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σ-1 Receptor at the Mitochondrial-Associated Endoplasmic Reticulum Membrane Is Responsible for Mitochondrial Metabolic Regulation[S]

Karla-Sue C. Marriott, Manoj Prasad, Veena Thapliyal, and Himangshu S. Bose

Department of Natural Sciences, College of Science and Technology, Savannah State University, Savannah, Georgia (K.-S.C.M.); and Departments of Biochemistry and Biomedical Sciences, Mercer University School of Medicine and Memorial University Medical Center, Anderson Cancer Institute, Savannah, Georgia (M.P., V.T., H.S.B.)

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ABSTRACT

The mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) is a small section of the outer mitochondrial membrane tethered to the ER by lipid and protein filaments. One such MAM protein is the σ-1 receptor, which contributes to multiple signaling pathways. We found that short interfering RNA-mediated knockdown of σ-1 reduced pregnenolone synthesis by 95% without affecting expression of the inner mitochondrial membrane resident enzyme, 3-β-hydroxysteroid dehydrogenase 2. To explore the underlying mechanism of this effect, we generated a series of σ-receptor ligands: 5,6-dimethoxy-3-methyl-*N*-phenyl-*N*-(3-(piperidin-1-yl)propyl)benzofuran-2-carboxamide (KSCM-1), 3-methyl-*N*-phenyl-*N*-(3-(piperidin-1-yl)propyl)benzofuran-2-carboxamide (KSCM-5), and 6-methoxy-3-methyl-*N*-phenyl-*N*-(3-(piperidin-1-yl) propyl)benzofuran-2-carboxamide (KSCM-11) specifically bound to σ-1 in the nanomolar range, whereas KSCM-5 and KSCM-11 also bound to σ-2. Treatment of cells with the KSCM ligands led to decreased cell viability, with KSCM-5 having the most potent effect followed by KSCM-11. KSCM-1 increased σ-1 expression by 4-fold and progesterone synthesis, whereas the other compounds decreased progesterone synthesis. These differences probably are caused by ligand molecular structure. For example, KSCM-1 has two methoxy substituents at C-5 and C-6 of the benzofuran ring, whereas KSCM-11 has one at C-6. KSCM ligands or σ-1 knockdown did not alter the expression of ER resident enzymes that synthesize steroids. However, coimmunoprecipitation of the σ-1 receptor pulled down voltage-dependent anion channel 2 (VDAC2), whose expression was enhanced by KSCM-1. VDAC2 plays a key role in cholesterol transport into the mitochondria, suggesting that the σ-1 receptor at the MAM coordinates with steroidogenic acute regulatory protein for cholesterol trafficking into the mitochondria for metabolic regulation.

Introduction

The physical interaction that occurs between subcellular organelles provides an efficient mechanism for modulating cellular processes. For example, plasma membrane associates with the endoplasmic reticulum (ER), thereby promoting the rapid reuptake of extracellular Ca^{2+} to replenish ER stores (Blaustein and Golovina, 2001; Várnai et al., 2007). The mitochondria, another major Ca^{2+}-buffering organelle, also associates with the plasma membrane, and by doing so, interacts with the ER (Malli et al., 2003). The physical interaction between the ER and mitochondria forms a structural element referred to as the mitochondria-associated ER membrane (MAM) (Vance, 1990), which plays a significant role in cellular functions, including lipids and cholesterol transport, energy metabolism, and apoptosis (Miller and Bose, 2011).

The MAM, present in many cell types, typically consists of only a small section of the outer mitochondrial membrane (OMM) that associates with the ER (Csordás et al., 2006).

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ABBREVIATIONS: ER, endoplasmic reticulum; MAM, mitochondria-associated ER membrane; Ab, antibody; IMM, inner mitochondrial membrane; IP3, inositol 1,4,5-triphosphate; KSCM-1, 5,6-dimethoxy-3-methyl-*N*-phenyl-*N*-(3-(piperidin-1-yl)propyl) benzofuran-2-carboxamide**;** KSCM-5, 3-methyl-*N*-phenyl-*N*-(3-(piperidin-1-yl)propyl)benzofuran-2-carboxamide; KSCM-11, 6-methoxy-3-methyl-*N*-phenyl-*N*-(3-(piperidin-1-yl)propyl) benzofuran-2-carboxamide; OMM, outer mitochondrial membrane; SCC, side-chain cleavage; siRNA, short interfering RNA; SKF-10047, 2*S*-[2α,6α,11*R**]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol; StAR, steroidogenic acute regulatory protein; TLC, thin-layer chromatography; VDAC, voltage-dependent anion channel; 3βHSD2, 3-β-hydroxysteroid dehydrogenase.

Initially, the literature reported a distance of approximately 100 nm between the ER and the OMM; however, a study using electron tomography demonstrated that the distance is actually within 10 to 25 nm (Csordás et al., 2006). Given the roles of MAM in cellular physiology, it is not surprising that this membrane interface is enriched with a variety of proteins, including enzymes associated with lipid and glucose metabolism, such as phosphotidylserine synthase, phosphotidylethanoloamine methyltransferase 2, acyl-CoA cholesterol acyltransferase, diacylglycerol acyltransferase, and glucose 6-phosphate (Hayashi et al., 2009). Other proteins shown to localize to the interface include the chaperones calnexin and calreticulun, which both bind Ca^{2+} and help with the quality control of protein folding; phosphofurin acidic cluster sorting protein 2, which plays a role in apoptosis, and the σ-1 receptor, which has been linked to a multitude of signaling pathways (Simmen et al., 2005; Hayashi and Su, 2007; Hayashi et al., 2009; Kornmann et al., 2009).

σ-Receptors consist of two subtypes, σ-1 and σ-2; however, only σ-1 has been characterized at the molecular level. Initially classified as a type of opioid receptor, the σ-1 receptor is now considered a nonopioid receptor (Su et al., 1988), but a definitive identity of its endogenous ligand remains somewhat elusive. The σ-1 receptor can be found in the central nervous system as well as in peripheral organs, including the lung, liver, adrenal gland, spleen, and pancreas (Hayashi and Su, 2007). Studies have shown that the σ-1 receptor, which has two transmembrane-spanning regions (Jbilo et al., 1997; Aydar et al., 2002), translocates during the activation of multiple signaling pathways and modulates the function or production of various intracellular secondary messengers (Hayashi and Su, 2001; Aydar et al., 2002), including binding to neuroactive steroids, such as progesterone and dehydroepiandrosterone (Valade et al., 2011). The σ-1 receptor also helps proteins fold appropriately at the ER to regulate the metabolic activity of receptor-mediated Ca^{2+} influx (Hayashi and Su, 2007).

