Sigma-1 Receptor Regulation of Voltage-Gated Calcium Channels Involves a Direct Interaction

Kissaou T. Tchedre,[1] Ren-Qi Huang,[2] Adnan Dibas,[2] Raghu R. Krishnamoorthy,[2] Glenn H. Dillon,[2] and Thomas Yorio[2]

Purpose. The σ-1 receptor belongs to a recently discovered family of transmembrane proteins expressed in the central nervous system, including the eye, and mediates the regulation of ion channels. The exact function of sigma receptors remains to be elucidated. The purpose of this study was to investigate the effect of σ-1 receptor ligands on calcium homeostasis in a retinal ganglion cell line (RGC)-5 and rat primary RGCs.

Methods. Calcium imaging was used to assess the effect of σ-1 receptor agonist (+)-N-allylnormetazocine ((+)-SKF10047) on potassium chloride (KCl)-induced calcium influx in RGC-5. The whole-cell patch clamp technique was used to analyze the effect of (+)-SKF10047 on calcium currents in primary RGCs. Coimmunoprecipitation assessed the interaction between the σ-1 receptor and the L-type voltage-gated calcium channel.

Results. The σ-1 receptor agonist (+)-SKF10047 inhibited potassium chloride (KCl)-induced calcium influx. The σ-1 receptor antagonist, BD1047, reversed the inhibitory effect of (+)-SKF10047. Whole-cell patch clamp recordings of rat cultured primary RGCs demonstrated that (+)-SKF10047 inhibited calcium currents. Coimmunoprecipitation studies demonstrated an association between L-type calcium channels and the σ-1 receptors.

Conclusions. These results suggest that σ-1 receptor activation can regulate calcium homeostasis and signaling in RGCs, likely by directly influencing the activity of L-type voltage-gated calcium channels. Regulation of calcium influx in RGCs by σ-1 receptor ligands may represent in part the neuroprotective effect of σ-1 receptors. (*Invest Ophthalmol Vis Sci.* 2008;49: 4993–5002) DOI:10.1167/iovs.08-1867

Sigma-1 receptors (accession number NP 005857) were first described as one of the subtypes of opioid receptors, but further studies have led to the distinction between sigma receptors and opioid receptors.[1] The σ-1 receptor was first cloned from guinea pig liver.[2] Through a sequence homology approach, human,[3] mouse, and rat σ-1 receptors were subsequently cloned with the use of guinea pig σ-1 receptor cDNA.[3–6] σ-1 Receptors are localized on both the endoplasmic reticulum (ER) and the plasma membrane and are known to translocate from the ER-associated reticular network to the cell periphery on stimulation.[7] Sigma receptors are nonopiate and nonphencyclidine membrane-bound protein receptors expressed in different tissues, including liver, endocrine glands, and the central nervous system.[8] The endogenous ligand of the σ-1 receptor is unknown. However, it has been suggested that neurosteroids may be the endogenous ligand of sigma receptors.[9] In the eye, the lachrymal gland, retina, iris-ciliary body, cornea, and lens also express σ-1 receptors.[10–12]

Two sigma receptor subtypes have been identified by ligand-binding assays and photoaffinity labeling. The σ-1 receptor has a molecular weight of approximately 25 kDa, and the σ-2 receptor has a molecular weight of approximately 18 to 21 kDa.[2,13] σ-1 Receptors have been linked to the regulation of intracellular calcium mobilization. σ-1 Receptors regulate calcium entry at the plasma membrane level (through the *N*-methyl D-aspartate [NMDA] receptor, potassium channels, voltage-sensitive calcium channels) and calcium mobilization from endoplasmic stores.[14]

σ-1 Receptor neuroprotective effects have been shown to be linked to the ability of these receptors to control calcium homeostasis in neuronal cells.[14] A major receptor linked to calcium signaling is the NMDA receptor, which plays a key role in synaptic plasticity.[15] However, overstimulation of NMDA receptors could trigger an excessive entry of Ca^{2+}, which can initiate a cascade of cellular events that culminate in neuronal cell death. In addition, calcium can activate endonucleases that may degrade DNA and contribute to cell injury.[16] Together, these mechanisms can induce cell death through necrosis and apoptosis. Various pharmacologic and physiologic functions have been proposed for σ-1 receptors and include antipsychotic activity, modulation of opioid analgesia,[17] and neuroprotection.[18–20] σ-1 Receptor ligands inhibit the NMDA receptor-ion channel complex.[21,22]

In vitro exposure of cultured rat brain neurons to selective sigma receptor agonists protects cells against glutamate or NMDA-mediated neurotoxicity.[23] Similarly, sigma receptor activation protects retinal ganglion cells (RGCs) from glutamate and homocysteine excitotoxicity.[20] The neuroprotective mechanism of σ-1 receptors in vivo involves the attenuation of neuronal nitric oxide synthase activity and ischemia-evoked NO production.[24,25] Moreover, σ-1 receptors reduce cell death in vitro by a mechanism involving receptor-dependent preservation of protective genes such as bcl_2.[26]

Recently, a complex of σ-1 receptors with a chaperone, BiP, was found at the mitochondria-associated ER membrane. σ-1 Receptors dissociate from BiP when ER Ca^{2+} depletion occurs or through ligand stimulation. This dissociation leads to prolonged Ca^{2+} signaling into mitochondria through IP3 receptors, which can lead to ER stress.[27] Under ER stress or calcium overload, σ-1 receptors translocate to the cell periphery. The translocation of σ-1 receptors in cells counteracts the ER stress response to promote cell survival.[27] In addition, sigma receptors have been shown to inhibit voltage-gated calcium channels (VGCCs) and Kv1.4 potassium channels[28,29] in intact neuronal cells.[30] Knockdown of σ-1 receptor expression by siRNA

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increases cell death in lens cells.[31] σ-1 Receptor antagonists have been shown to induce apoptotic cell death that can be rescued by caspase-3 inhibitors[32] or by σ-1 agonists.[33] However, the molecular mechanism of σ-1 receptor–induced neuroprotection in RGCs remains to be elucidated. We investigated the activation of σ-1 receptors and their effects on calcium influx in RGCs through interaction with VGCCs.

Materials and Methods

Nifedipine was purchased from Sigma Chemical (St. Louis, MO). (+)-*N*-allylnormetazocine ((+)-SKF10047) and ([2-(3,4-dichlorophenyl) ethyl]-*N*-methyl-2-(diamino) ethylamine (BD1047) were purchased from Tocris Bioscience (Ellisville, MO). An immunoprecipitation kit (F: sc-45043) was purchased from Santa Cruz Biotechnology (Exacta Cruz; Santa Cruz, CA). Rabbit anti–σ-1 receptor was a gift from Teruo Hayashi (National Institutes of Health/National Institute on Drug Abuse/Intramural Research Program, Baltimore, MD). Rabbit normal IgG was purchased from Sigma Chemical. Secondary antibodies including donkey anti–rabbit IgG and donkey anti–mouse IgG conjugated to horseradish peroxidase were purchased from Amersham Biosciences (Piscataway, NJ). Thy-1.1 polyclonal antibody was purchased from Santa Cruz Biotechnology. Monoclonal anti–neurofilament-200 and voltage-dependent L-type α1C subunit antibody (anti–human α1C) were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescent probes, including goat anti–rabbit Alexa 488, goat anti–mouse Alexa 633, and fura-2-AM, were purchased from Molecular Probes (Eugene, OR).

