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Full Paper

Analysis of the Effects of Anesthetics and Ethanol on μ-Opioid Receptor

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Abstract. G protein–coupled receptors, in particular, Ca^{2+}-mobilizing G_q-coupled receptors have been reported to be targets for anesthetics. Opioids are commonly used analgesics in clinical practice, but the effects of anesthetics on the opioid μ-receptors (μOR) have not been systematically examined. We report here an electrophysiological assay to analyze the effects of anesthetics and ethanol on the functions of μOR in *Xenopus* oocytes expressing a μOR fused to chimeric Gα protein G_{qi5} (μOR-G_{qi5}). Using this system, the effects of halothane, ketamine, propofol, and ethanol on the μOR functions were analyzed. In oocytes expressing μOR-G_{qi5}, the μOR agonist DAMGO ([D-Ala^2,*N*-MePhe^4,Gly-ol]-enkephalin) elicited Ca^{2+}-activated Cl^- currents in a concentration-dependent manner (EC_{50} = 0.24 μM). Ketamine, propofol, halothane, and ethanol themselves did not elicit any currents in oocytes expressing μOR-G_{qi5}, whereas ketamine and ethanol inhibited the DAMGO-induced Cl^- currents at clinically equivalent concentrations. Propofol and halothane inhibited the DAMGO-induced currents only at higher concentrations. These findings suggest that ketamine and ethanol may inhibit μOR functions in clinical practice. We propose that the electrophysiological assay in *Xenopus* oocytes expressing μOR-G_{qi5} would be useful for analyzing the effects of anesthetics and analgesics on opioid receptor function.

Keywords: μ-opioid receptor, $G_{i/o}$-coupled receptor, ketamine, ethanol, *Xenopus* oocyte

Introduction

Opioids are commonly used analgesics in clinical practice, but the role of opioid receptor (OR) in anesthetic action has still been unclear. It has been reported that the OR antagonist naloxone does not affect the anesthetic potency of volatile anesthetics halothane in animals (1, 2). On the other hand, Sarton et al. reported that S(+) ketamine interacts with the μ-opioid system at supraspinal sites (3). In order to clarify the role of ORs in anesthetic action, it would be necessary to study the direct effects on OR function.

Several lines of studies have been reported that metabotropic G protein–coupled receptors (GPCRs) are now recognized as targets for anesthetics and analgesics (4). We and others have previously reported that functions of G_q protein–coupled receptors, including muscarinic type1 receptors (M_1R) (5), metabotropic type 5 glutamate receptors (mGluR5) (6), 5-hydroxytryptamine (5HT) type 2A receptors (7), and substance P receptors (8), are inhibited by anesthetics and analgesics. The ORs belong to the GPCR family and three types of ORs, μ, δ, and κ, have been identified by molecular cloning (9). Within three subtypes of these receptors, μORs are the major receptor to mediate the analgesic effects of opioids (9). On the basis of second messenger signaling, μOR couple to $G\alpha_{i/o}$ protein to cause inhibition of adenylate cyclase, inhibition of voltage-dependent Ca^{2+} channels, or activation of G protein–coupled inwardly rectifying K^+ channels (GIRKs) (9). Functions of G_q-coupled receptors have been reported to be modified by some anesthetics and analgesics (4, 10); as far as the functions of $G_{i/o}$-coupled receptors including μOR are concerned, much less is known about the direct effects of anesthetics and analgesics.

The *Xenopus* oocyte expression system has widely

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been employed to study functions of a number of GPCRs (4, 10). In the case of G_q-coupled receptors, stimulation of the receptors result in activation of Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes by G_q-mediated activation of phospholipase C (PLC) and subsequent formation of IP_3 and diacylglycerol (4, 11). The IP_3 formed causes release of Ca^{2+} from the endoplasmic reticulum by activation of IP_3 receptors (IP_3R), which in turn, triggers the opening of Ca^{2+}-activated Cl^- channels endogenously expressed in the oocytes (4, 11). However, in the case of $G_{i/o}$-coupled receptors, analysis has been difficult due to lack of appropriate analytical output in oocytes. We have established the assay method for $G_{i/o}$-coupled receptors by using G_{qi5} chimeric G protein to switch the $G_{i/o}$ signal to a G_q signal (12). By using this assay system, we reported that halothane inhibited the function of $G_{i/o}$-coupled muscarinic M_2 receptor (M_2R) in oocytes coexpressing M_2R and G_{qi5} (13). Recently, in order to improve the $G_{i/o}$-coupled–receptor assay system, we made a μOR fused to G_{qi5} (μOR-G_{qi5}) and expressed it in *Xenopus* oocytes (13).

By using this assay system, we examined the effects of halothane, ketamine, propofol, and ethanol on the function of μOR.

Materials and Methods

Materials

Adult *Xenopus laevis* female frogs were purchased from Kato Kagaku (Tokyo); halothane, from Dinabot Laboratories (Osaka), and the Ultracomp *E. coli* Transformation Kit, from Invitrogen (San Diego, CA, USA). Purification of cDNAs was performed with a Qiagen purification kit (Qiagen, Chatworth, CA, USA). Gentamicin, sodium pyruvate, [D-Ala^2,*N*-MePhe^4,Gly-ol]-enkephalin (DAMGO), and propofol were purchased from Tokyo Kagaku (Tokyo), and ketamine was purchased from Sigma (St. Louis, MO, USA). Other chemicals are analytical grade and were from Nacalai Tesque (Kyoto). The rat μOR was provided by Dr. N. Dascal (Tel Aviv University, Ramat Aviv, Israel). The chimeric $G\alpha_{qi5}$ was a kind gift from Dr. B.R. Conklin (The University of California, San Francisco, CA, USA). Each of the cRNAs was prepared by using an mCAP mRNA Capping Kit and transcribed with a T7 RNA Polymerase in vitro Transcription Kit (Stratagene, La Jolla, CA, USA).

