S(+)-Ketamine Suppresses Desensitization of γ-Aminobutyric Acid Type B Receptor-mediated Signaling by Inhibition of the Interaction of γ-Aminobutyric Acid Type B Receptors with G Protein–coupled Receptor Kinase 4 or 5

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ABSTRACT

Background: Intrathecal baclofen therapy is an established treatment for severe spasticity. However, long-term management occasionally results in the development of tolerance. One of the mechanisms of tolerance is desensitization of γ-aminobutyric acid type B receptor ($GABA_BR$) because of the complex formation of the $GABA_{B2}$ subunit (GB_2R) and G protein–coupled receptor kinase (GRK) 4 or 5. The current study focused on *S*(+)-ketamine, which reduces the development of morphine tolerance. This study was designed to investigate whether *S*(+)-ketamine affects the $GABA_BR$ desensitization processes by baclofen.

Methods: The G protein–activated inwardly rectifying K^+ channel currents induced by baclofen were recorded using *Xenopus* oocytes coexpressing G protein–activated inwardly rectifying K^+ channel 1/2, $GABA_{B1a}$ receptor subunit, GB_2R, and GRK. Translocation of GRKs 4 and 5 and protein complex formation of GB_2R with GRKs were analyzed by confocal microscopy and fluorescence resonance energy transfer analysis in baby hamster kidney cells coexpressing $GABA_{B1a}$ receptor subunit, fluorescent protein–tagged GB_2R, and GRKs. The formation of protein complexes of GB_2R with GRKs was also determined by coimmunoprecipitation and Western blot analysis.

Results: Desensitization of $GABA_BR$-mediated signaling was suppressed by *S*(+)-ketamine in a concentration-dependent manner in the electrophysiologic assay. Confocal microscopy revealed that *S*(+)-ketamine inhibited translocation of GRKs 4 and 5 to the plasma membranes and protein complex formation of GB_2R with the GRKs. Western blot analysis also showed that *S*(+)-ketamine inhibited the protein complex formation of GB_2R with the GRKs.

Conclusion: *S*(+)-Ketamine suppressed the desensitization of $GABA_BR$-mediated signaling at least in part through inhibition of formation of protein complexes of GB_2R with GRK 4 or 5.

What We Already Know about This Topic:

- Tolerance to intrathecal baclofen for treatment of spasticity is produced by desensitization of the γ-aminobutyric acid type B receptor ($GABA_BR$).

What This Article Tells Us That Is New:

- In cell culture, *S*(+)-ketamine suppressed the desensitization of $GABA_BR$-mediated signaling at least in part through inhibition of formation of protein complexes of $GABA_{B2}$ subunit (GB_2R) with GRK 4 or 5.

BACLOFEN, a selective γ-aminobutyric acid type B receptor ($GABA_BR$) agonist, has been widely used as an antispasticity agent. Intrathecal baclofen (ITB) therapy is an established treatment for severe spasticity of both spinal and

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cerebral origin.[1] Recently, increasing reports have shown that ITB therapy has powerful antinociceptive effects in patients with spasticity and in patients without spasticity who experience chronic pain,[1] such as somatic pain,[2] central pain,[2,3] and complex regional pain syndrome.[4,5]

However, long-term management of ITB therapy occasionally results in the development of tolerance,[6] which makes treatment difficult with respect to both pain and spasticity. Such decreased responsiveness to baclofen, so-called baclofen tolerance, is, in part, because of the desensitization of $GABA_BR$.[7,8] In addition, the desensitization of $GABA_BR$ occurred by the formation of complexes of $GABA_BR$ and either G protein–coupled receptor kinase (GRK) 4[7,8] or 5,[7] which is a member of the GRK family consisting of GRKs 1 through 7.[9]

Until today, several agents (*e.g.*, morphine, baclofen, ketamine, clonidine, and local analgesics) have been administered intrathecally for effective chronic pain management or spinal anesthesia clinically.[10,11] Among them, intrathecal ketamine coadministration has a synergistic analgesic effect with opioids.[12] In addition, ketamine administration prevented the development of tolerance against morphine in several animal models,[13,14] although the mechanism has not yet been clearly elucidated. Regulation of tolerance of μ-opioid receptor–mediated cellular signaling, receptors to which morphine mainly act, is known to be mediated by GRKs, particularly GRK 2[15] or 3.[16,17] GRKs 2 and 3 are reported to play in desensitization processes of μ-opioid receptors[15,17] or development of tolerance to opioids in an animal model.[16] In case of $GABA_BR$, it was previously demonstrated that the desensitization of $GABA_BR$-mediated responses was associated with the formation of protein complexes of $GABA_{B2}$ receptor subunit (GB_2R) with GRK 4 or 5.[7] Our hypothesis is that ketamine would interact with GRK 4 or 5. Thus, we focused on the effects of ketamine on the modification of GRKs 4 and 5 in $GABA_BR$-mediated desensitization processes. Ketamine consists of two enantiomers, *S*(+)-ketamine and *R*(−)-ketamine, that have distinct pharmacologic properties.[18] *S*(+)-Ketamine has a three times higher anesthetic potency than that of the racemic mixture, the incidence of adverse effects is equal at the same concentration for both enantiomers,[18] and both are clinically available.[18] Thus, in the current study, we used *S*(+)-ketamine and investigated whether *S*(+)-ketamine has effects on $GABA_BR$ desensitization and the formation of complexes of $GABA_BR$ with GRK 4 or 5.

Materials and Methods

Drugs and Chemicals

Baclofen was purchased from Tocris Cookson, Bristol, United Kingdom; and *S*(+)-ketamine, gentamicin, and sodium pyruvate were obtained from Sigma, St Louis, MO. All other chemicals used were of analytic grade and were obtained from Nacalai Tesque, Kyoto, Japan.