Retinal Ganglion Cell Culture

The RGC-5 cell line was developed specifically to establish a permanent rat RGC culture.[34] RGC-5 cell cultures were grown in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin and were incubated at 37°C in 5% CO_2. All RGCs (RGC-5) used in this report were differentiated as described by Tchedre et al.[33]

Primary Rat RGC Isolation: Modified Immunopanning Protocol

All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center. Wistar Kyoto rats (3–4 weeks old, either sex) were purchased from Harlan Laboratory (Indianapolis, IN). The rats were anesthetized with isoflurane and then decapitated. The eyes were enucleated, and the retinas were gently peeled off with fine forceps and placed in sterile phosphate-buffered saline (PBS). Retinas were collected in DMEM and incubated at 37°C in 3 mL dispase for 1 hour. After 1-hour incubation, the retinas were gently washed once with complete neurobasal medium. RGCs were obtained by trituration of the retina in neurobasal medium (Gibco, Carlsbad, CA) with a fire-polished Pasteur pipette. The cell suspension collected was seeded on coverslips previously coated first with poly-D-lysine (overnight) and second coated with Thy 1.1 monoclonal antibody (Chemicon International, Temecula, CA) for 2 hours. After 30 minutes' incubation, the coverslips were gently washed once with complete neurobasal medium, covered with 2 mL complete neurobasal medium supplemented with B27 and penicillin/streptomycin (Pen-Strep; Gibco, Carlsbad, CA), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic growth factor (CTNF; PHC 7074; Biosource), and forskolin (F6886; Sigma-Aldrich). Cell culture dishes were subsequently incubated in a 5% CO_2 incubator at 37°C for up to 7 days in vitro (7 DIV). Isolated RGCs were characterized by immunocytochemistry for normally expressed RGC markers Thy-1[35] and neurofilament-200 (Fig. 1).

Intracellular Calcium ($[Ca^{2+}]_i$) Measurement

Intracellular calcium in RGC-5 was measured at 37°C by the ratiometric technique using fura-2-AM (excitation at 340 nm and 380 nm, emission at 510 nm) according to Prasanna et al.[36] Intracellular calcium ($[Ca^{2+}]_i$) in nanomolar (nM) was calculated using the Grynkiewicz equation.[37] In calcium imaging studies, cells were pretreated with drugs such as (+)-SKF10047, BD1047, and nifedipine for 30 minutes before they

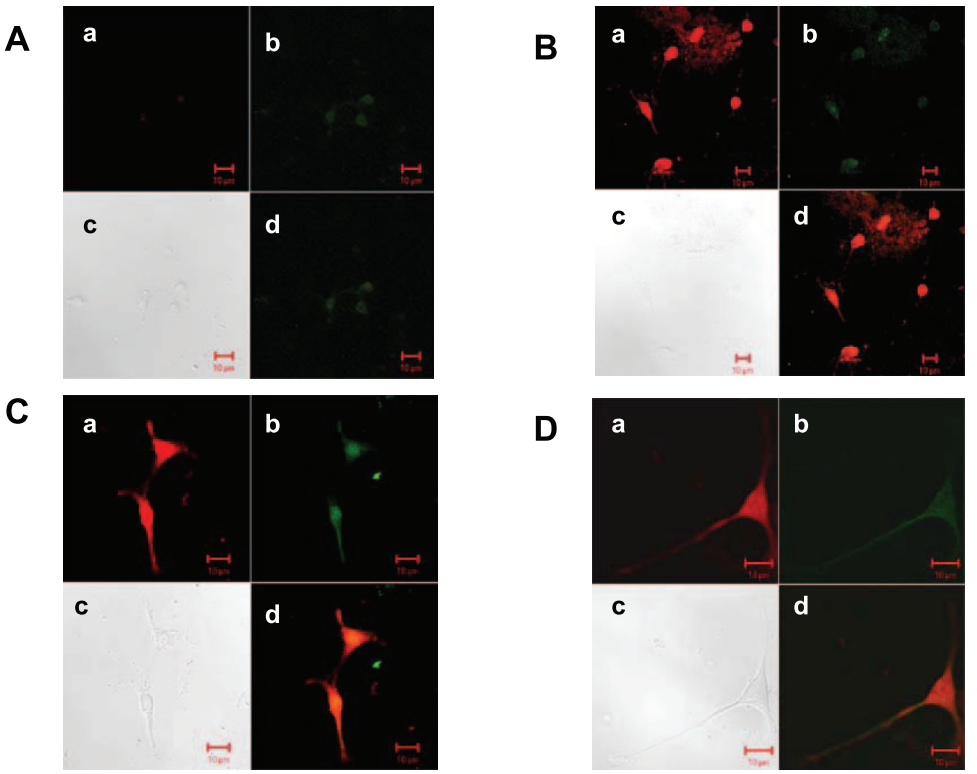

Figure 1. Characterization of the isolated primary RGCs. Modified immunopanning protocol for RGC isolation. Cells were characterized by immunocytochemistry for a normally expressed RGC marker Thy-1 [35] and neurofilament-200. (**a**) Thy-1 labeling (*red*). (**b**) Neurofilament-200 (*green*). (**c**) DIC picture. (**d**) Merge. The RGC isolation method yielded pure primary RGC cultures. (**A–D**) Isolated RGCs but with different magnifications. Scale bar, 10 μm.

were stimulated with KCl. In addition, the bath solution contained (+)-SKF10047, BD1047, or nifedipine.

Preparation of Stably Transfected RGC-5

Sigma receptor agonists have been shown to protect RGCs from glutamate-induced excitotoxicity.[20] To test whether the neuroprotective effect of sigma ligands was mediated through the σ-1 receptor, we overexpressed σ-1 receptors in RGC-5. Overexpressing the receptor served two roles. It allowed us to provide excess receptor to the system to determine whether we could influence channel activity without added ligand, and it provided greater opportunity for our exogenous agonist to interact with the receptor.

σ-1 Receptor stably transfected RGC-5 were established as previously described by Tchedre et al.[33] Briefly, a rat σ-1 receptor cDNA cloned in the pSPORT-1 plasmid vector was a gift from Ganapathy (Medical College of Georgia, Augusta, GA). *Escherichia coli*-competent strain SURE-2 (Stratagene, La Jolla, CA) cells were transformed with a plasmid pSPORT-σ-1R. Transformed cells were plated on ampicillin (100 μg/mL) containing Luris-Bertani (10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar in 1 L dH_2O) plates. Many colonies were obtained after overnight incubation. Colonies were picked and grown in Luria-Bertani (10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1 L dH_2O; adjusted pH to 7.5 with NaOH) media for large-scale preparation of plasmid DNA. Plasmid DNA was purified through the equilibrium cesium chloride gradient method. After the plasmid preparation, the purity of the DNA was checked on agarose gel. The lipofectamine 2000 (Invitrogen, Carlsbad, CA) method was used for transfection of RGC-5. Because RGC-5 are neomycin (G418) resistant, cotransfection of pSPORT-σ-1 and pZeoSV2 (Invitrogen) was used. After transfection, zeocin-resistant clones were selected, and the expression of σ-1 receptors was assessed by Western blot.

