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

Yuko Ando, M.D.,* Minoru Hojo, M.D.,† Masato Kanaide, M.D., Ph.D.,‡ Masafumi Takada, M.D., Ph.D.,† Yuka Sudo, B.S.,§ Seiji Shiraishi, M.D., Ph.D.,|| Koji Sumikawa, M.D., Ph.D.,# Yasuhito Uezono, M.D., Ph.D.**

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 (GABAγR) because of the complex formation of the GABAβ2 subunit (GB2R) 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 GABAγR 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 potassium channel 1/2, GABAβ1a receptor subunit, GABAγR, and GRK. Translocation of GRKs 4 and 5 and protein complex formation of GB2R with GRKs were analyzed by confocal microscopy and fluorescence resonance energy transfer analysis in baby hamster kidney cells coexpressing GABAβ1a receptor subunit, fluorescent protein–tagged GB2R, and GRK. The formation of protein complexes of GB2R with GRKs was also determined by coimmunoprecipitation and Western blot analysis.

Results: Desensitization of GABAγR-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 GB2R with the GRKs. Western blot analysis also showed that S(+)-ketamine inhibited the protein complex formation of GB2R with the GRKs.

Conclusion: S(+)-Ketamine suppressed the desensitization of GABAγR-mediated signaling at least in part through inhibition of protein complexes of GB2R with GRK 4 or 5.

What This Article Tells Us That Is New:

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

What We Already Know about This Topic:

• In cell culture, S(+)-ketamine suppressed the desensitization of GABAγR-mediated signaling at least in part through inhibition of formation of protein complexes of GABAβ2 subunit (GB2R) with GRK 4 or 5.

BACLOFEN, a selective γ-aminobutyric acid type B receptor (GABAγR) agonist, has been widely used as an antispasticity agent. Intrathecal baclofen (ITB) therapy is an established treatment for severe spasticity of both spinal and
cerebral origin. 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<sub>2</sub>B<sub>R</sub>.7,8 In addition, the desensitization of GABA<sub>2</sub>B<sub>R</sub> occurred by the formation of complexes of GABA<sub>2</sub>B<sub>R</sub> and either G protein–coupled receptor kinase (GRK) 4 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 angesitics) 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 acts, is known to be mediated by GRKs, particularly GRK 2 or 3.15,16,17 GRKs 2 and 3 are reported to play in desensitization processes of μ-opioid receptors5,17 or development of tolerance to opioids in an animal model.16 In case of GABA<sub>2</sub>B<sub>R</sub>, it was previously demonstrated that the desensitization of GABA<sub>2</sub>B<sub>R</sub>–mediated responses was associated with the formation of protein complexes of GABA<sub>2</sub>B<sub>R</sub> receptor subunit (GB<sub>B</sub>1<sub>R</sub>) 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<sub>2</sub>B<sub>R</sub>–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 the 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<sub>2</sub>B<sub>R</sub> desensitization and the formation of complexes of GABA<sub>2</sub>B<sub>R</sub> 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<sup>+</sup> channel (GIRK) 1 and mouse GIRK2 were provided by Henry A. Lester, Ph.D. (Professor of Biology, Caltech, Pasadena, CA). GABA<sub>1</sub>B<sub>R</sub>, receptor subunit (GB<sub>B</sub>1<sub>R</sub>), GB<sub>B</sub>2<sub>R</sub>, and anti-hemagglutinin (HA)–tagged GB<sub>B</sub>1<sub>R</sub> 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-DYKDPPPDK-C (FLAG) epitope tag (5′-GAACAAAAACTCATCTCAGAAGGATGTG-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<sub>B</sub>1<sub>R</sub> was created by ligating the receptor cDNA into HindIII 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 NorI or BamHI 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<sub>2</sub>, 1-mM MgCl<sub>2</sub>, 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<sub>B</sub>1<sub>R</sub> and GB<sub>B</sub>2<sub>R</sub> (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 tocicidity was adjusted to reduce concentrations of NaCl (48 mM NaCl, 49 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 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 × 10⁵ 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 the presence of the acceptor, and FRET was measured by imaging Cerulean 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.23

Before and after this bleaching, Cerulean images were collected to assess changes in donor fluorescence.

Coloinmunoprecipitation 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 GB₄R (HA-GB₄R), and nontagged GB₄R 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 re-acted with chemiluminescence Western blot detection reagents (Nacalai Tesque).
Results

S(+)—Ketamine Inhibits the Desensitization of GABA<sub>B</sub>R 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<sub>B</sub>R (G<sub>B1a</sub>R and HA-tagged G<sub>B2</sub>R [HA-GB2R]) with GRKs 1 and 2 (GIRK1/2). In addition, GABA<sub>B</sub>R desensitization was observed after repeated application of baclofen at 100 μM, which was a submaximum concentration to elicit inward K<sup>+</sup> current through GIRK1/2 to oocytes, coexpressing GRK 4 or 5 but not 2, 3, or 6.

