1　趣旨

がん対策推進基本計画は，がん対策基本法に基づき政府が策定するものであり，具体的には，長期的視点に立ちつつ，平成19(2007)年度から平成23(2011)年度までの5年間を対象として，がん対策の総合的かつ計画的な推進を図るため，がん対策の基本的方向について定めるとともに，都道府県がん対策推進計画の基本となるものである。

今後は，「がん患者を含めた国民が，がんを知り，がんと向き合い，がんに負けることのない社会」の実現を目指すこととする。

2　基本方針

●「がん患者を含めた国民」の視点に立ったがん対策を実施すること。
●全体目標の達成に向け，重点的に取り組むべき課題を定め，分野別施策を総合的かつ計画的に実施すること。

3　重点的に取り組むべき課題

(1)放射線療法及び化学療法の推進並びにこれらを専門的に行う医師等の育成

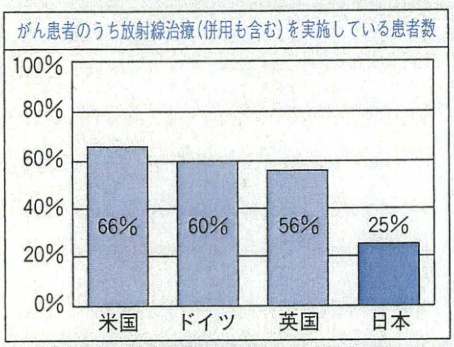
我が国のがん医療については，手術の水準が世界の中でもトップクラスであるのに対して，相対的に放射線療法及び化学療法の提供体制等が不十分であることから，これらの推進を図り，手術，放射線療法及び化学療法を効果的に組み合わせた集学的治療を実現する。

(2)治療の初期段階からの緩和ケアの実施

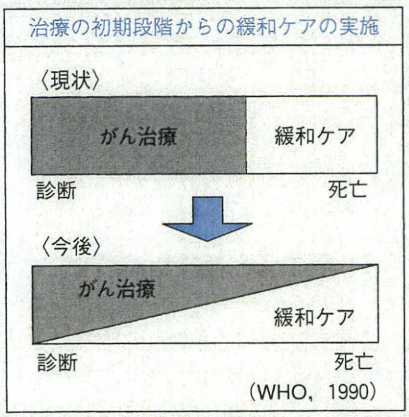
がん患者の多くは，がんと診断された時から身体的な苦痛や精神心理的な苦痛を抱えており，また，その家族も様々な苦痛を抱えていることから，治療の初期段階から緩和ケアが実施されるようにする。

(3)がん登録の推進

がん登録は，がん対策の企画立案や評価に際しての基礎となるデータを把握・提供するために必要不可欠なものであるが，我が国では，諸外国と比較してもその整備が遅れていることから，がん登録を円滑に行うための体制を整備する。



出典)第3回がん対策推進協議会における中川恵一委員(東京大学)からの提出資料をもとに作成



4　全体目標【10年以内】

●がんによる死亡者の減少(75歳未満の年齢調整死亡率の20%減少)
●すべてのがん患者及びその家族の苦痛の軽減並びに療養生活の質の維持向上

図2．がん対策推進基本計画の概要
(厚生労働省ホームページ(http://www.mhlw.go.jp/shingi/2007/06/dl/s0615-1b.pdf)より)

に立ったがん対策を実施すること，②全体目標達成のために重点的に取り組む課題を具体的に定め，最終的に(1)75歳未満のがん年齢調整死亡率の20%減少，(2)すべてのがん患者およびその家族の苦痛を軽減することと療養生活の質(QOL)の維持向上を目指すものです。

さらに，がん対策推進基本計画では重点的に取り組むべき3課題が取り上げられました。

①放射線療法及び化学療法の推進並びにこれらを専門的に行う医師等の育成
②治療の初期段階からの緩和ケアの実施
③がん登録の推進

このなかで，特に②治療の初期段階からの緩和ケアの実施とそれに加えて全体目標であるすべてのがん患者とその家族の苦痛軽減とQOL向上の達成に至る計画を紹介し，5年計画の3年が過ぎた2010年6月15日に発表されたがん対策推進基本計画中間報告書を通して，緩和ケア推進ならびに緩和ケア研究の何が順調に進捗しており何が未達成なのか，そして今後の計画(第二次がん対策推進基本計画)立案にあたっての課題などについて，中間報告書をもとに話を進めていきたいと思います。