Preparation of Cell Lysate and Western Blotting of Proteins

Nonoverexpressing and σ-1 receptor–overexpressing RGC-5 cell lysates were prepared as described by Bu et al.[38] Protein concentrations of cell lysates were determined with a protein kit (BioRad DC; Bio-Rad Laboratories, Hercules, CA). Samples were run on SDS-PAGE gel, and immunoblot analysis was carried out according to standard methods as described by Laemmli[39] and Towbin et al.[40] using anti–σ-1 receptor rabbit polyclonal antibody. The blots were developed with an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunofluorescence Microscopy

Primary retinal ganglion (7 DIV) cells were grown on coverslips and treated for 30 minutes. Coverslips were fixed with 4% paraformaldehyde and blocked with blocking solution (5% BSA + 5% normal goat serum) for 1 hour at room temperature. The blocking buffer was discarded, and the coverslips were washed three times with 1× PBS before the addition of the affinity-purified σ-1 receptor polyclonal antibody (1/200 dilution; Santa Cruz Biotechnology) and voltage-dependent L-type α1C subunit antibody (1/200; Sigma-Aldrich) or *Thy1.1* monoclonal antibody (1/500 dilution; Santa Cruz Biotechnology) and monoclonal anti–neurofilament-200 (Sigma-Aldrich) with 1% normal goat serum and 5% bovine serum albumin (Sigma-Aldrich). Incubation was performed overnight at 4°C. Coverslips were washed three times, and 1/500 secondary antibodies (Alexa Fluor 633) and (Alexa Fluor 488) were added and incubated for 1 hour in the dark. After incubation, the coverslips were washed again three times with 1× PBS. Mounting was performed on glass slides using antifade reagent with DAPI (Prolong Gold; Invitrogen) and allowed to dry for 20 minutes in the dark. Cells were viewed with a confocal laser scanning microscope (LSM 510; Zeiss, Thornwood, NY).

Whole-Cell Patch Clamp

Macroscopic calcium currents were measured using a whole-cell patch clamp technique.[41] Patch pipettes of borosilicate glass (1B150F; World Precision Instrument, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC; Sutter Instrument, Novato, CA) to a tip resistance of 5 to 7 MΩ. The pipette solution contained 140 mM CsCl, 10 mM EGTA, 4 mM Mg-ATP, and 0.2 mM Na_3-GTP, pH 7.2. The external bath solution contained 125 mM NaCl, 1 mM MgCl, 10 mM HEPES, 5 mM $CaCl_2$, 10 mM glucose, and 0.5 mM TTX (μM), pH 7.3. Coverslips containing cells were placed in a small chamber (approximately 1.5 mL) and superfused continuously (7–10 mL/min). Cells were visualized under an upright, fixed-stage microscope (Optiphot-2UD; Nikon, Tokyo, Japan) with a 12-V, 100-W halogen lamp equipped with standard Hoffman modulation contrast optics and video camera system (XC-75 CCD Tandy video monitor; Sony, Tokyo, Japan). Whole-cell Ca^{2+} currents were obtained using a patch clamp amplifier (PC-505B; Warner Instruments, Hamden, CT) equipped with a 201B head stage. Currents were low-pass filtered at 2 kHz, sampled at 50 kHz, and stored on a computer (pCalmp6.0.2; Axon Instruments) for subsequent analysis. Sixty percent to 80% series resistance compensation was applied at the amplifier. Leak current was subtracted using a P/4 protocol. Cells were voltage clamped at −90 mV. Currents were evoked with voltage step from −90 to 0 mV for 55 or 200 ms. All recordings were carried out at room temperature (20°-21°C).

Coimmunoprecipitation of σ-1 Receptor and L-Type VGCCs

Nonoverexpressing and σ-1 receptor overexpressing RGC-5 were collected and lysed with ice-cold nondenaturing lysis buffer (1% [wt/vol] Triton X-100, 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.02% [wt/vol] sodium azide). Immediately before use, fresh protease inhibitors (20 μg/mL leupeptin, 20 μg/mL aprotinin, 20 μg/mL soybean trypsin inhibitor, and 40 μg/mL phenylmethylsulfonyl fluoride)

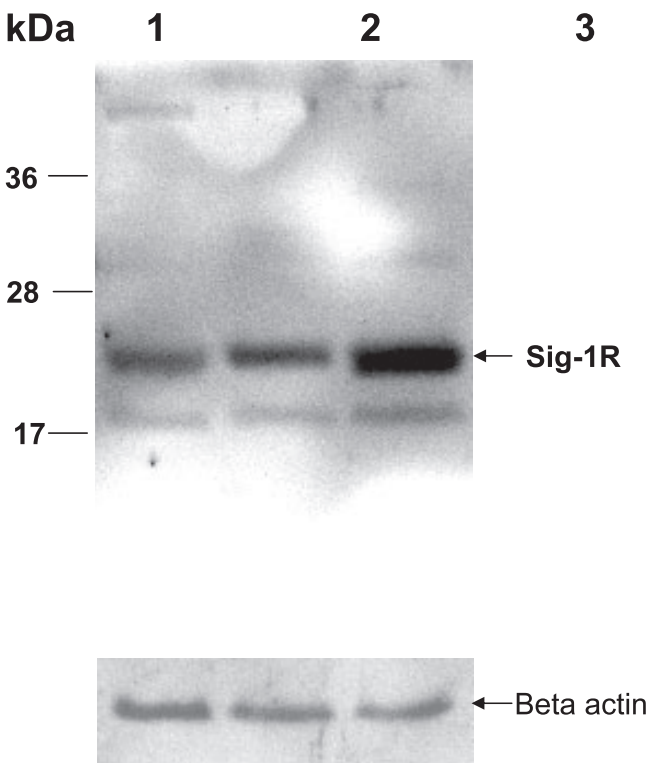

FIGURE 2. Western blot analysis of σ-1 receptor protein expression in RGC-5. Cells lysates from the nonoverexpressing and σ-1 receptor overexpressing RGC-5 were subjected to SDS-PAGE, followed by immunoblotting using specific antibodies for the σ-1 receptor. *Lanes 1* and *2*: RGC-5; *lane 3*: RGC-5 overexpressing σ-1 receptor (clone 4). Western blot results also showed the expression and overexpression of σ-1 receptor in RGC-5 and clone 4 RGC-5, respectively.