Construction of Complementary DNA and Preparation for Complementary RNAs

Complementary DNA (cDNA) for rat G protein–activated inwardly rectifying K^+ channel (GIRK) 1 and mouse GIRK2 were provided by Henry A. Lester, Ph.D. (Professor of Biology, Caltech, Pasadena, CA). $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), GB_2R, and anti-hemagglutinin (HA)–tagged GB_2R were provided by Niall. J. Fraser, Ph.D. (Glaxo Wellcome, Stevenage, United Kingdom). Cerulean, a brighter variant of cyan fluorescent protein, was obtained from David W. Piston, Ph.D. (Professor of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN); and Venus, a brighter variant of yellow fluorescent protein, was obtained from Takeharu Nagai, Ph.D. (Professor of Nanosystems Physiology, Hokkaido University, Sapporo, Japan). Human GRK4 was provided by Antonio De Blasi, Ph.D. (Professor of Istituto Neurologico Mediterraneo Neuromed, Pozzilli, Italy); and rat GRK5 was obtained from Yuji Nagayama, M.D., Ph.D. (Professor of Medical Gene Technology at Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan). For receptor construction, the N-DYKDDDDK-C (FLAG) epitope tag (5′-GAACAAAAACTCATCTCAGAAGAGGATGTG-3′) was engineered to ligate the N-terminus of GRK 4 or 5 by using standard molecular approaches that use polymerase chain reaction. Venus-fused GB_2R was created by ligating the receptor cDNA into *Hin*dIII sites into the corresponding sites of Venus cDNA. Venus- or Cerulean-fused GRKs 4 and 5 were created by ligating the GRK cDNA sequences into the *Not*I or *Bam*HI sites of corresponding Venus or Cerulean sites. All cDNAs for transfection in baby hamster kidney (BHK) cells were subcloned into pcDNA3.1 (Invitrogen, San Diego, CA). For expression in *Xenopus* oocytes, all cDNAs for the synthesis of complementary RNAs (cRNAs) were subcloned into the pGEMHJ vector, which provides 5′- and 3′-untranslated regions of the *Xenopus* β-globin RNA, ensuring a high concentration of protein expression in the oocytes.[19] Each of the cRNAs was synthesized with a messenger RNA kit (mCAP messenger RNA Capping Kit; Ambion, Austin, TX) and with a T7 RNA polymerase *in vitro* transcription kit (Ambion) from the respective linearized cDNAs.[20]

Oocyte Preparation and Injection

Immature V and VI oocytes from *Xenopus* were enzymatically dissociated, as previously described.[21,22] Isolated oocytes were incubated at 18°C in ND-96 medium (containing 96-mM NaCl, 2-mM KCl, 1-mM $CaCl_2$, 1-mM $MgCl_2$, and 5-mM HEPES, pH 7.4) containing 2.5-mM sodium pyruvate and 50-μg/ml gentamicin. For measurement of GIRK currents induced by baclofen, cRNAs of GIRKs 1 and 2 (0.2 ng each) and $GB_{1a}R$ and GB_2R (5 ng each) were coinjected into the oocytes, together with or without GRKs (4 or 5) or FLAG-tagged GRKs (FLAG-GRK4 or FLAG-GRK5) (3 ng each). The final injection volume was less than 50 nl in all

cases. Oocytes were incubated in ND-96 medium and used 3–8 days after injection, as previously reported.[21]

Electrophysiologic Recordings

Electrophysiologic recordings were performed using the two-electrode voltage clamp method with an amplifier (Geneclamp 500; Axon Instruments, Foster City, CA) at room temperature. Oocytes were clamped at −60 mV and continuously superfused with ND-96 medium or 49 mM K^+ (high potassium) solution, in which tonicity was adjusted to reduce concentrations of NaCl (48-mM NaCl, 49-mM KCl, 1-mM $CaCl_2$, 1-mM $MgCl_2$, and 5-mM HEPES, pH 7.4) in a 0.25-ml chamber at a flow rate of 5 ml/min. Then, baclofen alone or *S*(+)-ketamine and baclofen were added to the superfusion solution. Voltage recording microelectrodes were filled with 3 M potassium chloride, and their tip resistance was 1.0–2.5 MΩ. Currents were continuously recorded and stored with a data acquisition system (PowerLab 2/26; AD Instruments, Castle Hill, Australia) and a computer (Macintosh; Apple, Cupertino, CA), as previously described.[21,22] All test compounds applied to oocytes were dissolved into the ND-96 medium or 49-mM K^+ media.

Cell Culture and Transfection

The BHK cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. For confocal microscopic assay, BHK cells were seeded at a density of 1×10^5 cells/35-mm glass-bottomed culture dish (World Precision Instruments, Sarasota, FL) and cultured for 24 h. Transient transfection was then performed with a transfection reagent (Effectene; Qiagen, Tokyo, Japan) in 0.2 μg each cDNA, as previously described,[7,20] and according to the protocol provided by the manufacturer. Cells were used in confocal microscopy and fluorescence resonance energy transfer (FRET) analysis 16–24 h after transfection.

Confocal Fluorescence Microscopy

For translocation studies of GRKs and protein complex formation of $GABA_BR$ with each GRK (4 or 5) using confocal microscopy and the FRET assay, GB_2R and each of the GRKs (4 and 5) were fused through the carboxyl terminus to Cerulean or Venus. The BHK cells cultured in 35-mm glass-bottomed dishes were cotransfected with 0.2 μg Venus-fused $GABA_BR$ and Venus- or Cerulean-fused GRKs. A ×63 magnification 1.25–numerical aperture oil immersion objective was used with the pinhole for visualization. Both Venus and Cerulean were excited by a 458-nm laser, and images were obtained by placing the dish onto a stage in a confocal microscope (Zeiss LSM510 META; Carl Zeiss, Jena, Germany).

Photobleaching and Calculation of FRET Efficiency

To confirm FRET between Venus and Cerulean, we monitored acceptor photobleaching analysis in BHK cells that coexpressed $GB_{1a}R$, Venus-fused GB_2R, and Cerulean-fused GRKs. FRET was measured by imaging Cerulean before and after photobleaching Venus with the 100% intensity of a 514-nm argon laser for 1 min, a duration that efficiently bleached Venus with little effect on Cerulean. An increase of donor fluorescence (Cerulean) was interpreted as the evidence of FRET from Cerulean to Venus. All experiments were analyzed from at least six cells with three independent regions of interest. As a control, we examined the FRET efficiency of the unbleached area of membrane in the same cells in at least three areas. In some cases, we performed a photobleaching assay using fixed BHK cells. Cells were fixed as previously described.[23]

FRET efficiency was calculated using emission spectra before and after acceptor photobleaching of Venus.[24] According to this procedure, if FRET is occurring, then photobleaching of the acceptor (Venus) should yield a significant increase in fluorescence of the donor (Cerulean). Increase of donor spectra because of desensitized acceptor was measured by taking the Cerulean emission (at 488 nm) from spectra before and after acceptor photobleaching. FRET efficiency was then calculated using the following equation: $E = 1 - I_{DA}/I_D$, where I_{DA} is the peak of donor (Cerulean) emission in the presence of the acceptor, and I_D is the peak in the presence of the sensitized acceptor, as previously described.[25] Before and after this bleaching, Cerulean images were collected to assess changes in donor fluorescence.

Coimmunoprecipitation and Western Blotting

Monoclonal anti–FLAG M2 was obtained from Sigma; monoclonal anti-HA (12CA5), from Roche, Mannheim, Germany; and polyclonal anti-HA (Y-11), from Santa Cruz Biotechnology, Santa Cruz, CA. The BHK cells were transiently cotransfected with each of the FLAG-tagged GRK cDNAs, HA-tagged GB_2R (HA-GB_2R), and nontagged $GB_{1a}R$ cDNAs. Twenty-four hours later, the cells were harvested, sonicated, and solubilized in a protein extraction buffer containing a combination of protease inhibitor cocktail (PRO-PREP; iNtRON Biotechnology, Sungnam, Korea) for 1 h at 4°C. The mixture was centrifuged (at 15,000 rpm for 30 min), and the supernatants were incubated with FLAG or HA (12CA5) antibody at 5 μg/ml overnight at 4°C. The mixture was centrifuged, and the pellets were washed five times by centrifugation and resuspension. Immunoprecipitated materials were dissolved in sample buffer (Lammeli) containing 0.1-M dithiothreitol subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and subjected to immunoblotting using monoclonal antibodies against FLAG (1:10,000) and polyclonal HA (Y-11) (1:10,000); then, bovine mouse or goat rabbit anti-IgG was conjugated with horseradish peroxidase at 1:5,000 and reacted with chemiluminescence Western blot detection reagents (Nacalai Tesque).