Preperartion of chimeric μOR-Gqi5

The tandem cDNAs of chimeric μOR-G_{qi5} was created by ligating the receptor cDNA sequences into the NheI site of G_{qi5} cDNAs. The sequences of all PCR products were confirmed by sequencing with ABI3100 (Applied BioSystems, Tokyo). All cDNAs for the synthesis of cRNAs were subcloned into the pGEMHJ vector, which provides the 5′- and 3′-untranslated region of the *Xenopus* β-globin RNA (14), ensuring a high level of protein expression in the oocytes. Each of the cRNAs was synthesized using the mCAP mRNA Capping Kit, with the T7 RNA polymerase in vitro Transcription Kit (Ambion, Austin, TX, USA) from the respective linearized cDNAs.

Recording and data analyses

Isolation and microinjection of *Xenopus* oocytes were performed as previously described (12, 13). *Xenopus* oocytes were injected with appropriate amounts of cRNAs (50 ng, μOR-G_{qi5}) and incubated with ND 96 medium composed of 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES (pH 7.4, adjusted with NaOH), supplemented with 2.5 mM sodium pyruvate and 50 μg/ml gentamicin for 3 – 7 days until recording. Oocytes were placed in a 100-ml recording chamber and perfused with MBS (modified Barth's saline) composed of 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 7.4 adjusted with NaOH) at a rate of 1.8 ml/min at room temperature. Recording and clamping electrodes (1 – 2 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 of oocytes, 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 component of the transient inward currents induced by receptor agonists because this component is dependent on the concentrations of the receptor agonist applied and is quite reproducible, as described by Minami et al. (15). Anesthetics (halothane, ketamine, propofol) and ethanol were applied for 2 min before and during the application of test compounds to allow complete equilibration in the bath. The solutions of halothane were freshly prepared immediately before use. We calculated the final concentration of halothane in the recording chamber as reported previously (16), and accordingly, the concentrations of halothane represent the bath concentrations.

Statistical analyses

Results are expressed as percentages of control responses. The control responses were measured before and after each drug application, to take into account possible shifts in the control currents as recording proceeded. The "n" values refer to the number of oocytes studied. Each experiment was carried out with oocytes from at

least two different frogs. Statistical analyses were performed using a one-way ANOVA (analysis of variance) and the Dunnet correction. Curve fitting and estimation of EC_{50} values for the concentration–response curves were performed using Graphpad Inplot Software (San Diego, CA, USA).

Results

DAMGO-induced Ca^{2+}-activated Cl^- currents in Xenopus oocytes expressing μOR-G_{qi5}

We first determined the effects of the μOR agonist DAMGO on the Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes expressing μOR-G_{qi5}. As shown in Fig. 1A, DAMGO at 0.1 μM elicited a robust Ca^{2+}-activated Cl^- current. There were no Cl^--currents in oocytes expressing μOR not fused to G_{qi5} even at 10 μM DAMGO (data not shown), as reported previously (13). The EC_{50} of the DAMGO-induced Cl^- currents was 0.24 ± 0.01 μM (Fig. 1B).

Analysis of ketamine and propofol on DAMGO-induced Ca^{2+}-activated Cl^- currents in Xenopus oocytes expressing μOR-G_{qi5}

By using this assay, we examined the effects of the intravenous anesthetic ketamine on the μOR function in *Xenopus* oocytes expressing μOR-G_{qi5}. Ketamine by itself did not elicit any currents in oocytes expressing μOR-G_{qi5} but significantly inhibited DAMGO-induced Ca^{2+}-activated Cl^- currents in a concentration-dependent manner (Fig. 2A). Ketamine at 0.1, 1, and 10 μM inhibited the

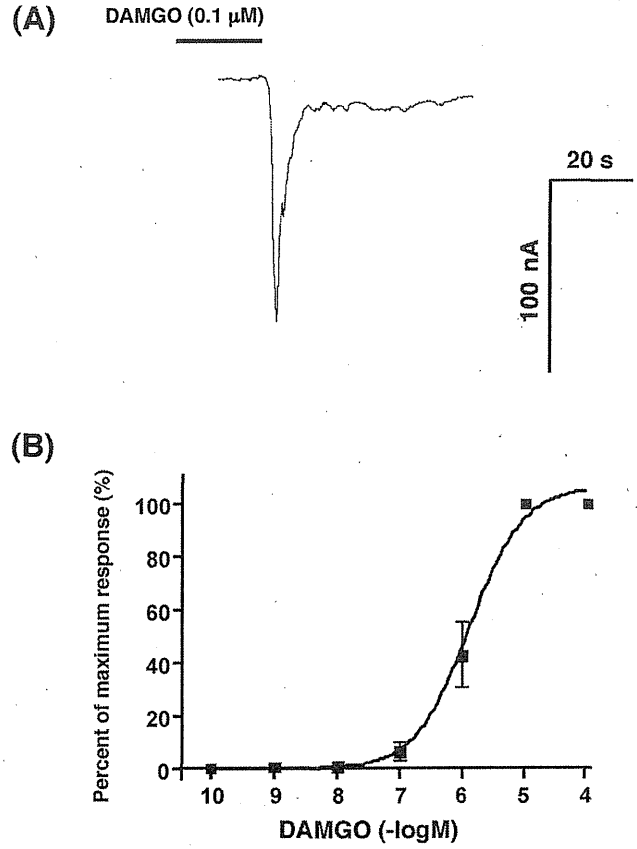

Fig. 1. Electrophysiological μOR assay induced by the μOR agonist DAMGO in *Xenopus* oocytes expressing μOR-G_{qi5}. A: Typical tracing of DAMGO (0.1 μM)–induced Ca^{2+}-activaed Cl^- current in an oocyte expressing μOR-G_{qi5}. B: Concentration–response curves of DAMGO-induced Ca^{2+}-activated Cl^- currents in oocytes. Oocytes were voltage-clamped at −70 mV and DAMGO was applied for 20 s.