Tissues generate steroid hormones on demand after transport of cholesterol, which serves as the initial substrate, into the mitochondria. Fractions of MAM that contain σ-1 receptors (Hayashi and Su, 2007) are exceptionally enriched in cholesterol and neutral lipids (Hayashi and Su, 2003). Moreover, acyl-CoA cholesterol acyltransferase, the enzyme responsible for the synthesis of cholesteryl esters, also resides in the MAM. Thus, MAM might serve as a site for cholesterol loading through the OMM. The fact that cholesterol transport into the mitochondria represents the rate-limiting step in steroidogenesis supports the potential importance of MAM. Cholesterol is stored in lipid droplets as cholesterol esters and is transported first from the OMM to the inner mitochondrial membrane (IMM), where it is catalyzed by the cytochrome P450 side-chain cleavage (SCC) enzyme, to form pregnenolone, the first steroid in the biosynthetic pathway. Pregnenolone is then transported back to the ER for the synthesis of several other steroids (Miller and Bose, 2011). Transport of cholesterol to the site of steroidogenesis is facilitated by steroidogenic acute regulatory protein (StAR), which interacts with voltage-dependent anion channel 1 (VDAC1) at the OMM (Bose et al., 2008). VDAC may also contribute to the association of inositol 1,4,5-triphosphate (IP3) receptors with the OMM at the MAM. During times of calcium depletion in the ER, the σ-1 receptor induces prolonged calcium signaling into the mitochondria via these IP3 receptors. There are possible explanations where the IP3 receptors are connected from the MAM through VDAC with the OMM, because of the short distance of 20 nm. Interaction of σ-1 receptor is facilitated with the Ca^{2+} channel at the mitochondrial membrane either directly or through the VDAC (Hayashi and Su, 2007). It is noteworthy that progesterone synthesis in the bovine placentome is calcium-dependent (Shemesh et al., 1984). Together, this evidence points to a possible role for σ-1 receptors in steroidogenesis.

Selective ligands for the σ-1 receptor can help elucidate the physiological roles of this protein. σ-1 Ligands must contain a basic amine and two hydrophobic appendages (Costantino et al., 2005). Hence, a common pharmocophoric feature of σ-1 ligands is an *N*-alkyl, *N*,*N*-dialkyl, or *N*-arylalkyl amine moiety (Ablordeppey et al., 2000), which poses a challenge in the synthesis of these ligands. It is noteworthy that progesterone, which lacks a basic nitrogen, is considered one of the putative endogenous ligands for the σ-1 receptor (Walker et al., 1990). *N*,*N*-dimethyltryptamine has been identified as a potential endogenous σ-1 receptor ligand, but the role of *N*,*N*-dimethyltryptamine as a σ-1 receptor modulator is unclear because of the low abundance at physiological concentrations in brain tissues (Fontanilla et al., 2009). We synthesized ligands that have a benzofuran-2-carboxamide structural moiety, which has been N-arylated and N-alkylated to include both *N*-phenyl and *N*-(3-(piperidin-1-yl) propyl substituents. These ligands have a defined conformation and can be delivered to the ER in the cell. Using these ligands, we have identified an interaction between the σ-1 receptor and VDAC2 that contributes to the regulation of mitochondrial pregnenolone synthesis.

Materials and Methods

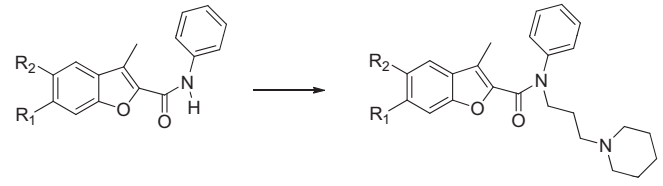
Cell Culture, Isolation, and Purification of Mitochondria. The mouse Leydig cell line (MA-10) was grown in Waymouth media containing 15% horse serum, 5% fetal bovine serum, and 1× gentamycin, which was replaced with 250 mg/ ml G418 (geneticin) for VDAC1 knockdown (ΔVDAC1) MA10 cells (Bose et al., 2008). Cells were maintained at 37°C in a humidified incubator under 5% CO_2. To isolate mitochondria, cells were removed from tissue culture plates by gentle scrapping in phosphate-buffered saline at room temperature, incubated in hypotonic buffer (10 mM HEPES, pH 7.4) for 30 to 40 min, and mitochondria were isolated following previously described procedures (Bose et al., 2007; Pawlak et al., 2011b). The mitochondrial preparation was verified by Western blotting with the cytochrome P450 SCC enzyme (Corgen, Tapai, Taiwan) and calnexin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Ligand Concentration and Cell Viability Assay. The σ-receptor compounds act as ligands and are named "ligands" in this article. Stock solutions (1 mM) of the ligands were made in 1× phosphate-buffered saline, filter-sterilized, and then further diluted up to 1 μM with serum-free medium. Cell viability was determined by the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide assay using a commercially available kit following the manufacturer's instructions (Promega, Madison, WI). MA-10 cells were plated in a 96-well plate at an initial density of 4×10^3 cells per well for 24 h at 37°C in 5% CO_2 atm. After 24 h of serum starvation, the culture media were changed to serum-free media containing 1, 5, 10, 50, and 100 nM concentration for each KSCM ligand. Next, the cells were incubated for 2 to 3 h with 0.5 mg/ml of 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide reagent and lysed with dimethyl sulfoxide, and the absorbance was measured at 550 nm (FlexStation 3; Molecular Devices, Sunnyvale, CA).

Western Blot Analysis. Protein (12.5 μg) was separated by 15% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidine difluoride membrane (Millipore Corporation, Billerica, MA). The membrane was blocked with 3% nonfat dry milk for 45 min, probed overnight with the primary antibodies, and then incubated with peroxide-conjugated goat anti-rabbit or anti mouse IgG (Thermo Fisher Scientific, Waltham, MA). Signals were developed with chemiluminescent reagent (Thermo Fisher Scientific). For loading control of all Western blots we stained the membrane with β-actin antibody, which was previously independently stained with StAR or 3-β-hydroxysteroid dehydrogenase (3βHSD2) or VDAC1 and VDAC2 antiserum. The band intensity was determined by using Image Quant 5.2 (Phosphorimager; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The densitometric calculation in each figure reflects the ratio of σ-1 or VDAC2 and β-actin following an established procedure (Pitre et al., 2007).

Metabolic Conversion Assays and Analysis of Steroids. To measure the conversion of [^{3}H]pregnenolone to [^{3}H]progesterone, isolated mitochondria from steroidogenic MA-10 cells (300 μg) were incubated in phosphate buffer along with the substrate, and the reaction was initiated by the NAD (Pawlak et al., 2011a). For metabolic conversion of [^{14}C]cholesterol to pregnenolone, the reaction was initiated by the addition of NADPH (Pawlak et al., 2011a), and for complete conversion we used 5-fold excess of cold carrier to reach the saturation point. The steroids were extracted with ether/acetone (9:1 v/v), and equal amounts of a cold pregnenolone-progesterone (50:50; Sigma, St. Louis, MO) mixture in CH_2Cl_2 was added as a carrier. The extracts were concentrated under a stream of nitrogen or air and then separated by thin-layer chromatography (TLC) (Whatman, Clifton, NJ) using a chloroform/ethyl acetate (3:1) mobile phase. The spots extracted from TLC plates were subjected to gas chromatography-mass spectrometry analysis on an Agilent 7890 GC with 5975C mass spectrometer (Agilent Technologies, Santa Clara, CA) and analyzed as described previously (Prasad et al., 2012).

