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Volatile anesthetics inhibit calcitonin gene-related peptide (CGRP) receptor-mediated responses in pithed rats and human neuroblastoma cells

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Running title: Volatile anesthetics inhibit CGRP mediated responses

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Abbreviations: CGRP, calcitonin gene-related peptide; ^{125}I-CGRP, (2-[^{125}I]iodohystidyl^{10}) CGRP; cAMP, adenosine 3',5'-cyclic monophosphate; GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate; GTPγS, guanosine 5'-O-(3-thio)triphosphate; G protein, guanine nucleotide (GTP)-binding protein; Gs, stimulatory G protein; Gsα, α-subunit of Gs; GPCR, G protein-coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; MAP, mean arterial pressure; MEM, minimal essential medium; NANC, nonadrenergic, noncholinergic; SK-N-MC cells, human neuroblastoma cells; SVR, systemic vascular resistance

Cardiovascular section

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Abstract

Calcitonin gene-related peptide (CGRP) has a potent vasodilatory effect, which is mediated by specific receptors predominantly coupled to the activation of adenylate cyclase. The effects of volatile anesthetics on CGRP-induced vasodilation are unclear. We studied the effects of sevoflurane and isoflurane on CGRP-induced vasodilation in pithed rats and CGRP receptor-mediated responses in SK-N-MC cells, which are used as a model system to study the CGRP receptor and its downstream pathways. Male Wistar rats were pithed by inserting a stainless steel rod into the spinal cord. Mean arterial pressure (MAP) and cardiac output (CO) were maintained at approximately 100 mmHg and 50 ml · min^{-1}, respectively, with continuous infusion of noradrenaline. After 30 min inhalation of anesthetics, CGRP (0.1, 0.3, 1.0, and 3.0 μg/kg) was administered intravenously. In SK-N-MC cells, CGRP-, forskolin-, or cholera toxin-induced cAMP production was measured with or without anesthetics using radioimmunoassays. CGRP receptor binding density and affinity for the agonist were determined with (2-[^{125}I]iodohystidyl^{10}) CGRP with or without the anesthetics. Sevoflurane (4%) and isoflurane (2%) significantly inhibited the decrease in MAP and systemic vascular resistance (SVR). Furthermore, both anesthetics significantly inhibited CGRP-, but not forskolin-, induced cAMP production. Sevoflurane (4%) and isoflurane (4%) significantly inhibited cholera toxin-induced cAMP production. Both anesthetics did not affect ligand binding. These data suggest

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that sevoflurane and isoflurane inhibit CGRP-induced vasodilation at the site between the CGRP receptor and adenylate cyclase activation. The inhibitory site of volatile anesthetics on the CGRP receptor-mediated response involves Gs protein.

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Introduction

Peripheral vascular tone is maintained primarily by norepinephrine released from sympathetic adrenergic nerve terminals. Nonadrenergic nerves also directly innervate regional vascular beds, and nonadrenergic vasodilation occurs in many species (Bevan et al., 1987). Perivascular nerve stimulation in mesenteric vascular beds induces neurogenic vasodilation; this effect is mediated by nonadrenergic, noncholinergic (NANC) nerves (Kawasaki et al., 1988). Primary afferent neurons, originating from the dorsal root ganglia, provide a perivascular network of fibers around the arterial system throughout the body. Stimulation of these fibers causes NANC vasodilation via the release of calcitonin gene-related peptide (CGRP) (Bell et al., 1996).

CGRP is a 37-amino acid peptide that is generated by alternative RNA splicing processes during calcitonin gene expression in rat and human tissues (Bell et al., 1996). CGRP binds to a specific cell surface receptor, a seven-transmembrane domain receptor protein, which belongs to a subgroup of the G-protein-coupled receptor family (McLatchie et al., 1998). The receptor is primarily coupled to the activation of adenylate cyclase, which produces cyclic adenosine monophosphate (cAMP), leading to smooth muscle relaxation (Bell et al., 1996). CGRP is widely distributed in perivascular nerves throughout the cardiovascular system. It has a potent vasodilatory and positive inotropic and chronotropic effects in rat and human (Bell et al., 1996). Intravenous administration of

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CGRP causes a significant decrease in the mean arterial blood pressure (MAP), total peripheral resistance, and sustained tachycardia in rats and humans (Bell et al., 1996). CGRP is implicated in the regulation of circulatory homeostasis and pathophysiology of various diseases. For example, CGRP is known as a potent vasodilator in brain vessels and has been implicated in the pathogenesis of migraine (Moreno et al., 2002). Increased plasma levels of CGRP have been demonstrated in endotoxemia, congestive heart failure (Bell et al., 1996), hepatic cirrhosis (Henriksen et al., 2000), and during cardio-pulmonary bypass (Terazawa et al., 2003). The development of autonomic dysfunction in a mouse model of spinal cord injury is associated with sprouting of CGRP fibers (Jacob et al., 2001). These data suggest that CGRP is responsible for the hemodynamic alterations in these disorders. Because general anesthetics are often used for patients with such orders, it is important to elucidate the effects of general anesthetics on the CGRP-induced hemodynamic response for better hemodynamic management of complicated patients.

The pithed rat model is used to investigate cardiovascular responses mediated by autonomic outflow from the spinal cord *in vivo* (Gillespie et al., 1967). In pithed animals, there is no intrinsic sympathetic nerve activity or centrally mediated compensatory reflex (Gray et al., 1990). The depressor response during spinal cord stimulation in the pithed rat is attributed to endogenous CGRP released from CGRP-containing nerves (Taguchi et al., 1992).

