibition of thapsigargin-evoked Ca\(^{2+}\) entry in HEK cells expressing a dominant-negative form of Orai1 (Orai1\(^{E106Q}\)) (Prakriya et al., 2006) and the enhancement of SOCE after over-expression of Orai1 with STIM1 (Fig. 1 A and B). The initial Ca\(^{2+}\) release evoked by thapsigargin was unaffected by these effects of Orai1 and STIM on SOCE. Stable expression of a V5-tagged σ1R in HEK cells (HEK-σ1R cells) attenuated the Ca\(^{2+}\) signals evoked by thapsigargin without affecting expression of Orai1 or STIM1 (103 ± 5% and 91 ± 8% of wild-type cells, respectively) (Fig. 1 C), or the basal cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_c\)]\(_c\)) (45 ± 7 nM and 50 ± 3 nM in wild-type and HEK-σ1R cells, respectively). The increase in [Ca\(^{2+}\)\(_c\)]\(_c\) after addition of thapsigargin in Ca\(^{2+}\)-free medium was reduced by 65 ± 9%, and the SOCE detected after restoration of extracellular Ca\(^{2+}\) was reduced by 86 ± 4% in HEK-σ1R cells (Fig. 1 D and E). The rate of increase of [Ca\(^{2+}\)\(_c\)]\(_c\) during SOCE decreased from 8.8 ± 0.3 nM.s\(^{-1}\) in wild-type HEK to 2.8 ± 0.3 nM.s\(^{-1}\) in HEK-σ1R cells. SOCE in HEK-σ1R cells was similarly reduced across a range of extracellular Ca\(^{2+}\) concentrations (Fig. S2 A). The inhibition of both thapsigargin-evoked Ca\(^{2+}\) release and SOCE in HEK-σ1R 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 min to 20 min (Fig. 1 E). Indeed, both the initial Ca\(^{2+}\) content of the stores (determined by addition of ionomycin in Ca\(^{2+}\)-free medium, the effects of which are not restricted to ER) and the residual content after
thapsigargin-treatment were reduced in HEK-σ1R cells (Fig. 1G 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-σ1R cells (Fig. 1 I and J). The lesser Ca²⁺ release evoked by ATP and carbachol in HEK-σ1R cells (52 ± 8% of wild-type cells) matched the reduced Ca²⁺ content of the stores (59 ± 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 period (2 h) that later experiments (see Fig. 3) show is sufficient for σ1R agonists to inhibit SOCE. This CPA treatment caused a more substantial depletion of the intracellular Ca²⁺ stores than was observed in HEK-σ1R 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-σ1R cells.

The smaller increase in [Ca²⁺]c evoked by SOCE in HEK-σ1R 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⁺]c) were unaffected by expression of σ1R (half-times, t₁/₂ = 36 ± 5 s and 32 ± 6 s for wild-type and HEK-σ1R cells, respectively). The smaller [Ca²⁺]c increases in HEK-σ1R 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-σ1R cells there was no change in the rate of
Mn\textsuperscript{2+} entry in response to either stimulus (Fig. 2 D). Together these results establish that stable expression of σ1R inhibits SOCE.

Selection of polyclonal HEK cells stably expressing σ1R might have propagated cells with different Ca\textsuperscript{2+} signalling behaviors. However, the thapsigargin-evoked increase in [Ca\textsuperscript{2+}]\text{c} and SOCE, and the Ca\textsuperscript{2+} content of the intracellular stores were also reduced in HEK cells transiently expressing σ1Rs (Fig. S2 C-E). The reduced SOCE correlated with the level of expression of σ1R (Fig. S2 F and G). Translocation of GFP-tagged nuclear factor of activated T cells (GFP-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 σ1R (Fig. 2 E and F).

We conclude that expression of σ1R inhibits SOCE by reducing the coupling of empty stores to activation of SOCE.

**Agonists and antagonists of σ1R regulate SOCE**

The σ1R agonist (+)SKF10047 (Navarro et al., 2012; Su et al., 2010) and the antagonist BD1047 (Gromek et al., 2014; Skuza and Rogóz, 2006) (Fig. S1) were used to investigate the acute effects of σ1Rs in Chinese hamster ovary (CHO) cells and HEK-σ1R cells. In CHO cells, σ1Rs are endogenously expressed (Hayashi and Su, 2007). As in HEK cells, SOCE was inhibited by transient expression of Orai1\textsuperscript{E106Q}, although in CHO cells the thapsigargin-evoked Ca\textsuperscript{2+} release was also inhibited (Fig. S3 A). In both CHO and HEK-σ1R cells, pre-incubation with BD1047 increased the amplitude of the Ca\textsuperscript{2+} signals evoked by SOCE, while 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 σ1Rs. The temperature-dependence and slow equilibration of ligand binding to σ1Rs (Yamamoto et al., 2001; Chu and Ruoho, 2016), together with the need to load cells
with Ca\textsuperscript{2+} indicator at 20°C, limited opportunities to investigate the time course of the effects of σ1R ligands. Nevertheless, it is clear that treatment with ligands for at least 1 h at 37°C before loading cells with Ca\textsuperscript{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 σ1R almost abolished expression of endogenous σ1R, but this was accompanied by reduced expression of Orai1 and increased expression of STIM1 (Fig. 3 G and H). The loss of Orai1 could reflect a chaperone role for σ1R, similar to the stabilization of HERG K\textsuperscript{+} channels by σ1R (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 σ1Rs. Over-stimulation of SOCE by constitutively active STIM1 was shown previously to reduce Orai1 expression (Kilch et al., 2012). Despite the reduced expression of Orai1, SOCE was increased in CHO cells lacking σ1Rs, and it was now unaffected by (+)SKF10047 or BD1047 (Fig. 3 I and J). The enhanced SOCE in CHO cells lacking σ1Rs 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\textsuperscript{2+} entry was also increased in CHO cells treated with siRNA to σ1R (Fig. S3 E and F). In normal CHO cells, (+)SKF10047 reduced the Ca\textsuperscript{2+} content of the stores, while the σ1R antagonist, BD1047, increased their content to a level that matched that of cells without σ1Rs. Neither ligand affected the Ca\textsuperscript{2+} stores in CHO cells lacking σ1Rs (Fig. 3 K and L). While comparison of SOCE-mediated Ca\textsuperscript{2+} signals in CHO cells with and without σ1Rs is compromised by accompanying changes in STIM1 and Orai1 expression (Fig. 3 G and H), the analyses demonstrate that σ1R ligands are effective only in cells expressing σ1Rs, and they establish a constitutive inhibition of SOCE by endogenous σ1Rs, and an associated reduction in ER Ca\textsuperscript{2+} content in CHO cells (Fig. 3 I-L). Similar results were obtained in HEK-σ1R cells: siRNA to σ1R abolished the effects of
σ1R ligands on both SOCE and the Ca\textsuperscript{2+} content of the stores; it also increased the basal Ca\textsuperscript{2+} content of the stores and the rate of Mn\textsuperscript{2+} entry evoked by either thapsigargin or by the more physiological stimuli, ATP and carbachol (Fig. S3 H and J).