がん対策推進基本計画中間報告書

がん対策推進基本計画中間報告書においては，基本計画で掲げた大きな2つの全体目標についての進捗度，3つの重点課題についての進捗度などが報告され，それらの結果に基づいた今後のあり方についての提言が行われています。そのなかで，緩和ケア領域に絞って内容を簡単に説明します。まず，全体目標については，

・75歳未満のがん死亡率については，おおむね順調に減少していること
・がん患者とその家族の苦痛軽減ならびにがん患者のQOL向上については，まずその評価法を確立し，それに基づき評価を行うべきであること，また目標達成には緩和ケアの推進および医療と介護の連携といった生活支援が重要であること

が挙げられました。

また，3つの重点項目のうちの②治療の初期段階からの緩和ケアの実施については，

個別目標①：緩和ケア研修を行うこと
個別目標②：10年以内に緩和ケアの知識，技術を習得した医師を増やすこと
個別目標③：医療用麻薬の消費量の増加を緩和ケアの提供体制の整備状況を計るための参考指標とすること

が挙げられました。

個別目標①，②については，

・10年以内(運用において5年以内と定められた)にすべてのがん診療に関わる医師が研修会などで緩和ケアの基本知識を得るための方策として，緩和ケア研修会の回数と質を確保するための「がん診療に携わる医師に対する緩和ケア研修会の開催指針」が策定され，それに沿った研修会が実施されました。研修会の修了証書を取得した医師は，2010年3月で11,254人にのぼりました。
・2008年3月にがん拠点病院の指定要件を見直し，身体症状緩和に携わる医師，精神症状の緩和に携わる医師および看護師などから構成される緩和ケアチームを整備し，組織上明確にすることが定められました。さらに，外来において専門的な緩和ケアを行うこと，緩和ケア医師と主治医が話し合えるカンファレンスを開催すること，緩和ケア相談窓口を開設すること，緩和ケア研修を毎年行うことが指定要件に位置づけられました。また，2007年に286施設であったがん拠点病院は2010年4月には377施設に達しました。

・緩和ケア研修会の指導者育成を目指した研修会が，独立行政法人国立がん研究センターがん対策情報センターにより行われました。
・診療報酬改定において，がん緩和ケア関連の診療加算項目が増えたこと，さらに緩和ケア研修会修了の医師が配属されていることがその算定条件となりました。
・国立がん研究センターならびに日本緩和医療学会が開催する緩和ケア指導者研修会，精神腫瘍学指導者研修会での2010年5月末での修了者が，それぞれ836人，445人にのぼりました。
・緩和ケアチーム設置医療機関数が，2007年5月で326施設であったものが，2008年10月で612施設に増えました。

個別目標③については，
・わが国でのモルヒネ換算消費量が，2007年は3,835kgであったものが2008年には4,152kgに増えたことが報告されました。

また，緩和ケア推進に連関した在宅医療の推進においては，在宅療養支援診療所数が2008年7月時点で11,450施設に増えたこと，がん患者の在宅での死亡割合が2005年は自宅5.7%，老人ホーム0.5%，介護老人保健施設0.1%であったものが，2008年ではそれぞれ7.3%，0.8%，0.2%と増えたことなどが報告されました。

中間報告を受けての緩和ケア施策の今後と基本計画の見直し

中間報告を受け，そのうちで緩和ケアに関する施策では，改善点，見直しとして，
・5年以内の緩和ケアに関する基本的知識の習得については，さらに研修を増やしていかないと間に合わないため，普及をさらに促すべきである
・緩和ケア研修の質そのものを向上させることが重要である（座学から実地研修へ）
・緩和ケアチーム設置数を増やすとともに，在宅医療における緩和ケアの推進を行うこと。このことにより，病院から地域への切れ目ない緩和ケア体制の確保を行う
・医師に加え，薬剤師，看護師などの育成を行うことが重要である。また，在宅医療では「家での看取り」は最終ではなく，「患者の希望する療養場所」を提供できることが重要であること。その達成のため，地域医療連携体制を整備することが必須である
・地域ごとに在宅医療推進協議会を置き，在宅医療に関する提供者など，関係者と当事者が集まり，地域におけるネットワークを構築することが重要である
・訪問看護ステーションの充実
・がん患者の療養生活の向上という点から，がん患者の就労支援に関する取り組みを行うべきである