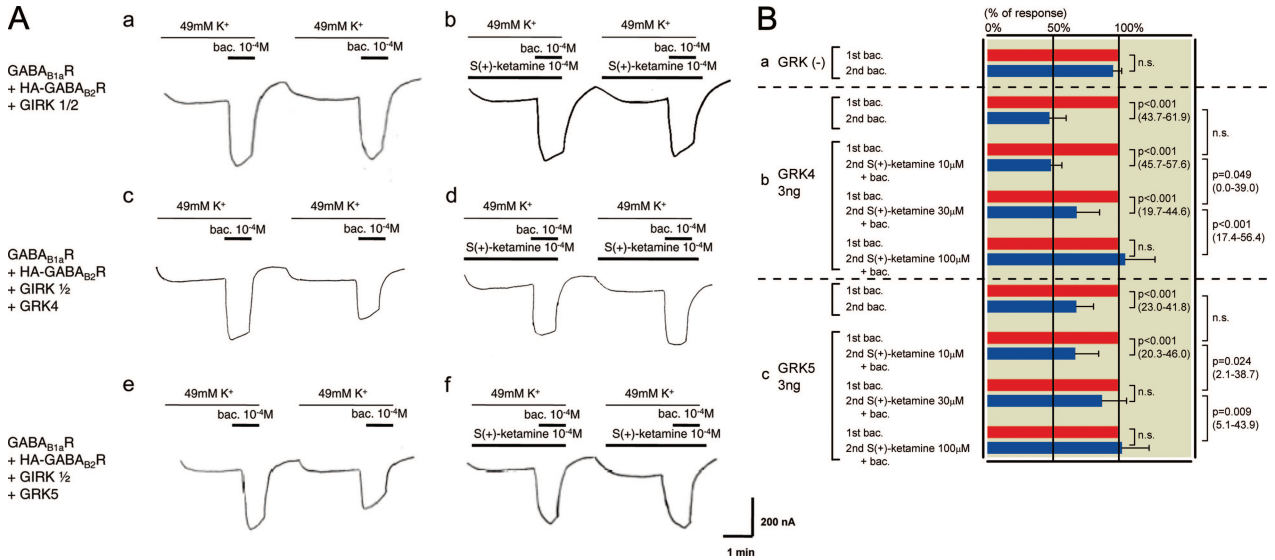

Fig. 1. Effects of *S*(+)-ketamine on the desensitization of γ-aminobutyric acid type B receptor ($GABA_BR$)–mediated G protein–activated inwardly rectifying K^+ channel (GIRK) currents in *Xenopus* oocytes. (*A*) Typical tracing of GIRK currents induced by the first and second application of baclofen (bac) (100 μM) for 1 min in a time lag of 4 min in oocytes coexpressing $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), hemagglutinin (HA)–$GABA_{B2}$ subunit (GB_2R), and GIRK1/2 without (*a*) or with (*b*) *S*(+)-ketamine (100 μM) before (2 min) and during (1 min) application of a second preapplication of bac. Typical tracing of GIRK currents induced by the first and second application of bac (100 μM) for 1 min in a time lag of 4 min in oocytes coexpressing $GB_{1a}R$, HA-GB_2R, GIRK1/2, and G protein–coupled receptor kinase (GRK) 4 or 5 without (*c* and *e*) or with (*d* and *f*) *S*(+)-ketamine (100 μM) before (2 min) and during (1 min) application of a second preapplication of bac 49 mM k^+: 49 mM K^+ (high potassium) solution. (*B*) Summary of the effects of *S*(+)-ketamine on $GABA_BR$ desensitization. Each bar represents the mean ± SD of the peak GIRK currents induced by second application, expressed as percentage to each current induced by first application of bac in oocytes. (*a*) A group coexpressing $GB_{1a}R$, HA-GB_2R, and GIRK1/2, n = 8, (*b*) groups coexpressing $GB_{1a}R$, HA-GB_2R, GIRK1/2, and GRK 4 (n = 10 for each group), (*c*) groups coexpressing $GB_{1a}R$, HA-GB_2R, GIRK1/2, and GRK5 (n = 10 for each group). Statistical results are represented as *P* values (95% confidence interval for the differences in the two conditions). ns = not significant.

Statistical Analysis

Data are expressed as mean ± SD. For comparisons of the peak GIRK currents induced by second application of baclofen with those by first application of baclofen in *Xenopus* oocytes coexpressing $GB_{1a}R$, HA-GB_2R, and GIRK1/2 with or without GRK 4 or 5, two-tailed paired *t* tests were performed and the 95% confidence intervals (CIs) are depicted. The effects of *S*(+)-ketamine on the percentages of GIRK currents induced by second application of baclofen to each current induced by first application of baclofen were compared using one-way ANOVA, followed by the Tukey test. For comparison of FRET efficiency in BHK cells coexpressing $GB_{1a}R$, GB_2R-Venus, and GRKs-Cerulean, with or without *S*(+)-ketamine application before and during baclofen stimulation, two-tailed unpaired *t* tests were performed. Statistical significance was accepted at $P < 0.05$. All analyses were performed using computer software (IBM SPSS Statistics 18; IBM Corp, Armonk, NY).

Results

S(+)-Ketamine Inhibits the Desensitization of $GABA_B$ Receptor-Mediated Signaling by GRK 4 or 5 in *Xenopus* Oocytes

It was previously reported that baclofen elicited a GIRK conductance in *Xenopus* oocytes coexpressing heterodimeric $GABA_BR$ ($GB_{1a}R$ and HA-tagged GB_2R [HA-GB_2R]) with GIRKs 1 and 2 (GIRK1/2).[7] In addition, $GABA_BR$ desensitization was observed after repeated application of baclofen at 100 μM, which was a submaximum concentration to elicit inward K^+ current through GIRK1/2 to oocytes, coexpressing GRK 4 or 5 but not 2, 3, or 6.[7]

As previously demonstrated,[7] no desensitization was observed after repeated application of baclofen at 100 μM (for 1 min, each application) to oocytes coexpressing the $GB_{1a}R$ and HA-GB_2R with GIRK1/2 (fig. 1, A and B). When either GRK 4 (3 ng) or 5 (3 ng) cRNA was coinjected with heterodimeric $GABA_BR$ and GIRK1/2 cRNA, the amplitude of first baclofen-induced K^+ currents was almost the same as that in oocytes coexpressing $GABA_BR$ and GIRK1/2 without GRKs, whereas that of the second K^+ currents induced by baclofen was attenuated to 47.2 ± 12.7% (n = 8) in oocytes coexpressing GRK4 and to 67.6 ± 13.1% (n = 8) in oocytes coexpressing GRK5. This indicates that GRK 4 or 5 induced $GABA_BR$ desensitization (fig. 1, A and B). *S*(+)-Ketamine (100–300 μM) by itself had no effects on both the 49-mM K^+- and baclofen-induced K^+ currents in oocytes expressing $GABA_BR$ and GIRK1/2 without GRKs (fig. 1A and data not shown).