Determining whether ligands of σ1R are more effective before or after depletion of Ca\textsuperscript{2+} stores was frustrated by the need for prolonged pre-incubations at 37°C for optimal effects (Fig. S3 B-D). In a modified protocol, fluo 4-loaded HEK-σ1R cells in Ca\textsuperscript{2+}-free 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 σ1R ligands affect an early step in the activation of SOCE.

Breast cancer cells express high levels of σ1Rs (Aydar et al., 2006; Spruce et al., 2004; Wang et al., 2004). In MDA-MB-231 human breast cancer cells, which also express σ1Rs, SOCE was enhanced by BD1047 and inhibited by (+)SKF10047 (Fig. S4). The Ca\textsuperscript{2+} content of the stores was also reduced by (+)SKF10047. Hence in three cell types, HEK-σ1R, CHO and MDA-MB-231 cells, σ1Rs both inhibit SOCE and decrease the Ca\textsuperscript{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\textsuperscript{2+} content by endogenous σ1Rs.

σ1R and STIM1 associate and move to ER-PM junctions after store depletion

Interactions between σ1R and STIM1 in unstimulated cells were investigated using HEK cells transiently expressing STIM1-Myc and σ1R-FLAG. Anti-Myc beads pulled down σ1R-FLAG from solubilized cell extracts, but only in cells expressing STIM1-Myc. Conversely, anti-FLAG beads pulled down STIM1-Myc, but only in cells expressing σ1R-FLAG (Fig. 4 A
and B). Co-immunoprecipitation of σ1R-FLAG with STIM1-Myc was enhanced by (+)SKF10047 and reduced by BD1047 (Fig. 4 C-E). These results show that STIM1 and σ1R are associated in unstimulated cells, and that their interaction is regulated by σ1R ligands. Furthermore, the increased association of σ1R with STIM1 evoked by the σ1R agonist (Fig. 4) correlates with the inhibition of SOCE (Fig. 3).

To investigate the intracellular dynamics of σ1R 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 σ1Rs (Fig. 5 A). In cells expressing σ1R-EGFP with mCh-STIM1, σ1R-EGFP and mCh-STIM1 colocalized within the ER (Mander’s correlation coefficient 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 σ1R-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 panel). 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 σ1R-EGFP expressed alone (Fig. 5 C, lower panel). However, when mCh-STIM1 and σ1R-EGFP were co-expressed, thapsigargin caused both proteins to accumulate in sub-PM puncta, within which the proteins colocalized (Mander’s correlation coefficient: 0.77 ± 0.04, n = 8) (Fig. 5 D), but expression of σ1R 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 σ1R were not due to non-specific accumulation of ER proteins. Furthermore, in cells co-expressing Orai1-EGFP, σ1R-mKate and HA-STIM1, Orai1-EGFP and σ1R-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, σ1R accompanies STIM1 to ER-PM junctions, but σ1R slows the accumulation of STIM1.

In related experiments, HeLa cells expressing different combinations of σ1R-EGFP, mCh-STIM1 and Orai1-Myc were fixed for immunolabeling, and confocal images were analysed to assess colocalization of the proteins before and after treatment with thapsigargin. As expected, in cells co-expressing 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 σ1R and Orai1 were co-expressed, 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 σ1R to the same junctions.

σ1R reduces the association of STIM1 with PM Orai1

The requirement for STIM1 in recruiting σ1R to ER-PM junctions containing Orai1 was investigated further by expressing σ1R-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 σ1R within the biotinylated sample was significantly increased in cells over-expressing STIM1 and Orai1, but not when only Orai1 was over-expressed (Fig. 6 A and B). This indicates that STIM1 either promotes trafficking of σ1R to the PM, where it is directly biotinylated or it promotes association of σ1R with a biotinylated PM protein. Similar analyses established that expression of σ1R-FLAG reduced the amount of STIM1 in the biotinylated sample to 47 ± 12 % (n = 3) of that
measured without σ1R (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 σ1R are associated with the PM Orai1 channel complex (and that there is no need to invoke cell-surface expression of σ1R to account for its presence in the biotinylated sample). The amount of STIM1 within this complex was again reduced by σ1R to 51 ± 7 % of that measured without σ1R (Fig. 6 C and D). These results indicate that σ1R reduces the amount of STIM1 bound to PM Orai1. This was confirmed by purifying HA-STIM1 with anti-HA beads: the amount of Orai1 that co-purified 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 over-expressing 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 percentage 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 Ca^{2+} stores: over-expression of STIM1 increased the Ca^{2+} 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 over-expressing 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 Ca\(^{2+}\) 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 over-expressing 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 Ca\(^{2+}\) stores (Balasuriya et al., 2014b; Hou et al., 2012). We examined extracts from thapsigargin-treated HEK cells expressing Orai1-Myc/His and HA-STIM1, with or without σ1R-FLAG, in which cell-surface proteins had been biotinylated and complexes 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 nm\(^3\), Fig. 7 B). A volume distribution of bound peripheral particles for the Orai1-Myc/HA-STIM1 sample had two peaks at 131 ± 2 nm\(^3\) and 235 ± 4 nm\(^3\) (Fig. 7 C), consistent with the expected volumes of STIM1 monomers and dimers. For the Orai1-Myc/HA-STIM1/σ1R-FLAG sample, the volume distribution of the peripheral particles had 3 peaks (62 ± 13 nm\(^3\), 130 ± 20 nm\(^3\) and 220 ± 22 nm\(^3\)) corresponding to STIM1 monomers and dimers, and a smaller peak consistent with the expected volume of σ1R monomers (~63 nm\(^3\)) (Fig. 7 D). Of the 300 Orai1 complexes analysed 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 analysed when co-expressed with STIM1 and σ1R, 76 had bound particles: there were 59 bound STIM1 and 46 bound σ1R. So the total number of bound STIM1 was
reduced by 39% in the presence of σ1R. AFM images of Orai1 isolated from cells expressing Orai1 and STIM1 revealed, albeit with low frequency, Orai1 bound to strings of STIM1 (Fig. 7 E). These assemblies were never seen in images from cells co-expressing σ1R. These results provide evidence for a PM complex of Orai1, STIM1 and σ1R, and for reduced binding of STIM1 to Orai1 in the presence of σ1R.