σ-Receptor Ligand Synthesis. Our novel carboxamide ligands [5,6-dimethoxy-3-methyl-*N*-phenyl-*N*-(3-(piperidin-1-yl)propyl) benzofuran-2-carboxamide (KSCM-1), 3-methyl-*N*-phenyl-*N*-(3-(piperidin-1-yl) propyl)benzofuran-2-carboxamide (KSCM-5), and 6-methoxy-3-methyl-*N*-phenyl-*N*-(3-(piperidin-1-yl) propyl)benzofuran-2-carboxamide (KSCM-11)] maintain the characteristic pharmacophore of documented σ-1 receptor ligands, such as a basic alkylamine moiety, the *N*-(3-(piperidin-1-yl) propyl with a protonatable nitrogen, and an aromatic phenyl ring and aromatic benzofuran moiety as the two hydrophobic residues. Syntheses of KSCM-11 and KSCM-1 and the unsubstituted KSCM-5 were achieved by treating the corresponding carboxamides (Scheme 1) with NaH followed by N-alkylation with iodopropylpiperidine outlined in Scheme 1. A halogen exchange reaction was used to convert chloropropylpiperidine hydrogen chloride salt in the presence of potassium iodide, tetrabutylammonium bromide, and potassium carbonate into the more reactive iodopropylpiperidine in situ, which then reacted with anions obtained from treating carboxamides (Scheme 1) with sodium hydride producing KSCM-11, KSCM-1, and KSCM-5, respectively.



1a, $R_1 = OCH_3$ $R_2 = H$
1b, $R_1 = OCH_3$ $R_2 = OCH_3$
1c, $R_1 = H$ $R_2 = H$

KSCM-11 $R_1 = OCH_3$ $R_2 = H$
KSCM-1 $R_1 = OCH_3$ $R_2 = OCH_3$
KSCM-5 $R_1 = H$ $R_2 = H$

Reagents: NaH, 1-(3-chloropropyl)piperidine.HCl, K_2CO_3, KI, TBAB, CH_2Cl_2

Scheme 1. Synthesis of different KSCM ligands from carboxamides via N-alkylation. The substitution of side chains is indicated above, generating KSCM-1, KSCM 5, and KSCM-11 ligands.

The structures of the new ligands were confirmed by ^{1}H and ^{13}C NMR spectra on a JEOL (Tokyo, Japan) 300-MHz spectrometer, and high-resolution mass spectrometry data were obtained in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois (Urbana IL). Chemical shifts for ^{1}H and ^{13}C NMR spectra are reported in δ values (ppm) relative to an internal standard of tetramethylsilane in $CDCl_3$. Multiplicities are presented as follows: s = singlet, d = doublet, t = triplet, q = quartet, qt = quintuplet, and m = multiplet. Solvents were purified by using standard procedures. TLC analyses were performed on Fluka (Buchs, Switzerland) 200-μm silica gel, particle size 25 μm, F_{254} plates and visualized by quenching of UV fluorescence (λ_{max} = 254 nm). All new ligands were purified on a Biotage (Charlotte, NC) Isolera-4 flash purification system using the indicated SNAP cartridges and solvents as eluents.

Radioligand Binding Assay. The primary binding screening assays were performed by using concentrations ranging from 50 μM (initial) to 10 μM (final) concentration of reference and experimental ligands following standard procedure (Kovács and Larson, 1998), at the National Institute of Mental Health-Psychoactive Drug Screening Program (http://pdsp.med.unc.edu/pdspw/function.php). The percentage of inhibition of specific binding by the experimental ligand was determined. Secondary binding assays were used to determine the inhibitor constant (K_i) of each of the ligands for σ-1 and σ-2 receptors. K_i represents the concentration of the competitor that binds to half the enzyme sites at equilibrium in the absence of substrate or other competitors (Kakkar et al., 2000). The log IC_{50} (the log of the ligand concentration that reduces radioligand binding by 50%) was thus estimated from the data and used to obtain the K_i by applying the Cheng-Prusoff approximation (Cheng and Prusoff, 1973).

σ-1 Receptor Knockdown and Coimmunoprecipitations. siRNA oligonucleotides for σ-1 were obtained from Ambion (Austin, TX). COS-1 and MA-10 cells were transfected with 30 and 60 pmol of siRNA1 (5′-GGACAUACAUACUUGUACAtt- 3′); siRNA2 (5′-GGAGAGACAGUUGUACACGtt-3′); siRNA3 (5′-CGAUACUGGGCUGAGAUUUtt-3′), and siRNA4 (5′-GCUUACCACCUACCUCUUUtt-3′), using oligofectamine (Invitrogen, Carlsbad, CA). Nontargeting siRNA served as a control in all experiments. For coimmunoprecipitation experiments, specific antibodies were preincubated with protein A Sepharose CL-4B (0.5 μg/μl; GE Healthcare) in 100 μl of 1× coimmunoprecipitation buffer (1% Triton X-100, 200 mM NaCl, and 0.5% sodium deoxycholate) for 2 h at 4°C, and then beads were washed with buffer. The antibody-coated beads were processed for coimmunoprecipitation as described previously (Pawlak et al., 2011b), and immune complexes were analyzed by Western blotting.

Results

Effect of σ-1 Receptor Knockdown on Mitochondrial and ER Protein Expression. To evaluate the potential role of σ-1 receptor in steroid synthesis (Fig. 1A), we first generated four siRNAs (siRNA 1–4) targeting σ-1 receptor expression and tested their effects at 30 and 60 pmol in MA-10 cells. Although all four reduced expression of the σ-1 receptor at 60 pmol, only siRNA4 had an effect at 30 pmol (Fig. 1B), thus we continued our experiments using siRNA4. We next asked whether loss of the σ-1 receptor would modulate the expression of other proteins associated with the ER and mitochondria, such as β-actin (Fig. 1B, right), 3βHSD2 (Pawlak et al., 2011b; Prasad et al., 2012), and OMM-associated Tom20 (Bose et al., 2002). Figure 1B, right shows the indicated corresponding control experiment for Fig. 1B, left. The results (Fig. 1B) showed that σ-1

