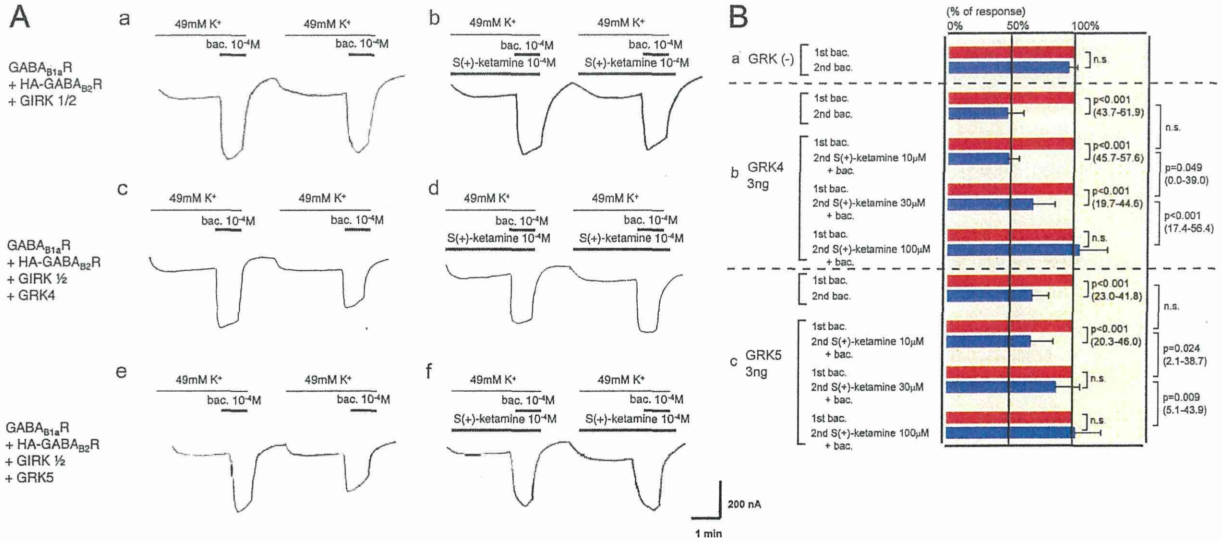

Fig. 1. Effects of *S*(+)-ketamine on the desensitization of γ-aminobutyric acid type B receptor (GABA$_B$R)–mediated G protein–activated inwardly rectifying K$^+$ channel (GIRK) currents in *Xenopus* oocytes. (*A*) Typical tracing of GIRK currents induced by the first and second application of baclofen (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$_2$R), 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$_2$R, 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$_B$R 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$_2$R, and GIRK1/2, n = 8, (*b*) groups coexpressing GB$_{1a}$R, HA-GB$_2$R, GIRK1/2, and GRK 4 (n = 10 for each group), (*c*) groups coexpressing GB$_{1a}$R, HA-GB$_2$R, 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$_2$R, 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$_2$R-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$_B$R (GB$_{1a}$R and HA-tagged GB$_2$R [HA-GB$_2$R]) with GIRKs 1 and 2 (GIRK1/2).[7] In addition, GABA$_B$R 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$_2$R with GIRK1/2 (fig. 1, A and B). When either GRK 4 (3 ng) or 5 (3 ng) cRNA was coinjected with heterodimeric GABA$_B$R and GIRK1/2 cRNA, the amplitude of first baclofen-induced K$^+$ currents was almost the same as that in oocytes coexpressing GABA$_B$R 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$_B$R 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$_B$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$_B$R and GIRK1/2 with GRK 4 or 5, the attenuation of the second baclofen-induced K$^+$ currents was