σ1R inhibits SOCE via STIM1 rather than by direct effects on Orai1

Reduced binding of STIM1 to Orai1 caused by σ1R is expected to reduce SOCE, but σ1R might also directly inhibit gating of Orai1 channels. To address this possibility, we used the channel-activating domain of STIM1 (CAD), which directly activates Orai1 (Gudlur et al., 2014; Muik et al., 2009; Park et al., 2009; Yuan et al., 2009). mCh-CAD expressed alone in HeLa cells was diffusely distributed in the cytoplasm, but it was peripherally distributed when co-expressed with Orai1, consistent with constitutive association of CAD and Orai1 (Fig. 8 A). Addition of extracellular Ca\(^{2+}\) to HEK or HEK-σ1R cells in Ca\(^{2+}\)-free HBS had no significant effect on [Ca\(^{2+}\)]\(_c\), but there was a substantial increase in [Ca\(^{2+}\)]\(_c\) 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-σ1R cells suggesting that σ1R does not directly modulate either PM expression of Orai1 or its activity.

Discussion

We have shown that σ1Rs inhibit SOCE by decreasing the effectiveness with which empty stores stimulate Orai1. The target for regulation of SOCE by σ1R appears to be STIM1 (Fig. 8 D). σ1R and STIM1 colocalize in the ER; they can be co-immunoprecipitated before and after depletion of Ca\(^{2+}\) stores, and their interaction is regulated by σ1R ligands. The agonist, (+)SKF10047, increases binding of STIM1 to σ1R and further inhibits SOCE, while the
antagonist, BD1047, has the opposite effects. After store depletion, σ1R translocates with STIM1 to ER-PM junctions, but σ1R 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 σ1R 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) (Vármai et al., 2007) to be bridged by the short cytosolic loop of σ1R (Hayashi and Su, 2007) (Fig. S1 A). The association of σ1Rs with PM Orai1 is therefore likely to be mediated by STIM1. Reduced binding of STIM1 to Orai1 in the presence of σ1R may be due to σ1R inhibiting oligomerization of STIM1 or directly reducing the affinity of STIM1 for Orai1.

There are interesting similarities between the behavior of σ1Rs and that of other ER membrane proteins, including SARAF (SOCE-associated regulatory factor) (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 de-activation 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 (PMCA) 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 σ1Rs, the effects of ER luminal Ca\(^{2+}\) on these delivery mechanisms may operate at two levels. Loss of ER Ca\(^{2+}\) (or a σ1R agonist) releases σ1R from its interaction with the ER luminal protein, BiP (Hayashi and Su, 2007) (Fig. S1 A). Store-depletion also causes STIM1 to oligomerize and thereby gain affinity for ER-PM junctions. Depletion of Ca\(^{2+}\) stores may therefore both release σ1R 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 σ1Rs to ER-PM junctions by STIM1
might also be involved in regulation of other PM channels by σ1Rs (Kourrich et al., 2013; Maurice and Su, 2009; Pabba, 2013; Su et al., 2010). For example, the L-type Ca^{2+} channel is inhibited by σ1R (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 σ1R to ER-PM junctions may inhibit voltage-gated Ca^{2+} entry and may also deliver σ1Rs to additional PM targets (Fig. 8 D).

Inhibition of SOCE by σ1Rs was invariably accompanied by a decrease in the Ca^{2+} content of the ER with no evident change in [Ca^{2+}]_{c}. By contrast, and consistent with other reports (Lopez 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 σ1Rs on ER Ca^{2+} content. It may be that σ1Rs 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 SERCA, as was shown for orosomucoid like 3 (Cantero-Recasens et al., 2010), or 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 (Schauble et al., 2012). Expression of σ1R might sequester BiP (Fig. S1 A) and thereby enhance the Sec61-mediated Ca^{2+} leak. The decreased Ca^{2+} content of the ER might also arise from σ1R stabilizing IP_{3}R3 and thereby enhancing Ca^{2+} transport from ER to mitochondria (Hayashi and Su, 2007).

The pathophysiological effects σ1Rs 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 (Giorgi et al., 2012; Maurice and Su, 2009). The effects of σ1Rs on mitochondrial Ca^{2+} uptake are probably finely balanced because σ1Rs enhance delivery of Ca^{2+} to mitochondria at MAMs
by stabilizing MAM-associated IP₃Rs (Hayashi and Su, 2007), while our results show that
σ1Rs reduce the ER Ca²⁺ content. The latter could explain the otherwise surprising anti-
apoptotic effects of σ1Rs (Crottès et al., 2013; Decuypere et al., 2011; Maurice and Su,
2009; Wang et al., 2005). The neuroprotective effects of σ1R agonists after ischemic injury
(Katnik et al., 2006) and in patients with amyotrophic lateral sclerosis arising from loss-of-
function mutations in σ1R (Ono et al., 2014; Al-Saif et al., 2011) may also, at least in part,
be due to inhibition of SOCE. Hyperactive SOCE may contribute to the motor deficiencies in
σ1R-knockout mice (Maurice and Su, 2009; Mavlyutov et al., 2010; Sabino et al., 2009) and
to neurodegeneration in Alzheimer’s (Hashimoto, 2009; Hyrskyluoto et al., 2013; Mishina et
al., 2008) and Parkinson’s diseases (Francardo et al., 2014; Hyrskyluoto et al., 2013;
Mishina et al., 2005), where expression of σ1R is reduced. These suggestions prompt
consideration of whether σ1R also interacts with STIM2, because it appears to play the
major role in regulating SOCE in central neurons (Berna-Erró et al., 2009).

We conclude that σ1Rs inhibit SOCE because they associate with STIM1, slow
STIM1 recruitment to ER-PM junctions and reduce its binding to Orai1 after depletion of
Ca²⁺ stores. Our study highlights a role for STIM1 in translocating σ1Rs to the PM and
establishes σ1Rs and their ligands as important regulators of SOCE, a ubiquitously
expressed Ca²⁺ entry pathway (Fig. 8 D).