などが挙げられました。

中間報告提言を受けての今後のがん研究について

がん対策推進基本計画のなかで謳われているがん研究は，「がんによる死亡者の減少，すべてのがん患者およびその家族の苦痛の軽減ならびにQOLの維持向上を実現するためのがん対策に資する研究」を推進することを目標として掲げました。

今後の研究推進については，以下のような指摘がなされました。
・がん研究については予算がつけられ，基礎研究は進んでいるが，その結果ががん医療開発，革新的がん予防の確立につながっていない。したがって，研究予算の充実に加え，研究進展，発展についてわかりやすい評価指標を示すべきである。
・がん研究をさらに推進するために，がん対策推進協議会とリンクしたがん研究特化国家戦略的

調整機能が不可欠である。
- 国内のがん研究全体を把握し，それに応じて国家レベルのがん研究戦略を立案できるシステムが必要である。
- がん研究推進のため，基礎研究の成果をシーズとしてがん医療のイノベーションを起こせる基礎→臨床の橋渡し機能を強化することが必要である。
- がん研究の成果発表にがん患者を含む国民が参加する機会を設け，がん医療推進に患者，市民の視点を取り入れることが重要である。

おわりに

がん対策を総合的に推進するためには，関係者の連携・協力を進めることのできる体制整備や，都道府県による計画策定を充実させること，またがん対策関係者の意見を集約し把握できる体制を整備すること，さらにがん登録などにおける国民の協力も不可欠であることが提言されています。加えて，緩和ケアにおいては特に，緩和ケア自身をしっかりと評価できる系を策定すること，そしてその進捗管理を行うことが必須であると提言されています。

中間報告で挙げられた未達成の部分，重要と思われる新たな施策提言への対応，「取り組むべき課題」の推進など，修正し行うべきことはたくさんあります。これらのことを勘案しながら，2012年から始まる「第二次がん対策推進基本計画」を実効あるものにし，国民のがんからの解放を目指してさらに注意深く，積極的に計画を進めていく必要があると考えます。

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).

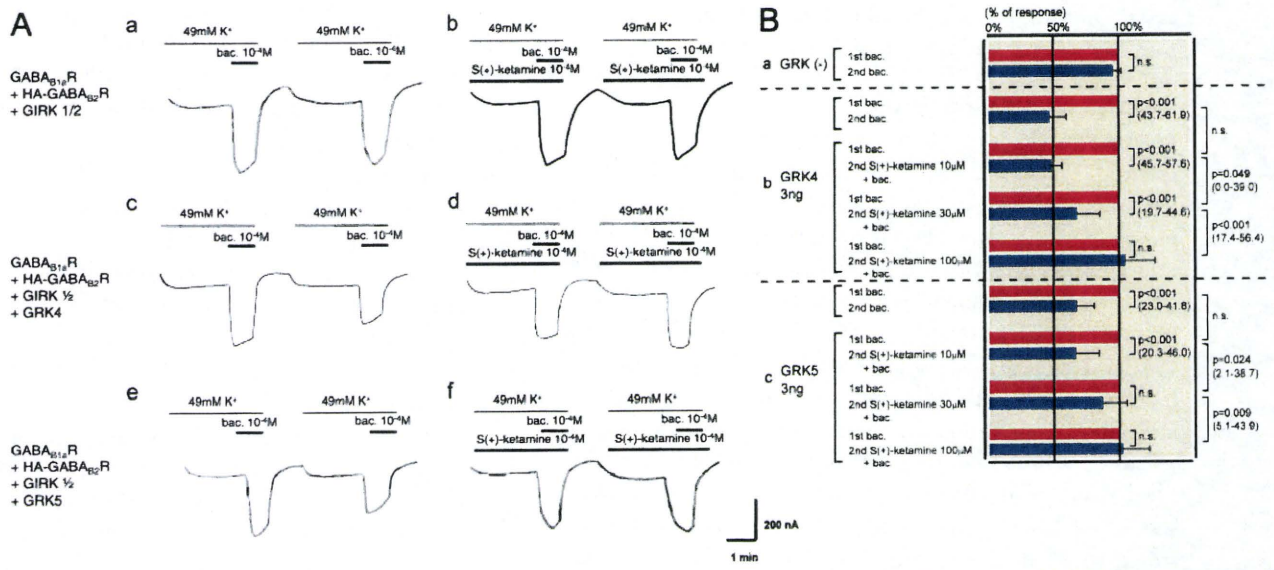

Fig. 1. Effects of *S*(+)-ketamine on the desensitization of γ-aminobutyric acid type B receptor ($GABA_BR$)–mediated G protein–activated inwardly rectifying K^+ channel (GIRK) currents in *Xenopus* oocytes. (*A*) Typical tracing of GIRK currents induced by the first and second application of baclofen (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