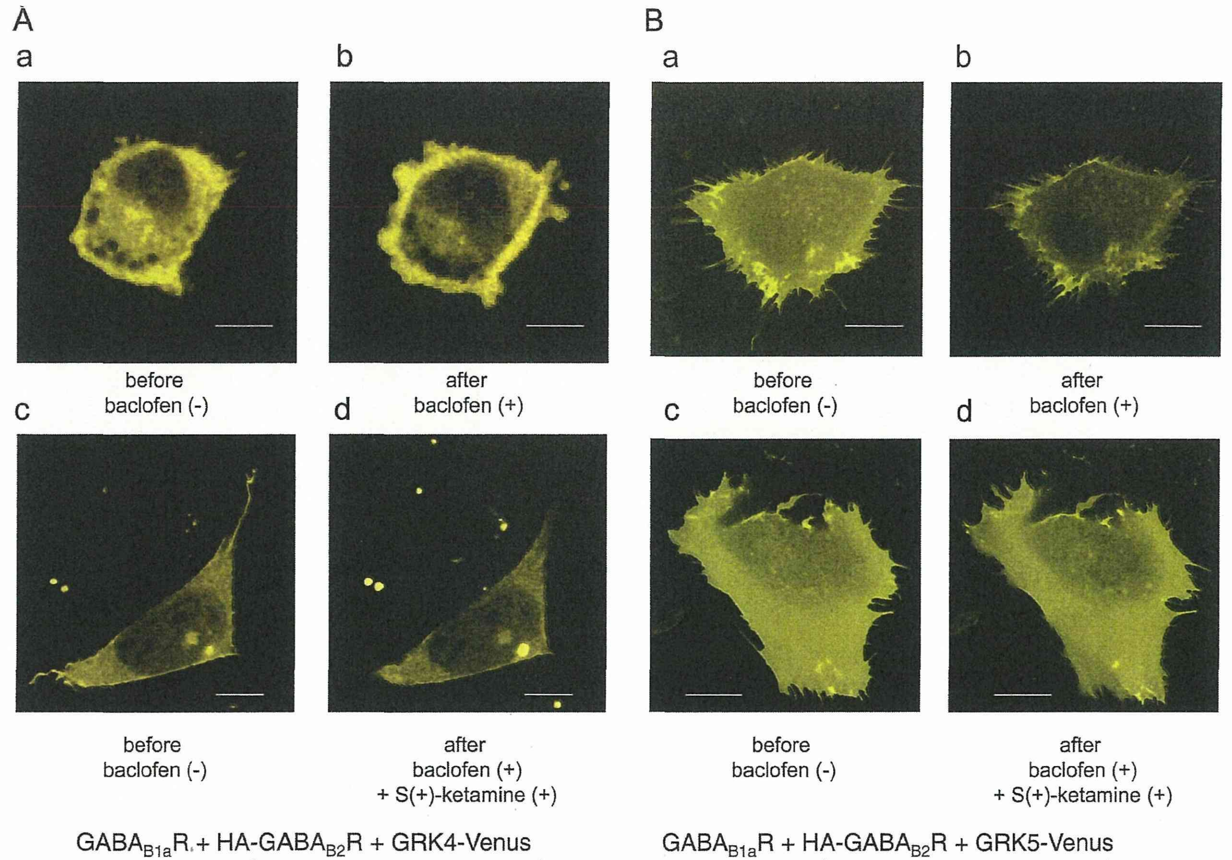

Fig. 2. Confocal imaging showing the effects of *S*(+)-ketamine on the translocation of G protein–coupled receptor kinase (GRK) 4–Venus or GRK5-Venus to the plasma membranes in baby hamster kidney (BHK) cells coexpressing the γ-aminobutyric acid (GABA)$_{B1a}$ receptor subunit (GB$_{1a}$R), hemagglutinin (HA)–GABA$_{B2}$ subunit (GB$_2$R), 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$_2$R, 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$_2$R, 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$_B$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 GB$_{1a}$R and HA-GB$_2$R 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$_B$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$_B$R

Previously, we showed that functional GABA$_B$R formed heterodimers with GB$_{1a}$R and GB$_2$R by analysis with FRET and