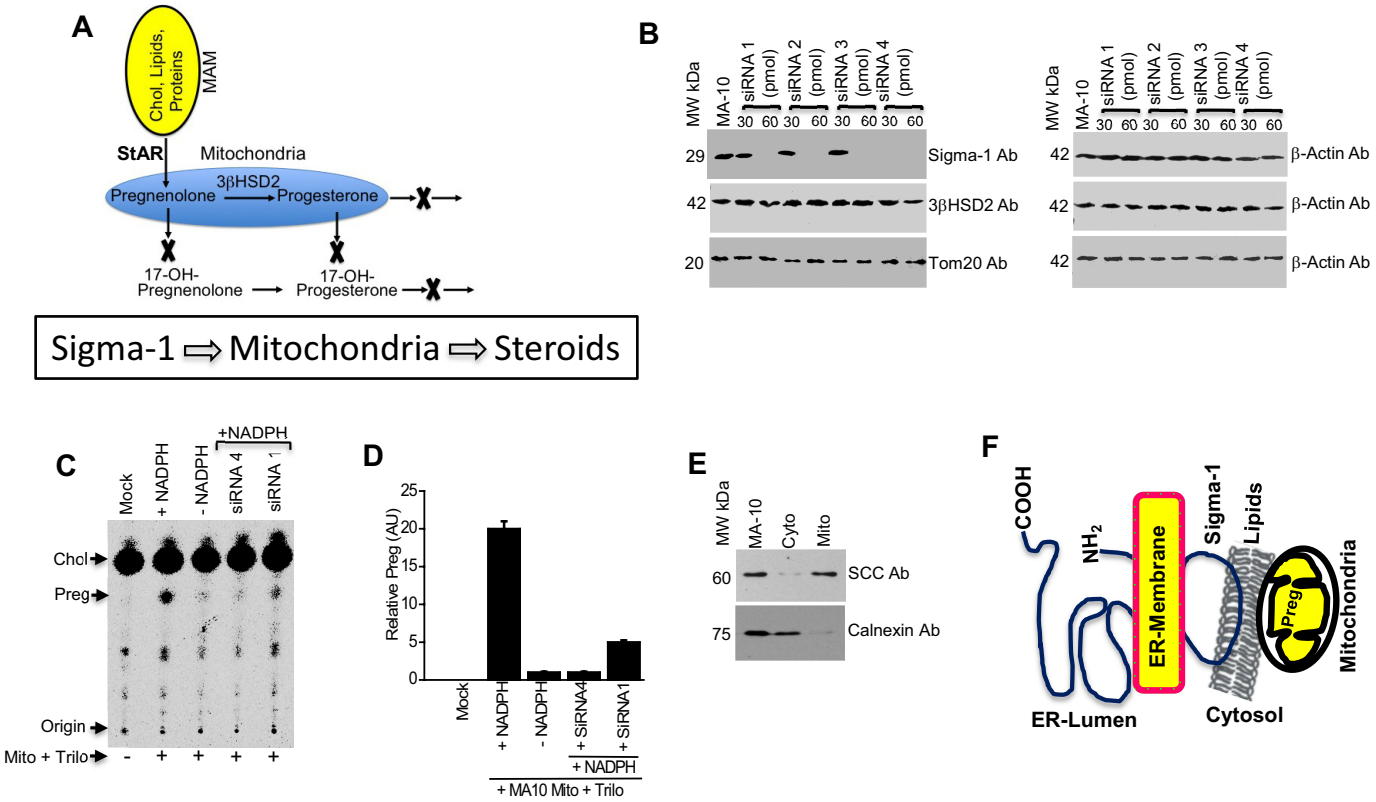


Fig. 1. Expression and activity of the σ-1 receptor in σ-1 receptor-knockdown MA-10 cells. A, schematic presentation of a simplified version of the biosynthesis pathway and its relationship to the MAM with σ-1 receptor. B, left, all four siRNA oligonucleotides at 60 pmol reduced expression of the σ-1 receptor in MA-10 cells, but only siRNA4 reduced expression at 30 pmol. Western blot shows unchanged expression of IMM resident 3βHSD2 and OMM-associated Tom20 in MA-10 cells. Right, loading control of probing the corresponding left with β-actin. C, isolated mitochondria from MA-10 control and siRNA knockdown cells were incubated with [^{14}C]cholesterol and NADPH in a metabolic conversion assay, and metabolites were separated by thin-layer chromatography. D, quantitative estimation of the metabolites from C. E, Western blot of the cellular fractions with the indicated antibodies, showing the purity of our mitochondrial fraction. Data presented are the mean ± S.E.M. of three independent experiments. F, proposed model of interaction between ER and mitochondria through the MAM resident σ-1 receptor.

receptor knockdown did not alter the expression of ER and mitochondrial resident proteins.

Effect of σ-1 Receptor Knockdown on Mitochondrial Pregnenolone Synthesis. Given the role of σ-1 receptor as a chaperone at the ER-mitochondria interface to regulate Ca^{2+} signaling, and the requirement of Ca^{2+} for progesterone synthesis in the bovine placentome (Shemesh et al., 1984), we next wanted to explore the effect of σ-1 knockdown on pregnenolone synthesis. We treated MA-10 cells with 30 pmol of siRNA4 or siRNA4-isolated mitochondria, and then determined the conversion of cholesterol to pregnenolone in an in vitro metabolic conversion assay (Fig. 1, C and D). Cytochrome P450 SCC enzyme (Chung et al., 1986) is an inner mitochondrial resident protein, and calnexin is associated with the ER. Thus Western blotting with the mitochondrial marker SCC and cytosolic maker calnexin antibodies showed purity of the mitochondria used (Fig. 1E), confirming that the pregnenolone activity identified in the earlier experiments was generated from the metabolic activity of mitochondria. The steroid conversion was confirmed by mass spectrometric analysis (Supplemental Fig. 1, A-E). Synthesis of pregnenolone was reduced by 75% with siRNA1- and 95% with siRNA4-mediated knockdown of σ-1 (Fig. 1C), with quantitation of the spots (Fig. 1D). As such, the loss of progesterone is before the Δ4 (progesterone onwards) step of steroid synthesis. Taken together, these results strongly suggested that the σ-1 protein plays a substantial role in steroid hormone synthesis.

Chemical Structure Elucidation of Ligands. We next elucidated the chemical structure of the ligands by using NMR. The NMR of KSCM-1 showed ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38 to 1.56 (m, 6H), 1.81 to 1.91 (qt, J = 7.58 Hz, 2H), 2.31 to 2.36 (m, 6H), 2.34 (s, 3H), 3.80 (s, 3H), 3.87 (s, 3H), 3.86 to 3.93 (m, 2H), 6.52 (s, 1H), 6.81 (s, 1H), 7.10- 7.30 (m, 5H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 9.9, 10.1, 24.5, 25.3, 26.0, 29.7, 49.0, 54.6, 56.2, 56.3, 56.6 94.7, 100.8, 120.8, 122.4, 126.5, 126.9, 128.9, 143.2, 143.6, 146.7, 148.4, 149.9, 161.4. The calculated and observed molecular mass [mass spectrometry (electrospray ionization)$^{+}$] for $C_{26}H_{33}N_2O_4$ $[M+H]^{+}$was 437.2440.

The NMR of KSCM-5 showed ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.36 to 1.56 (m, 6H), 1.82 to 1.92 (qt, J = 7.58 Hz, 2H), 2.31 to 2.36 (m, 6H), 2.34 (s, 3H), 3.90 to 3.95 (t, J = 7.64 Hz, 2H), 7.03 to 7.05 (d, J = 8.03 Hz, 1H), 7.11- 7.26 (m, 7H), 7.43 to 7.45 (d, J = 6.85 Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 9.1, 24.5, 25.3, 26.0, 49.0, 54.6, 56.6, 111.4, 120.3, 121.2, 122.6, 126.2, 126.7, 127.0, 128.9, 129.0, 142.8, 144.4, 153.4, 161.5. The calculated molecular mass for $C_{24}H_{29}N_2O_2$ $[M+H]^{+}$ [mass spectrometry (electrospray ionization)$^{+}$] was 377.2229, and the observed was 377.2227.

The NMR of KSCM-11 showed ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.39 to 1.57 (m, 6H), 1.82 to 1.92 (qt, J = 7.14 Hz, 2H), 2.33

to 2.37 (m, 6H), 2.35 (s, 3H), 3.74 (s, 3H), 3.90 to 3.95 (t, J = 7.57 Hz, 2H), 6.52 (s, 1H), 6.78 to 6.80 (d, J = 8.61 Hz, 1H), 7.12- 7.33 (m, 6H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 9.2, 24.5, 25.3, 26.0, 49.0, 54.6, 55.6, 56.6, 95.3, 112.3, 120.6, 122.1, 122.4, 126.6, 127.0, 129.0, 143.1, 143.6, 154.6, 159.6, 161.4. The molecular calculated for $C_{25}H_{31}N_2O_3$ $[M+H]^+$ was 407.2335, and observed was 407.2339.