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Volatile anesthetics inhibit NANC depressor responses through inhibition of the CGRP depressor effect, not through inhibition of CGRP release after NANC nerve stimulation in the pithed rat model (Yoshikawa et al., 2003), suggesting that volatile anesthetics inhibit vascular responses to CGRP. Anesthetic actions on cardiac output (CO), systemic vascular resistance (SVR), and CGRP receptor-mediated cellular responses, however, have not yet been studied.

The purpose of the present study was to investigate the effects of volatile anesthetics on 1) CGRP-induced vasodilation by evaluating the changes in hemodynamic parameters, including MAP, CO, and SVR *in vivo*, and 2) the CGRP receptor-mediated signal transduction pathway *in vitro*. In the *in vivo* study, we used a pithed rat model to evaluate the direct vascular effect of drugs without interference of centrally mediated circulatory reflexes. In the *in vitro* study, we used SK-N-MC human neuroblastoma cells, as a model system to study the CGRP receptor and its down-stream pathways (Van Valen et al., 1990; Muff et al., 1992; Poyner et al., 1998; Choksi et al., 2002).

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Methods

All surgical procedures and experimental protocols were approved by the Gunma University Institutional Animal Care and Use Committee.

Animal preparation

Surgical procedures were performed as described previously (Shiga et al., 1995; Yoshikawa et al., 1998; 2003). Male Wistar rats weighing 300 to 360 g were anesthetized with isoflurane in oxygen. The animals were ventilated artificially using a Harvard respirator (Harvard Apparatus, South Natick, MA) via a tracheal cannula at a rate of 50 to 60 breaths · min^{-1} with a stroke volume of 1 ml · 100 g body $weight^{-1}$. Respiration rate was adjusted to maintain a $PaCO_2$ of 35 to 40 mmHg. A polyethylene (PE 50) catheter was placed in the left carotid artery to measure MAP. Both vagus nerves were severed in the neck. The rats were pithed by inserting a stainless steel rod (diameter, 1.5mm) through the right orbit and foramen magnum down the spinal canal to its sacral terminus. This procedure destroys the entire central nervous system. Isoflurane anesthesia was discontinued immediately after pithing. Following a median sternotomy, a flow probe (Transonic Systems Inc., Ithaca, NY) was placed around the ascending aorta to measure aortic blood flow, i.e., CO. The right jugular vein was cannulated with PE-50 for the

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administration of norepinephrine. A 24-gauge Teflon cannula (Angiocath; Desert Medical, Sandy, UT) was placed in the tail vein for the administration of CGRP. Body temperature was maintained between 36 to 37°C using a heated blanket placed beneath the animal and controlled by a rectal thermistor probe (CMA/150; Stockholm, Sweden).

Experimental measurements

MAP was recorded with a Gould (Cleveland, OH) pressure transducer connected to the left carotid artery via a PE-50 catheter. Aortic blood flow was measured using a volume flow meter (Transonic Systems Inc.). CO and SVR were normalized by the body weight using the following formulae: CO = ml $\cdot$ min^{-1} $\cdot$ kg body weight^{-1}, and SVR (mmHg $\cdot$ ml^{-1} $\cdot$ min^{-1} $\cdot$ kg body weight^{-1}) = MAP / CO. SVR was determined when MAP and CO reached their minimal values.

Cell culture

The SK-N-MC cell line was obtained from the American Type Culture Collection. The cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C, 95% humidity, and

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5% CO_2. For cAMP assays, the cells were seeded on 100-mm culture dishes and grown to confluence. For binding studies, the cells were seeded on 24-well plates at a density of approximately 5×10^4 cells / well and grown to confluence.

Assay of cAMP production

Equilibration with volatile anesthetics

Preincubation with volatile anesthetics was performed as described by Still et al. (1993) with minor modifications. Serum-free MEM without cells at 37°C (4 ml) was aerated in semiclosed glass vials (5ml) for 20 min with 95% O_2 and 5% CO_2 gas mixture at 50 ml/min to which volatile anesthetic was added using a calibrated anesthetic vaporizer. After 20 min equilibration with anesthetics, the gas mixture containing an appropriate concentration of anesthetic was passed continuously over the medium to minimize the loss of anesthetic. The cells (5×10^6 cells / ml) were gently added to the medium at 37°C and preincubated with anesthetic for 20 min before the addition of CGRP or forskolin, a direct adenylate cyclase activator or cholera toxin, a Gs protein activator. Gas chromatographic analysis (GC-7A, Shimadzu, Kyoto, Japan) of the media at the end of the protocol indicated that exposure to 1% (v/v) sevoflurane, 2% sevoflurane, 4% sevoflurane, 0.5% isoflurane, 1% isoflurane and 2% isoflurane corresponded to

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actual concentrations of 0.969 ± 0.116, 2.181 ± 0.211, 3.991 ± 0.119, 0.482 ± 0.015, 1.086 ± 0.154 and 1.978 ± 0.155 % (v/v), respectively. Cell suspension for control experiments was prepared in the same manner, but without anesthetics.

Measurement of cAMP

Suspensions of SK-N-MC cells were used for determining cAMP production. At the end of each exposure protocol, incubation was terminated by adding 400 µl 1N HCl to a 4-ml cell suspension and strong vortexing. The mixture was frozen quickly in liquid nitrogen and stored at –70°C until cAMP determination. After thawing, the cell debris in the mixture was pelleted by centrifugation (13000 g for 10 min at 4°C), and the supernatant was transferred to a test tube for the cAMP assay. A cAMP [^{125}I]- radioimmunoassay kit (YAMASA, Choshi, Japan) was used to determine the cAMP levels. All assay procedures were performed according to the manufacturer's instructions. Data were normalized for protein content using Bradford's method (Bradford., 1976).