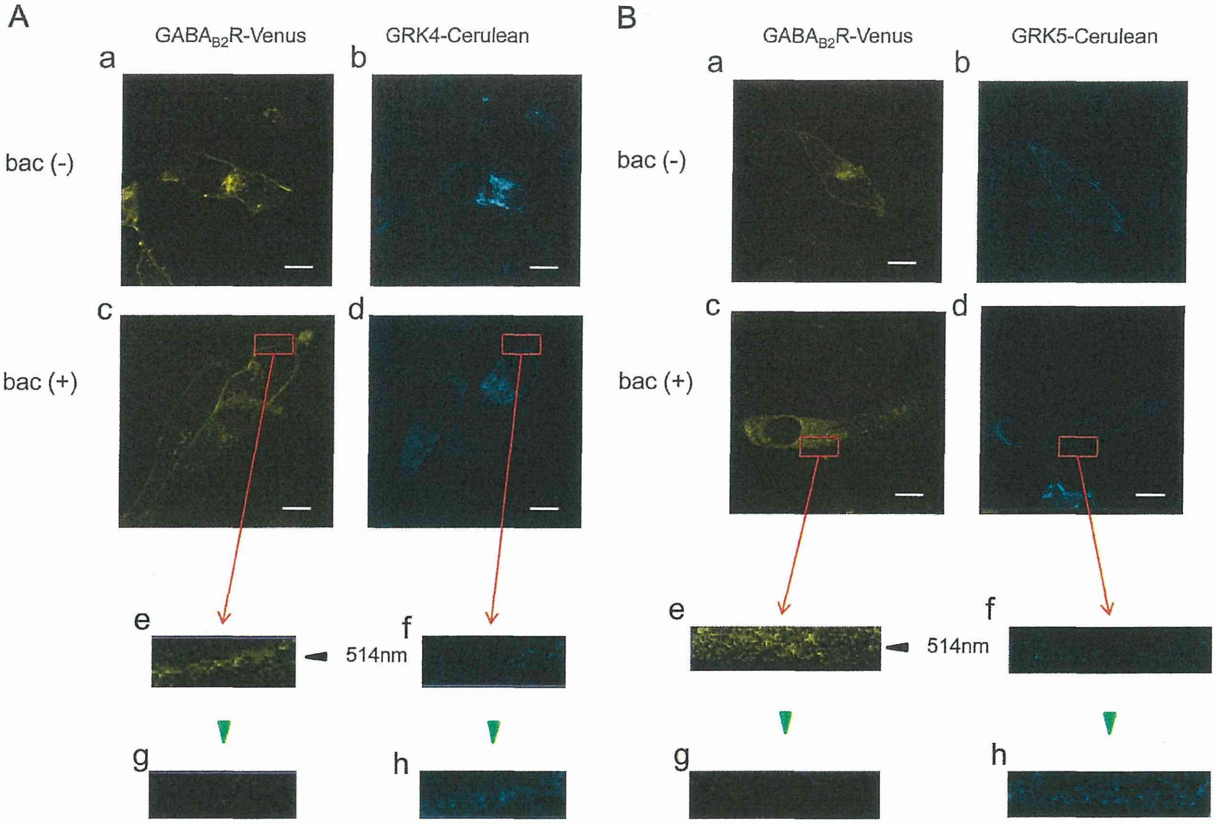

Fig. 3. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the protein complex formation of the γ-aminobutyric acid (GABA)$_{B2}$ subunit (GB$_2$R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing the GABA$_{B1a}$ receptor subunit (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 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$_2$R-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$_2$R-Cerulean.[7,20] We also showed that GRK 4 or 5, but not GRK 2, 3, or 6, formed protein complexes with the GB$_2$R subunit after GABA$_B$R activation in the cells coexpressing Venus-fused GB$_{1a}$R or GB$_2$R 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$_2$R in BHK cells coexpressing GB$_{1a}$R, GB$_2$R-Venus, and GRK4-Cerulean (fig. 3A) or GRK5-Cerulean (fig. 3B). The fluorescence from GB$_2$R-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$_2$R-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$_2$R-Venus was mostly detected on the plasma membranes. Acceptor photobleaching demonstrated the reduction of the fluorescence from GB$_2$R-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