As previously demonstrated, no desensitization was observed after repeated application of baclofen at 100 μM (for 1 min, each application) to oocytes coexpressing the G<sub>B1a</sub>R and HA-GB2R with GIRK1/2 (fig. 1, A and B). When either GRK 4 (3 ng) or 5 (3 ng) cRNA was coinjected with heterodimeric GABA<sub>B</sub>R and GIRK1/2 cRNA, the amplitude of first baclofen-induced K<sup>+</sup> currents was almost the same as that in oocytes expressing GABA<sub>B</sub>R and GIRK1/2 without GRKs, whereas that of the second K<sup>+</sup> 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<sub>B</sub>R desensitization (fig. 1, A and B). S(+)—Ketamine (100–300 μM) by itself had no effects on both the 49-mM K<sup>+</sup>- and baclofen-induced K<sup>+</sup> currents in oocytes expressing GABA<sub>B</sub>R 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<sub>B</sub>R and GIRK1/2 with GRK 4 or 5, the attenuation of the second baclofen-induced K<sup>+</sup> currents was...
significantly restored in a concentration-dependent manner (fig. 1, A and B). The amplitude of K\textsuperscript{+} currents induced by the second application of baclofen with 10-, 30-, or 100 M S(-)-ketamine was 48.3 ± 8.4%, 67.9 ± 17.4%, and 104.8 ± 22.7% in oocytes coexpressing GRK4 (n = 10 each) 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 GB1aR, HA-GB2R, and GRK4-Venus. 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\textsubscript{B1a}R with GRK4-Venus or GRK5-Venus (data not shown).

**Translocation of Venus-Fused GRK 4 or 5 to the Plasma Membranes after Activation of GABA\textsubscript{B}R 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 GB1aR and HA-GB2R 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 distributed diffusely 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\textsubscript{B1a}R 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\textsubscript{B}R**

Previously, we showed that functional GABA\textsubscript{B}R formed heterodimers with GB1aR and GB2R by analysis with FRET and acceptor photobleaching (Figs. 3 and 4). The results of FRET analysis are consistent with the observed translocation properties of GRK4-Venus or GRK5-Venus in BHK cells coexpressing heterodimeric GABA\textsubscript{B}R with GRK4-Venus or GRK5-Venus (data not shown).
acceptor photobleaching in BHK cells coexpressing GB₁aR-Venus and GB₂R-Cerulean. We also showed that GRK 4 or 5, but not GRK 2, 3, or 6, formed protein complexes with the GB₂R subunit after GABA BR activation in the cells coexpressing Venus-fused GB₁aR or GB₂R and Cerulean-fused GRKs. We examined the effects of (−)-ketamine on the formation of protein complexes of GRK 4 or 5 with GB₂R in BHK cells coexpressing GB₁aR, GB₂R-Venus, and GRK4-Cerulean (fig. 3A) or GRK5-Cerulean (fig. 3B). The fluorescence from GB₂R-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-Cerulean or GRK5-Cerulean and GB₂R-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 (−)-ketamine on the protein complex formation of GRK4-Cerulean or GRK5-Cerulean with GB₂R-Venus plus GB₁aR, we applied (−)-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 (−)-ketamine. The fluorescence from GRK4-Cerulean or GRK5-Cerulean was detected diffusely in the cytosol and on the plasma membranes, whereas the fluorescence from GB₂R-Venus was mostly detected on the plasma membranes. Acceptor photobleaching demonstrated the reduction of the fluorescence from GB₂R-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. 3.** Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the protein complex formation of the γ-aminobutyric acid (GABA)₂ receptor subunit (GB₂R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing the GABA₁a receptor subunit (GB₁aR), GB₂R-Venus, and GRKs-Cerulean. Each bar represents 10 μm. (A) Visualization of GB₂R-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 GB₂R-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).

Anesthesiology 2011; 114:401–11 Ando et al.

406

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GB2R-Venus do not form baclofen-induced protein complexes in the presence of $S^\dagger$-ketamine.

Coimmunoprecipitation and Western Blot Analysis of GRK 4 or 5 Using BHK Cells Coexpressing FLAG-GRKs, HA-GB2R, and GB1aR

Previously, it was shown that FLAG-GRK 4 or 5, but not GRK 2, 3, or 6, formed protein complexes with HA-GB2R after baclofen stimulation (100 $\mu$M, 5 min) in BHK cells determined with coimmunoprecipitation and Western blot analysis. We investigated whether $S^\dagger$-ketamine has an effect on the protein complex formation of GRK 4 or 5 with GB2R induced by baclofen. Western blot analysis was performed with proteins extracted from BHK cells coexpressing FLAG-GRK4 or FLAG-GRK5, GB1aR, and HA-GB2R after immunoprecipitation with anti-HA. In the precipitate using anti-HA from the BHK cells coexpressing FLAG-GRK4 or FLAG-GRK5, GB1aR, and GB1aR, the band intensity of the immune complex determined with anti-HA was similar in nonstimulated and baclofen-stimulated (100 $\mu$M, 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 $S^\dagger$-ketamine on the protein complex formation of FLAG-GRK4 or FLAG-GRK5 with GB2R, we treated $S^\dagger$-ketamine (100 $\mu$M) to the cells coexpressing FLAG-GRK4 or FLAG-GRK5, HA-GB2R, and GB1aR 5 min before and during the stimulation of baclofen (5 min, 100 $\mu$M). In the precipitate using anti-HA from the cells coexpressing either FLAG-GRK4 or FLAG-GRK5 with HA-GB2R and GB1aR, the intensity of the immune complex with anti-HA was similar among nonstimulated and baclofen-stimulated cells with or without $S^\dagger$-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 $S^\dagger$-ketamine treatment than in baclofen-stimulated cells without $S^\dagger$-ketamine treatment; and the intensity in baclofen-stimulated cells with $S^\dagger$-ketamine treatment.