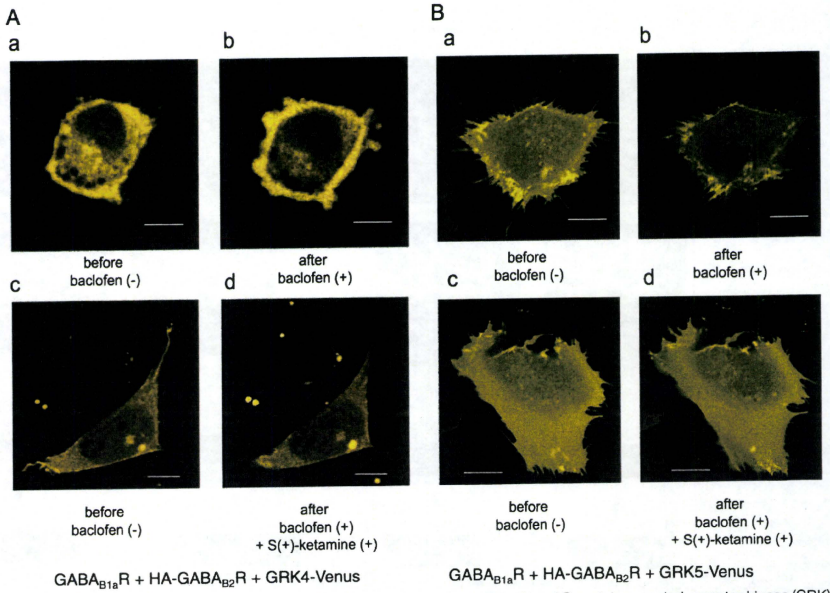

Fig. 2. Confocal imaging showing the effects of *S*(+)-ketamine on the translocation of G protein–coupled receptor kinase (GRK) 4–Venus or GRK5-Venus to the plasma membranes in baby hamster kidney (BHK) cells coexpressing the γ-aminobutyric acid ($GABA)_{B1a}$ receptor subunit ($GB_{1a}R$), hemagglutinin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

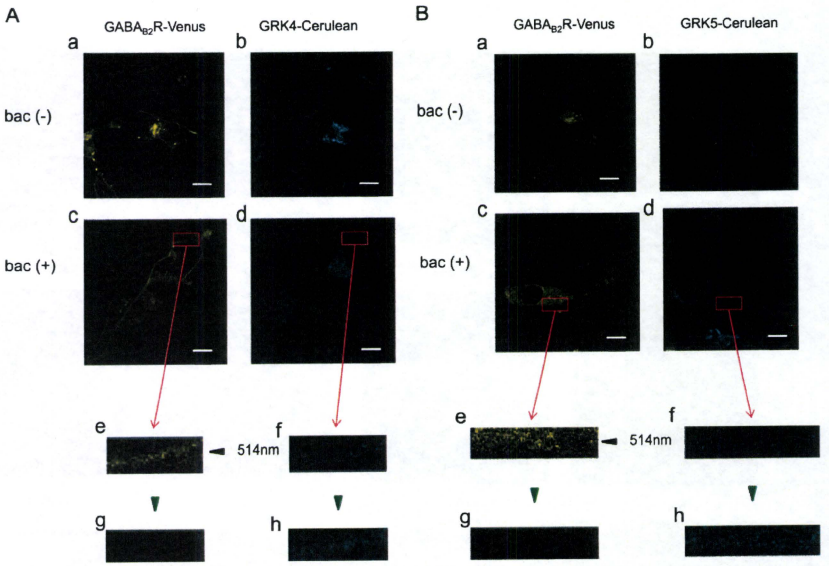

Fig. 3. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the protein complex formation of the γ-aminobutyric acid $(GABA)_{B2}$ subunit (GB_2R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing the $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), 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 co-expressing 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