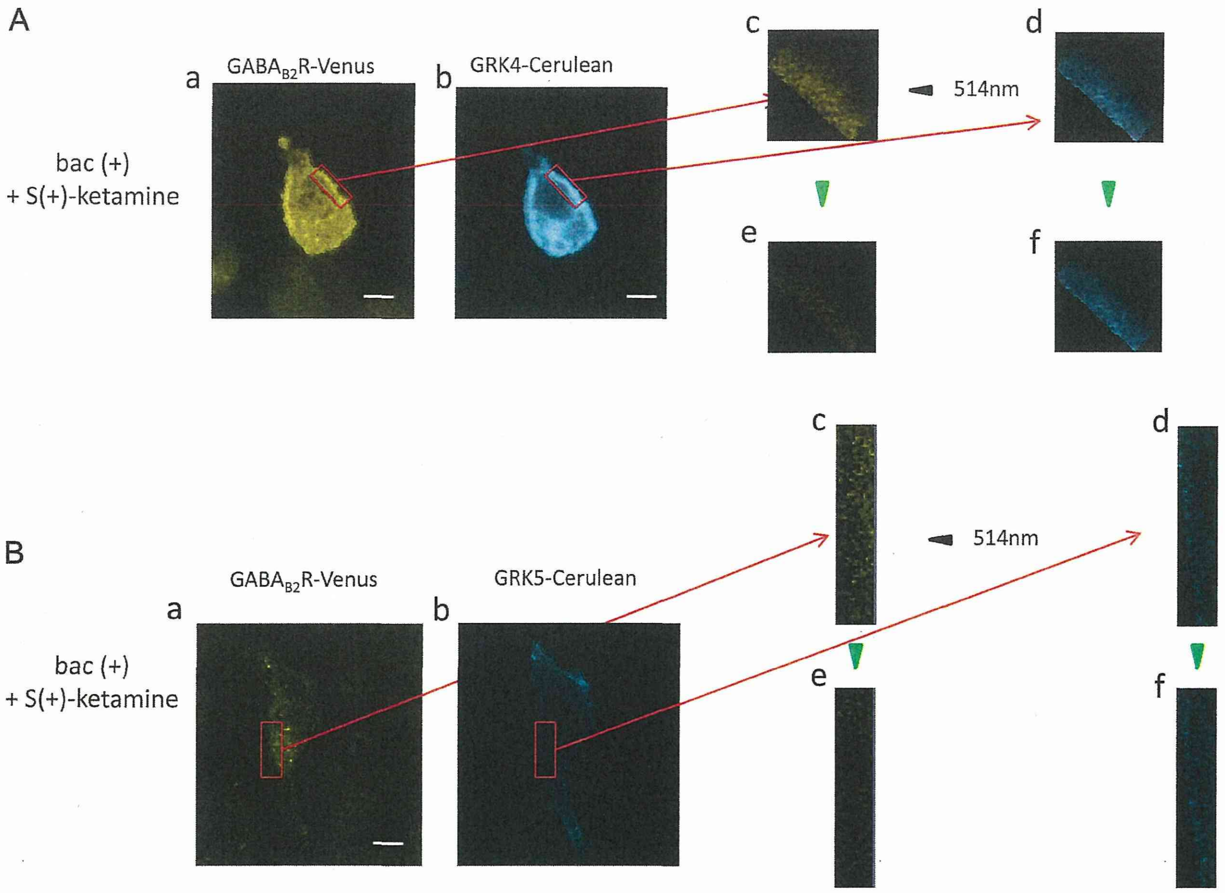

Fig. 4. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the effects of $S(+)$-ketamine on the interaction of γ-aminobutyric acid (GABA)$_{B2}$ subunit (GB$_2$R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing GABA$_{B1a}$ 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

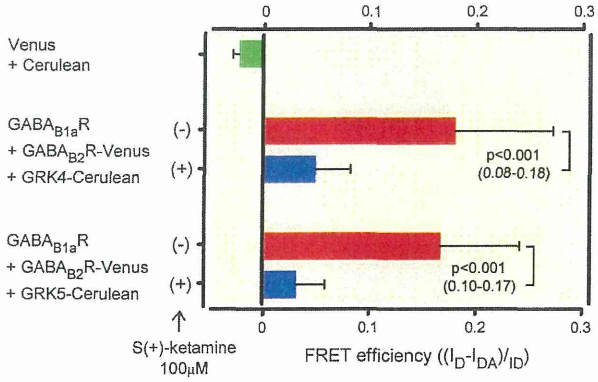

Fig. 5. Comparison of fluorescence resonance energy transfer (FRET) efficiency in baby hamster kidney (BHK) cells expressing γ-aminobutyric acid (GABA)$_{B1a}$ receptor subunit (GB$_{1a}$R), GABA$_{B2}$ subunit (GB$_2$R)–Venus, and G protein–coupled receptor (GRK) 4–Cerulean or GRK5-Cerulean, with or 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$_B$R-mediated responses was associated with the formation of protein complexes of the GB$_2$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.[7] In the current study, the same desensitization was observed by the second application of baclofen in *Xenopus* oocytes coexpressing heterodimeric GABA$_B$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 GB$_2$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-GB$_2$R, GB$_{1a}$R, and FLAG-