Fig. 4. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the effects of $S^\dagger$-ketamine on the interaction of $\gamma$-aminobutyric acid (GABA)$B_2$ subunit (GB2R) with G protein–coupled receptor kinase (GRK) in a BHK cell coexpressing GABA$B_2$ receptor subunit (GB2R), GB2R-Venus, and GRKs-Cerulean. Each bar represents 10 $\mu$m. (A) Visualization of GB2R-Venus and GRK4-Cerulean in a BHK cell treated by $S^\dagger$-ketamine (100 $\mu$M) 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 GB2R-Venus and GRK5-Cerulean in a BHK cell pretreated with $S^\dagger$-ketamine (100 $\mu$M) 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).
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<sub>B2</sub>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<sub>B</sub>R-mediated responses was associated with the formation of protein complexes of the G<sub>B2</sub>R 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. In the current study, the same desensitization was observed by the second application of baclofen in *Xenopus* oocytes coexpressing heterodimeric GABA<sub>B</sub>R 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 G<sub>B2</sub>R-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-G<sub>B2</sub>R, G<sub>B1a</sub>R, and FLAG-
GRK4 or FLAG-GRK5. Collectively, these results suggest that S(+)-ketamine could suppress the GRK 4– or 5–induced GABABR desensitization, at least in part, by interfering with the protein complex formation of GRK 4 or 5 with the GB2R subunit.

The selective GABABR agonist baclofen is widely used as a spasmylytic drug. ITB therapy, proposed by Penn and Kroin 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. Recently, it was reported that ITB therapy is also effective in the management of various forms of chronic pain, with or without spasticity. There is no doubt that ITB therapy will play a greater part in the management of chronic pain; however, long-term management of ITB therapy has been reported to occasionally result in the development of tolerance to baclofen in both clinical and animal studies. Several reports have shown that intrathecal administration of morphine in place of baclofen for some period (the so-called baclofen holiday) or a shift in treatment to continuous intrathecal morphine administration 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 GABABR neuron. In addition, cellular responses mediated by GABABR are attributed to the development of baclofen tolerance. In the rat model, ITB down-regulated the number of GABABR binding sites in the spinal cord. Desensitization of GABABR-mediated signaling is one of the mechanisms of development of baclofen tolerance. The desensitization of GABABR was induced after protein complex formation of GB2R with GRK 4 or 5. Ketamine is an agent that has widely been used as an analgesic for postoperative pain, chronic non-cancer pain, and cancer pain. 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, opioid receptors, substance P receptors, and voltage-dependent Na+ and K+ channels). In animal studies, intrathecal 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. 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 GABABR-mediated desensitization. It is not known how S(+)-ketamine interferes the baclofen-induced protein complex formation of GB2R 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 GABABR-mediated signaling was significantly attenuated by pretreatment of S(+)-ketamine, suggesting that S(+)-ketamine suppresses baclofen-induced GABABR 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. Although some reports have shown no neurotoxic damage after intrathecal administration of preservative-free ketamine using pig and rabbit models, recent animal studies have shown the severe neurotoxicity of intrathecal administration of ketamine with canine and rabbit. 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. 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. A recent report indicates that the neurotoxicity of S(+)-ketamine is produced by blockade of N-methyl-D-aspartate receptors on the inhibitory neurons, resulting in an excitotoxic injury through hyperactivation of muscarinic M3 receptors and non-N-methyl-D-aspartate glutamate receptors in the cerebral cortex. Recently, 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-
tion, 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\(_B\)R-mediated signaling, at least in part, through inhibition of protein complex formation of the GB\(_R\) 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.

The authors thank Kohtaro Taniyama, M.D., Ph.D., Department of Technology, Nagasaki Institute of Applied Science, Nagasaki, Japan, for their skilled technical assistance.

**References**

17. Kvoor A, Nappcy Y, Kieffler BL, Chavkin C: \(\mu\) And \(\delta\) opioid receptors are differentially desensitized by the coexpression of \(\beta\)-adrenergic receptor kinase 2 and \(\beta\)-arrestin 2 in Xenopus oocytes. J Biol Chem 1997; 272:27605–11
34. Sarton E, Tepperma LJ, Olivier C, Nieuwenhuijs D, Matthes