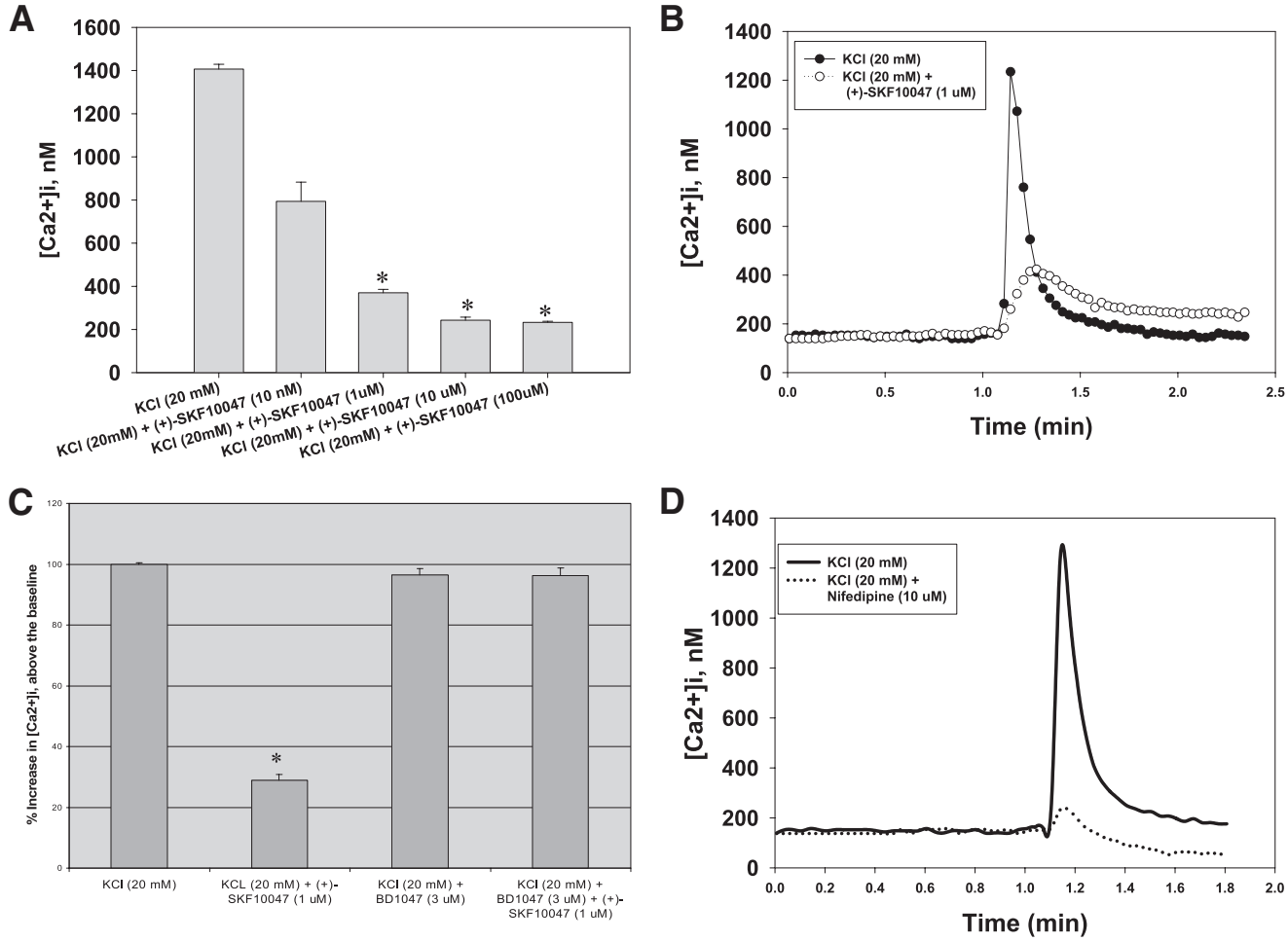

FIGURE 3. Sigma-1 receptor ligand (+)-SKF10047 effect on KCl-induced calcium influx in RGC-5. KCl-induced calcium influx was measured by fura-2-AM imaging. Cells were preincubated for 30 minutes with fura-2 before stimulation with 20 mM KCl. In calcium imaging studies, cells were pretreated with fura-2 and with drugs such as (+)-SKF10047, BD1047, and nifedipine for 30 minutes before the cells were stimulated with KCl. In addition, the bath solution contained (+)-SKF10047, BD1047, or nifedipine. Once added, all drugs remained in the bath for the duration of the experiment. (**A**) Sigma-1 receptor agonist (+)-SKF10047 inhibited KCl-induced calcium influx in a concentration-dependent manner. Data were analyzed by one-way analysis of variance (ANOVA) and all pairwise multiple comparison procedures (Student-Newman-Keuls method) between baseline, peak, and 1-minute after peak. Changes are mean ± SEM. *Statistically significant ($P < 0.05$) compared with baseline. (**B**) Representative $[Ca^{2+}]_i$ trends in response to KCl in RGC-5. KCl (20 mM; *arrow*) induced a calcium influx in RGC-5 (*black dotted line curve*) and (+)-SKF10047 (1 μM) inhibited KCl-induced calcium influx in RGC-5 (*clear dotted line curve*). (**C**) Summary of KCl-induced calcium influx measured by fura-2-AM imaging in RGC-5. Cells were preincubated with fura-2-AM for 30 minutes with the σ-1 receptor antagonist (BD1047) before stimulation with KCl. Data were analyzed by one-way ANOVA and all pairwise multiple comparison procedures (Student-Newman-Keuls method) between baseline, peak, and 1-minute after peak. Values are mean ± SEM ($n = 44$ for KCl, 20 mM; $n = 44$ for (+)-SKF10047, 1 μM; $n = 36$ for BD1047 3 μM; and $n = 30$ for KCL + BD1047 + (+)-SKF10047). *Statistically significant ($P < 0.05$) compared with control KCl (20 mM). (**D**) Representative $[Ca^{2+}]_i$ trends in response to KCl (*solid line*) in the presence of nifedipine (*dotted line*). Nifedipine significantly inhibited the KCl-induced calcium influx (*arrow*), similar to (+)-SKF10047.

were added. Coimmunoprecipitation was achieved using the immunoprecipitation kit method (Exacta Cruz [F: sc-45043], as described in datasheet: http://datas-heets.scbt.com/sc-45043.pdf). After coimmunoprecipitation, the samples were resolved on an SDS-PAGE gel and immunoblotted by standard methods using anti–σ-1 receptor antibody (1/500) or anti–human α1C antibody (1/500). The blots were developed with an ECL kit (Amersham Pharmacia Biotech).

RESULTS

σ-1 Receptors and RGC-5 Cells

σ-1 Receptors are expressed in many organs, including the eye.[12] In this study, with the use of immunoblot analysis, we examined the expression of σ-1 receptors in control and σ-1 receptor overexpressing RGC-5. The Western blot shows the expression of σ-1 receptor in RGC-5 cells (Fig. 1, lanes 1, 2), and the overexpression of σ-1 receptor in RGC-5 cells (clone 4, Fig.1, lane 3; see Materials and Methods for the preparation of stably transfected RGC-5), respectively (Fig. 2).

KCl-Induced Calcium Influx in RGC-5

σ-1 Receptor Agonist (+)-SKF10047 and KCl-Induced Calcium Influx in RGC-5. Intracellular calcium mobilization in RGC-5 was measured by a ratiometric technique using fura-2-AM. Depolarization with KCl (20, 40, and 80 mM) dose dependently induced calcium influx in RGC-5 (data not shown). The σ-1 receptor agonist (+)-SKF10047 (10 nM, 1, 10, and 100 μM) was able to significantly inhibit the KCl-induced calcium influx peak dose dependently (Fig. 3A). (+)−SKF10047 (1 μM) inhibited the KCl-induced calcium in-

flux peak from 100% to 22% (Figs. 3B, 3C). The σ-1 receptor antagonist BD1047, while not having any significant effect on KCl-induced calcium influx in RGC-5 on its own, was able to block the effect of (+)-SKF10047 (Fig. 3C).

Nifedipine, a selective L-type calcium channel blocker, was used to determine whether the calcium response observed was mainly mediated through L-type calcium channels. Nifedipine significantly inhibited the KCl-induced calcium influx (Fig. 3D).