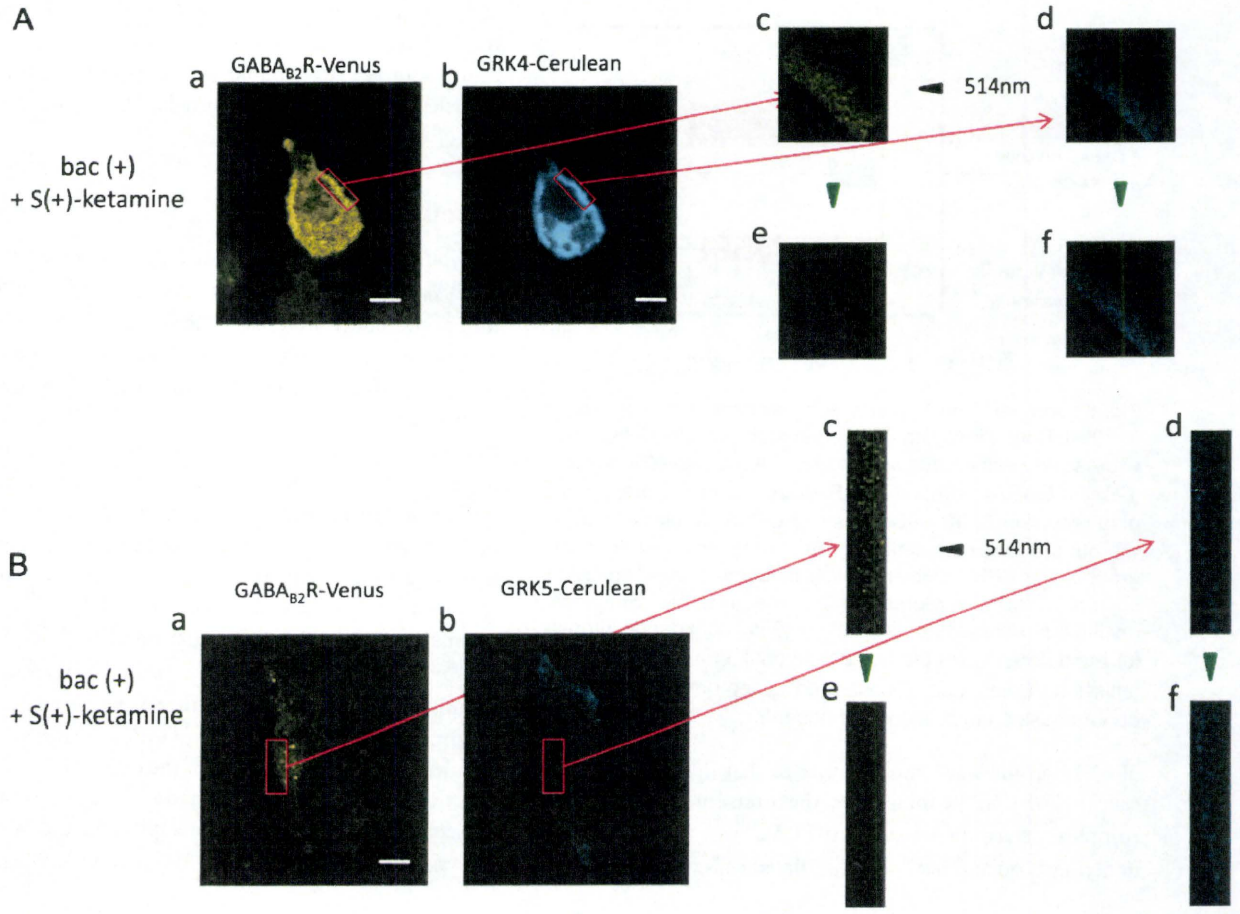

Fig. 4. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the effects of *S*(+)-ketamine on the interaction of γ-aminobutyric acid $(GABA)_{B2}$ subunit (GB_2R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing $GABA_{B1a}$ 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 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_2R-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_2R-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_2R, 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_2R 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_2R 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_2R after immunoprecipitation with anti-HA. In the precipitate using anti-HA from the BHK cells coexpressing FLAG-GRK4 or FLAG-GRK5, HA-GB_2R, 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_2R, we treated *S*(+)-ketamine (100 μM) to the cells coexpressing FLAG-GRK4 or FLAG-GRK5, HA-GB_2R, 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_2R 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

