Laboratory Reports

Halothane facilitates the translocation of GRK-2 and phosphorylation of β 2- adrenergic receptor in rat synaptosomes

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Purpose: To examine the effect of halothane on β 2-adrenergic receptor phosphorylation and on G-protein coupled receptor kinase (GRK), responsible for β 2-receptor downregulation.

Methods: Rat forebrain synaptosomes were incubated for 30 min with halothane 1 or 2%. The cytosolic and membrane fractions were separated, and phosphorylation activity of recombinant β 2-adrenergic receptor was quantified autoradiographically using 32 P labeled adenosine triphosphate. Phosphorylation activity of a specific GRK-2 substrate, was examined by measuring 32 P binding. Subcellular localization of the enzyme was immunologically analyzed by Western blotting.

Results: Halothane 2% decreased the phosphorylation activity of the recombinant receptor in the cytosol fraction, regardless of $10 \mu\text{M}$ isoproterenol (ISP) ($P < 0.01$), which activity in the membrane fraction was increased ($P < 0.01$). Phosphorylation activity of the synthetic peptide decreased in the cytosol obtained from synaptosomes exposed to halothane 2% ($P < 0.05$). In contrast, activity in the membrane increased by exposure to halothane 2% ($P < 0.01$). The concentration of GRK-2 decreased in the cytosol obtained from synaptosomes exposed to halothane 1% or 2% (decreases of $8.3 \pm 1.2\%$ @ 1%, and $18.0 \pm 2.1\%$ @ 2%, $P < 0.05$). In the membrane, exposure to halothane 1% or 2% increased the GRK-2 amount dose dependently ($22.5 \pm 3.1\%$ @ 1%, and by $45.7 \pm 6.1\%$ @ 2%, $P < 0.01$).

Conclusion: Halothane could facilitate translocation of GRK-2 and possibly promote the downregulation of β 2-adrenergic receptors in the synaptic membrane. The anesthetic action and hemodynamic suppressive action of halothane may be related to this phenomenon.

Objectif : Évaluer l'effet de l'halothane sur la phosphorylation des récepteurs β 2-adrénergiques et sur la kinase du récepteur couplé avec la protéine G (KRG), responsables de la régulation restrictive des récepteurs β 2.

Méthode : Des synaptosomes cérébraux antérieurs de rat ont été mis en incubation pendant 30 min avec de l'halothane à 1 ou 2 %. Les fractions cytosoliques et membranaires ont été séparées et l'activité de phosphorylation du récepteur recombinant β 2-adrénergique a été quantifiée par autoradiographie en utilisant l'adénosine triphosphate marquée avec du ^{32}P . L'activité de phosphorylation d'un substrat spécifique de KRG-2 a été examinée en mesurant la fixation de ^{32}P . La localisation subcellulaire de l'enzyme a été immunologiquement analysée par buvardage de Western.

Résultats : L'halothane à 2 % a réduit l'activité de phosphorylation du récepteur recombinant dans la fraction cytosolique, malgré $10 \mu\text{M}$ d'isoprotérénol (ISP) ($P < 0,01$), et augmenté l'activité dans la fraction membranaire ($P < 0,01$). L'activité de phosphorylation du peptide de synthèse a baissé dans le cytosol obtenu des synaptosomes exposés à l'halothane à 2 % ($P < 0,05$). Par ailleurs, l'activité dans la membrane a baissé lors de l'exposition à l'halothane à 2 % ($P < 0,01$). La concentration de KRG-2 a diminué dans le cytosol obtenu à partir des synaptosomes exposés à l'halothane à 1 % ou 2 % (baisse de $8,3 \pm 1,2\%$ @ 1 %, et de $18,0 \pm 2,1\%$ @ 2 %, $P < 0,05$). Dans la membrane, l'exposition à l'halothane à 1 % ou 2 % a augmenté la quantité de KRG-2 de manière dépendante de la dose ($22,5 \pm 3,1\%$ @ 1 %, et $45,7 \pm 6,1\%$ @ 2 %, $P < 0,01$).

Conclusion : L'halothane peut faciliter la translocation de KRG-2 et activer sans doute la régulation restrictive des récepteurs β 2-adrénergiques dans la membrane synaptique. L'action anesthésique et l'action hémodynamique suppressive de l'halothane peuvent être reliées à ce phénomène.