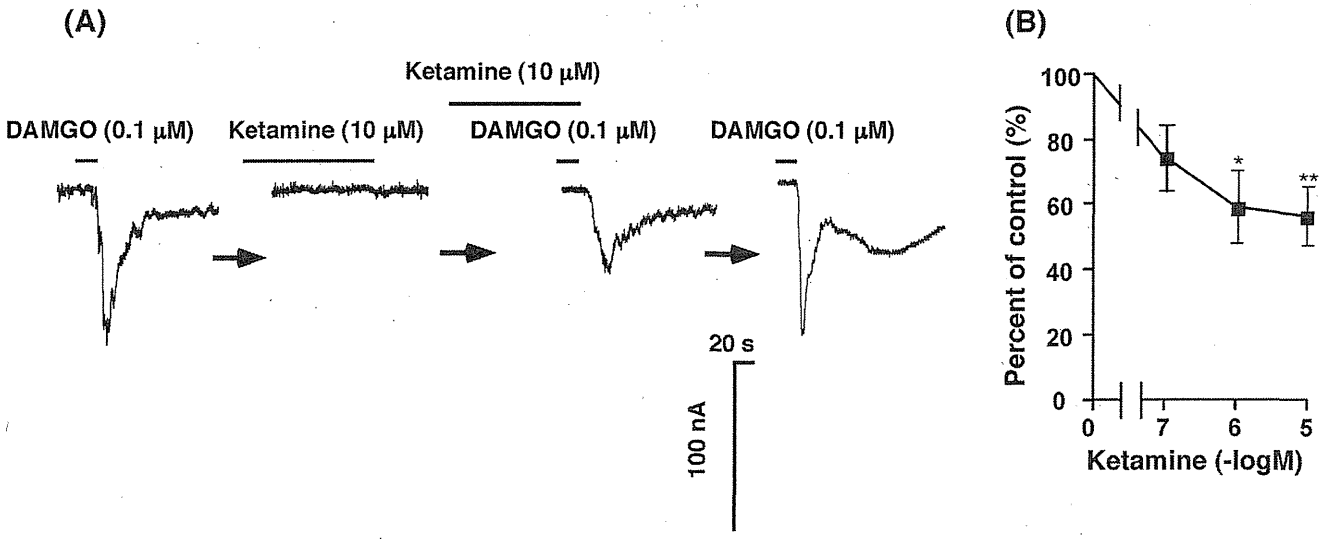

Fig. 2. Effects of ketamine on the basal and DAMGO-induced Ca^{2+}-activated Cl^- currents in oocytes expressing μOR-G_{qi5}. A: Typical tracings of the effect of 10 μM ketamine on the Cl^- current evoked by 0.1 μM DAMGO in an oocyte expressing μOR-G_{qi5}. B: Concentration–response curve for the inhibitory effects of ketamine on DAMGO (0.1 μM)–induced Cl^- currents in oocytes expressing μOR-G_{qi5}. $^*P < 0.05$ and $^{**}P < 0.01$ vs. control.

DAMGO-induced Cl^- currents to 74 ± 10.3%, 59.1 ± 11.3%, and 56.2 ± 9.3% of the control value, respectively (n = 6 for each) (Fig. 2B).

We next determined the effects of another intravenous anesthetic propofol on the function of μOR in oocytes expressing μOR-G_{qi5} (Fig. 3). Propofol by itself elicited no currents, but inhibited DAMGO-induced Cl^- currents in oocytes expressing μOR-G_{qi5} in a concentration-dependent manner (Fig. 3A). Propofol at concentrations of 0.1, 1, 10, and 100 μM inhibited the DAMGO-induced Cl^- currents to 93.3 ± 3.7%, 73.5 ± 7.9%, 72.8 ± 5.7%, and 53.7 ± 7.5% of the control value, respectively (n = 6 for each) (Fig. 3B).

Analysis of halothane and ethanol on the DAMGO-induced Ca^{2+}-activated Cl^- currents in Xenopus oocytes expressing μOR-G_{qi5}

We then examined the effects of the volatile anesthetic halothane on the function of μOR in oocytes expressing μOR-G_{qi5} (Fig. 4). Halothane by itself did not elicit any currents in oocytes expressing μOR-G_{qi5} at concentrations up to 2 mM, (Fig. 4A). Higher concentrations of halothane more than 1 minimum alveolar concentration (MAC, 0.25 mM) had inhibitory effects on the DAMGO-induced Cl^- currents in a concentration-dependent manner; 1MAC concentration of halothane did not suppress DAMGO-induced Cl^- currents. Halothane at concentrations of 0.25, 0.5, 1, and 2 mM inhibited the current to 75.1 ± 12.4%, 57.8 ± 10.3%, 54.7 ± 10.3%, and 48.6 ± 9.4% of the control value, respectively (n = 6 for each) (Fig. 4B).

We finally examined the effects of ethanol on the function of μOR in oocytes expressing μOR-G_{qi5} (Fig. 5). Ethanol by itself had no effects in oocytes expressing μOR-G_{qi5}, but it significantly inhibited DAMGO-induced Cl^- currents in a concentration-dependent manner (Fig. 5B). Ethanol at concentrations of 25, 50, 100, and 200 mM inhibited the currents to 53.1 ± 10.1%, 47 ± 13.3%, 43.3 ± 9.6%, and 35 ± 5.3% of the control value, respectively (n = 6 for each) (Fig. 5B).

Discussion

We previously proposed an electrophysiological assay of the $G_{i/o}$-coupled receptors in *Xenopus* oocytes expressing the receptors and chimeric G protein G_{qi5} (12, 13). By using this system, we examined the effects of several anesthetics and ethanol on the μOR function in oocytes expressing fused μOR-G_{qi5}.