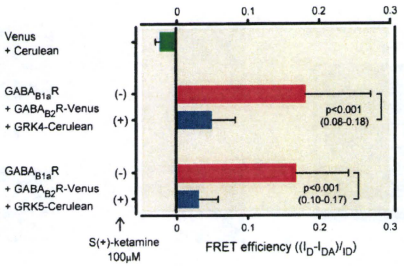

Fig. 5. Comparison of fluorescence resonance energy transfer (FRET) efficiency in baby hamster kidney (BHK) cells expressing γ-aminobutyric acid $(GABA)_{B1a}$ receptor subunit ($GB_{1a}R$), $GABA_{B2}$ subunit (GB_2R)–Venus, and G protein–coupled receptor (GRK) 4–Cerulean or GRK5-Cerulean, with or without 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-

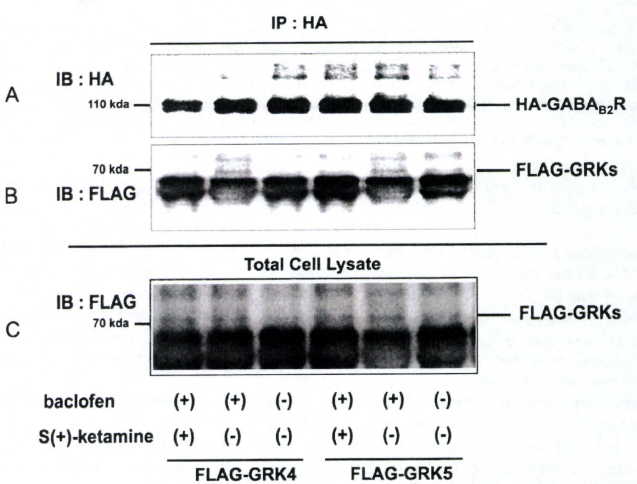

Fig. 6. Immunoprecipitation and Western blot analysis of hemagglutinin (HA)–γ-aminobutyric acid $(GABA)_{B2}$ subunit (GB_2R) and N-DYKDDDDK-C (FLAG)–G protein–coupled receptor (GRK) proteins extracted from nonstimulated cells, baclofen-stimulated cells (100 μM, 5 min), or baclofen-stimulated cells (100 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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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-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

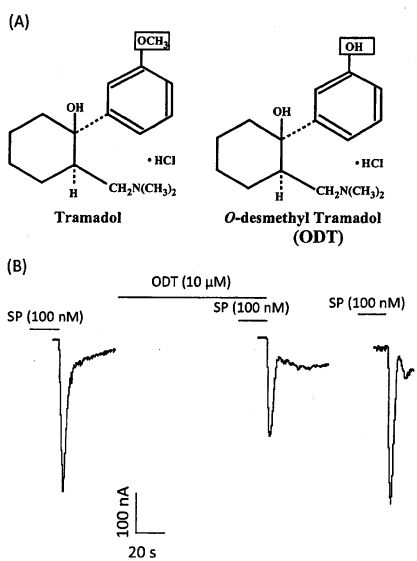

Fig. 1. Effects of *O*-desmethyl tramadol (ODT) on substance P (SP)-stimulated currents in *Xenopus* oocytes expressing SP receptors (SPR). A) Chemical structures of tramadol and *O*-desmethyl tramadol (ODT). B) ODT suppresses the SP-induced Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes expressing 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.

100 nM SP to 71.0 ± 12.3%, 73.6 ± 9.2%, and 56.7 ± 8.6% of the control at concentrations of 0.1, 1, and 10 μM, respectively (Figs. 1 and 2). After washout of the ODT, the size of SPR-induced currents was reversed to almost the same as the control levels.

We previously reported that treatment with the PKC inhibitor GF109203X (200 nM), which has a Ki value of 20 nM for the inhibition of PKC activity (10), produced the enhancement of the initial Cl^- currents activated by 100 nM SP (4, 5). The control currents before ODT treatment was 35.1 ± 27.6 nA. GF109203X enhanced the currents to 398 ± 86% of the control currents (119 ± 79.6 nA), which was similar to our previous report. The inhibitory effects of ODT on SP-induced currents were observed in the oocytes pretreated with GF109203X (Fig. 3). ODT (10 μM) inhibited the action of 100 nM SP to 52.0 ± 9.7%, while treatment of GF109203X resulted in the action of ODT (10 μM) to 45.9 ± 14.6% of control (Fig. 3), although the effect was not significantly different.