KCl-induced increases in $[Ca^{2+}]_i$ could also have resulted from the inhibition of Ca^{2+} reuptake into SR or the stimulation of Ca^{2+}-induced Ca^{2+} release from intracellular Ca^{2+} stores.[42] Therefore, we used thapsigargin to investigate whether the KCl-stimulated increase in $[Ca^{2+}]_i$ in RGC-5 was caused by mobilization from IP_3-sensitive Ca^{2+} stores. Adding thapsigargin to RGC-5 resulted in an increase in $[Ca^{2+}]$i, followed by a drop to a new steady state calcium level. When the cells were subsequently stimulated with KCl after the addition of thapsigargin, there was an increase in $[Ca^{2+}]_i$ with a profile normally observed only in KCl-treated cells (Figs. 4A, 4B). The thapsigargin result was confirmed by a second experiment, which was conducted in calcium-free buffer. No calcium response was seen in the absence of extracellular calcium; however, when 2.5 mM extracellular calcium was added to the media and cells were stimulated with KCl, a peak of calcium response was seen (Fig. 4C). This experiment demonstrated that extracellular calcium is required for the KCl-induced calcium influx in RGC-5. Taken together, the calcium data suggest that activation of σ-1 receptors leads to the inhibition of L-type calcium channels. These data also suggest that the KCl-induced calcium influx is mediated mainly through extracellular calcium. Overall, these findings suggest that the activation of σ-1 receptors, through the regulation of L-type calcium channels, may play an important role in RGCs.

σ-1 Receptors and L-Type VGCCs in Rat Primary RGCs. σ-1 Receptors and VGCCs are expressed in the central nervous system,[30] in the eye,[12] and in many other systems.[14,15] Through immunocytochemistry we confirmed the expression of σ-1 receptors and L-type VGCCs in our system (Fig. 5B). In addition, the σ-1 receptor colocalized with L-type VGCCs. Given the interaction between σ-1 receptors and L-type VGCCs, it may be that σ-1 receptors control calcium influx through the channel. Figure 5A is a negative control that was

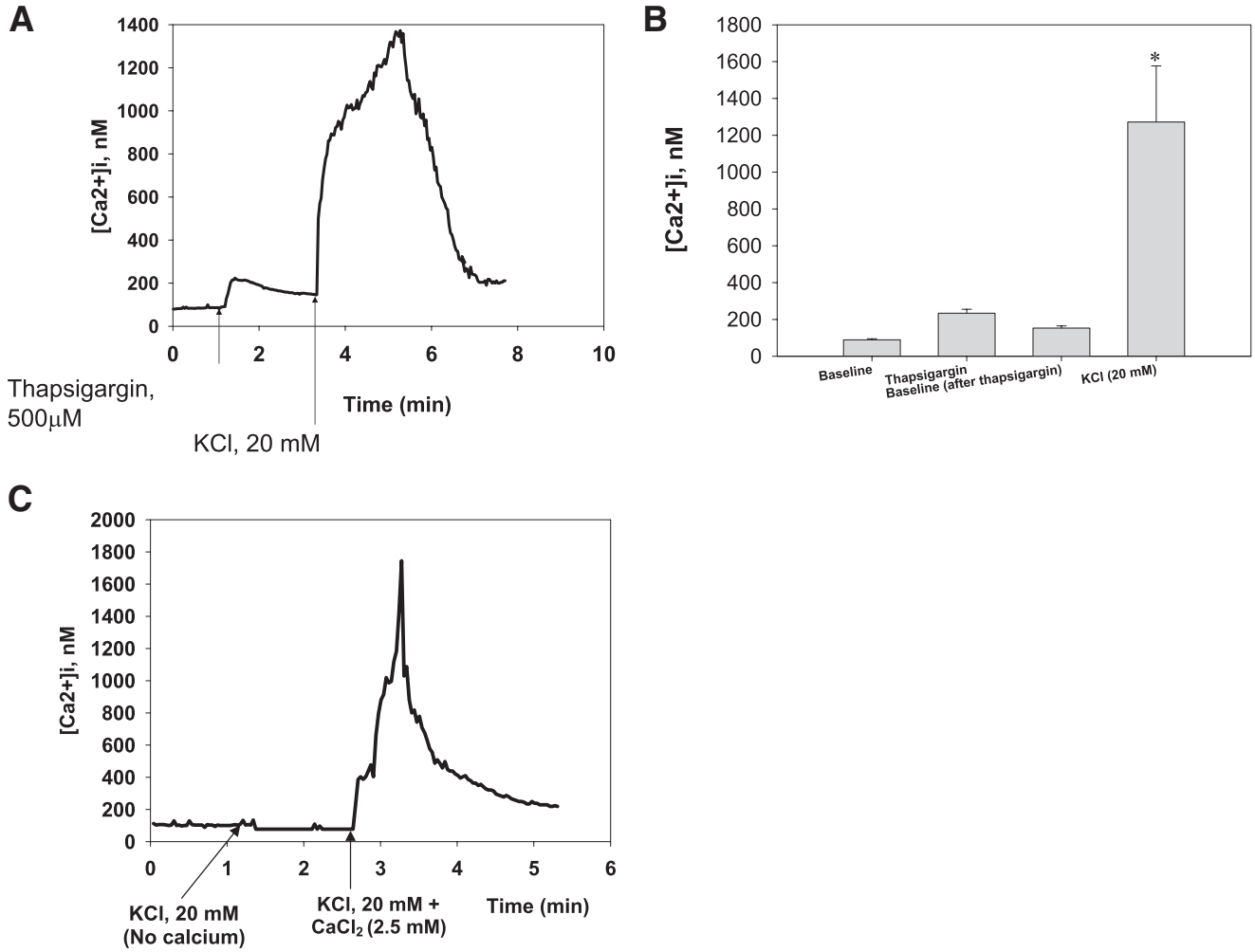

FIGURE 4. Extracellular calcium is required for the KCl-induced calcium influx in RGC-5. (**A**) Representative $[Ca^{2+}]_i$ trends in response to thapsigargin followed by KCl. The calcium response is measured by fura-2-AM imaging. RGC-5 were preincubated with fura-2-AM in Hanks balanced salt solution (HBSS) for 30 minutes before stimulation with thapsigargin, then by KCl alone. Once added, all drugs remained in the bath for the duration of the experiment. (**B**) Summary of thapsigargin and KCl-induced calcium influx measured by fura-2-AM imaging in RGC-5. Data were analyzed by one-way (ANOVA) and all pairwise multiple comparison procedures (Student-Newman-Keuls method) between baseline, peak, and 1 minute after peak. Values are the mean ± SEM ($n = 36$). *Statistically significant ($P < 0.05$) compared with thapsigargin (500 nM). (**C**) Representative $[Ca^{2+}]_i$ trends in response to KCl. The calcium response was measured by fura-2-AM imaging. RGC-5 were preincubated with fura-2-AM in a calcium-free HBSS for 30 minutes before stimulation with KCl alone, without or with calcium (2.5 mM).