In general, $G_{i/o}$-coupled receptors such as μOR are known to inhibit adenylate cyclase to decrease cAMP levels in the cells (9). Numerous reports have shown that ketamine, halothane, and ethanol increase basal cAMP levels in a variety of the cells, possibly by direct activation of adenylate cyclases (17 – 20); thus it might be difficult to estimate the effects of anesthetics and ethanol

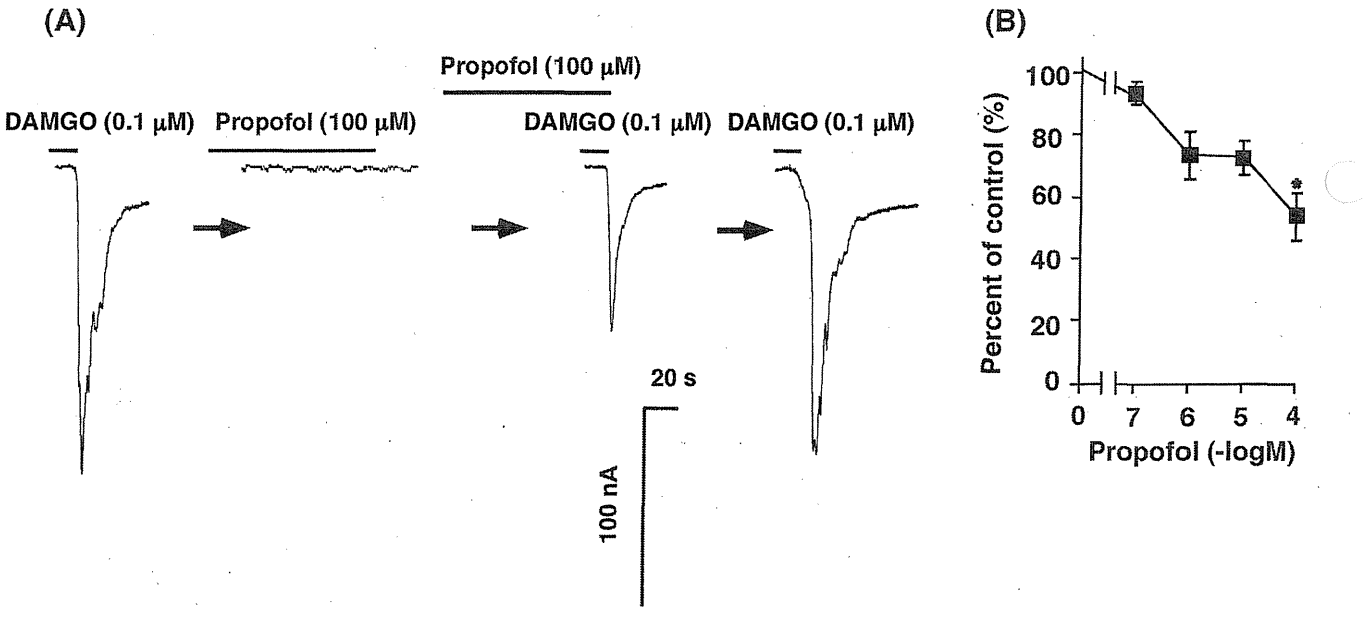

Fig. 3. Effects of propofol on the basal and DAMGO-induced Ca^{2+}-activated Cl^- currents in oocytes expressing μOR-G_{qi5}. A: Typical tracings of the effect of 100 μM propofol on the Cl^- current evoked by 0.1 μM DAMGO in an oocyte expressing μOR-G_{qi5}. B: Concentration–response curve for the inhibitory effects of propofol on DAMGO (0.1 μM)–induced Cl^- currents in oocytes expressing μOR-G_{qi5} *$P < 0.05$ and vs. control.

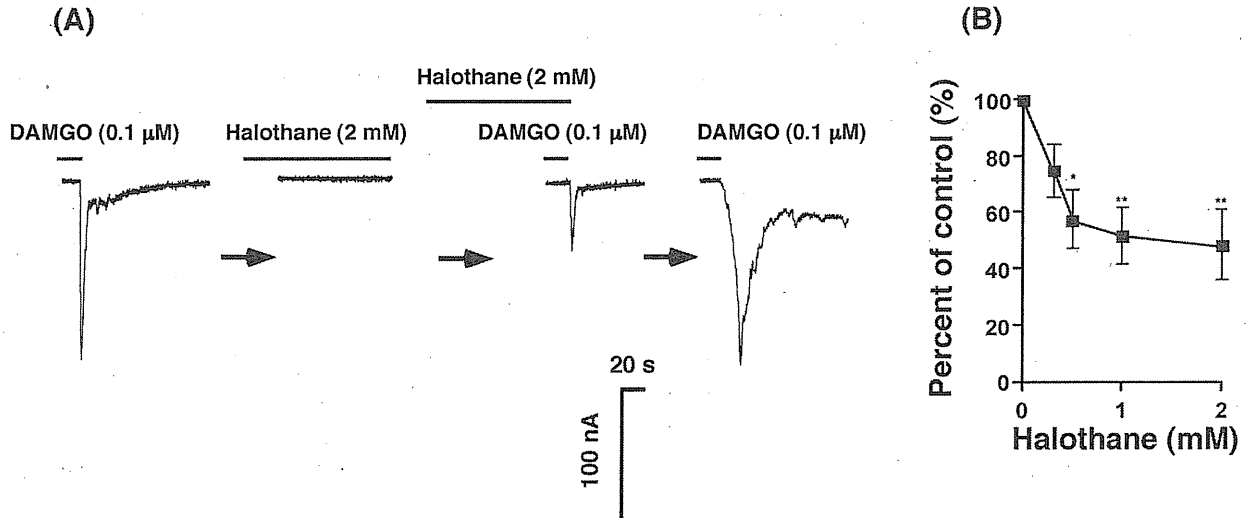

Fig. 4. Effects of halothane on the basal and DAMGO-induced Ca^{2+}-activated Cl^- currents in oocytes expressing μOR-G_{qi5}. A: Typical tracings of the effect of 2 mM halothane on the Cl^- current evoked by 0.1 μM DAMGO in an oocyte expressing μOR-G_{qi5}. B: Concentration–response curve for the inhibitory effects of halothane on DAMGO (0.1 μM)–induced Cl^- currents in oocytes expressing μOR-G_{qi5}. $^*P < 0.05$ and $^{**}P < 0.01$ vs. control.