σ-1 Receptor Binding Assays. Pharmacological characterization of the synthesized ligands were performed by radioligand binding assays. Of the compounds screened, three compounds (KSCM-1, KSCM-5, and KSCM-11) showed selectivity for σ-1 and σ-2 receptors. σ-1-Selective ligands usually possess a primary and secondary hydrophobic site separated by an amine functionality. The σ-1 receptor site displays some bulk tolerance, so this proved to be quite amenable to the introduction of a benzofuran moiety to produce the general molecular framework. The inclusion of methoxy substituents at both C-5 and C-6 of the benzofuran moiety resulted in both high affinity and selectivity at the σ-1 receptor preferentially. For σ-1 receptors, the K_i of KSCM-1, KSCM-5, and KSCM-11 was 27, 7.8, and 34 nM, respectively, suggesting that KSCM-5 had the maximum affinity followed by KSCM-1 and then KSCM-11 (Fig. 2, A-C). KSCM-1 had a K_i of 527 nM at σ-2 receptors, which indicated selectivity for σ-1. Moreover, these compounds were specific for the σ-receptors because the secondary binding assays revealed insignificant binding to non-σ-receptors. The exclusion of a methoxy substituent at C-5 of the benzofuran moiety to produce KSCM-11 resulted in a slightly decreased affinity at σ-1 (K_i = 34 nM); however, we observed a significant increase in affinity at σ-2 (K_i = 41 nM) in comparison with KSCM-1. The exclusion of both methoxy substituents at C-5 and C-6 of the benzofuran moiety to produce KSCM-5 resulted in significantly increased affinity at both σ-1 (K_i = 7.8 nM) and σ-2 (K_i = 16 nM) in comparison with KSCM-1 and KSCM-11 (Fig. 2). However, selectivity of KSCM-5 for σ-1 over σ-2 was only 2-fold. In summary, the dimethoxybenzofuran-2-carboxamide KSCM-1 molecular structure produced a selective, high-affinity σ-1 receptor ligand.

σ-Selective Ligand Structural Changes Affects Protein Expression. To understand the metabolic regulation in steroidogenic cells we compared MA-10 with the nonsteroidogenic COS-1 cells. We were surprised that we did not find any expression of σ-1 in COS-1 cells, although it is possible that the level was too low to detect or the protein was efficiently proteolyzed. We next evaluated the effect of the ligands on MA-10 cell viability. Incubation with increasing concentrations of KSCM ligands decreased cell viability, although to different extents (Fig. 3). For example, 1 nM KSCM-1 produced a 3.5% decrease in cell viability, whereas 100 nM led to a 16% decrease (Fig. 3A). In the case of KSCM-5, 1 nM decreased cell viability by 28%, whereas 100 nM resulted in a 74% decrease (Fig. 3B). Finally, like KSCM-1, KSCM-11 at lower concentrations did not greatly affect cell viability (a decrease of 3.3% with 1–5 nM); however, at 100 nM there was a 62% decrease (Fig. 3C). It seems that KSCM-11 had a greater cytotoxic effect than KSCM-1, perhaps caused by increased affinity at the σ-2 receptor. KSCM-5 produced the most potent decrease in cell viability. This compound differs from the other two ligands in that it has no methoxy groups at C-5 and C-6 of

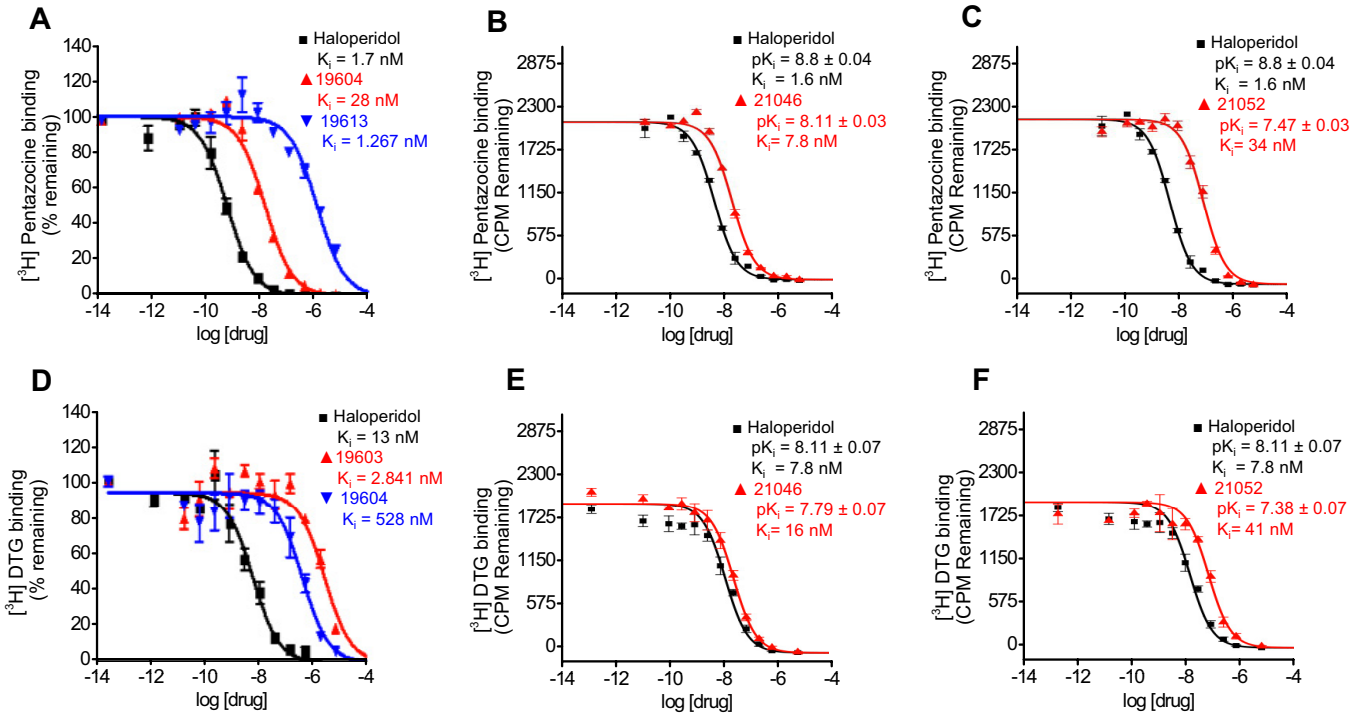


Fig. 2. A to C, pharmacokinetics of KSCM ligands. Ligand binding of KSCM-1 (A), KSCM-5 (B), and KSCM-11 (C) to σ-1 receptors. Affinities K_i (nM) were determined in rat brain homogenate in σ-binding buffer. σ-1 Receptors were labeled with [^{3}H](+)- pentazocine, and haloperidol served as the reference compound. KSCM-5 exhibited the strongest binding followed by KSCM-1 and KSCM-11. D to F, ligand binding of KSCM-1 (D), KSCM-5 (E), and KSCM-11 (F) to σ-2 receptors. Affinities K_i (nM) were determined in PC12 cells in σ-binding buffer. σ-2 Receptors were labeled with [^{3}H]1,3-di(2-tolyl) guanidine and haloperidol as the reference compound. KSCM-5 exhibited the strongest binding followed by KSCM-11 and then very weak binding by KSCM-1. Data represent K_i (nM) values obtained from nonlinear regression of radioligand competition binding isotherms. K_i values are calculated from best-fit IC_{50} values using the Cheng-Prusoff equation. Data presented are the mean ± S.E.M. of three independent experiments. PDSP compound 19613 (control), compound 19603 (control).