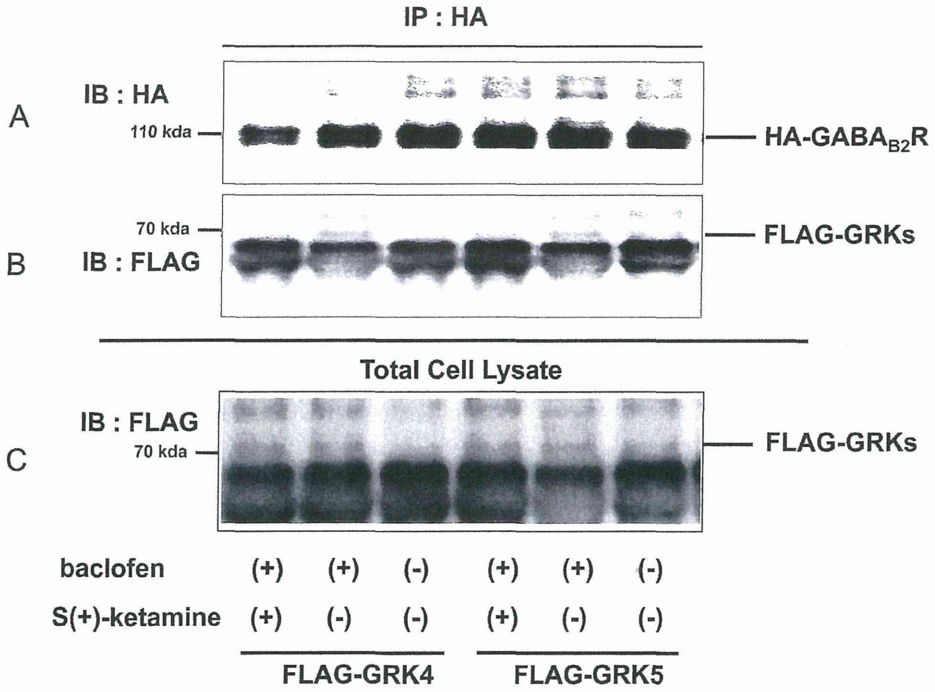

Fig. 6. Immunoprecipitation and Western blot analysis of hemagglutinin (HA)–γ-aminobutyric acid (GABA)$_{B2}$ subunit (GB$_2$R) and N-DYKDDDDK-C (FLAG)–G protein–coupled receptor (GRK) proteins extracted from nonstimulated cells, baclofen-stimulated cells (100 μM, 5 min), or baclofen-stimulated cells (100 μM, 5 min) with previous stimulation of *S*(+)-ketamine (100 μM, 5 min), coexpressing GABA$_{B1a}$ receptor subunit (GB$_{1a}$R), HA-GB$_2$R, 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 administration, 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$_2$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.

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Short Communication

The Tramadol Metabolite *O*-Desmethyl Tramadol Inhibits Substance P–Receptor Functions Expressed in *Xenopus* Oocytes

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Abstract. Tramadol has been widely used as analgesic. *O*-Desmethyl tramadol (ODT) is one of the main metabolites of tramadol, having much greater analgesic potency than tramadol itself. Substance P receptors (SPR) are well known to modulate nociceptive transmission within the spinal cord. In this study, we investigated the effects of ODT on SPR expressed in *Xenopus* oocytes by examining SP-induced Ca^{2+}-activated Cl^- currents. ODT inhibited the SPR-induced Cl^- currents at pharmacologically relevant concentrations. The protein kinase C (PKC) inhibitor bisindolylmaleimide I did not abolish the inhibitory effects of ODT on SP-induced Ca^{2+}-activated Cl^- currents. The results suggest that the tramadol metabolite ODT inhibits the SPR functions, which may be independent of activation of PKC-mediated pathways.