Binding studies

Equilibration with volatile anesthetics

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Binding studies were performed at 37°C in an air-tight glass chamber (50 l) under a continuous flow of a 95% O_2 and 5% CO_2 gas mixture at 3 l/min supplemented with 4% sevoflurane or 2% isoflurane using a calibrated anesthetic vaporizer. The anesthetic concentrations in the chamber were monitored continuously with a calibrated gas analyzer (Capnomac Ultima; Datex, Helsinki, Finland). Eagle's MEM was equilibrated by continuous bubbling with the corresponding concentration of anesthetic for 20 min at 37°C in semiclosed glass vials. Following the preparation, a gas mixture containing the anesthetic was passed continuously over the medium to minimize the loss of anesthetic. Then, 1% bovine serum albumin was added to the medium and incubated with anesthetic for 30 min at 37°C. The solution equilibrated with anesthetic was used as the binding buffer. The anesthetic concentrations in the buffer were measured by gas chromatography (GC-7A, Shimadzu, Kyoto, Japan). Control experiments were performed in the same manner without anesthetic.

Analysis of binding data

Saturation binding studies were performed using (2-[^{125}I]iodohystidyl^{10}) CGRP (2000 Ci/mmol; Amersham, UK) at concentrations ranging from 5 to 200 pM per well in the absence (total binding) or presence (nonspecific binding) of excessive unlabeled human alpha CGRP (1 μM) (Peptide Institute, Inc. Osaka, Japan). Specific binding was

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determined as the difference of total binding and nonspecific binding. Scatchard analysis was used to determine receptor density (B_{max}) and the ligand dissociation constant (K_d) (Scatchard, 1949).

Experimental protocols

Protocol 1: The effects of volatile anesthetics on hemodynamic changes induced by CGRP in pithed rat

Pithed rats were allowed to stabilize for 30 min before any experimental intervention. MAP and CO were maintained at approximately 100 mmHg and 50 ml · min^{-1} by continuous infusion of norepinephrine (2-3 μg · kg^{-1} · min^{-1}) throughout the protocol. Rats were randomly assigned to anesthetic groups (n = 7, each), no anesthetic group (n = 7), and there was a 1 h waiting period after the termination of isoflurane administration to avoid the effects of isoflurane. In the anesthetic groups, 1 h after pithing, a volatile anesthetic (2% sevoflurane, 4% sevoflurane, 1% isoflurane or 2% isoflurane) was administered for 30 min. Concentrations of inspired anesthetics were monitored continuously with a calibrated gas monitor (Capnomac Ultima; Datex, Helsinki, Finland). In the no - anesthetic group, no volatile anesthetic was administered. Rat alpha CGRP (0.1, 0.3, 1.0, and 3.0 μg · kg^{-1}; Peptide Institute, Inc.) was administered intravenously for 30 s in 0.1 ml of saline.

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Protocol 2: The effects of volatile anesthetics on cAMP production in SK-N-MC cells

SK-N-MC cells were preincubated for 20 min with or without anesthetics in media containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemical Co., St. Louis, MO) to limit cAMP breakdown. Human alpha CGRP (10^{-7} M), forskolin ($10^{-6.5}$ M; Sigma Chemical Co.), or cholera toxin (10^{-9} M; Wako, Osaka, Japan) was added to the cells. Cell suspensions were incubated for 15 min (the appropriate times for cholera toxin) at 37°C with or without anesthetics, then used for cAMP assay. CGRP induced maximal cAMP production at 10^{-7} M in SK-N-MC cells, and $10^{-6.5}$ M forskolin and 10^{-7} M CGRP induced comparable cAMP production in our preliminary studies. The dose of cholera toxin and the incubation time were determined as described previously (Orlandi et al., 1993). Incubation with 10^{-9} M cholera toxin for 2h induced maximal cAMP production. The amount was comparable to that of 10^{-7} M CGRP or $10^{-6.5}$ M forskolin in the current study.

Protocol 3: The effects of volatile anesthetics on ligand-receptor binding

Binding studies were performed as described by Muff et al. (1992) with minor modifications. Confluent cell monolayers (approximately 2×10^5 cells per well), grown on 24-well plates, were washed once with binding buffer; MEM supplemented with 1% bovine serum albumin. They were then incubated with binding buffer

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equilibrated with volatile anesthetic in the experimental chamber, saturated with the anesthetic, and incubated for 20 min at 37°C. ^{125}I-CGRP (with or without unlabeled CGRP) was added to each well, and incubated for 15 min at 37°C in the chamber. Unbound label was then removed by aspiration of binding buffer. Cells were washed once with 500 µl ice-cold binding buffer and subsequently lysed with 500 µl 0.5% sodium dodecylsulfate. Lysates were then counted for radioactivity in a gamma counter. The procedures in the experimental chamber were performed using manipulation arms that were tightly sealed to the side of the chamber. The total incubation volume was 300 µl. The binding buffer contained 30 µl of ^{125}I-CGRP solution.

Statistical analysis

Statistical comparisons within groups were assessed using analysis of variance. When differences were significant, multiple intergroup comparisons were performed with Scheffe's test. All values are expressed as the mean ± SD. A p value of less than 0.05 was considered statistically significant.

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Results

The effects of volatile anesthetics on CGRP-induced hemodynamic changes in pithed rats

Base-line values of hemodynamic parameters and the infusion rates of norepinephrine before the administration of CGRP at 0.1 μg / kg in control group were as follows; MAP = 109.0 ± 4.6 mmHg, CO = 154.1 ± 6.3 ml · min^{-1} · kg^{-1}, SVR = 0.709 ± 0.05 mmHg · ml^{-1} · min^{-1} · kg^{-1}, and norepinephrine = 2.2 ± 0.9 μg · kg^{-1} · min^{-1}, respectively. There was no significant difference in these data among groups (data not shown). Consistent with our previous reports, intravenously administered CGRP induced a transient increase in MAP followed by a persistent decrease. MAP and CO changed similarly. MAP and CO maximally increased immediately after injection of CGRP, and reached the minimal value approximately 1 min after injection of CGRP. SVR also decreased 1 min after administration of CGRP. Injection of saline had no effect.