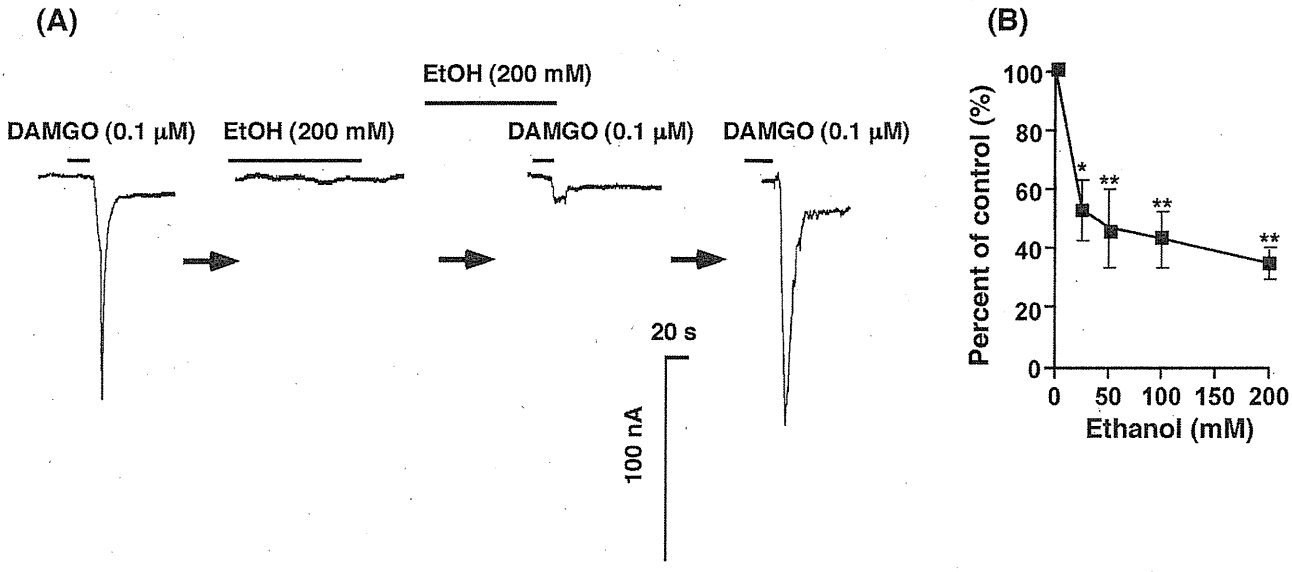

Fig. 5. Effects of ethanol on the basal and DAMGO-induced Ca^{2+}-activated Cl^- currents in oocytes expressing μOR-G_{qi5}. A: Typical tracings of the effect of 200 mM ethanol on the Cl^- current evoked by 0.1 μM DAMGO in an oocyte expressing μOR-G_{qi5}. B: Concentration–response curve for the inhibitory effects of ethanol on DAMGO (0.1 μM)–induced Cl^- currents in oocytes expressing μOR-G_{qi5}. $^*P < 0.05$ and $^{**}P < 0.01$ vs. control.

on the functions of $G_{i/o}$-coupled receptors by using a cAMP inhibition assay. Alternatively we and others have used *Xenopus* oocytes expressing GIRK channels for the analysis of functions of $G_{i/o}$-coupled receptors such as μOR, $GABA_BR$, or cannabinoid CB_1 and CB_2 receptors (13, 21 – 23); GIRKs have been demonstrated to be excellent reporter channels for assay of the activity of $G_{i/o}$-coupled receptors (21). However, recent reports have revealed that GIRKs are possible targets for several anesthetics including halothane and ethanol (24 – 26). In such a situation, it should be taken into consideration that functions of either $G_{i/o}$-coupled receptors, GIRKs, or both could be affected by anesthetics or alcohol if GIRKs are used as reporters (24 – 26). In this study, we thus employed μOR-G_{qi5} in a *Xenopus* oocyte expression assay system. Accordingly, this system makes it possible

to study the direct effects of anesthetics and alcohols on μOR functions.

In the present study, we demonstrated that ketamine and ethanol inhibited the DAMGO-induced Cl^- currents at clinically equivalent concentrations, while propofol and halothane inhibited the DAMGO-induced currents only at higher concentrations. In our experimental system, the inhibitory effects of the anesthetics and ethanol are considered due to specific inhibition of μOR or the inhibition of the downstream steps in the μOR-induced G_{qi5}–PLC–IP_3–IP_3R–Ca^{2+} mobilization pathways. There are numerous reports showing that ketamine, propofol, halothane, and ethanol did not inhibit such downstream pathways after activation of GPCRs in the *Xenopus* oocyte expression system. In the case of ketamine and halothane, they inhibit muscarinic M_1R-mediated Ca^{2+}-activated Cl^- currents in clinically relevant concentrations (5, 27) without affecting angiotensin II receptor (AT_1R)–induced Cl^- currents, although activation of M_1R and AT_1R consequently activate the same G_q–PLC–IP_3–IP_3R–Ca^{2+} mobilization pathways (5, 27). These results suggest that ketamine and halothane affect functions of Ca^{2+}-mobilizing GPCRs possibly by receptor sites rather than the downstream pathway after GPCR activation. As for propofol, our previous study demonstrated that this anesthetic inhibited the functions of M_1R but not substance P receptors, although both receptors were considered to couple to the same Gq-mediated pathways (8, 28). In addition, we demonstrated that propofol (50 μM) did not inhibit the direct G protein activator AlF_4^-–induced Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes (28). In the case of ethanol, we previously reported that ethanol also selectively inhibited the glutamate mGluR5 but not mGluR1, although both receptors couple to Gq to activate Ca^{2+}-activated Cl^- currents in oocytes (6). Taken together, these findings indicate that anesthetics and ethanol employed in the present study may not inhibit the step of G protein–PLC–IP_3–IP_3R–Ca^{2+} mobilization in the μOR signaling pathway.