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VOLATILE anesthetics have multiple actions on inter- and intra-cellular signal transduction systems. These actions are thought to be closely related to the fundamental mechanism of anesthesia and also to the neurological and hemodynamic side effects of anesthetics.¹ For G protein coupled receptor families, receptor proteins, G proteins and intra-cellular signal transduction machineries are considered as potential targets of anesthetic action.^{1,2} Protein phosphorylation, which has essential roles in intra-cellular signal transduction systems including receptor modification,^{3,4} is a such potential target of anesthetics.

The β -adrenergic receptor system in the brain is known to control consciousness and alertness.^{5,6} In addition, prostaglandin biosynthesis⁷ and the production of corticotropin-releasing factor⁸ in the brain are also regulated by the β -adrenergic system. The neuro-depressive action of alcohol is antagonized by β -adrenergic stimulation. Thus, β -adrenergic neurotransmission may play a minor, yet to be identified modulator role in anesthetic-induced depression of consciousness.⁹ Furthermore, several anesthesia related neurological problems, such as local anesthetic toxicity,¹⁰ septic encephalopathy¹¹ and hypoxic brain damage,¹² and the cardiovascular side effects of anesthetics have been reported to have interactions with β -adrenergic signals.¹³⁻¹⁶ If the precise site of action of anesthetics in the β -adrenergic signal cascade could be identified, better use of anesthetics in neurologically and cardiologically complicated cases might be devised. There have been no reports of interactions between anesthetics and β -adrenergic signal transduction in brain.

The function of the β -adrenergic receptor is controlled by protein phosphorylation.⁶ Recent studies by Ferguson *et al.*¹⁷ and Goodman *et al.*¹⁸ demonstrated that phosphorylation leads to internalization of the receptor molecule and that phosphorylation of the β -adrenergic receptor is a crucial step in the cellular regulation of receptors.¹⁹ The hypothesis of the present study was that anesthetics might interfere with phosphorylation of the β -adrenergic receptor by affecting kinase activity and, thereafter, downregulation of the receptors. In the present study, we examined phosphorylation activity of the β -adrenergic receptor in rat forebrain synaptosome after exposure to halothane. The synaptosome is the simplest subcellular preparation that retains signal transduction mechanisms and electrophysiological properties of the synapse.²⁰ Thus, it is often utilized for the study of protein phosphorylation in the synapse.^{21,22} In addition, synaptosomal β -adrenergic receptor could be an experimental model for studying the effect of anesthetics on phosphorylation of

the G-protein coupled receptors. In the present study, we focused on phosphorylation of the β 2-adrenergic receptor, which has been extensively studied by a molecular biological approach for a decade.^{2,3} In the first experiment, we confirmed that halothane increased phosphorylation activity of the recombinant β 2-adrenergic receptor in membrane. In the second experiment, GRK-2 (G-protein coupled receptor kinase -2, previously named β -adrenergic receptor kinase) activity was assayed using a synthetic peptide, which is preferentially phosphorylated by GRK-2 and not by protein kinase C (PKC; calcium dependent protein kinases) or protein kinase A (PKA; cAMP dependent protein kinases). Since phosphorylation of the β 2-adrenergic receptor by membrane bound GRK-2 is a crucial step in regulating the number of receptors,¹⁹ subcellular localization of GRK-2 was analyzed by immuno-blotting in the third experiment.

Materials and methods

The recombinant β 2-adrenergic receptor was obtained from BioSignal Inc. (Montreal, Canada). [γ -³²P]ATP was purchased from NEN Life Science Products, Inc. (Boston, MA). Synthetic peptide, RRREEEEESAAA, was synthesized and purified by Accord Inc. (Tokyo, Japan). Peptide binding paper, P-81, was from Whatman Inc. (Maidstone, England). Anti-GRK-2 antibody was obtained from Wako Pure Chemical, Inc. (Tokyo, Japan). Chemicals for polyacrylamide gel electrophoresis and immunoblotting were from BioRad Inc. (Hercules, CA). Silver staining kit for polyacrylamide gel was obtained from Daiichi Kagakuyaku Inc. (Tokyo, Japan). (-) isoproterenol (ISP) was purchased from Sigma Inc. (St. Louis, MO).