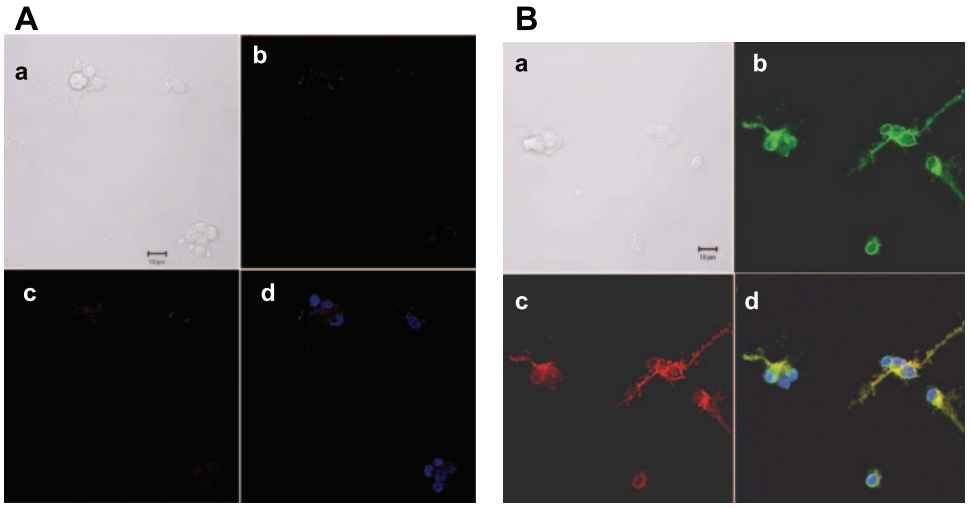

FIGURE 5. Expression of σ-1 receptor and L-type VGCCs in primary rat RGCs. Immunocytochemistry. Primary RGCs were grown on 35-mm coverslips for 7 days, fixed, and subjected to immunofluorescence staining for σ-1 receptors and L-type VGCCs. Cells were incubated (**A**, negative control) without primary antibodies or (**B**) with primary anti–goat σ-1 receptor polyclonal antibody and anti–rabbit L-type VGCC α1C subunit antibody, followed by incubation with secondary Alexa Fluor 488 donkey anti–goat and Alexa Fluor 633 goat anti–rabbit. Confocal laser scanning microscopy was used to detect σ-1 receptor (*green*) and L-type VGCCs (*red*). (**a**) DIC. (**b**) σ-1 receptor. (**c**) L-type VGCCs. (**d**) Merge. Scale bar, 10 μm.

conducted with secondary antibody without the addition of primary antibody.

σ-1 Receptor Ligand (+)-SKF10047 and Voltage-Gated Calcium Channel Currents in Rat Primary RGCs. To directly assess the modulation of VGCCs by σ-1 receptors, whole-cell calcium currents were measured using the patch clamp technique on rat-cultured RGCs (7 DIV). Currents were evoked with voltage steps from −90 to 0 mV for 55 ms. The average current amplitude was 284 ± 33 pA (n = 38). Extracellular application of the σ-1 receptor agonist (+)-SKF10047 (0.3–10 μM) inhibited calcium current amplitude in a dose-dependent manner (Figs. 6A, 6C). The onset of the effect, occurring approximately 3 minutes after (+)-SKF10047 perfusion, was relatively rapid. Furthermore, the (+)-SKF10047 effect was typically reversible in 3 to 5 minutes after washout.

To study the effects of (+)-SKF10047 on activation properties of calcium currents, current-voltage relationships (I-V curves) were determined from the amplitude of current elicited by 200 ms voltage ramp from a holding potential of −80 mV to 50 mV. As can be illustrated in representative raw data (Fig. 6B), at potentials more positive than −30 mV, an inward Ca^{2+} current was evoked. The maximum peak amplitude was obtained at approximately −10 mV, and the amplitude was reduced at more positive potentials. The inward Ca^{2+} current was reduced by (+)-SKF10047 (10 μM) at all values of membrane potential. For example, at 0 mV of membrane potential, the mean value of whole cell currents was 282 ± 75 pA at control and was reduced to 163 ± 47 pA in the presence of SKF (n = 5). However, (+)-SKF10047 did not significantly modify the properties of I-V curves. On average, the voltage for half-maximal activation ($V_{1/2max}$), maximal activation (V_{max}), half-maximal inactivation ($V_{1/2max}$), and reversal potential did not differ significantly between control and (+)-SKF10047 treatment (n = 5; $P > 0.05$, paired t-test). These data suggest

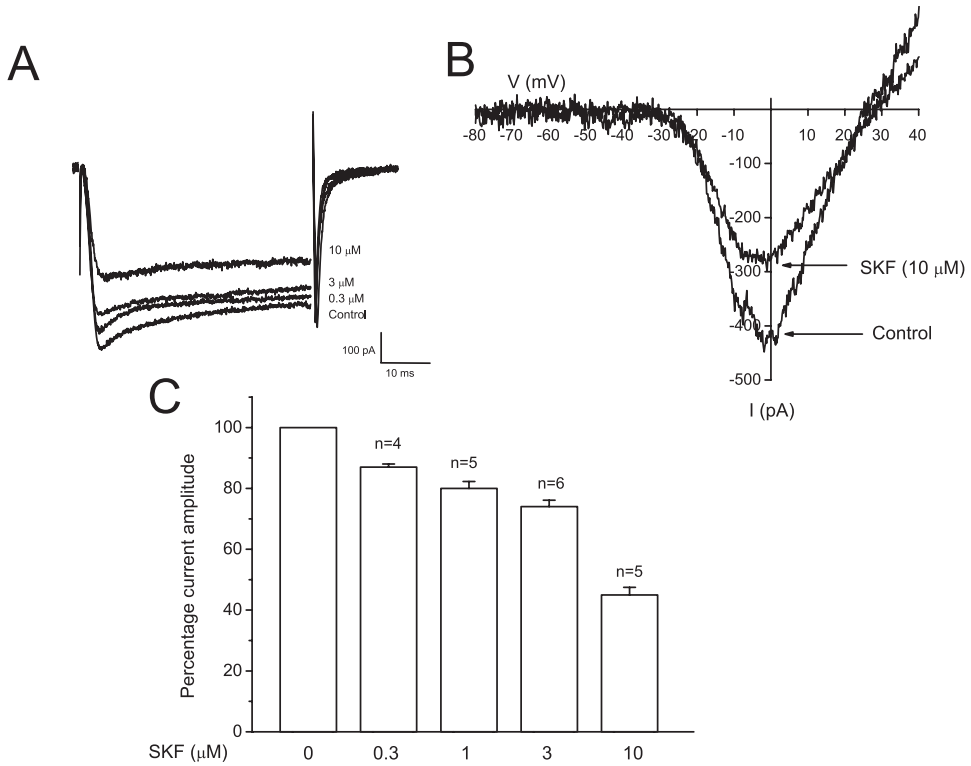

FIGURE 6. Effect of σ-1 receptor agonist (+)-SKF10047 on L-type VGCCs in rat RGC. (**A**) Representative traces showing that the σ-1 receptor agonist (+)-SKF10047 (SKF) inhibited voltage-gated Ca^{2+} currents in a concentration- dependent manner. 5 mM Ca^{2+} was added to external solution as the charge carrier. Whole-cell Ca^{2+} currents were recorded using patch clamp on cultured RGCs (2 DIV) prepared from adult rats. Currents were evoked with a depolarization voltage step from −90 to 0 mV for 55 ms. Ca^{2+} currents were measured in the absence (as control) or presence of SKF for 3 minutes and during 3 minutes of washout. (**B**) The current- voltage relationship was measured using a single voltage ramp from −80 mV to +50 mV of 200-ms duration (0.65 mV/ms). I-V curves exhibited similar properties in the control and in (+)-SKF10047 treatment. (**C**) Concentration-dependent inhibition of Ca^{2+} currents by (+)-SKF10047. All currents are normalized to the control (assigned as 100%). Each column indicates the mean of four to six observations.