The EC_{50} value of DAMGO of the μOR-induced Ca^{2+}-activated Cl^--currents through G_{qi5} was 0.24 μM in the present study. In our previous experimental study in *Xenopus* oocytes expressing μOR-G_{qi5} (13), the EC_{50} of DAMGO was approximately 0.1 μM. In *Xenopus* oocytes expressing cloned μOR and GIRKs, the EC_{50} values of DAMGO were 0.1 (13), 0.034 – 0.133 (29), and 0.02 – 0.09 μM (30) determined with the GIRK channel assay. These results suggest that our present EC_{50} value seems not too far from the previously reported EC_{50} values obtained in *Xenopus* oocytes expressing μOR.

We showed that ketamine had an inhibitory effect on DAMGO-induced Cl^- currents in oocytes expressing μOR-G_{qi5} at concentrations more than 1 μM. In clinical situations, the free plasma concentration of ketamine was approximately 10.5 – 60 μM (31, 32). Previous reports showed that higher concentration of ketamine than those in clinical usage (50 – 100 μM) displaced [^{3}H]diprenorphine binding to μORs expressed in Chinese hamster ovary cells (33). In an animal study, S(+)ketamine interacts with the μOR, which contributed to S(+)ketamine-induced respiratory depression and supraspinal antinociception (3). Consistent with these reports, our present results suggest that anesthetic concentrations of ketamine would have direct inhibitory effects on μOR.

The effects of propofol on the μOR functions have not been reported so far. In the present study, only high concentration (100 μM) of propofol (but less than 100 μM) had inhibitory effects on the DAMGO-induced Cl^- currents in oocytes expressing μOR-G_{qi5}. In humans, the peak plasma concentration of propofol after intravenous injection of the anesthetic dosage of 2.5 mg/kg was approximately 23 ± 0.24 μM (34). From our present results, it seems that propofol would have little effect on the μOR functions in its clinically used concentrations.

The direct effects of halothane on the μOR have not been studied. In the present study, clinical concentrations of halothane (0.25 mM) had no effect on basal- and DAMGO-induced Cl^- currents in *Xenopus* oocytes expressing μOR-G_{qi5}, whereas higher concentrations of halothane (0.5 – 2.0 mM) inhibited the DAMGO-induced Cl^- currents. To our knowledge, this is the first report that shows the direct effects of halothane on the function of μOR in the heterologous expression system. Lambert et al. have reported that binding of [^{3}H]DAMGO was unaffected by lower concentrations of halothane, but 5.0% (approximately 5.3 MAC) halothane reduced its affinity (35). From our present and previous reports, higher concentrations of halothane would inhibit the DAMGO-induced currents by reducing the affinity of DAMGO to μOR. Yamakura et al., on the other hand, reported that inhibition by halothane is likely caused by inhibition of GIRK channels, not by μOR (25). Furthermore, it was recently reported that the MACs for halothane are not different between wild-type and μOR–knock-out mice (36). Although further study would be necessary, our present result suggests that halothane would have little effect on μOR in the clinical situation.

Interaction between alcohol and the CNS opioid signaling system is well established in both basic and clinical research (37, 38). However, mechanisms involving direct ethanol interaction on the μOR have not been fully elucidated. We showed that ethanol at a concentration more than 25 mM inhibited DAMGO-induced Cl^- currents in oocytes expressing μOR-G_{qi5}. Several hypotheses of such inhibitory effects have been asserted; Vukojević et al. reported that relevant concentrations of ethanol

(10 – 40 mM) altered μOR mobility and surface density and affect the dynamics of plasma membrane lipids of pheochromocytoma PC12 cells, suggesting that ethanol modified μOR activity by sorting of μOR at the plasma membrane (39). Although further studies will be required, ethanol might inhibit the DAMGO-induced currents by reducing the affinity of DAMGO to the μOR.

In conclusion, we demonstrated that ketamine and ethanol have significant inhibitory effects on the function of μOR at clinically relevant concentrations. On the other hand, halothane and propofol seem not to suppress the μOR functions at least at clinically used concentrations. Further studies will be necessary to clarify the effects of these agents on opioid systems with other assay systems. The electrophysiological method for analysis of the function of μOR fused to the chimeric Gα protein shown in this study could be useful for investigating the effects of analgesics, anesthetics, and alcohol on other $G_{i/o}$-coupled receptors.