When *S*(+)-ketamine at a concentration of 10, 30, or 100 μM was applied before (2 min) and during the second application of baclofen (1 min) to oocytes coexpressing heterodimeric $GABA_BR$ and GIRK1/2 with GRK 4 or 5, the attenuation of the second baclofen-induced K^+ currents was

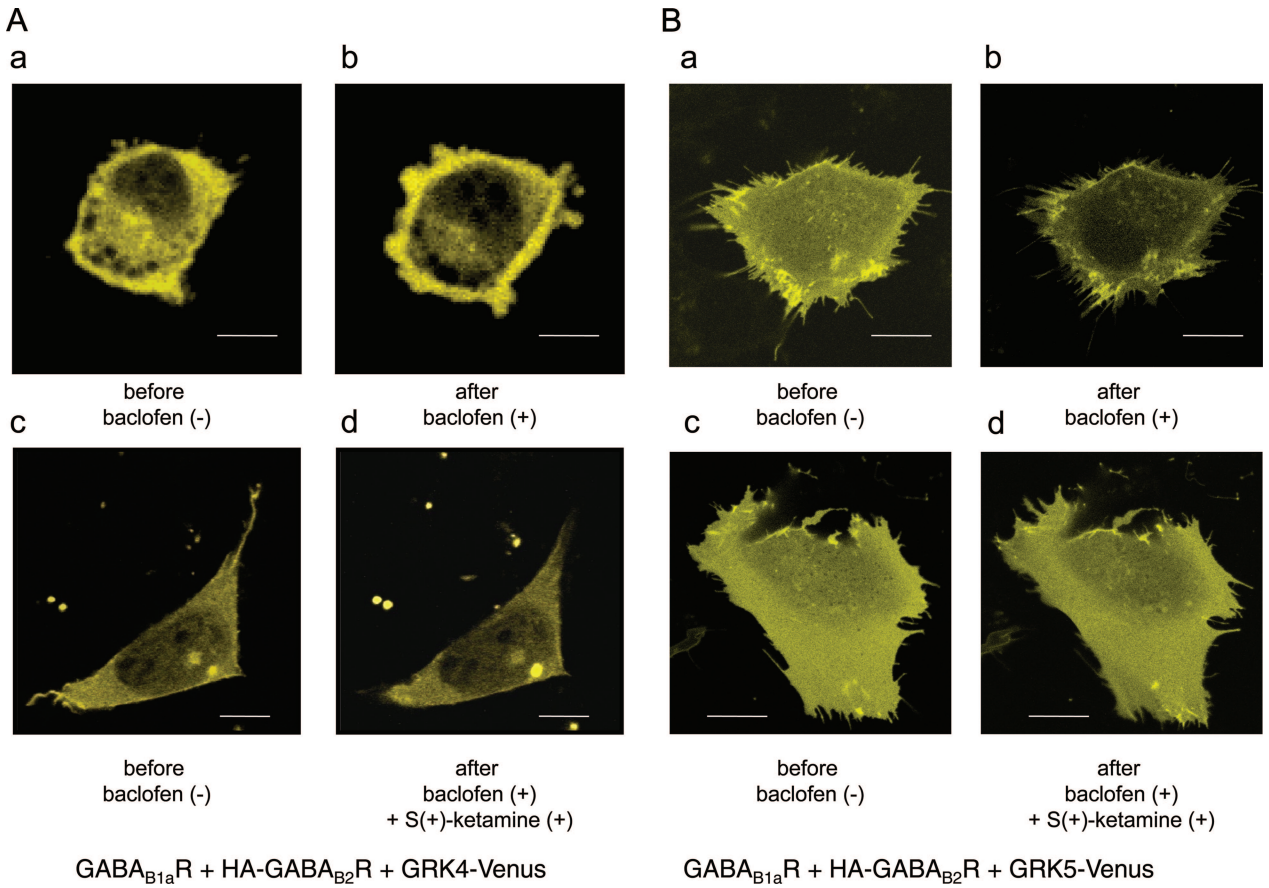

Fig. 2. Confocal imaging showing the effects of *S*(+)-ketamine on the translocation of G protein–coupled receptor kinase (GRK) 4–Venus or GRK5-Venus to the plasma membranes in baby hamster kidney (BHK) cells coexpressing the γ-aminobutyric acid $(GABA)_{B1a}$ receptor subunit ($GB_{1a}R$), hemagglutinin (HA)–$GABA_{B2}$ subunit (GB_2R), and GRKs-Venus. Each bar represents 10 μm. (*A*) Visualization of GRK4-Venus in the cells before (*a* and *c*) and after stimulation of baclofen (100 μM) for 5 min with (*d*) or without (*b*) previous application of *S*(+)-ketamine (100 μM) for 5 min in BHK cells coexpressing $GB_{1a}R$, HA-GB_2R, and GRK4-Venus. (*B*) Visualization of GRK5-Venus in BHK cells before (*a* and *c*) and after stimulation of baclofen for 5 min with (*d*) or without (*b*) previous application of *S*(+)-ketamine for 5 min in BHK cells coexpressing $GB_{1a}R$, HA-GB_2R, and GRK5-Venus.

significantly restored in a concentration-dependent manner (fig. 1, A and B). The amplitude of K^+ currents induced by the second application of baclofen with 10-, 30-, or 100-μM *S*(+)-ketamine was 48.3 ± 8.4%, 67.9 ± 17.4%, and 104.8 ± 22.7% in oocytes coexpressing GRK4 (n = 10 each) and 66.8 ± 17.9%, 87.2 ± 18.7%, and 102.4 ± 20.6% in oocytes coexpressing GRK5 (n = 10 each) of those induced by the first application of baclofen, respectively (fig. 1, A and B). When typical GIRK currents were not obtained by first application of baclofen, such data were excluded. Overall, approximately 67–83% of recording data in each group of oocytes were obtained for statistical analyses.