The effects of sevoflurane on CGRP-induced changes of the hemodynamic parameters are shown in Fig. 1. Sevoflurane significantly inhibited CGRP-induced increases in MAP ($p<0.01$, n = 7). On the other hand, CGRP induced a dose-dependent decrease in MAP, CO, and SVR. Sevoflurane concentration-dependently inhibited the CGRP-induced decrease in MAP and SVR ($p<0.05$, n = 7). The decrease in CO did not differ significantly among groups. Heart rate did not change significantly after administration of CGRP in either group. Isoflurane also

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inhibited CGRP-induced increases in MAP ($p<0.05$, n = 7) and dose-dependently inhibited CGRP-induced decreases in MAP and SVR ($p<0.05$, n = 7), without effects on CO reduction and HR (data not shown).

The effects of volatile anesthetics on CGRP, forskolin, or cholera toxin -induced cAMP production in SK-N-MC cells

Sevoflurane did not alter basal cAMP accumulation (Fig. 2A). The effect of sevoflurane on CGRP-induced cAMP production is shown in Fig. 2B. CGRP (10^{-7} M) increased cAMP production in SK-N-MC cells (20-fold increase over basal). Preincubation of cells with sevoflurane concentration-dependently inhibited CGRP-induced cAMP production. Sevoflurane (4%) significantly inhibited CGRP-induced cAMP production to 35% of no anesthetic ($p<0.01$, n = 6). Forskolin ($10^{-6.5}$ M) increased cAMP production in SK-N-MC cells (25-fold increase over basal), whereas preincubation with sevoflurane did not influence the forskolin-induced increase in cAMP (Fig. 2C). Preincubation of cells with isoflurane also caused a concentration-dependent reduction in CGRP-induced cAMP production. Isoflurane (2% or 4%) significantly inhibited CGRP-induced cAMP production to 46% of no anesthetic ($p<0.05$, n = 6), or to 27% ($p<0.01$, n = 6), respectively, whereas preincubation with isoflurane did not alter forskolin-induced cAMP production (data not shown).

The effects of sevoflurane on time dependent cholera toxin-induced cAMP production in SK-N-MC

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cells were shown in Fig. 3 (inset). After a lag of 15 min, cholera toxin (10^{-9} M) exhibited time dependent increases in cAMP production in each group. Sevoflurane (4%) significantly inhibited cholera toxin-induced cAMP production at 90 min and 120 min (Fig. 3 (inset); $p<0.05$, n = 6). Dose dependence of sevoflurane on cholera toxin-induced cAMP production was shown in Fig. 3. Sevoflurane did not affect basal cAMP production (data not shown). Cholera toxin (10^{-9} M) increased cAMP production in SK-N-MC cells (30-fold increase over basal). Sevoflurane dose-dependently inhibited cholera toxin-induced cAMP production. Sevoflurane (4%) significantly inhibited cholera toxin-induced cAMP production to 70% of no anesthetic ($p<0.05$, n = 6). Isoflurane dose-dependently inhibited cholera toxin-induced cAMP production. Although isoflurane (2%) did not significantly inhibit cholera toxin-induced cAMP production (83% of no anesthetic; $p = 0.10$), isoflurane (4%) significantly inhibited it to 58% of no anesthetic ($p<0.01$, n = 6).

Radioligand Binding Studies

Specific binding of ^{125}I-CGRP to SK-N-MC cells was saturable with increasing concentration of ^{125}I-CGRP (5-200 pM). The receptor density (B_{max}) and the ligand dissociation constant (K_d) calculated from plots according to the method of scatchard are shown in Table 1. There was no significant difference in B_{max} and K_d among groups.

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Preincubation with 4% sevoflurane did not affect CGRP-receptor density and affinity. Isoflurane (2%) had also no effect on B_{max} and K_d compared with no anesthetic and sevoflurane.

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Discussion

Major findings of the present study are summarized as follows. Sevoflurane and isoflurane, at clinically relevant concentrations, dose-dependently inhibited the CGRP-induced increase in MAP and the decrease in MAP and SVR in pithed rats. Sevoflurane and isoflurane inhibited CGRP-induced cAMP production in a dose-related manner in SK-N-MC cells. Neither anesthetic had an effect on forskolin-induced cAMP production in the cells. Neither anesthetic inhibited ligand binding to the CGRP-receptor. Furthermore, both anesthetics dose-dependently inhibited cholera toxin-induced cAMP production in the cells. These data suggest that both anesthetics inhibit CGRP-induced vasodilation by interfering with the signal transduction pathway between CGRP receptors and adenylate cyclase. The inhibitory site of volatile anesthetics on CGRP receptor-mediated response involves Gs protein.

The effects of volatile anesthetics on CGRP-induced hemodynamic changes in pithed rats

Yoshikawa et al. (2003) reported that isoflurane and halothane inhibited the NANC depressor response in pithed rats. Although isoflurane, halothane and sevoflurane did not affect CGRP release after spinal cord stimulation, isoflurane and halothane inhibited the depressor response to exogenously administered CGRP. These

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results suggest that volatile anesthetics inhibit the NANC depressor response by inhibiting the CGRP depressor effect, but not by inhibiting CGRP release.

In a pithed rat model, we investigated not only the effect of volatile anesthetics on CGRP-induced changes in blood pressure but also the CGRP-induced vasodilation by measuring SVR, which reflects the systemic vascular tone. Sevoflurane (4%) and isoflurane (2%) significantly inhibited the CGRP-induced decrease in SVR. Lower concentrations of volatile anesthetics (2% sevoflurane and 1% isoflurane) did not affect the decrease in SVR.