Materials and methods

Materials

(+)SKF10047 and BD1047 were from Tocris (Bristol, UK). Ionomycin was from
MerckEurolab (Nottingham, UK). Thapsigargin was from Alomone Labs (Jerusalem, Israel).
Anti-Myc monoclonal antibody (46-0603) (1:500 dilution for immunoblots), fura 2-AM and
fluo 4-AM were from Life Technologies (Paisley, UK). Anti-HA (16B12) (1:500) and anti-
FLAG (F3165) (1:500) monoclonal antibodies were from COVANCE (Pennsylvania, USA) and Sigma (Poole, UK), respectively. The anti-σ1R antibody (Ab53852) (1:200), which recognizes a sequence conserved in human and mouse σ1R, was from AbCam (Cambridge, UK). Custom-made rabbit polyclonal anti-peptide antisera to STIM1 (1:100) (CDPQHGHGSQRLTR, the Cys used for conjugation is underlined) and Orai1 (1:200) (CEFAWLQDQLDHRGD) were prepared by Sigma. Anti-actin (A5441) (1:500) antibody was from Sigma. Anti-mouse (1:1000) and anti-rabbit (1:1000) HRP-conjugated secondary antibodies were from Thermo Scientific (Loughborough, UK) and BioRad (Hemel Hempstead, UK), 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 (Balasuriya et al., 2014b; Willoughby et al., 2012). For mCh-STIM1, human STIM1 was sub-cloned into mCherry-C1 (Clontech, Saint-Germain-en-Laye, France) using Xba1 and Not1. For σ1R-FLAG, σ1R was sub-cloned into pcDNA3.1/FLAG using HindIII and Age1. For σ1R-GFP, σ1R was subcloned into GFP-N1 (Clonetech) using HindIII and Kpn1. For σ1R-V5, σ1R was subcloned into pcDNA3.1/V5-TOPO using HindIII and Age1. For σ1R-mKate, σ1R was subcloned into mKate2-N (Evrogen) using HindIII and Kpn1. The coding sequences of all new constructs were verified. pDsRed2-Mito was from Clontech. A pSIREN vector encoding siRNA for σ1R (5′-GATCCACACGTTGAGTGGAGTATTCAAGAGATACTCCACCATCCACGTGTTTTTTTGCTAGCG-3′) was used to inhibit expression σ1R. pSIREN encoding luciferase gene was used as a negative-control. Both pSIREN constructs were gifts from T-P Su (National Institutes of Health, USA) (Hayashi and Su, 2004). An expression plasmid (MO70)
encoding a dominant-negative form of Orai1 in which Glu-106 is replaced by Gln (Orai1\textsuperscript{E106Q}) was a gift from Y. Gwack and S. Srikanth (UCLA) (Srikanth et al., 2012). The expression plasmid for mouse GFP-NFAT1 was a gift from A. Parekh (Oxford, UK) (Kar et al., 2011). The mCh-STIM1 CAD expression plasmid was a gift from P. Hogan (La Jolla) (Gudlur et al., 2014).

**Cell culture and transfection**

All cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma) at 37°C in humidified air with 5% CO\textsubscript{2}. tsA201 cells were grown to 70% confluence in a 162-cm\textsuperscript{2} flask and transfected using calcium phosphate. Plasmid DNA (50 μg) was mixed with 5 ml of 250 mM CaCl\textsubscript{2} and diluted with 5 ml of medium comprising: 275 mM NaCl, 10 mM KCl, 1.4 mM Na\textsubscript{2}HPO\textsubscript{4} mM, 15 mM glucose, 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 one well of a 6-well plate, plasmid DNA (1 μg) was mixed with 2 μl of 7.5 mM polyethylenimine (Polysciences, Inc.) and then diluted with 150 μl 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 σ1R-V5 (HEK-σ1R cells) was performed as described previously (Xu et al., 2012). These cells were maintained in medium supplemented with 0.8 mg/ml G418 (Life Technologies).

HeLa cells were grown on poly-L-lysine-coated 25-mm glass coverslips and transfected using lipofectamine 2000 (Life Technologies). For 1 well of a 6-well plate,
plasmid DNA (2 μg) was diluted in 200 μl Opti-MEM and incubated at 20°C for 5 min. This was combined with 200 μl of Opti-MEM containing 4 μl 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 [Ca²⁺]_<sub>c</sub>**

For measurements of [Ca²⁺]_<sub>c</sub> in populations of cells, HEK cells were seeded into poly-L-lysine-coated 96-well plates. After 24 h, cells were incubated with fluo 4-AM (2 μM) in HEPES-buffered saline (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, 10 mM HEPES, pH 7.3. In Ca²⁺-free HBS, Ca²⁺ was omitted and 1 mM BAPTA was added. For treatments with (+)SKF10047 and BD1047, 10 mM stock solutions were prepared in dimethyl sulfoxide (DMSO) and water, respectively. Cells were pre-treated with (+)SKF10047 (25 μM) or BD1047 (10 μM) in serum-free DMEM for 1 h at 37°C before loading cells with fluorescent Ca²⁺ indicators. Drug treatments were continued during loading and throughout [Ca²⁺]_<sub>c</sub> measurements. After loading, cells were washed, and incubated in HBS for a further 30 min at 20°C. Fluorescence (excitation 490 nm, emission 520 nm) was measured at 20°C using a FlexStation 3 plate-reader that allows online additions (MDS Analytical Devices, Wokingham, UK). Fluorescence was calibrated to [Ca²⁺]_<sub>c</sub> from: [Ca²⁺]_<sub>c</sub> = K_D \left( \frac{F-F_{\text{min}}}{F_{\text{max}}-F} \right)

where K_D = 345 nM (K_D = 190 nM at 37°C, Fig. S2 B), F is the measured fluorescence, and Fₘₐₓ and Fₘᵢₙ are the fluorescence values determined after addition of Triton X-100 (0.1%) in HBS with 10 mM Ca²⁺ or 10 mM BAPTA, respectively.

**Measurements of Mn²⁺ entry**
Confluent cultures of HEK cells in 96-well plates were loaded with fura 2-AM (2 μM) using the method described for fluo 4. Fluorescence (excitation 360 nm, emission 510 nm) was measured using a FlexStation 3 plate-reader at 1.5-s intervals at 20°C. Quenching of fura 2 fluorescence (which reports unidirectional entry of Mn$^{2+}$) is reported as F/F₀, where F is the fluorescence intensity recorded at each time, and F₀ is the average fluorescence intensity measured in the 5 s before addition of MnCl₂. Monoexponential curve-fits to the time course of the changes in F/F₀ were used to compute half-times (t½) 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 thapsigargin (5 μM) to cells at 37°C in HBS. Fluorescence (excitation at 488 nm, emission at 510-540 nm) was collected using a Leica SP5 confocal microscope with an oil-immersion 40x 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 plasmid DNA (50 µg) using polyethylenimine. Cells were extracted in ice-cold medium (138 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 7.5 mM glucose, 21 mM HEPES, 2 mM EDTA, pH 7.4 and centrifuged (1000 xg, 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, 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. Pre-treatments with (+)SKF10047 and BD1047 were for 2 h at 37°C, and stimulation with thapsigargin (5 µM) was for 30 min at 20°C. Cells were extracted in 25 ml ice-cold medium, and all subsequent steps were carried out at 4°C. The suspension was centrifuged (1000 xg, 5 min) and pelleted cells were solubilized for 60 min in 500 µl of TS. After centrifugation (50,000 xg, 60 min), 50 µl of the supernatant was removed for analysis of total expression (input) and 450 µl was incubated with EZ View Red anti-Myc or anti-FLAG beads (30 µl, Sigma) for 3 h with rotation. Protein-bead complexes were isolated (20,800 xg, 10 min), washed 3 times in TS, and proteins were eluted either with 50 µl of the peptides (1 mg/ml, Sigma) to which the anti-Myc or anti-FLAG antibodies had been raised, or with 50 µl Laemmli buffer. The eluted samples were analyzed by SDS-PAGE, followed by immunoblotting. For immunoblots, lanes were loaded with 10 µl of the 500-µl sample (2% of the entire sample) for the measurement of input, and with 10 µl or 20 µl of the 50-µl 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 biotin-sulfo-NHS (0.2 mg/ml, Thermo Scientific). After 60 min on ice, cells were washed 3 times with 15 ml Tris-buffered saline (25 mM Tris-HCl, 150 mM NaCl, 10
mM EDTA, pH 7.4), centrifuged (1000 xg, 5 min), and the pellet was solubilized in TS (500 µl, 60 min, 4°C). After centrifugation (50,000 xg, 60 min), the supernatant was incubated with 50 µl monomeric avidin-coated agarose beads (Thermo Scientific) at 4°C for 2 h. Protein-bead complexes were collected (20,800 xg, 10 min), washed 3 times in TS and either eluted with 50 µl Laemmli buffer for immunoblots, or biotin (1 mg/ml in 1 ml of TS) for further immunopurification using anti-Myc beads as described for immunoprecipitation analyses. 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 phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄), fixed with paraformaldehyde (4%, 20 min) and permeabilized with saponin (0.5 mg/ml, 60 min, Sigma) in blocking solution (5% goat serum, 3% BSA in PBS). Cells were stained with primary antibody in blocking solution (PBS containing 3% BSA and 5% goat serum) (60 min, 20°C), washed twice with PBS, then incubated in the dark with secondary antibody in blocking solution (60 min, 20°C), washed with PBS, dried, mounted onto a glass microscope slide and stored at 4°C. Cells were imaged using an oil-immersion 60x objective (NA 1.40) using a Leica SP5 confocal microscope. For both Pearson’s and Mander’s coefficient measurements, images were analysed with ImageJ using the JACoP plugin. For Mander’s coefficient, only pixels in which HA-STIM1 (or σ1R-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 (Olympus IX51 inverted microscope with a 100x oil immersion objective (NA 1.49) coupled to Andor iXon EMCCD camera, 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 σ1R-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 μM thapsigargin in Ca²⁺-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/F₀, where F and F₀ are the fluorescence intensities at each time and at the start of the experiment, respectively.