Translocation of Venus-Fused GRK 4 or 5 to the Plasma Membranes after Activation of $GABA_BR$ Is Inhibited in the Presence of S(+)-Ketamine

To determine the effects of *S*(+)-ketamine on the translocation of GRK 4 or 5 in response to baclofen in BHK cells, we cotransfected GRK4-Venus or GRK5-Venus cDNA with $GB_{1a}R$ and HA-GB_2R cDNAs and determined the intracellular distribution and translocation properties of GRK4-Venus or GRK5-Venus. We then applied baclofen with or without *S*(+)-ketamine application to living BHK cells. As shown in figure 2, A and B, GRK4-Venus or GRK5-Venus was diffusely distributed in the cytosol without agonist stimulation in BHK cells but was translocated to the plasma membranes gradually in 5 min after application of baclofen (100 μM). When *S*(+)-ketamine (100 μM) was applied to such cells 2.5 min before and during application of baclofen, the translocation of GRK4-Venus or GRK5-Venus to the plasma membranes was almost inhibited (fig. 2, A and B). Treatment of *S*(+)-ketamine (100 and 300 μM) alone for 10 min did not affect translocation properties of both GRK4-Venus and GRK5-Venus in BHK cells coexpressing heterodimeric $GABA_BR$ with GRK4-Venus or GRK5-Venus (data not shown).

FRET and Acceptor Photobleaching Analysis of BHK Cells Coexpressing GRK 4 or 5 with Heterodimeric $GABA_BR$

Previously, we showed that functional $GABA_BR$ formed heterodimers with $GB_{1a}R$ and GB_2R by analysis with FRET and

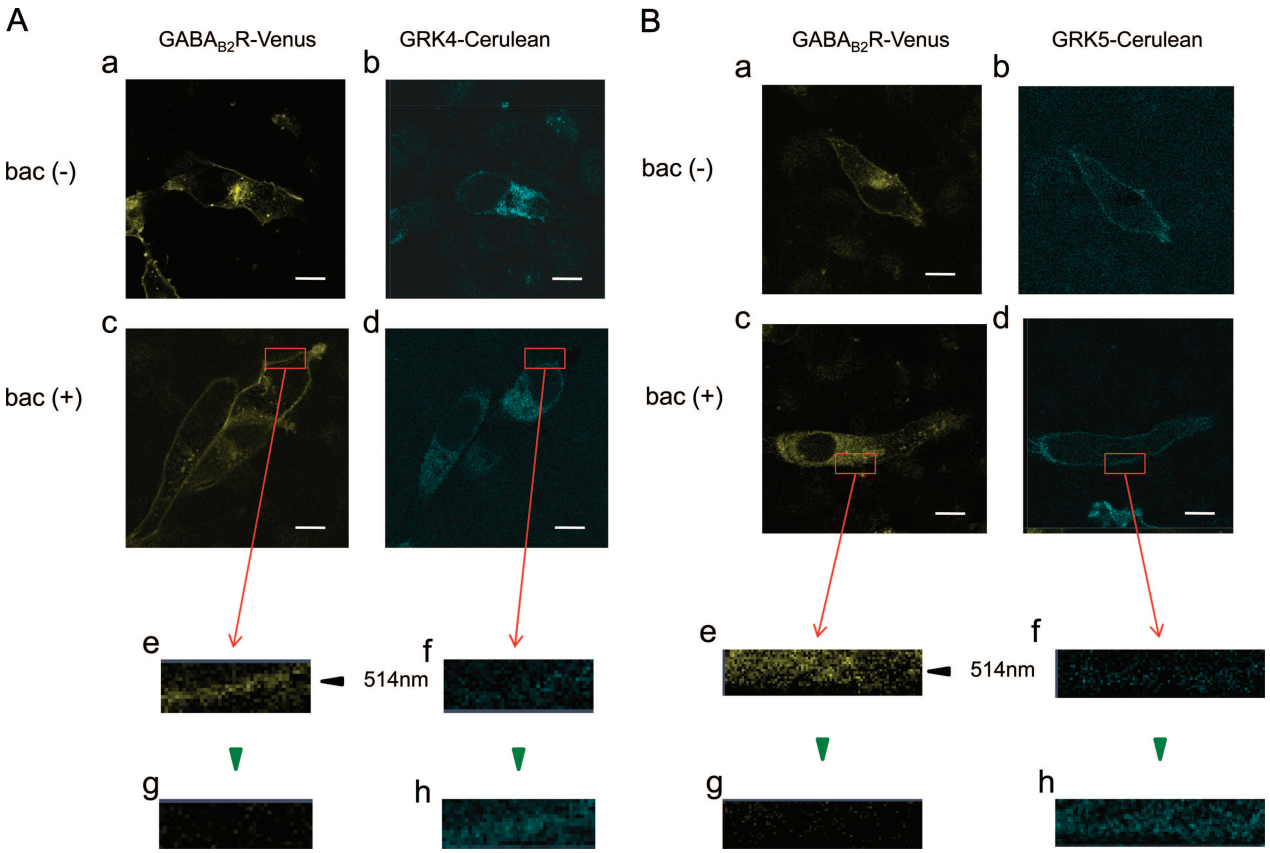

Fig. 3. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the protein complex formation of the γ-aminobutyric acid $(GABA)_{B2}$ subunit (GB_2R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing the $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), GB_2R-Venus, and GRKs-Cerulean. Each bar represents 10 μm. (*A*) Visualization of GB_2R-Venus and GRK4-Cerulean in nonstimulated (*a* and *b*) and baclofen (bac)-stimulated (100 μM, 5 min) BHK cells (*c* and *d*). Fluorescence changes by acceptor photobleaching (1-min application of 514-nm wavelength) in bac-stimulated BHK cells (*e–h*). (*B*) Visualization of GB_2R-Venus and GRK5-Cerulean in nonstimulated (*a* and *b*) and bac-stimulated (100 μM, 5 min) BHK cells (*c* and *d*). Fluorescence changes by acceptor photobleaching in bac-stimulated BHK cells (*e–h*).

acceptor photobleaching in BHK cells coexpressing $GB_{1a}R$-Venus and GB_2R-Cerulean.[7,20] We also showed that GRK 4 or 5, but not GRK 2, 3, or 6, formed protein complexes with the GB_2R subunit after $GABA_BR$ activation in the cells coexpressing Venus-fused $GB_{1a}R$ or GB_2R and Cerulean-fused GRKs.[7] We examined the effects of *S*(+)-ketamine on the formation of protein complexes of GRK 4 or 5 with GB_2R in BHK cells coexpressing $GB_{1a}R$, GB_2R-Venus, and GRK4-Cerulean (fig. 3A) or GRK5-Cerulean (fig. 3B). The fluorescence from GB_2R-Venus was mostly localized on the plasma membranes, whereas that from GRK4-Cerulean or GRK5-Cerulean was localized in the cytosol and to some extent on the plasma membranes (fig. 3A, a and b, and 3B, a and b). When cells were stimulated with baclofen (100 μM) for 5 min, the fluorescence of GRK4-Ceulean or GRK5-Cerulean and GB_2R-Venus was detected on and around the plasma membranes (fig. 3A, c and d, and 3B, c and d). Photobleaching analysis demonstrated that Venus fluorescence was reduced by application of a 514-nm wavelength at 100% intensity of the argon laser power to the indicated area (fig. 3A, e–h, and 3B, e–h). This application did not affect the fluorescent intensity of Venus and Cerulean in the unbleached area (data not shown). Acceptor photobleaching showed increased Cerulean fluorescence (donor) with decreased Venus fluorescence (acceptor) (fig. 3A, e–h, and 3B, e–h).