Keywords: *O*-desmethyl tramadol (ODT), tramadol, substance P

Substance P (SP) acts as a neurotransmitter released from C fibers located within nociceptive primary afferent neurons into the spinal cord and mediates a part of the excitatory synaptic input to nociceptive neurons at this level (1). SP and its receptors (SPR) are widely distributed in the central and peripheral nervous systems (2). Several studies showed that pain sensitivity is altered in mice lacking the gene encoding SPR; a reduction in nociceptive responses to certain somatic and visceral noxious stimuli occurs in SPR knockout mice (3).

SPR belongs to the family of Gq protein–coupled receptors that activate the protein kinase C (PKC) and Ca^{2+}-mobilization by stimulation of phospholipase C. Our recent reports have shown that the function of SPR is inhibited by volatile anesthetics and intravenous anesthetics. Halothane, isoflurane, enflurane, diethyl ether, and ethanol inhibit the function of SPR (4). Moreover, ketamine and pentobarbital inhibited the SPR-induced currents at pharmacologically relevant concentrations, whereas propofol had little effect on the currents in *Xenopus* oocytes expressing SPR (5). These results suggest that SPR is one of the targets of some anesthetics.

O-Desmethyl tramadol (ODT) is one of the metabolites of analgesic, tramadol. Only ODT among these metabolites has been shown to have analgesic activity in mice and rats, as assessed by the tail-flick responses. Analgesic potency of ODT is 2 – 4-times higher than that of tramadol (1, 3). In addition, ODT has more affinity for the μ-opioid receptor than does tramadol in biochemical receptor binding studies, although its chemical structure is quite similar to tramadol (1). There have been several reports suggesting that ODT, at pharmacologically relevant concentrations, inhibited 5-HT–evoked Ca^{2+}-activated Cl^- currents in oocytes expressing $5\text{-}HT_{2C}R$, and inhibited the functions of NMDA receptors, but not those of glycine and $GABA_A$ receptors (6). We have previously reported in *Xenopus* oocytes expressing SPR that tramadol had little effect on the SP-induced Ca^{2+}-actvivated Cl^- currents (5). However, a recent report has shown that tramadol, given intraperitoneally or intravenously, produced significant inhibition of the biting behavior induced by intrathecal injection of SP (7). We have previously reported the different effects on the Gq-coupled muscarinic M_3 receptors (M_3R) between ODT and tramadol: tramadol inhibited acetylcholine (ACh)-induced currents in oocytes expressing M_3R, whereas ODT did not. In the report we suggest that ODT does not affect the M_3R-mediated signaling in spite of having only a small difference in its structure compared with that of tramadol (8). Collectively these data suggest that inhibitory effects of

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tramadol on SP-induced biting behavior could be due to ODT, although the effects of ODT on SPR functions have not been studied in detail.

The *Xenopus* oocyte expression system has been used to study a multiplicity of receptors including Gq-coupled receptors (5). Stimulation of SPR results in activation of phospholipase C–mediated Ca^{2+}-activated Cl$^-$ currents in *Xenopus* oocytes (4, 5). In the present study we examined the effects of the ODT on the SP-induced Ca^{2+}-activated Cl$^-$ currents in SPR-expressing *Xenopus* oocytes.

Adult *Xenopus laevis* female frogs were purchased from Seac Yoshitomi (Yoshitomi, Fukuoka). SP was from Sigma (St. Louis, MO, USA). ODT hydrochloride was a kind gift from Nippon Shinyaku (Kyoto). Bisindolylmaleimide I (GF109203X) was from Calbiochem (La Jolla, CA, USA). The Ultracomp *E. coli* Transformation Kit was from Invitrogen (San Diego, CA, USA). A Qiagen (Chatsworth, CA, USA) Kit was used to purify plasmid cDNA. Rat SPR cDNA was kindly provided by Dr. J.E. Krause (Washington University School of Medicine, St. Louis, MO, USA). The cDNA for the SPR was inserted into the pBlueScriptIISK(−) vector and linearized with *Xba*I. The SPR synthetic RNA was prepared by using a mCAP mRNA Capping Kit and transcribed with a T7 RNA Polymerase in vitro Transcription Kit (Stratagene, La Jolla, CA, USA).