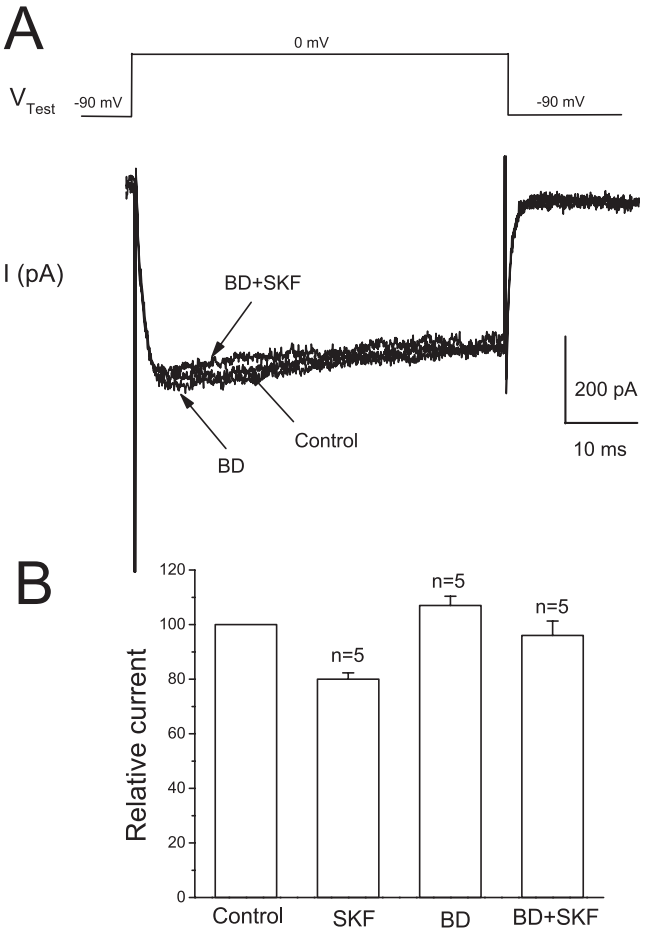

FIGURE 7. (+)-SKF10047 effect on Ca^{2+} currents is mediated by σ-1 receptors. (**A**) Representative traces were recorded in the control, at 3-minute perfusion with BD1047 (BD; 3 μM) or BD (3 μM) + SKF10047 (SKF; 1 μM). Treatment with BD1047 alone had little effect on whole-cell Ca^{2+} currents but prevented (+)-SKF10047- induced inhibition. (**B**) Summary of BD1047 blockade of (+)-SKF10047- induced inhibition of Ca^{2+} channel currents. Currents are normalized to the control (100%). Each bar in the graph point represents a mean of five cells.

that (+)-SKF10047 did not alter the I-V relationships of VGCCs.

BD1047 has already been identified as a selective σ-1 receptor antagonist.[17] To determine whether the observed effect of (+)-SKF10047 on VGCCs is mediated by σ-1 receptors, we tested the (+)-SKF100047 effect in the presence of BD1047. BD1047 (3 μM) alone had no effect on calcium channel currents (current amplitude 105% ± 7.8% of control; $n = 5$; $P > 0.05$, paired *t*-test, compared with control). The Ca^{2+} current amplitude was reduced to 80% ± 2.3% of control by 1 μM (+)-SKF10047 alone (Fig. 6C). However, as shown (Figs. 7A, 7B), pretreatment with BD1047 completely abolished the effect of (+)-SKF10047 (96% ± 5.3% of control; $n = 5$; $P > 0.05$, paired *t*-test, compared with control). These data suggest that the observed (+)-SKF10047 inhibitory effect on voltage-gated Ca^{2+} current is mediated by the activation of σ-1 receptors.

To characterize the Ca^{2+} channel subtypes inhibited by (+)-SKF10047, we examined the effect of (+)-SKF10047 in the presence of nifedipine, a selective blocker of L-type Ca^{2+} channels. We used nifedipine at 10 μM, which is sufficient to completely block L-type channels.[43] Nifedipine occlusion of the response to SKF10047 suggests that SKF targets L-type Ca^{2+} channels. Application of nifedipine significantly reduced the calcium current amplitude (to 74.25% ± 6.7% of control; Fig. 8A). In the presence of nifedipine, the application of SKF (3 μM) was still able to reversibly inhibit current amplitude. However, the magnitude of inhibition (11% ± 4.7%) was approximately half that observed when L-type calcium channels were not blocked (Fig. 8B). Thus, σ-1 receptors inhibit L-type VGCCs and potentially other VGCCs.

Sigma Receptor L-Type VGCC Coimmunoprecipitation

We performed coimmunoprecipitation experiments to detect whether σ-1 receptors were associated with L-type VGCCs.

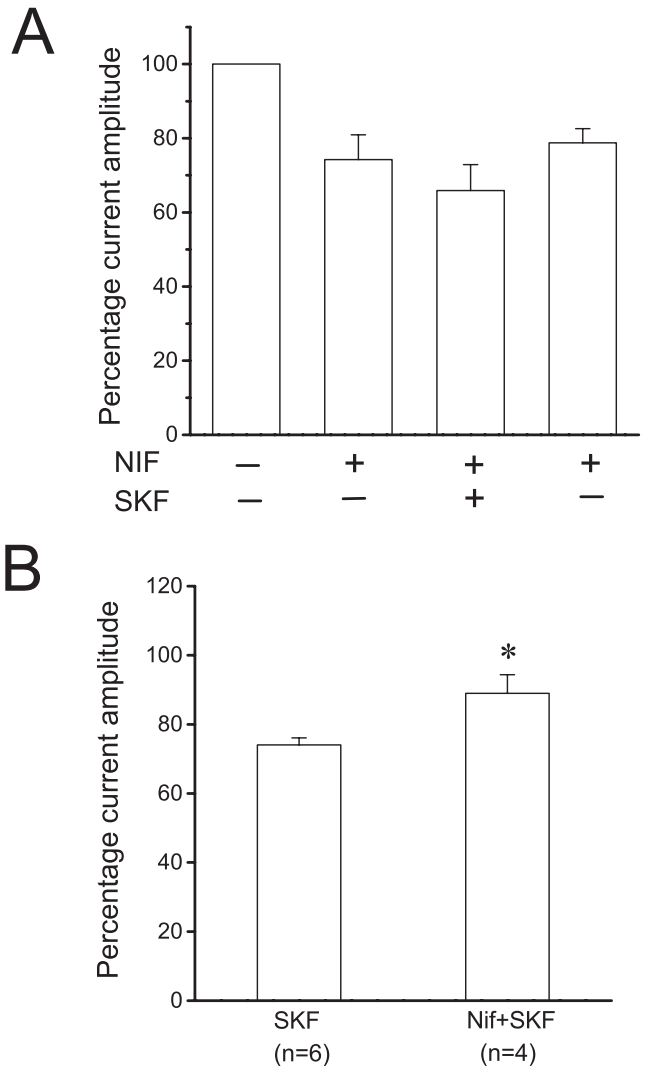

FIGURE 8. Effect of nifedipine, a specific L-type Ca^{2+} channel blocker, on (+)-SKF10047- induced inhibition of Ca^{2+} currents in rat cultured RGCs. (**A**) Whole-cell Ca^{2+} currents were recorded in the control, perfusion with nifedipine (Nif, 10 μM), or perfusion with Nif (10 μM) + SKF (3 μM). The data point represents a mean of four cells. All the currents were normalized to the control before drug administration. After the inhibition of whole-cell currents by nifedipine reached a steady sate (74.25% ± 6.7% of control), application of SKF (3 μM) was able to further inhibit the current to 65.9% ± 7% of control in the presence of nifedipine, though the magnitude of its effect was reduced. SKF-induced inhibition was reversible after washout of SKF. (**B**) Blockade of L-type Ca^{2+} channels with nifedipine significantly reduced SKF-induced inhibition ($P < 0.05$, unpaired *t*-test). For direct comparison, the SKF (3 μM) data were replotted from Figure 1C. All currents were normalized to the control before SKF treatment.