In general, there are four main mechanisms of the vasodepressor response elicited by intravenous drug administration: (i) central nervous system actions; (ii) inhibition of vascular sympathetic transmission; (iii) release of endothelium-derived relaxing factors; and (iv) direct vasorelaxing effects (Saxena et al., 1990). Both central and autonomic nervous system activity were excluded by using pithed, vagotomized rats. Therefore, CGRP-induced vasodilatory effects in the present study were mediated by actions on the vascular endothelium (releasing relaxing factors) and/or the vascular smooth muscle cells (producing direct relaxation). CGRP, among the most potent vasodilating substances known, also exerts positive inotropic effects (van Rossum et al., 1997). These biologic responses are mediated by specific cell surface receptors predominantly coupled to the activation of adenylate cyclase (van Rossum et al., 1997). In the current study, 4% sevoflurane and 2% isoflurane inhibited the CGRP-

induced vasodilatory effects. In addition, 4% sevoflurane and 2% isoflurane inhibited the increase in MAP after CGRP administration, suggesting that high concentrations of volatile anesthetics inhibit both vasodilatory and positive inotropic effects induced by CGRP. These data strongly suggest that volatile anesthetics inhibit the CGRP-induced response through an effect on CGRP-receptor mediated responses.

The effects of volatile anesthetics on CGRP receptor-mediated signal transduction in SK-N-MC cells

CGRP relaxes vascular smooth muscle cells via both endothelium-dependent and endothelium-independent signal transduction pathways (Bell et al., 1996). These responses are mediated by the specific cell surface family B Gs protein-coupled receptor (GPCR) and the calcitonin receptor-like receptor (CRLR) (McLatchie et al., 1998). The receptor is predominantly coupled to the activation of adenylate cyclase, which produces cAMP, leading to vascular relaxation in both pathways. In addition, there are other mechanisms by which CGRP elevates nitric oxide (NO) directly without the involvement of adenylate cyclase, or activates adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channels (Bell et al., 1996). On the other hand, CGRP-mediated inotropic action in the myocardium is mediated through CGRP receptors (van Rossum et al., 1997). In the present study, sevoflurane and isoflurane significantly inhibited CGRP-induced cAMP production but had no effect on forskolin-induced cAMP

production. Neither anesthetic had an effect on CGRP-receptor binding. Furthermore, sevoflurane and isoflurane significantly inhibited cholera toxin-induced cAMP production. Therefore, sevoflurane and isoflurane inhibited CGRP-receptor mediated signal transduction at the step between the CGRP receptor and adenylate cyclase activation, by interfering with the activation of Gs protein.

Volatile anesthetics are likely to affect several hydrophobic sites within the cell membrane, particularly membrane-binding proteins (Franks et al., 1994). Possible sites of the action of anesthetics in the GPCR-mediated signal transduction pathway are divided into four target groups: (a) agonist-receptor binding (b) G-protein function (c) effector activity (adenylate cyclase), and (d) other intracellular sites (e.g., Ca^{2+} stores, cellular kinases) (Lambert., 1993).

There are no previous reports demonstrating an effect of volatile anesthetics on CGRP receptor-mediated signal transduction, although several studies reported that volatile anesthetics affect the signal transduction pathway mediated by other GPCRs. For example, Halothane and isoflurane attenuate isoproterenol-induced vasodilation and interfere with β adrenoceptor-mediated responses in the rat aorta at a point between agonist-receptor binding and adenylate cyclase activation (Tanaka et al., 1998). Pentyala et al. (1999) reported that volatile anesthetics, including sevoflurane and isoflurane, at clinically relevant doses, modulate the binding of guanine nucleotides to purified Gα

sub-units in aqueous solution, inhibiting the exchange of a nonhydrolyzable analog of guanosine 5'-triphosphate (GTPγS) for bound guanosine 5'-diphosphate (GDP). This finding suggested that volatile anesthetics directly affect the guanine-nucleotide binding site, including the Gsα subunit. These studies are consistent with our current study and support the notion that sevoflurane inhibits GPCR-mediated signal transduction pathway at Gs protein level. Furthermore, cholera toxin affect Gs protein by ADP ribosylating the α subunit of Gs. ADP-rybosylation of the Gsα by cholera toxin stabilizes the GTP-bound conformation of Gsα and decreases its intrinsic GTPase activity, resulting in the persistent activation of adenylate cyclase (Neer and Clapham, 1988). In the current study, sevoflurane and isoflurane dose-dependently inhibited cholera toxin-induced cAMP production, suggesting that both anesthetics might affect the conformation and stability of the GTP bound state of Gsα and/or GTPase activity.

On the other hand, isoflurane interferes with muscarinic receptor-G protein interactions in rat brainstem (Anthony et al., 1989). Sevoflurane depresses the β adrenoceptor-mediated signal transduction in rat myocardium by reducing ligand-receptor binding and disrupting the relation between the receptor and G protein (Sanuki et al., 1994). Halothane, which inhibits receptor-activated Giα-coupled pathways in airway smooth muscle cells, must functionally target a component of the G protein-coupled receptor complex other than Giα (Streiff et al., 2003). These studies suggest that volatile anesthetics might inhibit GPCR-mediated signal transduction pathway at the

receptor itself or the interaction between the receptor and G protein. In the current study, isoflurane significantly inhibited CGRP-induced cAMP production at 2 %, whereas isoflurane significantly inhibited cholera toxin-induced cAMP production at 4 %. Therefore, in addition to Gs protein, isoflurane might inhibit CGRP receptor-mediated signal transduction between receptor and Gs protein.