Isolation and microinjection of *Xenopus* oocytes were performed as described by Sanna et al. (9). Briefly, *Xenopus* oocytes were injected with 50 ng of synthetic RNA encoding SPR and incubated for 2 days. Oocytes were placed in a 100-μl recording chamber and perfused with modified Barth's saline (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, and 0.91 mM $CaCl_2$ (pH 7.5) at a rate of 1.8 ml/min at room temperature. Recording and clamping electrodes (1 – 5 MΩ) were pulled from 1.2-mm outside diameter capillary tubing and filled with 3 M KCl. A recording electrode was imbedded in the animal's pole, and once the resting membrane potential stabilized, a clamping electrode was inserted and the resting membrane potential was allowed to restabilize. A Warner OC 725-B oocyte clamp (Hampden, CT, USA) was used to voltage-clamp each oocyte at −70 mV. We analyzed the peak of the transient inward current component of the SPR-induced currents because this component is dependent on SP concentration and is quite reproducible, as described by Minami et al. (4, 5). The ODT were pre-applied for 2 min to allow for complete equilibration in the bath. The solutions of ODT were freshly prepared immediately before use. The concentrations in the figures represent the bath concentrations.

To determine whether activation of PKC plays a role in ODT modulation of SPR-mediated events, oocytes were exposed to a PKC inhibitor, bisindolylmaleimide I (GF109203X) (200 nM) (10), in MBS for 120 min. We then compared the effects of anesthetics on SP-induced Ca^{2+}-activated Cl$^-$ currents in *Xenopus* oocytes expressing SPR between before and after the exposure to GF109203X.

Results were expressed as a percentage of control responses, due to the variable SPR expression rate in oocytes. The control responses were measured before and after application of each test compound to take into account possible shifts in the control currents as recording preceded. The "n" values refer to the number of oocytes studied. Each experiment was performed with oocytes from at least two different frogs. Statistical analyses were performed using either a *t*-test or a one-way ANOVA (analysis of variance).

The tramadol metabolite ODT inhibited the action of

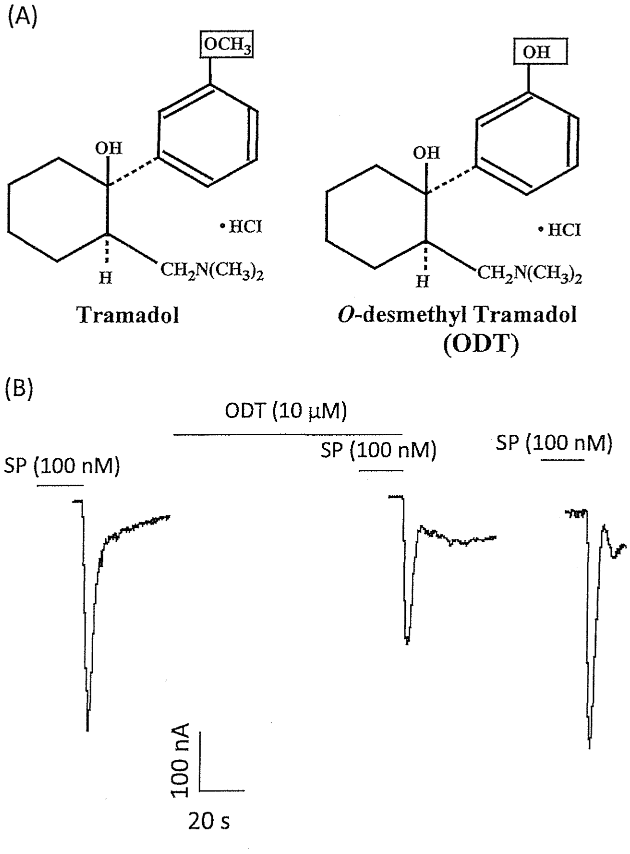

Fig. 1. Effects of *O*-desmethyl tramadol (ODT) on substance P (SP)-stimulated currents in *Xenopus* oocytes expressing SP receptors (SPR). A) Chemical structures of tramadol and *O*-desmethyl tramadol (ODT). B) ODT suppresses the SP-induced Ca^{2+}-activated Cl$^-$ currents in *Xenopus* oocytes expressing SPR. Tracings obtained from a single oocyte expressing SPR show the effect of ODT on currents induced by 100 nM SP. SP was applied for 20 s with or without 2-min ODT treatment.