Preparation of synaptosomes

After obtaining approval of the institutional animal experiment ethics committee, synaptosomes were prepared by the method of Booth and Clark.²⁴ Adult male Wistar rats were obtained with CO₂ 80% / O₂ 20% (vol / vol) and decapitated. The forebrain of the animal was rapidly removed, dropped into ice-cold isolation medium (0.32M sucrose, 1mM potassium EDTA, 1 μ g⁻¹ ml leupeptin, 10 mM Tris HCl, pH 7.4) and chopped into small pieces. Blood and other debris were washed off the brain tissue. The tissue was then homogenized in a Dounce-type glass homogenizer and spun at 1300 g for three minutes. The supernatant from this spin was centrifuged at 17,000 g for 10 min, producing the crude mitochondrial/synaptosomal pellet. This pellet was resuspended in isolation medium and diluted by six volumes of Ficoll 12/ 0.32M sucrose containing 50 μ M potassium EDTA, pH 7.4. This suspension was

introduced into a centrifuge tube and, above this, 7.5% Ficoll/0.32M sucrose containing 50 μ M potassium EDTA, pH 7.4 was carefully layered. Finally, on top of this, isolation medium without Ficoll was layered. The tubes were centrifuged at 99,000 g for 30 min in a swinging rotor. The synaptosomes suspended at the second interface were gently sucked off and diluted with a medium containing 140 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1 mM MgCl₂, 10 mM D-glucose, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4 with NaOH. Then, the suspension was spun at 5,500 g for 10 min and synaptosomes were collected as a pellet. Throughout the preparation, the temperature of the sample solution was kept at 4°C.

The synaptosome pellet was resuspended in incubation buffer containing 140 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1 mM MgCl₂, 10 mM D-glucose, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mg⁻¹ ml fatty acid free bovine serum albumin, pH 7.4 with NaOH, which was equilibrated with a gas mixture consisting of O₂ 95% and CO₂ 5% (vol/vol). In some experiments, 10 μ M ISP were added to the incubation medium. In the halothane exposure experiments, the gas mixture was applied to the suspension via vaporizer (Vaper 19; Dräger, Lübeck) as described previously.²⁵ The anesthetic concentration was monitored continuously by a gas monitor (Datex Instrument Corp, Helsinki), which was calibrated by gas chromatography (GC 7A; Shimadzu Corp, Kyoto). During the 30 min equilibration, the suspension was kept at 30°C and gently shaken.

Following incubation, the suspension was quickly cooled to 4°C, and the synaptosomes were lysed by sonication in ice-cold hypotonic buffer (1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 μ g⁻¹ ml leupeptin, 10 mM HEPES, pH 7.4). To separate the synaptosomal cytosol and membrane, the lysed synaptosomes were homogenized with a Dounce type glass homogenizer and centrifuged at 200 000 g for 15 min at 4°C. The supernatant was collected as cytosol fraction and the pellet was resuspended in the hypotonic buffer as membrane fraction. Protein concentrations in solutions were measured by the method of Bradford.²⁶

Major protein constitution in each fraction was determined by one-dimensional sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS / PAGE) according to Laemmli's method.²⁷ Proteins in gel were stained by Commassie brilliant blue.

Phosphorylation of recombinant β 2-adrenergic receptor by synaptic cytosol or membrane

Phosphorylation of recombinant β 2-adrenergic receptor was based on the method by Frederick *et al.*²⁸

Reaction mixture was containing 500pM receptor, 20 mM Tris-HCl (pH 8), 2 mM EDTA, 10 mM MgCl₂, 1 mM dithiothreitol and 100 μ M (-)ISP. After the pre-warming at 30°C, phosphorylation reaction was initiated by adding sample solution and 50 μ M [γ -³²P]ATP. The mixture was incubated at 30°C for 20 min and the reaction was stopped by adding double concentration Laemmli's sample buffer.²⁷ Phosphoproteins were analyzed by one- dimensional SDS/PAGE according to Laemmli's method and followed by autoradiography. The relative amounts of phosphorylation were quantified using an image analyzer (Personal Densitometer; Molecular Dynamics Inc., Sunnyvale, CA).