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Activation of the neurokinin-1 receptor in rat spinal astrocytes induces Ca^{2+} release from IP_3-sensitive Ca^{2+} stores and extracellular Ca^{2+} influx through TRPC3

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ABSTRACT

Substance P (SP) plays an important role in pain transmission through the stimulation of the neurokinin (NK) receptors expressed in neurons of the spinal cord, and the subsequent increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as a result of this stimulation. Recent studies suggest that spinal astrocytes also contribute to SP-related pain transmission through the activation of NK receptors. However, the mechanisms involved in the SP-stimulated $[Ca^{2+}]_i$ increase by spinal astrocytes are unclear. We therefore examined whether (and how) the activation of NK receptors evoked increase in $[Ca^{2+}]_i$ in rat cultured spinal astrocytes using a Ca^{2+} imaging assay. Both SP and GR73632 (a selective agonist of the NK1 receptor) induced both transient and sustained increases in $[Ca^{2+}]_i$ in a dose-dependent manner. The SP-induced increase in $[Ca^{2+}]_i$ was significantly attenuated by CP-96345 (an NK1 receptor antagonist). The GR73632-induced increase in $[Ca^{2+}]_i$ was completely inhibited by pretreatment with U73122 (a phospholipase C inhibitor) or xestospongin C (an inositol 1,4,5-triphosphate (IP_3) receptor inhibitor). In the absence of extracellular Ca^{2+}, GR73632 induced only a transient increase in $[Ca^{2+}]_i$. In addition, H89, an inhibitor of protein kinase A (PKA), decreased the GR73632-mediated Ca^{2+} release from intracellular Ca^{2+} stores, while bisindolylmaleimide I, an inhibitor of protein kinase C (PKC), enhanced the GR73632-induced influx of extracellular Ca^{2+}. RT-PCR assays revealed that canonical transient receptor potential (TRPC) 1, 2, 3, 4 and 6 mRNA were expressed in spinal astrocytes. Moreover, BTP2 (a general TRPC channel inhibitor) or Pyr3 (a TRPC3 inhibitor) markedly blocked the GR73632-induced sustained increase in $[Ca^{2+}]_i$. These findings suggest that the stimulation of the NK-1 receptor in spinal astrocytes induces Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores, which is positively modulated by PKA, and subsequent Ca^{2+} influx through TRPC3, which is negatively regulated by PKC.

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1. Introduction

Substance P (SP), a member of the tachykinin peptide family, is mainly expressed in primary afferent neurons (Severini et al., 2002). The centrally directed axonal terminals of SP-containing dorsal root ganglion (DRG) neurons project to the superficial lamina of the spinal horn, and their distally directed axonal terminals reside in peripheral tissues. In the spinal dorsal horn, SP released from the central terminals of primary afferent neurons by noxious stimuli activates the SP receptor, neurokinin (NK) receptor, which is expressed on the postsynaptic membrane, thus resulting in the transmission of nociceptive information to the central nervous system (Randic and Miletic, 1977; Hirota et al., 1985).

It is well known that SP binds to all subtypes of NK receptor; NK-1, -2 and -3 (Maggi, 1995). Among the three subtypes, SP has the highest affinity to NK-1 receptor (Maggi and Schwartz, 1997). The stimulation of NK receptors evokes the activation of phospholipase C (PLC), thus leading to phosphoinositol breakdown and an elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Maggi, 1995; Snijdelaar et al., 2000). In addition, NK receptors also activate adenylate cyclase in order to induce cyclic AMP production (Nakajima et al., 1992; Maggi, 1995; Snijdelaar et al.,

Abbreviations: 2-APB, 2-aminoethyl diphenylborinate; BIM, bisindolylmaleimide I; BTP2, N-{4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl}-4methyl-1,2,3-thiadiazole-5-carboxamide; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglion; fura-2 AM, fura-2 acetoxymethyl ester; GFAP, glial fibrillary acidic protein; Hank's buffer, Hanks' balanced salt solution; IP_3, inositol 1,4,5-triphosphate; NK, neurokinin; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; Pyr3, ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate; SP, substance P; TRPC channel, canonical transient receptor potential channel.

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2000). Recent studies have shown that spinal astrocytes, the major population of glia supporting neurons, also express functional NK-1 receptor (Marriott et al., 1991; Palma et al., 1997). Therefore, SP released from the nerve terminal may act not only neurons, but also on the astrocytes surrounding synaptic junctions in the spinal cord. In this manner, SP may induce the increase of $[Ca^{2+}]_i$ via spinal astrocytes. This successive event has an important role in communication between neurons and astrocytes, and might be essential to achieve the synaptic transmission (Fields and Stevens-Graham, 2002). It is therefore possible that NK receptors on spinal astrocytes may also be associated with SP-related pain transmission. Although it has been showed that the activation of the NK-1 receptor on spinal astrocytes produces inositol 1,4,5-triposphate (IP_3) (Marriott et al., 1991; Palma et al., 1997), the Ca^{2+} signaling induced by the activation of that receptor in spinal astrocytes has not yet been investigated.

Recently, activation of the PLC-linked receptor (histamine receptor and proteinase-activated receptor) was reported to induce Ca^{2+} release from the intracellular Ca^{2+} stores through the IP_3 receptor, and also has been shown to cause the influx of extracellular Ca^{2+} in human astrocytoma (Barajas et al., 2008; Nakao et al., 2008). Several reports have demonstrated that the family of canonical transient receptor potential (TRPC) channels is one of candidate receptors responsible for mediating the extracellular Ca^{2+} influx induced after the activation of PLC-linked receptors in vasucular smooth muscle and TRPC channel-expressing cells (Venkatachalam and Montell, 2007; Large et al., 2009). Moreover, functional TRPC channels are also expressed in human astrocytoma (Barajas et al., 2008; Nakao et al., 2008). Therefore, these reports indicate the possibility that the NK-1 receptor-stimulated increase in $[Ca^{2+}]_i$ by spinal astrocytes involves the Ca^{2+} influx through TRPC channels. However, it is unclear whether the stimulation of the NK-1 receptor causes Ca^{2+} influx though TRPC channels in spinal astrocytes. The present study is the first to demonstrate that the activation of the NK-1 receptor by SP or GR73632, a selective NK-1 receptor agonist, evoked an increase in $[Ca^{2+}]_i$ in cultured spinal astrocytes, which involved both Ca^{2+} release from intracellular Ca^{2+} stores, and extracellular Ca^{2+} influx through the TRPC channels.