To determine the effects of *S*(+)-ketamine on the protein complex formation of GRK4-Cerulean or GRK5-Cerulean with GB_2-Venus plus $GB_{1a}R$, we applied *S*(+)-ketamine (100 μM) to the cells 5 min before application of baclofen (100 μM) and then simultaneously treated the cells for 5 min with baclofen and *S*(+)-ketamine. The fluorescence from GRK4-Cerulean or GRK5-Cerulean was detected diffusely in the cytosol and on the plasma membranes, whereas the fluorescence from GB_2R-Venus was mostly detected on the plasma membranes. Acceptor photobleaching demonstrated the reduction of the fluorescence from GB_2R-Venus; however, the fluorescence from GRK4-Cerulean or GRK5-Cerulean hardly changed (fig. 4, A and B; and fig. 5), which indicates that GRK4-Cerulean or GRK5-Cerulean and

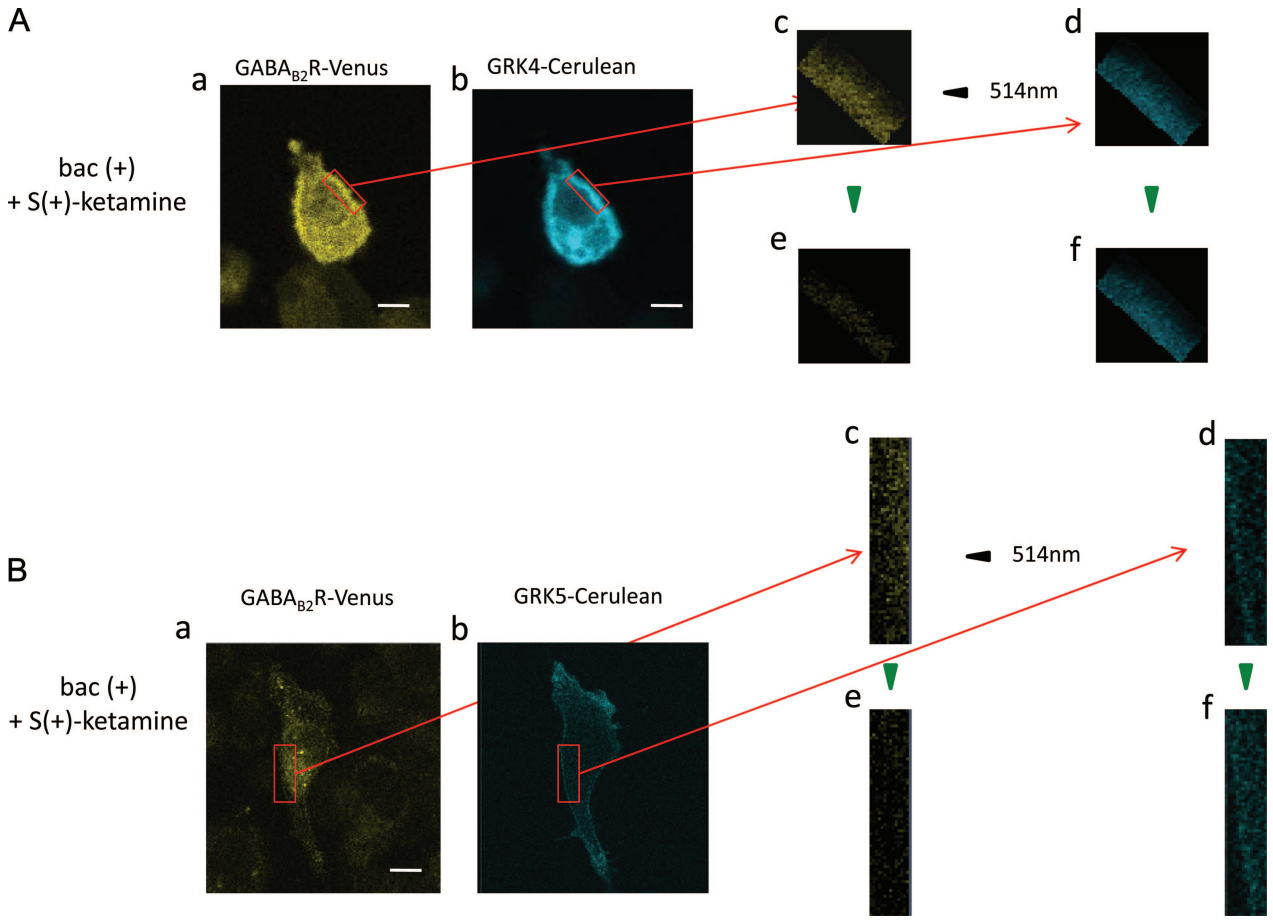

Fig. 4. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the effects of *S*(+)-ketamine on the interaction of γ-aminobutyric acid (GABA)$_{B2}$ subunit (GB$_2$R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing GABA$_{B1a}$ receptor subunit (GB$_{1a}$R), GB$_2$R-Venus, and GRKs-Cerulean. Each bar represents 10 μm. (*A*) Visualization of GB$_2$R-Venus and GRK4-Cerulean in a BHK cell treated by *S*(+)-ketamine (100 μM) before (5 min) and during (5 min) baclofen (bac) stimulation (*a* and *b*). Fluorescence changes by acceptor photobleaching in bac-stimulated BHK cells (*c–f*). (*B*) Visualization of GB$_2$R-Venus and GRK5-Cerulean in a BHK cell pretreated with *S*(+)-ketamine (100 μM) before (5 min) and during (5 min) bac stimulation (*a* and *b*). Fluorescence changes by acceptor photobleaching in bac-stimulated BHK cells (*c–f*).

GB$_2$R-Venus do not form baclofen-induced protein complexes in the presence of *S*(+)-ketamine.

Coimmunoprecipitation and Western Blot Analysis of GRK 4 or 5 Using BHK Cells Coexpressing FLAG-GRKs, HA-GB$_2$R, and GB$_{1a}$R

Previously, it was shown that FLAG-GRK 4 or 5, but not GRK 2, 3, or 6, formed protein complexes with HA-GB$_2$R after baclofen stimulation (100 μM, 5 min) in BHK cells determined with coimmunoprecipitation and Western blot analysis.[7] We investigated whether *S*(+)-ketamine has an effect on the protein complex formation of GRK 4 or 5 with GB$_2$R induced by baclofen. Western blot analysis was performed with proteins extracted from BHK cells coexpressing FLAG-GRK4 or FLAG-GRK5, GB$_{1a}$R, and HA-GB$_2$R after immunoprecipitation with anti-HA. In the precipitate using anti-HA from the BHK cells coexpressing FLAG-GRK4 or FLAG-GRK5, HA-GB$_2$R, and GB$_{1a}$R, the band intensity of the immune complex determined with anti-HA was similar in nonstimulated and baclofen-stimulated (100 μM, 5 min) BHK cells (fig. 6A). On the other hand, the immune complex determined with anti-FLAG was stronger in baclofen-stimulated cells than that in nonstimulated cells (fig. 6B).