Phosphorylation of synthetic peptide

Peptide phosphorylation was performed according to the method by Pitcher *et al.*²⁹ Peptide (1 mM) and synaptosome fraction were incubated in 20 mM Tris-HCl, pH 7.2, 2 mM EDTA, 7.5 mM MgCl₂, and 50 μ M [γ -³²P]ATP at 30°C for 15 min. Reactions were stopped by spotting onto P-81 phosphocellulose paper. Free [γ -³²P]ATP was subsequently removed from the paper by washing in 75 mM phosphoric acid as described by Cook *et al.*³⁰ The radioactivity trapped on the paper was measured using a liquid scintillation counter (Aloca 650; Aloca Inc., Tokyo). The GRK-2 mediated peptide phosphorylation was determined by subtracting the radioactivity count incorporated in the absence of peptide from the count incorporated in the presence of this substrate. Autophosphorylation of the peptide was measured by incubating the peptide in the reaction mixture without synaptosome fraction, and determined to be ignorable.

Immunodetection by anti-GRK-2 antibody

For protein blotting, 20 μ g proteins were electrophoresed on SDS/PAGE and transferred electrophoretically to a nitrocellulose membrane in blotting buffer containing 25 mM Tris hydroxymethyl aminomethane, 192 mM glycine, SDS 0.02%, methanol 20%, pH 8.3 using a standard blotting well (NA-1512; Nippon-Eido Inc.; Tokyo). After blotting, the membrane surface was blocked by non-fat milk solution 5% for one hour at room temperature and then blots were incubated with anti-GRK-2 polyclonal antibody in Tris buffered saline with 0.05% tween 20 at dilution ratio 1: 500. The immunoreactivity was detected by sequential application to biotinylated goat anti-rabbit IgG, streptavidin-biotinylated alkaline phosphatase complex and alkaline phosphatase color development buffer. Incubation time and washing condition were determined according to the manufacturer's recommendations (BioRad Inc.; Hercules, CA).

The relative densities of the blots were quantified by an image analyzer (Personal Densitometer; Molecular Dynamics Inc., Sunnyvale, CA).

Statistical analysis

The data from six independent animal experiments were expressed as mean $\pm$ SEM. Comparisons between groups were assessed by one-way analysis of variance followed by Scheffe's post-hoc test.

Results

There was no major alteration of cytosolic or membrane protein band pattern either after exposure to halothane or incubation with 10 μ M ISP (Figure 1). The 67kDa protein was the major phosphoprotein in samples containing the recombinant receptor. Although several other phosphoproteins were visible on the gels, the intensities of phosphorylation were minor. Exposure to halothane 2% with 10 μ M ISP, decreased the phosphorylation of the recombinant receptor in cytosol fraction, but increased in membrane fraction (Figure 2), implying that phosphorylation activity shifted from cytosol to membrane.

Phosphorylation of the synthetic peptide (which is preferentially phosphorylated by GRK-2, but not by PKA and PKC) decreased in the cytosol fraction obtained from synaptosomes exposed to halothane 2% in the presence of 10 μ M ISP. In contrast, activity in the membrane fraction increased by exposure to halothane 2% with or without ISP. (Figure 3)

The amount of GRK-2 decreased in the cytosol obtained from synaptosomes exposed to halothane 1% or 2% in the presence of 10 μ M ISP ($8.3 \pm 1.2\%$ decrease by halothane 1%, $18.0 \pm 2.1\%$ by halothane 2%). In the membrane fraction, the GRK-2 amount was increased simply by the presence of 10 μ M ISP ($19.5 \pm 3.1\%$ increase). Exposure to halothane 1% or 2% in the presence of 10 μ M ISP further increased the amount in the membrane dose dependently ($22.5 \pm 3.1\%$ increase by halothane 1%, $45.7 \pm 6.1\%$ by halothane 2%). Two percent halothane increased GRK-2 in synaptosomal membrane in the absence of 10 μ M ISP ($10.8 \pm 3.3\%$ increase). (Figure 4)

Discussion

Translocation of kinases

In our first experiment, we observed that phosphorylation of the β 2-adrenergic receptor decreased after exposure to halothane in synaptosomal cytosol and increased in the membrane fraction, implying that phosphorylation activity shifted from cytosol to membrane. Since the receptors are only phosphorylated in the membrane, this finding indicates that, after expo-

FIGURE 1 Synaptosomal proteins after exposure to halothane and isoproterenol.