Tramadol undergoes biotransformation in the liver by two metabolic pathways to form five *N*- or *O*-desmethylated metabolites. ODT is one of the five main metabolites of tramadol; and the others are mono-*N*-desmethyl tramadol, di-*N*-desmethyl tramadol, tri-*N*,*O*-desmethyl tramadol, and di-*N*,*O*-desmethyl tramadol. We have previously reported that tramadol had little effect on SPR function (5). In another paper, we reported that a low concentration (under 0.1 μM) of ODT did not suppress SP-induced currents in oocytes expressing the SPR (11). Grond et al. (12) reported the mean ODT concentrations after a 200 mg bolus IV infusion of tramadol and those after patient controlled analgesia with demand doses of 20 mg for 24 h in 92 patients. In our study, the mean concentration of ODT was 84.0 ± 34 ng/mL (approximately 0.3 μM). Sindrup et al. (13) also reported mean ODT concentrations of 5.0 – 122 ng/mL (maximally 0.4 μM) in patients who received 200 – 400 mg of tramadol. In the present study, 0.1 μM and higher concentrations of ODT actually inhibited SP-induced Ca^{2+}-activated Cl^- currents. From the present results, ODT at higher levels,

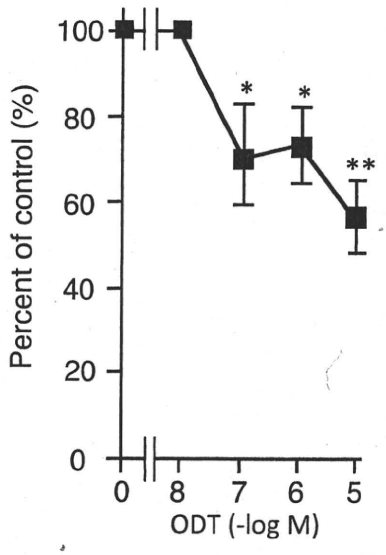

Fig. 2. Concentration–response relationship of *O*-desmethyl tramadol (ODT) on substance P (SP)-induced currents. ODT (10 μM – 10 nM) was applied to the oocytes for 2 min, and then 100 nM of SP was applied for 20 s. Data represent the mean ± S.E.M. of 40 oocytes. $*P < 0.05$ and $**P < 0.01$, compared with the control response using analysis of variance.

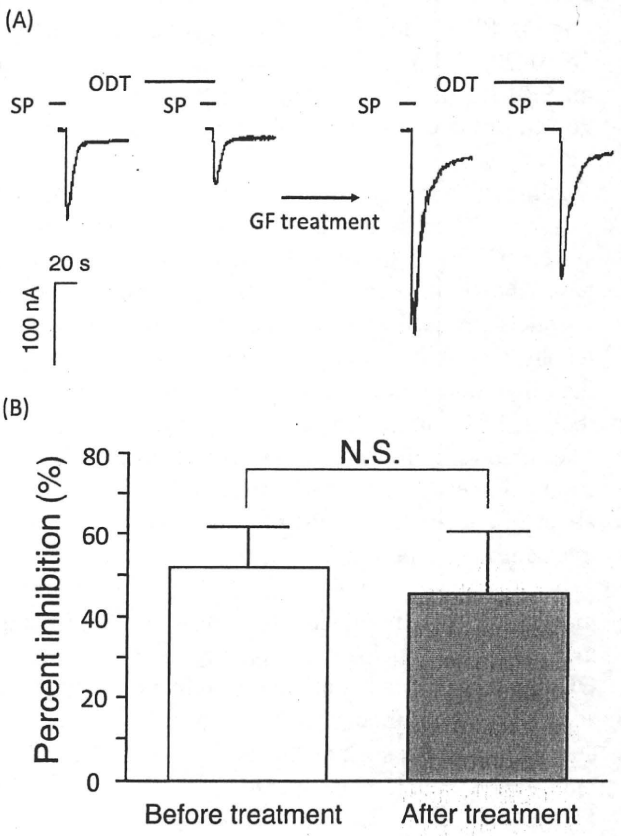

Fig. 3. The effects of GF109203X (protein kinase C inhibitor: PKCI) on inhibition by *O*-desmethyl tramadol (ODT) on substance P (SP)-stimulated currents in *Xenopus* oocytes expressing SP receptors (SPR). A) Tracings obtained from a single oocyte expressing SPR show the effect of ODT on SP (100 nM)-induced currents before and after treatment of PKCI. SP was applied for 20 s with or without 2-min ODT treatment. PKCI was treated for 120 min. B) Comparison of the effects of PKCI on the inhibitory effects of ODT. Oocytes were incubated with 200 nM GF109203X (PKCI) for 120 min. ODT (10 μM) shown was preapplied for 2 min before being co-applied with SP (100 nM) for 20s. "Before treatment" indicates the effects of ODT before application of bisindolylmaleimide. Data represent the mean ± S.E.M. for 10 separate determinations. A paired Student's *t*-test was used for the statistical analysis.

although within the clinically relevant concentrations, would inhibit SPR functions clinically.