To determine the effect of *S*(+)-ketamine on the protein complex formation of FLAG-GRK4 or FLAG-GRK5 with GB$_2$R, we treated *S*(+)-ketamine (100 μM) to the cells coexpressing FLAG-GRK4 or FLAG-GRK5, HA-GB$_2$R, and GB$_{1a}$R 5 min before and during the stimulation of baclofen (5 min, 100 μM). In the precipitate using anti-HA from the cells coexpressing either FLAG-GRK4 or FLAG-GRK5 with HA-GB$_2$R and GB$_{1a}$R, the intensity of the immune complex with anti-HA was similar among nonstimulated and baclofen-stimulated cells with or without *S*(+)-ketamine treatment (fig. 6A). On the other hand, the intensity of the immune complex determined with anti-FLAG was less in baclofen-stimulated cells with *S*(+)-ketamine treatment than in baclofen-stimulated cells without *S*(+)-ketamine treatment; and the intensity in baclofen-stimulated cells with

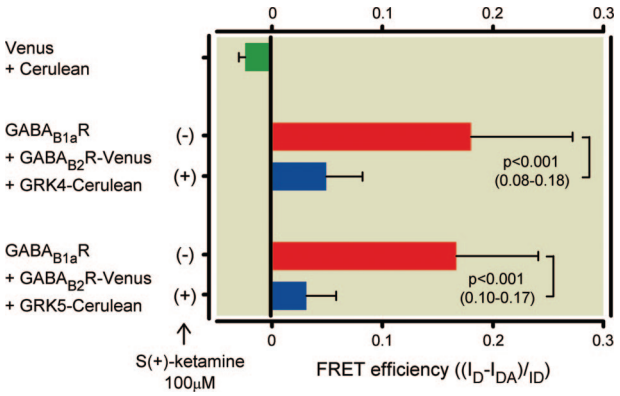

Fig. 5. Comparison of fluorescence resonance energy transfer (FRET) efficiency in baby hamster kidney (BHK) cells expressing γ-aminobutyric acid $(GABA)_{B1a}$ receptor subunit ($GB_{1a}R$), $GABA_{B2}$ subunit (GB_2R)–Venus, and G protein–coupled receptor (GRK) 4–Cerulean or GRK5-Cerulean, with or without previous stimulation of *S*(+)-ketamine (n = 8 for each group). The FRET efficiency was calculated from emission spectra. Each bar represents the mean ± SD. Statistical results are represented as *P* values (95% confidence interval for the differences in the two conditions). I_D = peak of donor emission in presence of sensitized acceptor; I_{DA} = peak of donor emission in presence of acceptor.

S(+)-ketamine was almost similar to that in nonstimulated cells (fig. 6B). In the total lysate, the intensity of the immune complex determined with anti-FLAG was similar among nonstimulated and baclofen-stimulated cells with or without *S*(+)-ketamine treatment (fig. 6C). *S*(+)-Ketamine treatment alone (100 μM) did not affect the intensity of the immune complex determined with anti-HA (HA-$GABA_{B2}R$) and that determined with anti-FLAG (FLAG-GRK4 and FLAG-GRK5) (data not shown).

Discussion

Previously, it was demonstrated that the desensitization of $GABA_BR$-mediated responses was associated with the formation of protein complexes of the GB_2R subunit with GRK 4 or 5 on the plasma membranes, which may cause signal disconnection from the receptors to downstream transducers, such as G proteins.[7] In the current study, the same desensitization was observed by the second application of baclofen in *Xenopus* oocytes coexpressing heterodimeric $GABA_BR$ and GIRKs in the presence of GRK 4 or 5. We demonstrated that pretreatment of *S*(+)-ketamine significantly suppressed such desensitization. Furthermore, our results showed that the translocation of GRK4-Venus or GRK5-Venus to the plasma membranes after stimulation of baclofen was inhibited by pretreatment of *S*(+)-ketamine in BHK cells. In addition, FRET analysis showed that *S*(+)-ketamine inhibited the protein complex formation of GB_2R-Venus with GRK4-Cerulean or GRK5-Cerulean in the cells. Such an inhibitory effect of protein complex formation by *S*(+)-ketamine was also confirmed by coimmunoprecipitation and Western blot analysis in cells coexpressing HA-GB_2R, $GB_{1a}R$, and FLAG-

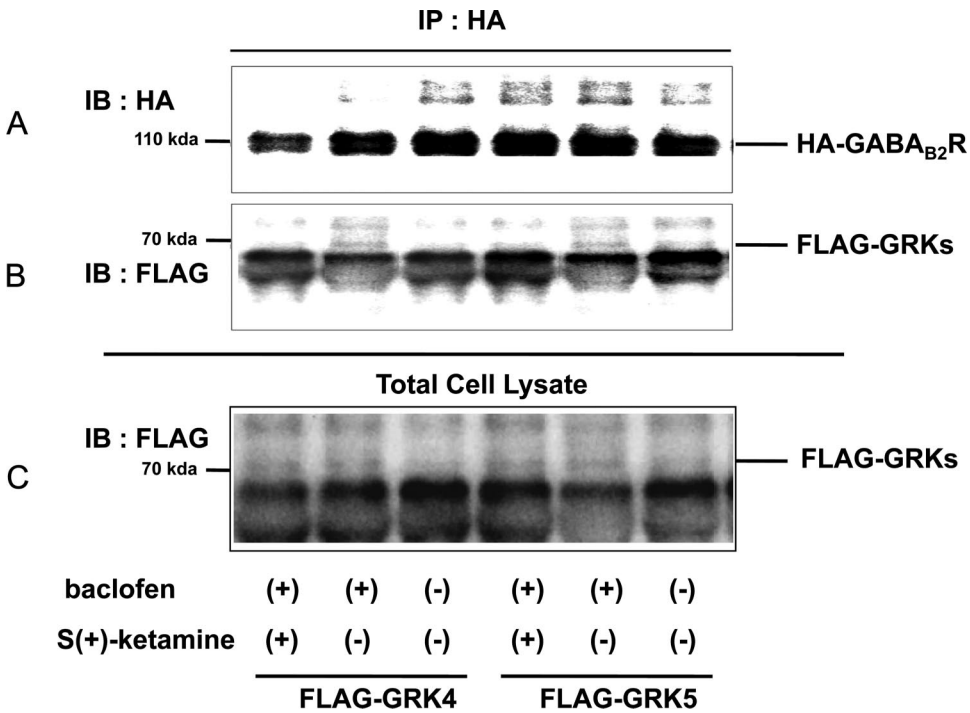

Fig. 6. Immunoprecipitation and Western blot analysis of hemagglutinin (HA)–γ-aminobutyric acid $(GABA)_{B2}$ subunit (GB_2R) and N-DYKDDDDK-C (FLAG)–G protein–coupled receptor (GRK) proteins extracted from nonstimulated cells, baclofen-stimulated cells (100 μM, 5 min), or baclofen-stimulated cells (100 μM, 5 min) with previous stimulation of *S*(+)-ketamine (100 μM, 5 min), coexpressing $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), HA-GB_2R, and FLAG-GRKs. Western blot of anti–HA immunoprecipitates from FLAG-GRK4– or FLAG-GRK5–expressing cells determined with anti-HA (*A*) and anti-FLAG (*B*) and with anti-FLAG in the total lysate (*C*).