**Atomic force microscopy**

tsA 201 cells expressing appropriate combinations of Orai1-Myc-His, σ1R-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 above. Proteins (~45 μl) were added to a 1-cm² mica disc, incubated at 20°C for 10 min, gently washed with water, and dried under nitrogen. Samples were imaged in air using a Bruker Multimode AFM. 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 0.7-1.0 V). Molecular volumes for individual particles were determined using the Scanning Probe Image Processor version 5. 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 cm$^3$/g), $V_2$ is the water specific volume (1 cm$^3$/g) and $d$ is the extent of hydration (assumed to be 0.4 g H$_2$O/g protein).

**Data analysis**

Most results are presented as means ± s.e.m. from $n$ independent experiments. Statistical analysis used Student’s t-test or ANOVA followed by Tukey’s post hoc test, as appropriate.

**Online supplemental material**

Fig. S1 illustrates key features of $\sigma$1R and its ligands. Fig. S2 shows the effects of expressing $\sigma$1Rs in HEK cells on SOCE and the Ca$^{2+}$ content of the intracellular stores. Fig. S3 shows the effects of $\sigma$1R ligands on SOCE in CHO and HEK cells. Fig. S4 shows the effects of $\sigma$1R ligands on SOCE and the Ca$^{2+}$ content of the intracellular stores in MDA-MB-231 human breast cancer cells. Fig. S5 shows the effects of $\sigma$1R ligands on SOCE in HEK-$\sigma$1R cells over-expressing STIM1 and Orai1.

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Figure 1. **Inhibition of SOCE by σ1R.** (A) Ca$^{2+}$ signals recorded from populations of fluo 4-loaded HEK cells transiently transfected with Orai1$^{E106Q}$, STIM1 and Orai1 or mock-transfected (control). Cells were stimulated with thapsigargin (5 μM) in Ca$^{2+}$-free HBS before restoration of extracellular Ca$^{2+}$ (final free \([\text{Ca}^{2+}]_c\), 4 mM). Results show average responses from 6 replicates. (B) Summary results (means ± s.e.m., \(n = 3\)) show peak increases in \([\text{Ca}^{2+}]_c\) evoked by thapsigargin (Ca$^{2+}$ release) and Ca$^{2+}$ restoration (SOCE). (C) Typical immunoblot of σ1R, STIM1, Orai1 and β-actin from 20 μg of solubilized protein from wild-type HEK (WT) and HEK-σ1R cells. (D) Ca$^{2+}$ signals evoked by thapsigargin in Ca$^{2+}$-free HBS and after restoration of extracellular Ca$^{2+}$ to wild-type and HEK-σ1R cells. (E) Summary shows responses to thapsigargin (Ca$^{2+}$ release) and SOCE detected after restoring Ca$^{2+}$ 10 or 20 min after thapsigargin (means ± s.e.m., \(n = 6\)). (F) Responses from single fura 2-loaded HEK cells show fluorescence ratios (\(F_{340}/F_{380}\)) after stimulation with thapsigargin (5 μM) and restoration of extracellular Ca$^{2+}$ (4 mM). Results show means ± s.e.m (\(n = 3\), each with approximately 45 cells). (G) Ca$^{2+}$ contents of the intracellular stores determined by measuring \([\text{Ca}^{2+}]_c\) after addition of ionomycin (5 μM) in Ca$^{2+}$-free HBS before or 10 min after treatment with thapsigargin. (H) Summary results (means ± s.e.m., \(n = 6\)). (I) Ca$^{2+}$ release and SOCE evoked by carbachol (100 μM) and ATP (100 μM). (J) Summary results (means ± s.e.m., \(n = 6\)). ***\(P < 0.001\), **\(P < 0.01\), *\(P < 0.05\), Student’s t test (E, H and J) or ANOVA with Tukey’s post-hoc analysis (B).

Figure 2. **Stable and transient expression of σ1R inhibit SOCE.** (A) Ca$^{2+}$ signals evoked by thapsigargin (1 μM) in Ca$^{2+}$-free HBS followed by restoration of extracellular Ca$^{2+}$ (4 mM) in HEK wild-type cells treated with CPA (0.5 μM or 1 μM, 2.5 h) or HEK-σ1R cells. (B) Summary results show peak increases in \([\text{Ca}^{2+}]_c\) evoked by SOCE or by addition of ionomycin in Ca$^{2+}$-free HBS (means ± s.e.m. \(n = 3\)). (C) Populations of fura 2-loaded
cells were treated with thapsigargin (5 μM, 10 min) in nominally Ca^{2+}-free HBS before addition of MnCl₂ (5 mM). Results show normalized fluorescence intensity (F/F₀) for 6 replicates. (D) Summary results (means ± s.e.m., n = 3) show half-times (t_{1/2}) for fluorescence quenching from unstimulated cells (basal) and cells treated with thapsigargin (5 μM, 10 min), or ATP and carbachol (100 μM each, 3.5 min). (E) Typical images of HEK cells expressing NFAT-GFP before and 30 min after addition of thapsigargin (5 μM) in normal HBS (upper panels). Scale bar, 10 μm. Images of larger fields (lower panels) show thapsigargin-treated HEK-WT and HEK-σ₁R cells. Asterisks indicate cells used for analysis. Scale bar, 20 μm. (F) Summary results show nuclear translocation of NFAT-GFP before and after treatment with thapsigargin (% cells, means ± s.e.m. from 6 independent fields, with between 595 and 660 cells counted for each condition). ***P < 0.001, **P < 0.01, *P < 0.05, Student’s t test (D) or ANOVA with Tukey’s post-hoc analysis (B and F).