2. Materials and methods

2.1. Materials

The following drugs and reagents were used for the present studies: bisindolylmaleimide I (BIM) and N-{4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl}-4methyl-1,2,3-thiadiazole-5-carboxamide (BTP2) (Calbiochem, La Jolla, CA, USA); Fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel); fura-2 acetoxymethyl ester (fura-2 AM) (Dojindo Laboratories, Kumamoto, Japan); 2.5% trypsin (Gibco-BRL, Gaithersburg, MD, USA); Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan); 2-aminoethyl diphenylborinate (2-APB), GR94800, H89, Hanks' balanced salt solution (Hanks' buffer), penicillin/streptomycin, poly-L-lysine, SB222200, thapsigargin, U73122 and xestospongin C (Sigma Chemical, St. Louis, MO, USA); SP (Peptide Institute, Osaka, Japan); CP96345 (Pfizer Central Research, Groton, CT, USA); DNase (Roche, Basel, Switzerland). Ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate (Pyr3) was kindly provided by Prof. Y. Mori of Kyoto University (Japan). All other reagents were of the highest purity available from commercial sources.

2.2. Cell culture

Spinal astrocytes were prepared from spinal cords of neonatal Wistar rats according to a previously reported method (Morioka et al., 2009). In brief, the isolated spinal cords were minced, and then incubated with trypsin and DNase. Dissociated cells were suspended in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Thereafter, cell suspensions were plated in 75 cm^2 tissue culture flasks (7.5 to 10 × 10^6 cells/flask) precoated with poly-L-lysine (10 μg/ml). The cells were maintained in a 10% CO_2 incubator at 37 °C. After 10 days, microglial cells were removed by vigorously shaking the growth flasks. Thereafter, the cells were harvested and replated to 35 mm diameter dishes at a density of 3 × 10^5 cells/dish, or glass coverslips with a silicon rubber wall (FlexiPERM; Heraeus Biotechnology, Hanau, Germany) at a density of 0.2 × 10^5 cells/slide. At 3 days post-seeding, the medium was replaced with serum-free DMEM. The cells were used for experiments overnight after the medium change. Prepared astrocytes showed a purity >95% as determined by glial fibrillary acidic protein (GFAP) immunoreactivity. All animal procedures were performed in accordance with the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University, Japan.

2.3. Measurement of $[Ca^{2+}]_i$ in spinal astrocytes

The measurement of $[Ca^{2+}]_i$ was performed using a previously described method (Miyano et al., 2009). All experiments were performed in Ca^{2+} (1.3 mM)-containing or Ca^{2+}-free Hanks' buffer. Spinal astrocytes were loaded with 5 μM of fura-2 AM in Ca^{2+}-containing Hanks' buffer for 50 min at 37 °C. After washing, the cells treated with either SP or GR73632 in Ca^{2+}-containing or Ca^{2+}-free Hanks' buffer, respectively. The fluorescence intensity was measured with the excitation wavelengths of 340 and 380 nm and the emission wavelength of 510 nm. The video image output was digitized by an Argus Hisca color image processor (Hamamatsu Photonics, Shizuoka, Japan).

2.4. RT-PCR analysis

According to a previously reported method (Morioka et al., 2009), total RNA in astrocytes was prepared and used to synthesize cDNA with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and a random hexamer primer (Takara Bio Inc., Shiga, Japan). PCR reactions were performed with the specific primers indicated in Table 1 and AmpliTaq Gold™ (Applied Biosystems) at 95 °C for 10 min followed by 35–40 cycles (Table 1) of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and elongation at 72 °C for 2 min, with a final extension at 72 °C for 5 min. The resulting PCR products were analyzed on a 1.5% agarose gel and had the size expected from the known cDNA sequence.

2.5. Immunofluorescence staining

Cells were washed with PBS(−), fixed with 4% paraformaldehyde, and permeabilized with 0.1% triton-X at room temperature. After blocking with 3% BSA, cells were incubated with a polyclonal antibody against the NK-1 receptor (1:100; Sigma), TRPC3 (1:100; AnaSpec Inc., San Jose, CA), or a monoclonal antibody against GFAP (1:200; Sigma) for 1 h at room temperature. Next, the cells were further incubated with Alexa 546-conjugated anti-rabbit IgG antibody, or Alexa 488-conjugated anti-mouse IgG antibody (1:500, Molecular Probes, Invitrogen, Carlsbad, CA) for 1 hour at room temperature. Immunolabeled cells were visualized under a Zeiss LSM510 META confocal microscope (Carl Zeiss, Jene, Germany).

2.6. Statistical analysis

The data are presented as the means ± S.E.M. of at least three independent experiments. The statistical analysis of all data except for Fig. 3F, was performed by a one-way analysis of variance (ANOVA) followed by Bonferroni's test. In Fig. 3F, *t*-test was used to analyze the differences between the two groups. A probability value (p) of less than 0.05 was considered to be statistically significant.

Table 1
The primer sequences and sizes of PCR products of rat TRPC channels.

Subtypes of TRPC	Forward primers (5′→3′)	Reverse primers (3′→5′)	Size (bp)
C1	TCTGGCCAGTCCAGCTCTAA	CCCTTCATACCACAGCCTCT	682
C2	CCCTGCAACCATGCTCATGT	CTTGAGCTGGACAACGGTCT	609
C3	CTTGATCCAGGCTGGGGAAA	CTTTGGCCCCAAGGTAGTAG	708
C4	CTCGCTCATTGCGCTGTCAA	GTCGATGTGCTGAGAGGCTA	547
C5	GCCAAGCTGAAGGTGGCAAT	AGATCTGCAGAGGCCCTAAG	664
C6	GACTCCTTCAGCCACTCTAG	ACGAGCAGCCCCAGGAAAAT	561
C7	TCCCTTTAACCTGGTGCCGA	TCACCCTCAGGTGGTCTTTG	449