In the present study, we focused on CGRP actions in rat hemodynamic system. CGRP is widely distributed in the body including nervous systems and gastrointestinal system. Further study elucidating CGRP actions in multiple organs may be indispensable to fully understand the roles of CGRP in the body and the importance of circulating cAMP levels in the plasma.

In conclusion, sevoflurane and isoflurane inhibit CGRP-induced positive inotropic and vasodilatory effects in pithed rats. Both anesthetics inhibited CGRP-, and cholera toxin-induced cAMP production. Both anesthetics did not affect forskolin-induced cAMP production and ^{125}I-CGRP ligand-receptor binding in SK-N-MC cells. These results suggest that the inhibitory site of volatile anesthetics on the CGRP receptor-mediated signal transduction involves Gs protein.

References

Anthony BL, Dennison RL and Aronstam RS (1989) Disruption of muscarinic receptor-G protein coupling is a general property of liquid volatile anesthetics. Neurosci Lett 99:191-196

Bell D and McDermott BJ (1996) Calcitonin gene-related peptide in the cardiovascular system: characterization of receptor populations and their (patho) physiological significance. Pharmacol Rev 48:253-288.

Bevan JA and Brayden JE (1987) Nonadrenergic neural vasodilator mechanisms. Circ Res 60:309-326.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Choksi T, Hay DL, Legon S, Poyner DR, Hagner S, Bloom SR and Smith DM (2002) Comparison of the expression of calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) with CGRP and adrenomedullin binding in cell lines. Br J Pharmacol 136:784-792.

Franks NP and Lieb WR (1994) Molecular and cellular mechanisms of general anaesthesia. Nature 367:607-614.

Gillespie JS and Muir TC (1967) A method of stimulating the complete sympathetic outflow from the spinal cord to blood vessels in the pithed rat. Br J Pharmacol 30:78-87.

Gray GA, Furman BL and Parratt JR (1990) Endotoxin-induced impairment of vascular reactivity in the pithed rat: role of arachidonic acid metabolites. Circ Shock 31:395-406.

Henriksen JH, Schifter S, Moller S and Bendtsen F (2000) Increased circulating calcitonin in cirrhosis. Relation to severity of disease and calcitonin gene-related peptide. Metabolism 49:47-52.

Jacob JE, Pniak A, Weaver LC and Brown A (2001) Autonomic dysreflexia in a mouse model of spinal cord injury. Neuroscience 108:687-693.

Kawasaki H, Takasaki K, Saito A and Goto K (1988) Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. Nature 335:164-167.

Lambert DG (1993) Signal transduction: G proteins and second messengers. Br J Anaesth 71:86-95.

McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG and Foord SM (1998) RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393:333-339.

Moreno MJ, Abounader R, Hebert E, Doods H and Hamel E (2002) Efficacy of the non-peptide CGRP receptor antagonist BIBN4096BS in blocking CGRP-induced dilations in human and bovine cerebral arteries: potential implications in acute migraine treatment. Neuropharmacology 42:568-576.

Muff R, Stangl D, Born W and Fischer JA (1992) Comparison of a calcitonin gene-related peptide receptor in a human neuroblastoma cell line (SK-N-MC) and a calcitonin receptor in a human breast carcinoma cell line (T47D). Ann NY Acad Sci 657:106-116.

Neer EJ and Clapham DE (1988) Roles of G protein subunits in transmembrane signalling. Nature 333:129-134.

Orlandi PA, Curran PK and Fishman PH (1993) Brefeldin A blocks the response of cultured cells to cholera toxin. Implications for intracellular trafficking in toxin action. J Biol Chem 268:12010-12016.

Pentyala SN, Sung K, Chowdhury A and Rebecchi MJ (1999) Volatile anesthetics modulate the binding of guanine nucleotides to the alpha subunits of heterotrimeric GTP binding proteins. Eur J Pharmacol 384:213-222.

Poyner DR, Soomets U, Howitt SG and Langel U (1998) Structural determinants for binding to CGRP receptors expressed by human SK-N-MC and Col 29 cells: studies with chimeric and other peptides. Br J Pharmacol 124:1659-1666.

Sanuki M, Yuge O, Kawamoto M, Fujii K and Azuma T (1994) Sevoflurane inhibited beta-adrenoceptor-G protein bindings in myocardial membrane in rats. Anesth Analg 79:466-471.

Saxena PR and Villalon CM (1990) Cardiovascular effects of serotonin agonists and antagonists. J Cardiovasc Pharmacol 15:17-34.

Scatchard G (1949) The attraction of proteins for small molecules and ions. Ann NY Acad Sci 51:660-672.

Shiga T and Yoshikawa D (1995) Platelet-activating factor-induced loss of vascular responsiveness to noradrenaline in pithed rats: involvement of nitric oxide. Eur J Pharmacol 282:151-156.

Sill JC, Eskuri S, Nelson R, Tarara J and Van Dyke RA (1993) The volatile anesthetic isoflurane attenuates Ca^{2+} mobilization in cultured vascular smooth muscle cells. J Pharmacol Exp Ther 265:74-80.

Streiff J, Jones K, Perkins WJ, Warner DO and Jones KA (2003) Effect of halothane on the guanosine 5' triphosphate binding activity of G-protein alpha i subunits. Anesthesiology 99:105-11.

Taguchi T, Kawasaki H, Imamura T and Takasaki K (1992) Endogenous calcitonin gene-related peptide mediates nonadrenergic noncholinergic depressor response to spinal cord stimulation in the pithed rat. Circ Res 71:357-364.

Tanaka S and Tsuchida H (1998) Effects of halothane and isoflurane on beta-adrenoceptor-mediated responses in the vascular smooth muscle of rat aorta. Anesthesiology 89:1209-1217.