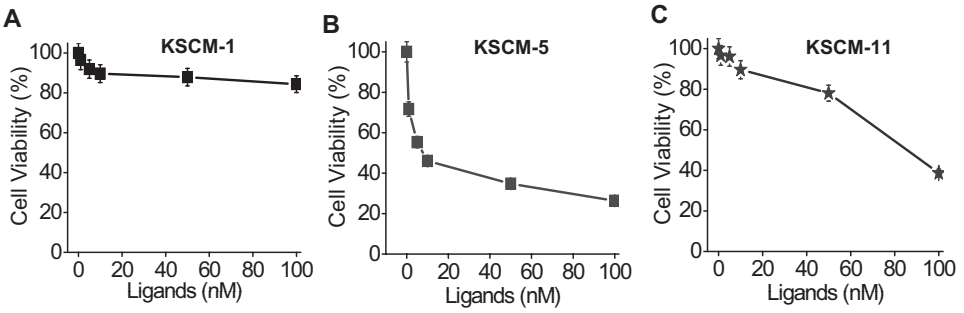


Fig. 3. Cell viability assay of the KSCM ligands after incubation for 24 h at varying concentrations in MA-10 cells. KSCM-5 (B) exhibited the greatest toxicity followed by KSCM-1 (A) and KSCM-11 (C). Data are the mean ± S.E.M. of at least three independent experiments.

the benzofuran ring and very high affinity at both σ-receptors. A nonplanar conformation is more stable than the planar conformation (Anderson et al., 1979), because of the stronger interaction with the protein conformation and charged phospholipids, perhaps resulting in overall less toxicity.

Expression and Steroidogenic Activity of KSCM-1, KSCM-5, and KSCM-11. We next wanted to evaluate whether KSCM ligand binding affected σ-1 receptor expression. Western analysis demonstrated that incubation of MA-10 cells for 24 h with KSCM-1 and KSCM-11 increased expression of the σ-1 receptor (Fig. 4A, left), whereas expression increased only minimally in the presence of KSCM-5 (Fig. 4A, left). The densitometric measurement (Fig. 4B) was determined comparing the ratio between the σ-1 (Fig. 4A, left) and β-actin (Fig. 4A, right). This result suggests an interaction of the ligands on the σ-1 receptor, which is a cytosolic protein localized at the junction of the ER and mitochondria. So, we next asked whether these ligands had an effect on the IMM resident protein 3βHSD2 (Pawlak et al., 2011b), which associates with the IMM without membrane integration because of molten globule conformation (Pawlak et al., 2011b; Prasad et al., 2012). Western blotting with antibodies directed against

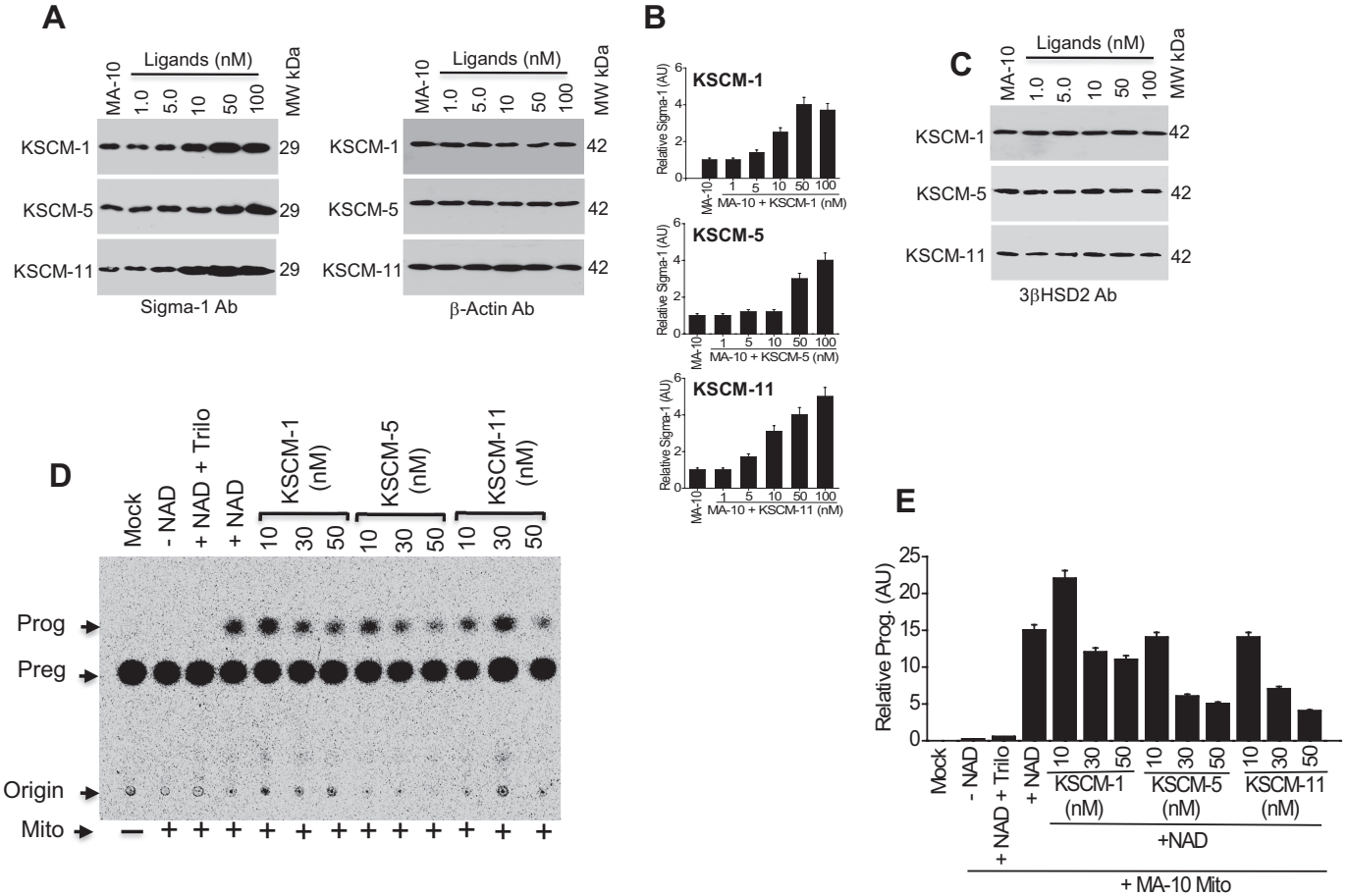


Fig. 4. Effect of different KSCM ligands on the σ-1 receptor expression. KSCM ligands were added to MA10 cells at concentrations ranging from 1 to 100 nM for 24 h. A, left, Western blots showed that expression of the σ-1 receptor increased with increasing the concentrations of KSCM-1 (top), moderately increased with increasing concentrations of KSCM-11 (bottom), but remained unchanged with KSCM-5 (middle). Right, Western blot of the same membranes probing with β-actin corresponding to each ligand. B, densitometric estimation of the Western blots in A illustrates the difference in expression with increasing concentrations of KSCM ligands. C, effect of incubating cells with KSCM-1, KSCM-5, and KSCM-11 on the expression of the inner mitochondrial steroidogenic enzyme 3-βHSD2. D, mitochondrial metabolic conversion of [^{3}H]pregnenolone to progesterone after the addition of NAD in the presence of various concentrations of KSCM-1, KSCM-5, and KSCM-11. E, quantitative estimation of the metabolic conversion from D, which shows 27% enhanced increase in activity in the presence of KSCM-1 at 10 nM. Data presented in B and E are the mean ± S.E.M. of three independent experiments.

with human 3βHSD2 (Prasad et al., 2012) and β-actin revealed no change in the levels of 3βHSD2 (Fig. 4C) and β-actin (Supplemental Fig. 2) levels at any concentrations with any of the ligands, suggesting that the KSCM series of ligands specifically targeted the cytosolic σ-1 receptor.