Membrane lysates were prepared from RGC-5 and σ-1 receptor overexpressing RGC-5 and were immunoprecipitated with rabbit antibodies against L-VGCC or σ-1 receptor. Probing the membrane with antibody against the σ-1 receptor revealed a distinct band of approximately 25 kDa in samples immunoprecipitated with anti–human L-type calcium channel (α1C) antibody (Fig. 9A, lanes 1, 2) or anti–σ-1 receptor (Fig. 9A, lane 3). Probing the membrane with anti–human α1C antibody indicated a distinct band of approximately 205 kDa in samples immunoprecipitated with anti–human α1C antibody (Fig. 9B, lanes 1, 2) or anti–σ-1 receptor (Fig. 9B, lane 3). These results suggest an association between σ-1 receptor and L-type VGCCs.

Discussion

Molecular characterization of the σ-1 receptor is just beginning to be assessed. The sigma receptor itself is ubiquitously distributed in vertebrates and has been implicated in a wide range of biological functions.[44,45] In neurons of the central nervous system, sigma receptors have been shown to produce various cellular effects, including inhibition of calcium influx, through voltage-activated calcium channels in rat sympathetic and parasympathetic neurons.[30]

In the present studies, we showed, with the use of coimmunoprecipitation, an association between L-type VGCCs and σ-1 receptors. These results indicate a novel relationship similar to that described for the Kv1.4 potassium channels and sigma receptors.[28] This effect of σ-1 receptors on L-type VGCCs supports the hypothesis that protein-protein interactions are characteristic of sigma receptor signal transduction. This form of signal transduction has already been proposed in previous studies, in which it was shown that sigma receptor-mediated signal transduction does not require G-protein activation or protein phosphorylation.[46] RGCs express L-type VGCCs and other types of VGCCs. Because we focused on the interaction of σ-1 receptors with L-type VGCCs, we cannot rule out the possibility that σ-1 receptors directly interact with other types of VGCCs. Further studies will focus on the direct interaction between other VGCCs and σ-1 receptors.

Overactivation of NMDA receptors leads to depolarization of the cell membrane, which in turn activates VGCCs. L-type calcium channels, through their long-conductance calcium currents, contribute to calcium overload, which in some cases becomes toxic to the cells.[16] Zhang et al.[30] have shown that σ-1 receptor ligands inhibit high voltage–activated calcium channels in rat sympathetic and parasympathetic neurons. We tested whether σ-1 receptor agonist (+)-SKF10047 can inhibit calcium currents in rat primary RGCs. Using the whole-cell patch clamp technique, we showed that σ-1 receptor agonist (+)-SKF10047 inhibited calcium currents in RGCs. In addition, our data demonstrated that σ-1 receptors inhibit not only L-type VGCCs but also other types of VGCCs. Our data are similar to those previously reported by Zhang et al.[29] and are consistent with the finding that σ-1 receptors inhibit multiple classes of Ca^{2+} channels in guinea pig ileum longitudinal muscle.[47] However, further studies will be needed to assess in detail the inhibitory effect of σ-1 receptors on other calcium channels in RGCs. Although we cannot rule out the involvement of σ-2 receptors[48,49] in the calcium influx inhibition in our system, we focused our studies on the σ-1 receptor, which has been cloned and well studied.. The calcium data suggest that σ-1 receptor activation contributes to the reduction of calcium overload in RGCs. By inhibiting calcium influx in RGCs, σ-1 receptors can reduce intracellular calcium, thereby preventing calcium overload and secondary signaling that can lead to apoptosis.[33]

Changes in the concentration of free intracellular calcium ions are linked to the induction of apoptosis, but the relationship between calcium and its linkage to the apoptotic program is complex because calcium can be a signal for life and death pathways.[50] Differential regulation of calcium channels and NMDA receptors have been shown to determine survival or death in rat primary cortical neurons.[51,52] Therefore, in addition to regulating proapoptotic genes,[25,35] σ-1 receptors prevent calcium overload in RGCs by regulating L-type calcium channel activation.

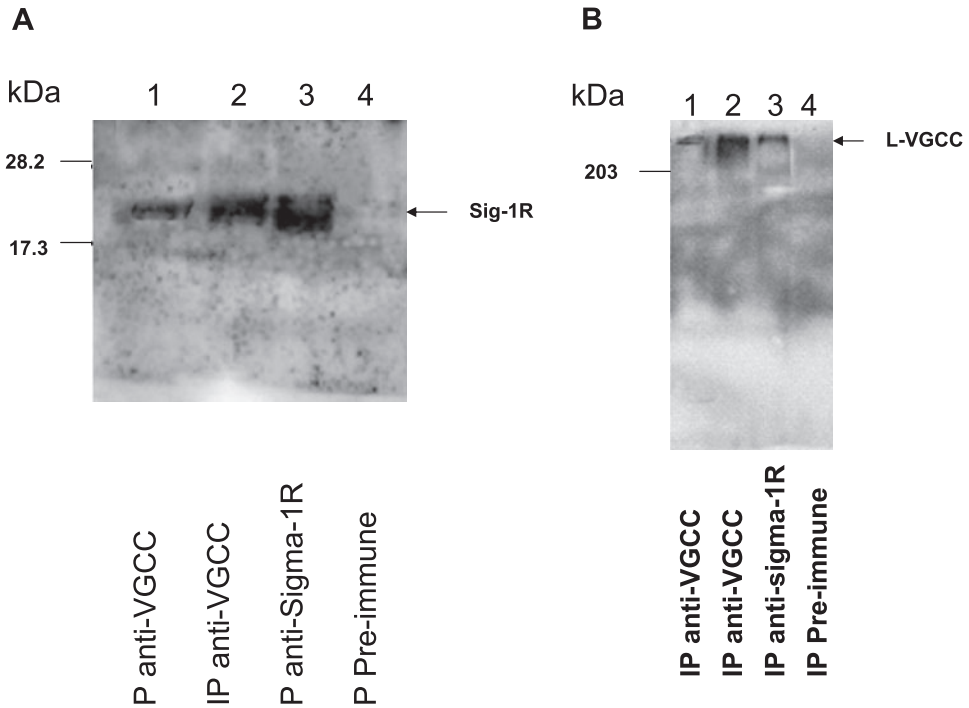

Figure 9. Coimmunoprecipitation of σ-1 receptor (σ-1R) and L-type VGCCs. Coimmunoprecipitation of σ-1 receptor (σ-1R) and L-type VGCCs. Cell lysates were prepared from the rat nontransfected and σ-1 receptor cDNA-transfected RGC-5 and were immunoprecipitated with anti–human α1C antibody or σ-1 receptor antibody. Eluates from the coimmunoprecipitation were resolved with SDS-PAGE. *Lane 1*: nontransfected RGC-5; *lanes 2–4*: RGC-5 overexpressing the σ-1 receptor. Immunoprecipitated samples were run on duplicate gels, and the blots were probed with anti–σ-1 receptor (**A**) or anti–human L-type VGCC (α1C) (**B**) antibodies. Coimmunoprecipitation results show a direct physical interaction between σ-1 receptor and L-type VGCCs.

In summary we have shown that in rat RGCs, σ-1 receptors in part control calcium homeostasis through the regulation of calcium influx. We reported a physical interaction between L-type calcium channels and σ-1 receptors. By controlling excess calcium influx in the cell, σ-1 receptor ligands may contribute to the control of calcium overload in RGCs and, therefore, may constitute a protective mechanism for these cells from undergoing apoptosis.

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