GRK4 or FLAG-GRK5. Collectively, these results suggest that *S*(+)-ketamine could suppress the GRK 4– or 5–induced $GABA_BR$ desensitization, at least in part, by interfering with the protein complex formation of GRK 4 or 5 with the GB_2R subunit.

The selective $GABA_BR$ agonist baclofen is widely used as a spasmolytic drug. ITB therapy, proposed by Penn and Kroin[26] in 1984, is a method for the treatment of spasticity and rigidity of spinal and cerebral origin, approved by the Food and Drug Administration in 1992.[1] Recently, it was reported that ITB therapy is also effective in the management of various forms of chronic pain, with or without spasticity.[1–5] There is no doubt that ITB therapy will play a greater part in the management of chronic pain[1]; however, long-term management of ITB therapy has been reported to occasionally result in the development of tolerance to baclofen in both clinical[6] and animal[27] studies. Several reports have shown that intrathecal administration of morphine in place of baclofen for some period (the so-called baclofen holiday)[28] or a shift in treatment to continuous intrathecal morphine administration[29] was effective for pain management in patients who had developed tolerance against ITB therapy. However, the preventive measures for the development of baclofen tolerance have not been established yet.

Baclofen tolerance is the condition in that gradually increased doses of baclofen are required to keep the therapeutic effects stable. Many processes underlie baclofen tolerance *in vivo*, including adaptations in neural circuitry (*e.g.*, descending excitatory pathways) and changes in neurotransmitter signaling pathways surrounding the $GABA_BR$ neuron. In addition, cellular responses mediated by $GABA_BR$ are attributed to the development of baclofen tolerance. In the rat model, ITB down-regulated the number of $GABA_BR$ binding sites in the spinal cord.[30] Desensitization of $GABA_BR$-mediated signaling is one of the mechanisms of development of baclofen tolerance. The desensitization of $GABA_BR$ was induced after protein complex formation of GB_2R with GRK 4 or 5.[7,8] Ketamine is an agent that has widely been used as an analgesic for postoperative pain,[18] chronic noncancer pain,[31] and cancer pain.[32] Although it has been commonly acknowledged that ketamine shows an analgesic effect by blocking the *N*-methyl-D-aspartate receptors in the central nervous system, many other prospective targets are reported (*e.g.*, muscarinic acetylcholine receptors,[33] opioid receptors,[34] substance P receptors,[35] and voltage-dependent Na^+ and K^+ channels).[36] In animal studies, intrathecal[13] or subcutaneous[14] administration of ketamine attenuated the development of tolerance to morphine. The precise mechanisms of such phenomena were not understood; however, tolerance of opioids to μ-opioid receptors could be attributed by receptor desensitization, in which GRKs 2 and 3 were involved.[15–17] One possibility is that ketamine would inhibit μ-opioid receptor–mediated desensitization by modulation of GRK 2 or 3. Likewise, we expected, and suggested, that *S*(+)-ketamine would attenuate the development of tolerance to baclofen to the sites where GRK 4 or 5 is involved in $GABA_BR$-mediated desensitization.[7,8] It is not known how *S*(+)-ketamine interferes the baclofen-induced protein complex formation of GB_2R with GRK 4 or 5. Because there are no *N*-methyl-D-aspartate, muscarinic, opioid, substance P receptors, and no voltage-dependent Na^+ and K^+ channels, expressed in our experimental system, we could say that we find another intracellular target site for ketamine that is independent of the previously reported receptors and ion channel modulation. Taken together, we showed, for the first time to our knowledge, that desensitization of $GABA_BR$-mediated signaling was significantly attenuated by pretreatment of *S*(+)-ketamine, suggesting that S(+)-ketamine suppresses baclofen-induced $GABA_BR$ desensitization, possibly followed by greater antinociceptive effects when used in ITB therapy for long-term pain management.

Clinically, our results propose the possibility that combination intrathecal administration of *S*(+)-ketamine with ITB therapy provides high-quality pain relief without tolerance of ITB to patients experiencing chronic pain. Intrathecal ketamine has been administered in an animal model and to humans, but the safety of preservative-free ketamine through the intrathecal route remains controversial.[37–40] Although some reports have shown no neurotoxic damage after intrathecal administration of preservative-free ketamine using pig[37] and rabbit[38] models, recent animal studies have shown the severe neurotoxicity of intrathecal administration of ketamine with canine[39] and rabbit.[40] Pathologic findings also demonstrated subpial spinal cord vacuolar myelopathy after intrathecal ketamine in a terminally ill cancer patient who received continuous-infusion intrathecal ketamine for 3 weeks.[41] Furthermore, the continuous intrathecal administration of *S*(+)-ketamine, in combination with morphine, bupivacaine, and clonidine, resulted in adequate pain relief in a patient experiencing intractable neuropathic cancer pain; however, postmortem observation of the spinal cord and nerve roots revealed severe histologic abnormalities, including central chromatolysis, nerve cell shrinkage, neuronophagia, microglial up-regulation, and gliosis.[42] A recent report[43] indicates that the neurotoxicity of *S*(+)-ketamine is produced by blockade of *N*-methyl-D-aspartate receptors on the inhibitory neurons, resulting in an exicitotoxic injury through hyperactivation of muscarinic M_3 receptors and non–*N*-methyl-D-aspartate glutamate receptors in the cerebral cortex. Yaksh *et al.*[39] recently reported the detailed toxicology profile of an *N*-methyl-D-aspartate antagonist, including ketamine, delivered through long-term (28-day) intrathecal infusion in the canine model and suggested needs for reevaluation of the use of these agents in long-term spinal delivery. Clinical and pathologic results from an animal or clinical study with intrathecal administration of a combination of baclofen and ketamine have not been reported. Thus, carefully designed studies with an animal model and a clinical trial should be required to know how ketamine (*i.e.*, timing of administration, concentration, duration of adminis-

tration, and ratio of doses of ketamine and baclofen) is safely administered without pathophysiologic findings and how it might suppress the development of baclofen-induced tolerance clinically.

In conclusion, we demonstrated that *S*(+)-ketamine suppressed the baclofen-induced desensitization of $GABA_BR$-mediated signaling, at least in part, through inhibition of protein complex formation of the GB_2R subunit and GRK 4 or 5. If the safety of intrathecal administration of *S*(+)-ketamine is established, it could be a candidate for preventing the development of tolerance against ITB therapy in long-term spasticity and pain management.

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