The protein band pattern on gel was not altered either by exposure to halothane or by incubation with 10 μ M ISP in cytosol or membrane subfractions. Ten μ g cytosolic proteins or 50 μ g membrane proteins were run on acrylamide 10% gel. The cytosolic proteins were detected by silver staining solution, and the membrane proteins were stained by Coomassie brilliant blue. Lane 1 shows protein pattern following control experiment. Lane 2, 3 and 4 shows protein pattern following the exposure to 10 μ M ISP, halothane 2% and the both, respectively. Molecular weights of standard proteins were indicated on the left.

sure to halothane, β 2-adrenergic receptors should be more easily phosphorylated and down-regulated. It has been reported that this receptor can be phosphorylated by three different types of kinase, PKA, PKC and GRK-2.³¹ Among two different types of PKC (conventional PKC and atypical PKC), conventional PKC is the major calcium dependent protein kinase acting in synapse.³² Considering conventional PKC is only activated in the presence of Ca^{2+} ,³² the role of conventional PKC may be minor in the present study, where Ca^{2+} was deleted from phosphorylation medium.

In the second experiment, a part of the β 2-adrenergic receptor was used as a phosphorylation substrate, which cannot be phosphorylated by PKA or PKC.³³ Again, phosphorylation of the substrate decreased after exposure to halothane in synaptosomal cytosol and increased in the membrane fraction. This means GRK-2 activity shifted from the cytosol to the membrane after exposure to halothane. From this experiment, however, it is impossible to clarify whether this phenomenon was derived from translocation of the enzyme molecule or from regulation of

FIGURE 2 Phosphorylation activity of the recombinant β_2 adrenergic receptor. In cytosol, exposure to halothane 2% with 10 μ M ISP reduced phosphorylation activity to the recombinant β_2 adrenergic receptor. In membrane, exposure to halothane 2% with 10 μ M ISP increased phosphorylation activity to the β_2 adrenergic receptor. * Differences compared with control phosphorylation activity ($P < 0.01$).

the enzymatic activity without movement of the molecule. In the third experiment, we examined the amount of GRK-2 in each fraction by Western analysis. It showed that the shifting of β_2 -adrenergic receptor phosphorylation activity was likely caused by the translocation of the enzyme from cytosol to membrane in synaptosome.

In all three experiments, the changes in membrane fraction were more prominent than those in the cytosol. According to Inglese *et al.*, GRK-2 molecules are stored primarily in the cytosol.³⁴ The enzymes are translocated and activated by homologous ligand-binding or PKC activation after heterologous ligand-binding.^{3,5} Because the total amount of enzyme in the membrane was less than in the cytosol, the translocation of a set of enzymes from the cytosol to the membrane should have more apparent effect in the membrane.

FIGURE 3 Phosphorylation activity to a synthetic substrate peptide. Phosphorylation activity of the synthetic peptide, which can be phosphorylated by β ARK but not by PKC and PKA, decreased in cytosol fraction obtained from synaptosomes exposed to halothane 2% ($P < 0.05$). In contrast, the activity in membrane fraction increased by the exposure to halothane 2% ($P < 0.01$).

Similar translocation of phosphorylation enzymes in the presence of inhalational anesthetics has been described for PKC by Hemmings and Adamo.^{21,22} They demonstrated that halothane 2.4% facilitated translocation of conventional PKC from cytosol to membrane and down-regulation in rat cerebrocortical synaptosomes. In their experiment, the simultaneous presence of phorbol ester was required for the action. In the present study, translocation of GRK-2 was accelerated by halothane in the presence of a β_2 -adrenergic activator, ISP, but minor facilitation was also observed in the absence of the ligand. This ligand independent effect might be explained by the physicochemical action of volatile anesthetics on lipid bilayer.^{3,6}

Function of GRKs

G-protein coupled receptor kinases (GRKs) are phosphorylation enzymes responsible for light- or ligand-

FIGURE 4 Comparative βARK amount densitometrically measured following Western blotting. Cytosolic fraction: amount in the control experiment is set at 100%. The βARK amount decreased in the cytosol obtained from synaptosomes exposed to halothane 1% (¶: $P < 0.05$) or 2% (*: $P < 0.01$) in the presence of 10 μM ISP. Dagger shows significant reduction compared to the βARK amount after the exposure to 10 μM ISP alone (†: $P < 0.01$). Membrane fraction: amount in the control experiment is set at 100%. The βARK amount increased simply by the presence of 10 μM ISP (§: $P < 0.05$). Exposure to halothane 1% or 2% further increased the amount, dose dependently (: $P < 0.01$). Two percent halothane increased βARK in synaptosomal membrane in the absence of 10 μM ISP (**: $P < 0.05$). Double dagger shows significant increase compared to the βARK amount after the exposure to 10 μM ISP alone (‡: $P < 0.01$). Inlet shows a representative Western blotting pattern. Lane 1 shows βARK following control experiment. Lanes 2, 3 and 4 shows the protein following the exposure to 10 μM ISP, halothane 2% and the both, respectively. Molecular weights of standard proteins were indicated on the left.