Terazawa E, Dohi S, Akamastsu S, Ohata H and Shimonaka H (2003) Changes in calcitonin gene-related peptide, atrial natriuretic peptide and brain natriuretic peptide in patients undergoing coronary artery bypass grafting. Anaesthesia 58:223-232.

Van Rossum D, Hanisch UK and Quirion R (1997) Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. Neurosci Biobehav Rev 21:649-678.

Van Valen F, Piechot G and Jurgens H (1990) Calcitonin gene-related peptide (CGRP) receptors are linked to cyclic adenosine monophosphate production in SK-N-MC human neuroblastoma cells. Neurosci Lett 119:195-198.

Yoshikawa D, Shiga T, Saito S, Morita T, Imai T and Goto F (1998) Platelet-activating factor receptor antagonist attenuates endotoxin-induced vascular hyporeactivity in the pithed rat. Eur J Pharmacol 342:241-245.

Yoshikawa D, Kuroda M, Tsukagoshi H, Takahashi K, Saito S, Nishikawa K and Goto F (2003) The effects of volatile anesthetics on nonadrenergic, noncholinergic depressor responses in rats. Anesth Analg 96:125-131.

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Legends for Figures

Figure 1. The effects of sevoflurane on CGRP-induced changes of hemodynamic parameters. Upper panel, peak increase in mean arterial pressure (MAP) and cardiac output (CO); lower panel, peak decrease in MAP, CO and systemic vascular resistance (SVR). *p<0.05, **p<0.01 vs no anesthetic. Data are mean ± SD; n = 7. CGRP = calcitonin gene-related peptide.

Figure 2. (A) The effects of sevoflurane on steady state cyclic adenosine monophosphate (cAMP) production in SK-N-MC cells. (B) The effect of sevoflurane on CGRP-induced cAMP production. (C) The effect of sevoflurane on forskolin-induced cAMP production.

*p<0.05, **p<0.01 vs CGRP. Data are the mean ± SD from 6 separate experiments performed in duplicate. CGRP = calcitonin gene related peptide, FSK = forskolin.

Figure 3. Dose-dependence relation of sevoflurane on cholera toxin-induced cAMP production in SK-N-MC cells. Cells were incubated in serum-free medium containing 0.5 mM IBMX with or without anesthetic for 20 min at 37°C. Then cholera toxin (10^{-9} M final) was added to the cells, and cells were incubated with or without anesthetic

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at 37°C for 2h and assayed for cAMP. *p<0.05 vs cholera toxin. Data are the mean ± SD from 6 experiments performed in duplicate. SEV = Sevoflurane. *Inset*, time course of cholera toxin-induced cAMP production in SK-N-MC cells: the effects of sevoflurane. Cells were incubated in serum-free medium containing 0.5 mM IBMX with no anesthetic (open circles) or 4% sevoflurane (filled circles) for 20 min at 37°C. Then cholera toxin (10^{-9} M final) was added to the cells, and cells were incubated with or without anesthetic at 37°C for the indicated times and assayed for cAMP. *p<0.05 vs no anesthetic. Data are the mean ± SD from 6 experiments performed in duplicate.

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Table 1. The effects of sevoflurane on maximal binding capacity (B_{max}) and dissociation constant (K_d) in SK-N-MC cells. Data are mean ± SD from 3 experiments performed in duplicate.

	B_{max} (fmol / 10^6 cells)	K_d (pM)
No anesthetic	7.6 ± 1.1	156.8 ± 28.3
4% sevoflurane	6.6 ± 0.9	146.4 ± 24.5