Given the potential role of σ-1 in steroidogenesis, we also performed conversion assays in the presence of ligands. The results (Fig. 4D) showed that 10 nM KSCM-1 increased progesterone conversion more than 25% (Fig. 4E), but, with further increases in ligand concentration, reduced conversion to the basal level seen without KSCM-1. At 10 nM KSCM-5 and KSCM-11 had no effect, but conversion decreased significantly at higher concentrations. The possibility exists that the σ-1 receptor transiently interacts with the OMM-associated porins. As such, the increased levels of σ-1 receptor with KSCM-1 may enhance the transport of substrate cholesterol into the mitochondria, thereby increasing progesterone synthesis. The reduced metabolic activity seen with KSCM-5 and KSCM-11 may possibly be caused by an alteration in cholesterol import or an unknown mechanism.

Role of VDAC in σ-1 Receptor Action. We had shown previously that steroid synthesis requires VDAC1, because it contributes to the appropriate folding of the StAR, the protein responsible for cholesterol fostering from the OMM to IMM (Bose et al., 2008). Nevertheless, treatment with the KSCM ligands did not alter VDAC1 expression levels (Fig. 5A) compared with β-actin (Supplemental Fig. 3), suggesting that the ligands have no effect on mitochondrial-associated protein expression. However, next we probed with VDAC2 antibody (Fig. 5B, left) and compared with β-actin (Fig. 5B, right) expression of the same membrane. The densitometric analysis (Fig. 5C) showed increased expression after the addition of KSCM-1 (Fig. 5C, top), whereas KSCM-5 (Fig. 5C, middle) and KSCM-11 (Fig. 5C, bottom) reduced VDAC2 expression.

We next asked whether the effects seen with σ-1 were independent of VDAC1. To address this, we examined the expression of σ-1 receptor in the presence and absence of KSCM ligands in our previously developed VDAC1 knockdown MA-10 cells (Bose et al., 2008). For accurate comparison we probed the same membrane with β-actin and σ-1 antibody independently (Fig. 5, D-F). The results showed (Fig. 5, D-F, top) that, in the absence of VDAC1, σ-1 receptor and β-actin (Fig. 5, D-F, bottom) expression remained unchanged compared with the parental cells. Moreover, the addition of KSCM ligands did not have any significant effect on σ-1 expression, confirming that the σ-1 receptor functioned independently of VDAC1.

σ-1 Associates with VDAC via StAR. StAR is active at the OMM in a partially open conformation (Bose et al., 1999, 2009a,b) and functions to transport cholesterol into the mitochondria. Knowing this, our next objective was to determine the role of KSCM ligands on the expression of StAR in MA-10 cells. In all the cases, we observed both the 37-kDa unimported and the 30-kDa mitochondrial-imported, mature protein, but not the 32-kDa transient intermediate protein (Fig. 6A). We probed the same membrane first with StAR (Fig. 6A) antibody and then with β-actin antibody (Supplemental Fig. 4A) independently. We were surprised to find that KSCM-1, but not KSCM-5 or KSCM-11 (Fig. 6A, middle and bottom), increased the expression of unimported 37-kDa StAR (Fig. 6A).

We next performed coimmunoprecipitation experiments to determine whether there was a direct interaction between σ-1 receptor and StAR or VDAC2. Coimmunoprecipitation of

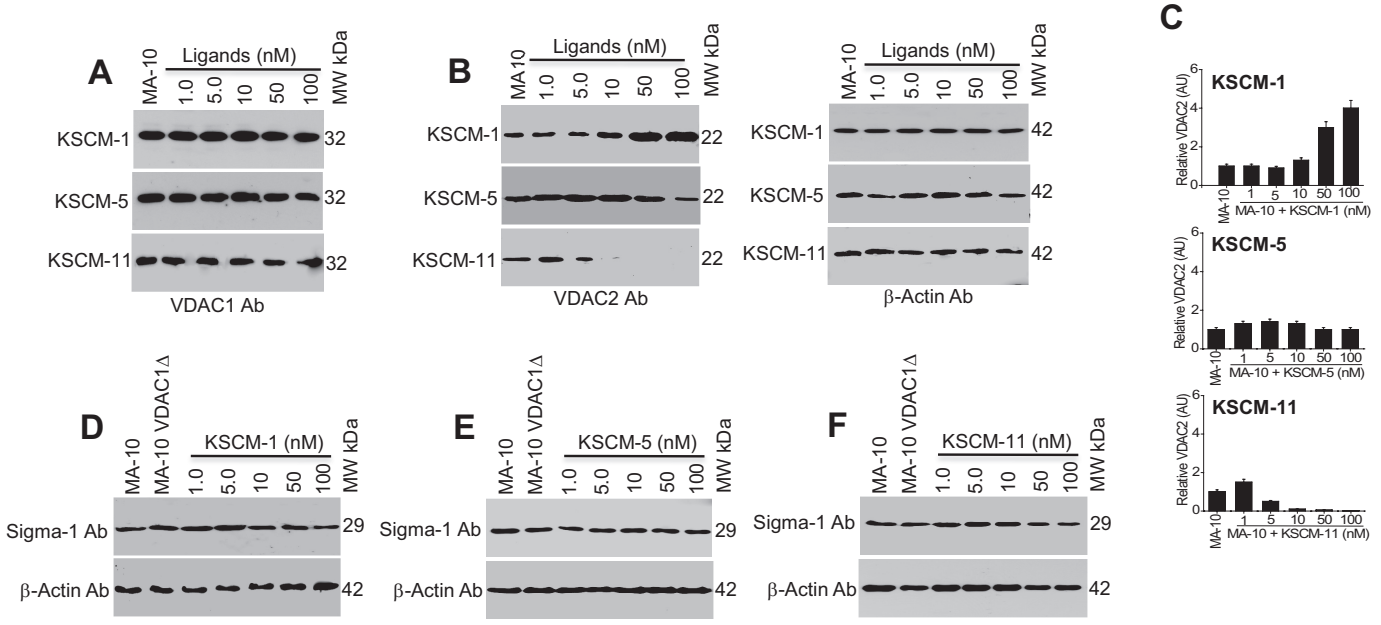


Fig. 5. Effect of σ-1 receptor on the outer mitochondrial proteins. A, effect of increasing concentrations of KSCM-1, KSCM-5, and KSCM-11 on VDAC1 expression as determined by Western blotting. B, left, effect of increasing concentrations of KSCM-1 (top), KSCM-5 (middle), and KSCM-11(bottom) on VDAC2 expression as determined by Western blotting. Right, the expression of β-actin when probed the same membrane presented at left under identical conditions. C, densitometric estimation of the effect of KSCM ligands on VDAC2 from B, left expression compared with β-actin in B, right. VDAC2 expression was increased with KSCM-1 up to 50 nM, and then partially decreased. KSCM-5 reduced expression at 100 nM, and KSCM-11 reduced expression at 5 nM. D, effect of KSCM-1 on σ-1 receptor expression in VDAC1 knockdown cells. Western blot with σ-1 antibody showed increased expression after addition of KSCM-1 at any concentration, except 100 nM. E and F, σ-1 expression in VDAC1 knockdown cells incubated with KSCM-5 and KSCM-11 ranging from 1 to 100 nM. Western blots indicated that σ-1 expression remained unchanged in VDAC1 knockdown cells. Unaffected expression of β-actin is presented (bottom).