Figure 3. **Ligands of σ₁R modulate SOCE.** (A-F) Populations of cells were treated with (+)SKF10047 (25 μM) or BD1047 (10 μM) before removal of extracellular Ca^{2+}, addition of thapsigargin (5 μM) and then restoration of extracellular Ca^{2+} (4 mM) to CHO (A and B), HEK-σ₁R (C and D) or wild-type (WT) HEK cells (E and F). Summary results (B, D and F) show peak increases in [Ca^{2+}]_c after restoration of extracellular Ca^{2+}. The color codes apply to all panels (A-F). (G) Representative immunoblot from CHO cells transfected with control plasmid or plasmid encoding siRNA for σ₁R (siσ₁R). (H) Summary results (means ± s.e.m., n = 3) 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 siσ₁R or control plasmid. (J) Summary shows peak [Ca^{2+}]_c after restoration of extracellular Ca^{2+} to thapsigargin-treated CHO cells treated with siσ₁R or control plasmid. Cells were pre-treated
with (+)SKF10047 (25 μM) or BD1047 (10 μM), as indicated. (K and L) Effects of σ1R or control plasmid and pre-treatment with σ1R ligands on the Ca\(^{2+}\) signals evoked by ionomycin (5 μM) in Ca\(^{2+}\)-free HBS. Typical traces (K) (color code as in I) and summary results (L) are shown. Legends for L are same as J. All summary results show means ± s.e.m., n = 3. ***P < 0.001, **P < 0.01, *P < 0.05, ANOVA with Tukey’s post-hoc analysis (B, D, F, J and L) or Student’s t test (H, and comparison of no treatment conditions in J and L).

Figure 4. **Ligands regulate association of σ1R with STIM1.** (A and B) Solubilized HEK cells expressing STIM1-Myc, σ1R-FLAG or both were immunoprecipitated (IP) with anti-Myc (A) or anti-FLAG (B) antibodies before immunoblotting. Input lanes were loaded with 10 μl of the 500-μl sample and IP lanes with 20 μl of the 50-μl eluate. (C and D) Similar IP analyses from cells expressing STIM1-Myc and σ1R-FLAG after pre-treatment with (+)SKF10047 (25 μM) or BD1047 (10 μM). (E) Summary results (normalized to control cells, means ± s.e.m., n = 3) show amounts of σ1R-FLAG immunoprecipitated by anti-Myc antibody in cells transiently expressing STIM1-Myc and σ1R-FLAG. **P < 0.01, ANOVA with Tukey’s post-hoc analysis.

Figure 5. **σ1R accompanies STIM1 to ER-PM junctions after store depletion.** (A) Immunoblot of lysates from wild-type HEK, HEK-σ1R or HeLa cells. The same amount of protein was loaded in each lane. (B) Confocal images of unstimulated HeLa cells transiently transfected with σ1R-EGFP and mCh-STIM1. Scale bar, 10 μm. Right panel shows an enlargement of the boxed area. Scale bar, 2.5 μm. (C and D) TIRF images of HeLa cells expressing mCh-STIM1 (C, upper panel), σ1R-EGFP (C, lower panel) or both (D) before and 10 min after addition of thapsigargin (5 μM) in Ca\(^{2+}\)-free HBS. (E) (upper panel) 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 $\sigma_1$R alone). (lower panel) Summary results show changes in mCh fluorescence (normalized to maximal intensity) after store depletion in cells with and without $\sigma_1$R (means ± s.e.m., $n = 87$). (F and G) TIRF images of HeLa cells expressing Orai1-EGFP and $\sigma_1$R-mKate either with (F) or without HA-STIM1 (G). Scale bars (C, D, F and G), 10 μm. (H) Confocal images of HeLa cells expressing $\sigma_1$R-EGFP (green), HA-STIM1 (red) and Orai1-Myc (blue), immunostained after treatment with thapsigargin (5 μM). Boxed areas in the left panels (scale bar, 5 μm) are enlarged on the right (scale bar, 2 μm). Arrows show colocalization of all three proteins as white puncta at the PM. (I) Summary results (means ± s.e.m., $n = 8$) show Mander’s overlap coefficient for colocalization of the indicated pairs of proteins in cells expressing only those tagged proteins or with $\sigma_1$R-EGFP or HA-STIM1 as indicated, with and without thapsigargin treatment. ***$P < 0.001$ and **$P < 0.01$, relative to resting cells, Student’s t test.

Figure 6. **STIM1, Orai1 and $\sigma_1$R interact within a macromolecular complex at the PM.** (A) HEK cells expressing $\sigma_1$R-FLAG alone, or with Orai1-Myc or Orai1-Myc and HA-STIM1 were treated with thapsigargin (5 μM, 30 min in Ca$^{2+}$-free HBS) and then cell-surface 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 $\sigma_1$R-FLAG detected in the avidin pull-downs (normalized to cells expressing only $\sigma_1$R-FLAG, means ± s.e.m., $n = 3$). (C) HEK cells expressing Orai1-Myc and HA-STIM1 with or without $\sigma_1$R-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 and anti-Myc pull-downs (normalized to Orai1-Myc pull-down in each condition, means ± s.e.m., n = 3). (E) HEK cells expressing Orai1-Myc and HA-STIM1 with or without σ1R-FLAG were immunoprecipitated (IP) with anti-HA antibody. (F) Peak [Ca^{2+}]_c signals evoked by SOCE were recorded from HEK or HEK-σ1R cells after treatment with thapsigargin (5 µM in Ca^{2+}-free HBS, 10 min) and then restoration of extracellular Ca^{2+} (4 mM). The effects of transiently over-expressing STIM1 or Orai1 are shown. (G) The Ca^{2+} contents of the intracellular stores of the same cells were measured by recording peak increases in [Ca^{2+}]_c from cells exposed to ionomycin (5 µM in Ca^{2+}-free HBS). Results (B, D, F and G) are means ± s.e.m. n = 3. ***P < 0.001, **P < 0.01, *P < 0.05, relative to control, ANOVA with Tukey’s post-hoc analysis (B, F and G) and Student’s t test for D. (B) The results suggest that STIM1 and σ1R within the ER are associated. When STIM1 is activated by depletion of the ER Ca^{2+} 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.