Fig. 1

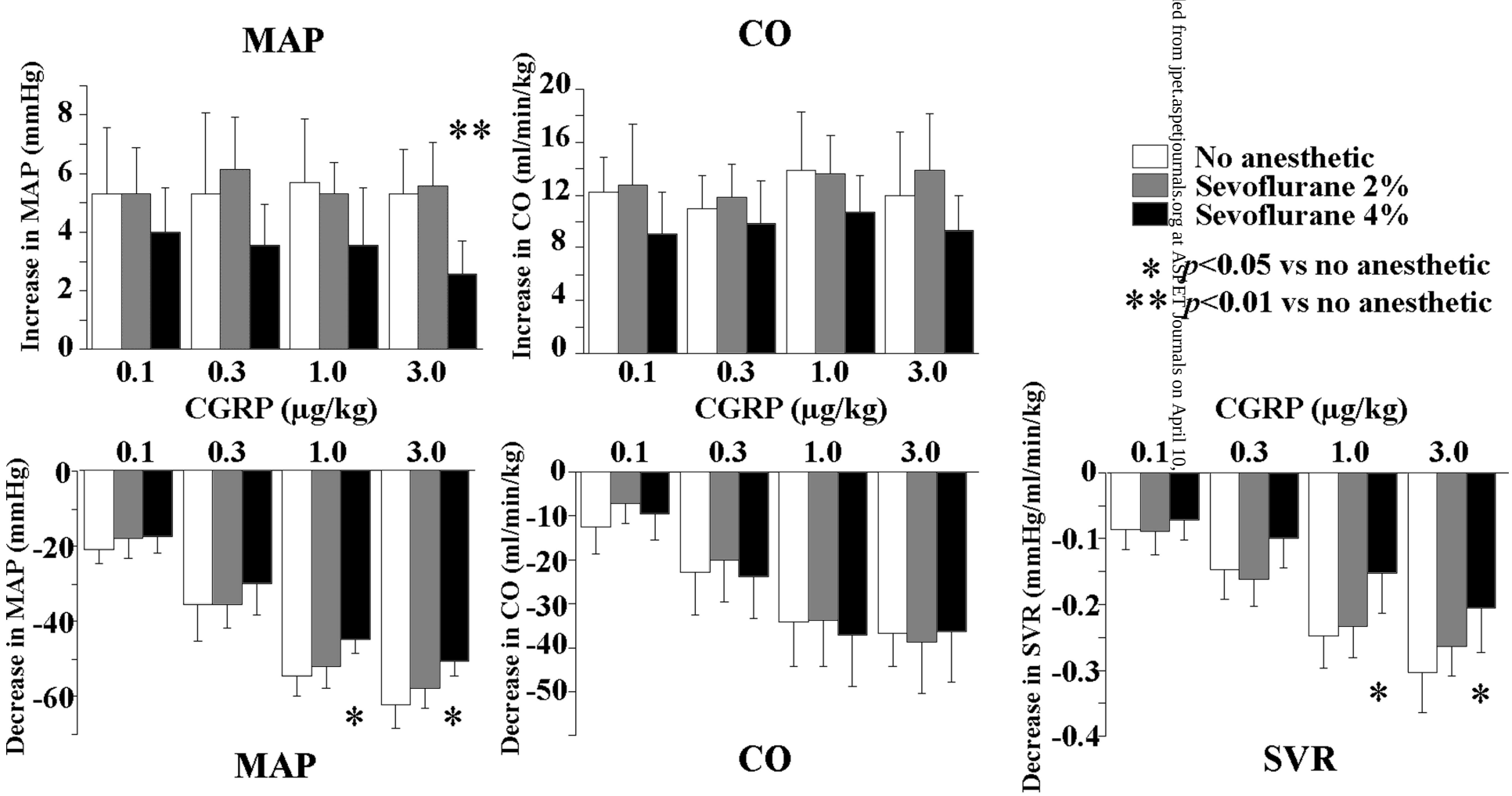

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Fig.2

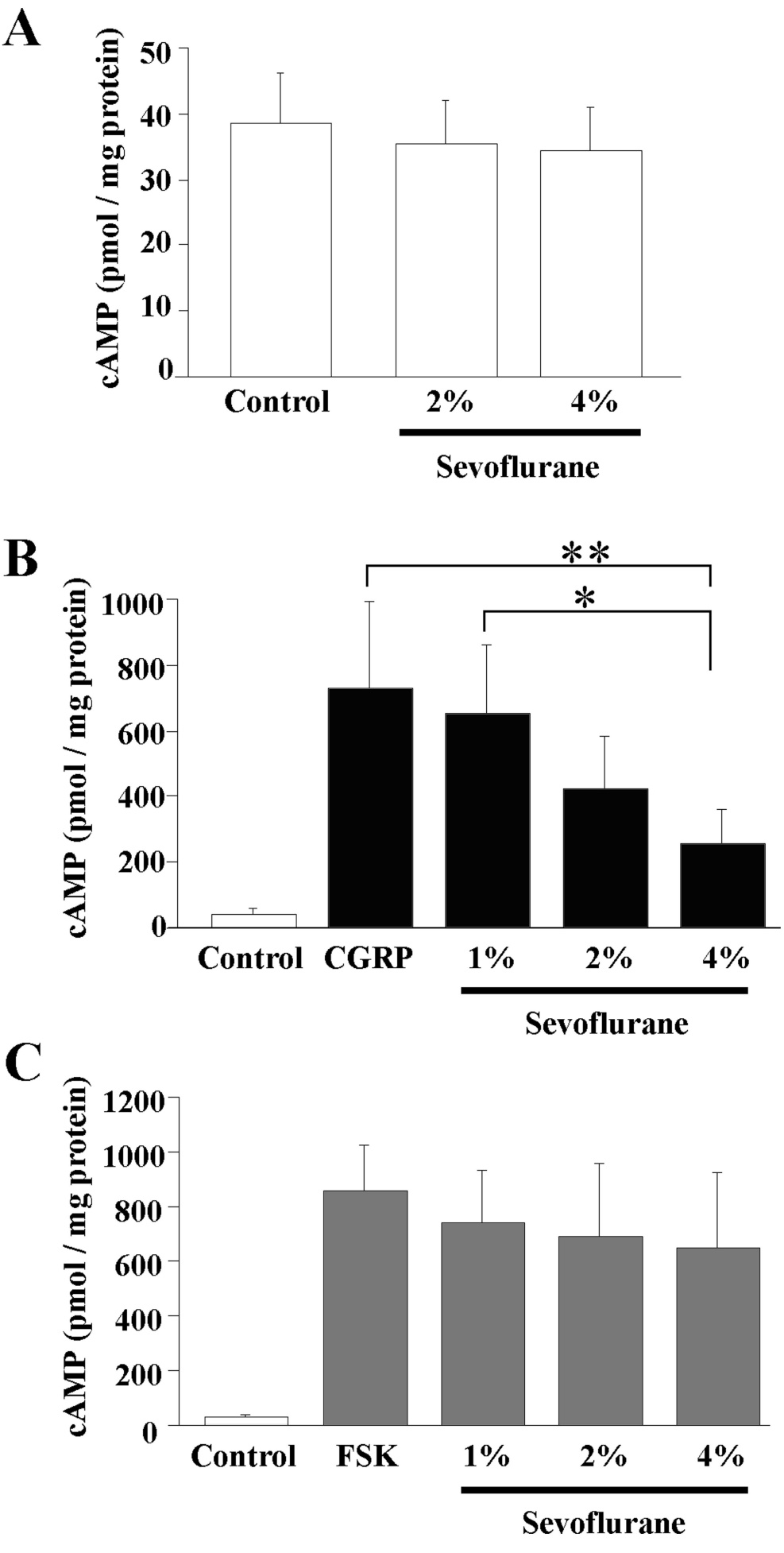

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Fig.3