dependent phosphorylation of receptors.^{3,7} Several GRKs have been cloned and sequenced by molecular biological approaches, and their unique function in receptor down regulation has been identified. GRK2, originally named β-adrenergic receptor kinase 1 (βARK1), is an 80 kDa protein that preferentially phosphorylates β2-adrenergic receptors in a ligand-depen-

dent manner.^{2,3} The binding of the ligand to the β2-adrenergic receptor promotes translocation of the enzyme from the cytosol to the membrane and activates the activity.^{3,7} Phosphorylation of β2-adrenergic receptor by GRK2 facilitates the binding of β-arrestins and leads to internalization of the receptor protein.^{3,8} By overexpressing βARK1 in mice, Koch *et al.* demonstrated that this enzyme was a key protein in β2-adrenergic receptor down-regulation in the heart.^{3,9} Recently, it was reported that the activity and content of this enzyme were closely related to clinical etiology of heart failure,⁴⁰ ischemic heart disease⁴¹ and hypertension.⁴² In the central nervous system, Terwilliger *et al.* showed that opioid tolerance correlates with the expression of GRK2 and β-arrestin.⁴³ Also, Nishimura *et al.* demonstrated that receptor phosphorylation through GRK-2 have important roles in pain sensation.⁴⁴

The translocation and activation of GRK-2 observed in the present study, implies that exposure to halothane should accelerate the phosphorylation and the desensitization of β2-adrenergic receptors. Therefore, anesthetics have the potential of modifying the G-protein mediated signal transduction by controlling phosphorylation status of receptor proteins. Since the β-adrenergic receptor system (both β1- and β2-) is known to regulate consciousness and alertness,^{5,6} the desensitization of the system may have some role in the anesthetic action of halothane. The two subtypes of β-adrenergic receptors often colocalize and share functions in the central nervous system.³ Although sympathetic synapses in heart were not examined in this study, it is possible that accelerated phosphorylation to β-adrenergic receptor is responsible for the suppressive action of volatile anesthetics on heart dynamics.^{4,5}

Anesthetics and G-protein coupled receptor signal transduction

The influence of volatile anesthetics on G protein coupled receptor signal transduction is relatively well documented for the muscarinic acetylcholine receptor family. Anthony *et al.*^{4,6} and Aronstam *et al.*^{5,3} demonstrated that liquid volatile anesthetics had the ability to disrupt the muscarinic receptor-G protein signal cascade, although the precise site of the inhibition has yet to be determined.² For β-adrenergic receptors, Sanuki *et al.* reported that anesthetics depressed the intracellular signal transduction evoked by isoproterenol.^{1,3} More recently, Schmidt *et al.*^{1,4} and Hanouz *et al.*^{15,16} demonstrated that volatile anesthetics affected adrenergic signal transduction systems in the heart. These results suggest that anesthetics interfere with G-protein coupled receptor signals in several tissues. G pro-

tein itself can be a target of anesthetics, but the experimental results concerning interaction between anesthetics and G protein are still controversial. For example, Puig *et al.* described that halothane may directly alter G protein function at clinically relevant concentrations.⁴⁷ In contrast, Morimoto *et al.* concluded that halothane did not affect pertussis toxin-sensitive G proteins.⁴⁸ Although they did not pay attention to phosphorylation enzymes surrounding G proteins, the results of this study implied that interaction between anesthetics and GRK could play an important role in their experimental model. GRKs consist of multiple subtypes, which have variable substrate specificity, phosphorylation efficiency and tissue distribution.^{38,39} Similar to the variability reported for the effects of anesthetics on G-proteins, the effects of anesthetics on GRKs may also vary. Further detailed studies concerning the interaction between anesthetics and each subtype in the GRK family, will be indispensable to understand the complex actions of anesthetics on cellular function.

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