Figure 7. **AFM analyses of interactions between σ1R, STIM1 and Orai1 at the PM.** (A) AFM images of Orai1 isolated with σ1R and STIM1, showing the central Orai1 (red arrow) channel decorated by either STIM1 (white arrow) or both STIM1 and σ1R (blue arrow). Scale bar, 20 nm; height scale, 0-3 nm. (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 (σ1R and STIM1) from cells expressing Orai1, STIM1 and σ1R (D). For these analyses, volume
ranges of 30-100 nm$^3$ and 120-300 nm$^3$ were set for σ1R and STIM1 particles, respectively; the intermediate volumes (100-120 nm$^3$) were disregarded. (E) AFM images showing strings of STIM1 (white arrow) connecting several Orai1 channels (red arrow). These infrequent structures were observed only in the absence of σ1Rs. Scale bar, 20 nm; height scale, 0-3 nm.

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 panel**) or with Orai1-CFP (**right panels**). Scale bars, 10 μm. (B) HEK or HEK-σ1R cells were mock-transfected or transfected with CAD, and [Ca$^{2+}$]$_c$ was recorded after addition of BAPTA (1 mM) and then restoration of extracellular Ca$^{2+}$ (4 mM). (C) Summary results show peak [Ca$^{2+}$]$_c$ signals evoked by restoration of extracellular Ca$^{2+}$ (means ± s.d., from 6 replicates). ***$P < 0.001$, relative to control, Student’s t test. (D) Several proteins, including POST, σ1R and SARAF, associate with STIM1 in ER membranes (Palty et al., 2012; Krapivinsky et al., 2011). Loss of Ca$^{2+}$ from 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 termination of SOCE (Palty et al., 2012). σ1R competes with STIM1 for binding to Orai1, thereby inhibiting SOCE. POST associates with and inhibits the PMCA, and thereby reduces local Ca$^{2+}$ extrusion (Krapivinsky et al., 2011).
Figure S1. **Key features of σ1R.** (A) σ1R comprises 223 residues. It is expressed predominantly in ER membranes, where it adopts the topology shown, with two transmembrane domains, a short cytosolic loop, and luminal N- and C-termini. The N-terminus includes an ER-retention signal. Residues important for ligand recognition are shown by the green oval. σ1Rs reside, along with some IP₃Rs, within mitochondria-associated ER membranes (MAMs), regions of close contact between ER and mitochondria that allow Ca²⁺ and lipid exchanges between them (Hayashi and Su, 2004, 2007). IP₃Rs associated with σ1Rs in MAMs are protected from degradation and can thereby deliver sustained physiological Ca²⁺ signals to mitochondria (Hayashi and Su, 2007). Within MAMs, σ1Rs also associate with the luminal Ca²⁺-binding protein and chaperone, BiP. The interaction between Ca²⁺-BiP and σ1R is disrupted by loss of Ca²⁺ from the ER or by σ1R agonists. This frees σ1Rs to move within ER membranes and establish contacts with different membranes (notably the PM) (Hayashi and Su, 2007). The present study demonstrates that STIM1 can then deliver σ1Rs to specific PM domains. (B) The list of ligands of σ1Rs is incomplete, but it illustrates the diversity of drugs that interact with σ1Rs, the overlap with drugs that are important in the clinic or as drugs of abuse, and drugs used in the present study (colored). The σ1R is probably unrelated to σ2R, although the two have overlapping pharmacology. **Abbreviations:** BB1047, (N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine); DTG, 1,3-di-o-tolylguanidine; Igmesine, ((R)-(+) -N-cyclopropylmethyl-α-ethyl-N-methyl-α-[(2E)-3-phenyl-2-propenyl]benzenemethanamine hydrochloride; MR22, (-)-methyl (1S,2R)-2-[[1-adamantyl(methyl)amino]methyl]-1-phenylcyclopropane-carboxylate); NE100, 4-methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeeneethanamine hydrochloride; PPBP, 4-phenyl-1-(4-phenylbutyl)-piperidine maleate; PPCC, (S*,R*)-2-[(4-hydroxy-4-phenyl-1-piperidinyl)methyl]-1-(4-methylphenyl)cyclopropanecarboxylic acid methyl ester; PRE-084, 2-(4-morpholinethyl) 1-
phenylcyclohexanecarboxylate hydrochloride; SA 4503, 1-[2-(3,4-dimethoxyphenyl)ethyl]-4-(3-phenylpropyl)-piperazine dihydrochloride; (+)SKF10047, [2S-(2α,6α,11R*]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol hydrochloride.  

*Drug actions: Cocaine,* inhibitor of catecholamine uptake (Schwartz et al., 2010). Drug of abuse. *Dimemorfan,* centrally-acting, cough suppressant (Shin et al., 2005). *Donepezil* (Aricept®), reversible inhibitor of acetylcholinesterase, used to treat Alzheimer's disease (Ramakrishnan et al., 2014). *DMT,* hallucinogen and possible endogenous ligand of σ1Rs (Fontanilla et al., 2009). *Fluvoxamine,* selective serotonin reuptake inhibitor (SSRI) used to treat depression (Hindmarch and Hashimoto, 2010). Many other SSRIs are also agonists of σ1Rs. *Haloperidol* (Dozic®, Serenace®), dopamine D2 receptor antagonist, used to treat schizophrenia and other psychotic disorders (Maurice and Su, 2009). *Ibogaine,* psychoactive natural product, potential utility in treatment of drug-craving (Mach et al., 1995). *Igmesine,* possible utility in treatment of depression (Kulkarni and Dhir, 2009). *Methamphetamine* (crystal meth, ice), stimulates release of catecholamines and blocks their reuptake (Nguyen et al., 2005). Drug of abuse. *Panamesine,* possible utility in treatment of schizophrenia (Kulkarni and Dhir, 2009). *Pentazocine,* opioid analgesic. Closely related to SKF10047 (Hayashi and Su, 2005). *Rimcazole,* adverse effects prevented use as a potential anti-cancer therapy (Happy et al., 2015).