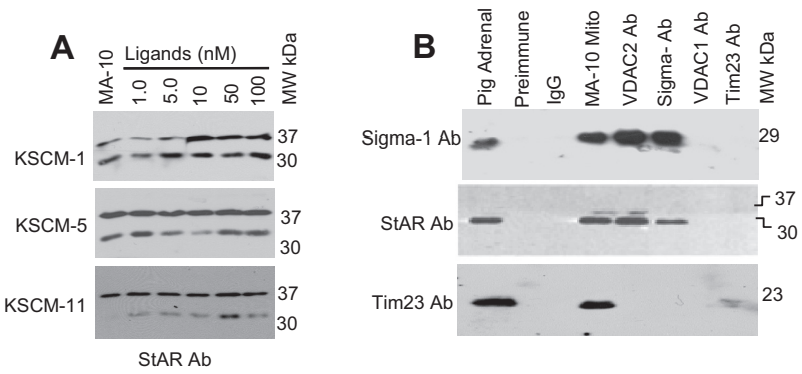


Fig. 6. Expression of StAR and its interaction with proteins involved in steroidogenesis. A, expression showing the unchanged expression of mitochondrial unimported 37-kDa and imported 30-kDa StAR after incubation with KSCM-5 and KSCM-11. KSCM-1, at or above 10 nM, stabilizes the expression of unimported StAR, but imported 30-kDa StAR expression was unaffected. B, coimmunoprecipitation of the MA-10 mitochondria with the indicated antibodies followed by Western blotting with σ-1 antibody (top) and StAR antibody (middle). The experiment shows the interaction of StAR with σ-1. Western blotting of the same coimmunoprecipitation samples with IMM resident protein Tim23 (bottom) antibody showed that Tim23 was present in MA-10 cells and pig adrenals; it did not interact with the other proteins.

the digitonin lysate from the mitochondria isolated from MA-10 cells showed that VDAC2 and σ-1 antibodies pulled down the σ-1 receptor (Fig. 6B, top). StAR is a 37-kDa protein (Bose et al., 2008) and thus it is higher than the 28-kDa σ-1 (Fig. 6A), confirming that StAR interaction with the σ-1 receptor. Probing the same membrane with the Tim23 antibody failed to show any interaction with the StAR or the σ-1 receptor (Fig. 6B, bottom), suggesting that the StAR interaction occurred before import into the mitochondria. Similar Western blotting of the flow-through from the previously mentioned coimmunoprecipitation experiments (Supplemental Fig. 4B) with σ-1 antibody showed minimal intensity when pulled down with VDAC2, which led us to conclude that the maximal interaction was between σ-1 and VDAC2 (Fig. 6B). Because StAR is expressed on hormonal stimulation in the adrenal and gonads, we conclude that StAR acts as a bridge between σ-1 and VDAC2 for cholesterol loading onto the OMM.

Discussion

A key criterion used to identify σ-receptors is their ability to bind several chemically unrelated drugs with high affinity. These drugs include psychotomimetic benzomorphans, 1-(1-phenylcyclohexyl)piperidine (phencyclidine) and its derivatives, cocaine and its derivatives, amphetamine, certain neuroleptics, many atypical antipsychotic agents, anticonvulsants, cytochrome P450 inhibitors, monoamine oxidase inhibitors, histaminergic receptor ligands, peptide from the neuropeptide Y or calcitonin gene-related peptide families, substance P, and neuroactive steroids. The σ-binding sites could be labeled by various specific radioligands, including [^{3}H]2*S*-[2α,6α,11*R**]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol (SKF-10,047), [^{3}H](+)3-(3-hydroxyphenyl)-*N*-(1-propyl)-piperidine, [^{3}H]haloperidol, [^{3}H]1,3-di-ortho-tolylguanidine, and [^{3}H](+) pentazocine. Pharmacological structure/activity studies led to the definition of two subclasses of σ sites, σ-1 and σ-2 (Quirion et al., 1992). The two sites were distinguished based on their different drug selectivity patterns and molecular weights. The σ-1 site shows a stereoselectivity with high affinity for dextrogyre isomers of benzomorphans, whereas σ-2 sites show the reverse stereoselectivity with a lower-affinity range (Hellewell et al., 1994). The σ-1-receptor sequence contains a 22-amino acid retention signal for the endoplasmic reticulum at its N-terminal region and the two short C-terminal hydrophobic amino acid sequences that may be involved in sterol binding (Hanner et al., 1996).

Steroid hormones are not stored in the tissue for future use. Rather, the steriodogenic tissues, including adrenals, gonads or ovaries, and brain, generate steroids acutely, when the need arises. This requires the transport of a huge amount of cholesterol from the cytoplasm into the mitochondria, where it serves as the sole substrate for the catalysis of pregnenolone, the first steroid in the pathway. However, the mechanism of enhanced cholesterol transport remains unknown. Studies have shown that the interaction of VDAC with StAR, a protein that acts exclusively on the OMM before import (Bose et al., 2002), enhances cholesterol import, and this requires StAR to undergo a structural transition to exert activity (Miller and Bose, 2011). In addition to the VDAC found at the OMM, the plasma membrane of various cell types contain this protein, termed plVDAC (Dermietzel et al., 1994; Bàthori et al., 1999; Buettner et al., 2000; Bahamonde et al., 2003; Elinder et al., 2005; Akanda and Elinder, 2006). The differential targeting is probably caused by an alternative first exon in the murine VDAC1 gene that codes a leader peptide at its N terminus (Buettner et al., 2000). This peptide serves as a signal to target the protein to the plasma membrane via the Golgi apparatus, and it is eventually cleaved away to produce the plVDAC protein. Because the N-terminal segment of VDAC is involved in voltage gating, it may adopt different conformations depending on the factors external to the protein (Ujwal et al., 2008). Using electron microscopy and NMR studies with a nanodisc technology, it has been demonstrated that the presence of phospholipid membrane facilitates this targeting to or interaction with different proteins (Raschle et al., 2009). Because VDAC1 knockout mice are viable (Anflous et al., 2001), it is likely that additional mechanisms promote cholesterol transport, possibly through the interaction of the σ-1 receptor associated with VDAC2 at the MAM region. Because a specific region of MAM anchors to the OMM, where the σ-1 receptor plays a crucial role for maintaining connectivity, we

suggest that VDAC2 may play a greater role than VDAC1 in mediating this connection. Because VDAC2 is expressed mostly with the presence of σ-1 receptor binding ligand KSCM-1, we propose that deletion of σ-1 receptor disrupts the bridge formed by VDAC2, resulting in an inhibition of cholesterol influx into the mitochondria.

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Authorship Contributions

Participated in research design: Marriott, Prasad, and Bose.

Conducted experiments: Marriott, Prasad, and Thapliyal.

Performed data analysis: Marriott, Prasad, and Bose.

Wrote or contributed to the writing of the manuscript: Marriott, Prasad, and Bose.

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Address correspondence to. Dr. Himangshu S. Bose, Department of Biochemistry, Mercer University School of Medicine and Memorial University Medical Center, Anderson Cancer Research Institute, Hoskins Research Building, Savannah, GA 31404. E-mail: bose_hs@mercer.edu or bosehi1@memorialhealth.com