The present study raises the question of how ODT inhibits SPR-mediated responses. We have reported that ODT had little effect on the function of muscarinic M_3R, which share the same downstream signaling steps as the SPR following Gq protein activation, expressed in *Xenopus* oocytes. These findings suggest that the inhibitory effect of ODT on the SP-induced Cl^- current is likely due to the inhibition of the SPR before activation of Gq proteins. There is considerable evidence that PKC plays an important role in regulating the function of GPCRs (14) and the functions of some GPCRs are inhibited by PKC activation. We reported that the inhibitory effects of halothane, isoflurane, enflurane, diethyl ether, and ethanol on SP-induced currents were suppressed in oocytes treated with the PKC inhibitor, suggesting that these anesthetics and ethanol inhibit SPR function via activation of PKC. However, in our present experiments, GF109203X did not alter the inhibitory effects of ODT on SPR function, suggesting that PKC may not be involved in the cases of the inhibitory effects of ODT on SPR.

Although much attention has been paid to the μ-opioid receptor and monoamine uptake in the central nervous system as targets for tramadol and ODT, several studies have shown that some GPCRs and ligand-gated ion channels are also targets for tramadol (15). In our present results, the inhibitory effects of ODT seem to be weaker than that on μ-opioid receptors and transporters. Nonetheless, SPR might also be one of the targets for ODT. The inhibitory effects of ODT on SPR might also contribute to the side effects of tramadol. More information about SPR may help to elucidate the role of SPR in the mechanisms of tramadol activity.

In conclusion, we demonstrated that the tramadol metabolite ODT inhibited SPR function. Our findings might help to elucidate the pharmacological basis of ODT and provide a better understanding of its neuronal action and the antinociceptive effects of tramadol. More definitive studies, such as the use of the SPR knockout mouse model, would be required.

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Short Communications

Possible Involvement of β-Endorphin in a Loss of the Coordinated Balance of μ-Opioid Receptors Trafficking Processes by Fentanyl

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BACKGROUND

It has been considered that opioid tolerance is, in part, the end result of a coordinated balance between processes that govern the desensitization, internalization, and resensitization of μ-opioid receptors (MOR) (Claing et al., 2002; Gainetdinov et al., 2004). However, a several line of evidence suggests that the trafficking properties of MORs driven by MOR agonists may depend on intrinsic characters of each agonist, and are still complicated. Previous biochemical studies on cultured enteric neurons have indicated that fentanyl induces either the functional desensitization or internalization of MORs (Minnis et al., 2003). In contrast, under the same condition, morphine does not promote the detectable internalization of MORs in cultured cells after prolonged or acute treatment in healthy animals, although it has been well-established that morphine causes the development of tolerance to its pharmacological actions (Minnis et al., 2003). However, recent studies have demonstrated that morphine activates MORs with promoting internalization of MORs via β-arrestin-2-dependent mechanisms in striatal neurons (Haberstock-Debic et al., 2005).

In the previous study, we demonstrated that repeated treatment with fentanyl, but not morphine, causes a rapid desensitization to its ability to block the hyperalgesia associated with the attenuation of MOR resensitization in mice with inflammatory pain (Imai et al., 2006). Based on this study, we hypothesized that released β-endorphin within the spinal cord under a chronic pain-like state may be implicated in the rapid development of tolerance to fentanyl, but not morphine and oxycodone. Namely, these findings raise the possibility that β-endorphin could attenuate the resensitization of MOR after the treatment with fentanyl, resulting in the high degree of tolerance to fentanyl-induced antihyperalgesic effects under long-lasting pain state. To further address this issue, this cell culture study was performed to investigate the effects of fentanyl on MOR internalization and resensitization in the presence or absence of β-endorphin.

MATERIALS AND METHODS

Baby hamster kidney (BHK) cells (Riken Cell Bank, Tsukuba, Japan) were grown in Dulbecco's

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