Figure S2. *σ1Rs inhibit SOCE-mediated Ca²⁺ signals.* (A) Experiments similar to those shown in Fig. 1 D were used to measure thapsigargin-evoked SOCE after restoration of different extracellular Ca²⁺ concentrations to wild-type HEK (WT) or HEK-σ1R cells. Results show peak increases in [Ca²⁺]c after restoration of extracellular Ca²⁺(concentrations refer to the free [Ca²⁺], means ± s.e.m., n = 3 with 6 replicates in each experiment). (B) Ca²⁺ signals evoked by thapsigargin and then restoration of extracellular at 20°C or 37°C. Results show
mean ± s.d. from 6 replicates. (C) Populations of fluo 4-loaded HEK cells transiently expressing σ1R-V5 (σ1Rtrans) or mock-transfected (control) were stimulated with thapsigargin (5 μM) in Ca²⁺-free HBS before addition of extracellular Ca²⁺ (4 mM). Results show the mean ± s.d. of 6 replicates. (D) Experiments similar to those shown in Fig. 1 G and H were used to assess the size of the intracellular Ca²⁺ stores in HEK cells transiently expressing σ1R-V5. Ionomycin (5 μM) was added to cells in Ca²⁺-free HBS, and the peak increases in [Ca²⁺]c were recorded (means ± s.d. from 6 replicates). (E) Populations of fluo 4-loaded HEK-σ1Rtrans or mock-transfected (control) cells were stimulated with ATP (100 μM) and carbachol (100 μM) in Ca²⁺-free HBS before addition of extracellular Ca²⁺ (4 mM). Results show means of 6 replicates. (F) Immunoblot showing σ1R expression at the indicated times after transfection. (G) Peak increases in [Ca²⁺]c evoked by SOCE recorded at the indicated times after transient transfection of HEK cells with σ1R. ***P < 0.001, **P < 0.01, relative to control (Student’s t test in A, D and ANOVA followed by Tukey’s post-hoc analysis in G). The results show that transient expression of σ1Rs has similar effects to stable expression, namely inhibition of SOCE and a reduction in the Ca²⁺ content of the intracellular stores.

Figure S3.  **Time course of the effects of σ1R ligands on SOCE.** (A) Populations of fluo 4-loaded CHO cells transiently transfected with Orai¹E¹⁰⁶Q or mock-transfected were stimulated with thapsigargin (5 μM) in Ca²⁺-free HBS before restoration of extracellular Ca²⁺ (4 mM). Results show means ± s.d. of 6 replicates. (B, C) HEK-σ1R cells were pre-treated with BD1047 (B, 10 μM) or (+)SKF10047, (C, 25 μM) for the indicated times, with the final 1.5 h at 20°C (during dye loading) and the preceding 1 h (for the 2.5-h incubation) at 37°C. Ca²⁺ signals evoked by thapsigargin in Ca²⁺-free HBS and then restoration of extracellular Ca²⁺ were recorded after the pre-incubations. (D) Summary results (means ± s.e.m., n = 3)
show peak increases in \([\text{Ca}^{2+}]_c\) evoked by SOCE. (E) Experiments similar to those shown in Fig. 2 C were used to measure quenching of fura 2 fluorescence in CHO cells treated with siRNA to σ1R or with control plasmid. Fura 2-loaded cells were treated with thapsigargin (5 μM, 10 min) in nominally Ca\(^{2+}\)-free HBS before addition of MnCl\(_2\) (5 mM). Results show normalized fluorescence intensity \((F/F_0)\) for 6 replicates. (F) Summary results show half-times \(t_{1/2}\) for fluorescence quenching before (basal) and after thapsigargin treatment (means ± s.e.m., \(n = 3\)). The results demonstrate that loss of σ1Rs in CHO cells increases unidirectional entry of Mn\(^{2+}\) through the SOCE pathway. (G) Summary results show peak increases in \([\text{Ca}^{2+}]_c\) evoked by SOCE after thapsigargin treatment (1 μM) in CHO cells transfected with siσ1R, Orai1\(^{E106Q}\) or both. (H) Typical traces show the effects of pre-treatment with ligands of σ1R ((+)SKF10047, 25 μM; BD1047, 10 μM) on thapsigargin-evoked Ca\(^{2+}\) signals in HEK-σ1R cells treated with siRNA to σ1R. Fluo 4-loaded cells were stimulated with thapsigargin (5 μM) in Ca\(^{2+}\)-free HBS before restoration of extracellular Ca\(^{2+}\) (4 mM). (I) Summary results (means ± s.e.m., \(n = 3\)) show peak increases in \([\text{Ca}^{2+}]_c\) evoked by ionomycin (5 μM). (J) Summary results show half-times \(t_{1/2}\) for fluorescence quenching (means ± s.e.m., \(n = 3\)) before (basal) and after treatment with thapsigargin (5 μM, 10 min) or carbachol with ATP (100 μM of each, 3.5 min). The results (means ± s.e.m., \(n = 3\)) demonstrate that the effects of σ1R ligands on thapsigargin-evoked Ca\(^{2+}\) release and SOCE are abolished after treatment with siRNA for σ1R. (K) Fluo 4-loaded HEK-σ1R cells were incubated with (+)SKF10047 (25 μM) or BD1047 (10 μM) for 2 h in Ca\(^{2+}\)-free HBS at 20°C. Thapsigargin (1 μM, 10 min) was added before or after addition of the σ1R ligands, and the increase in \([\text{Ca}^{2+}]_c\) evoked by SOCE was measured after restoration of extracellular Ca\(^{2+}\). The results (mean ± s.e.m., \(n = 3\)) show that the ligands have less effect on SOCE when added after store-depletion. ***\(P < 0.001\), **\(P < 0.01\), *\(P < 0.05\), relative to control.
(ANOVA followed by Tukey’s post-hoc analysis for D, G, I and J; Student’s t test for F and for comparison of no treatment conditions in I).

Figure S4. **Effects of ligands of σ1R on SOCE and intracellular Ca\(^{2+}\) stores in MDA-MB-231 breast cancer cells.** (A) Immunoblot showing detection of σ1R in a lysate of MDA-MB-231 cells. (B) Populations of fluo 4-loaded MDA-MB-231 cells were pre-treated with ((+)SKF10047 (25 μM) or BD 1047 (10 μM) before addition of thapsigargin (5 μM) in Ca\(^{2+}\)-free HBS and then measurement of SOCE following restoration of extracellular Ca\(^{2+}\) (4 mM) after 10 min. Results (means ± s.e.m., n = 3) show the peak [Ca\(^{2+}\)]\(_{c}\) evoked by the addition of extracellular Ca\(^{2+}\). (C) The Ca\(^{2+}\) content of the intracellular stores was assessed after the same pre-treatments with σ1R ligands by addition of ionomycin (5 μM) in Ca\(^{2+}\)-free HBS. Results (means ± s.e.m., n = 3) show the peak increases in [Ca\(^{2+}\)]\(_{c}\) evoked by ionomycin. ***P < 0.001, **P < 0.01, relative to control treatment (ANOVA followed by Tukey’s post-hoc analysis).

Figure S5. **Effects of ligands of σ1R on SOCE in cells over expressing STIM1 and Orai1.** Populations of fluo4-loaded HEK-σ1R cells were either mock-transfected (control) or transfected with STIM1 and Orai1 before pre-treatment with (+)SKF10047 (25 μM) or BD 1047 (10 μM). SOCE was then measured by addition of extracellular Ca\(^{2+}\) to cells treated with thapsigargin in Ca\(^{2+}\)-free HBS. Results (means ± s.d., 6 replicates) show the peak [Ca\(^{2+}\)]\(_{c}\) evoked by addition of extracellular Ca\(^{2+}\). **P < 0.01, relative to no treatment, using ANOVA followed by Tukey’s post-hoc analysis.
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