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Intrathecally Administered cGMP-dependent Protein Kinase Iα Inhibitor Significantly Reduced the Threshold for Isoflurane Anesthesia

Implication for a Novel Role of cGMP-dependent Protein Kinase Iα

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Background: **Inhalational anesthetics have been shown to inhibit the nitric oxide (NO)–cyclic guanosine monophosphate (cGMP) pathway. Previous studies indicated that inhibition of the NO–cGMP pathway decreased the level of consciousness and augmented anesthesia, analgesia, or sedation. The current study investigated the possible involvement of cGMP-dependent protein kinases (PKGs) as major effectors for the NO–cGMP pathway in the anesthetic state.**

Methods: **After initial baseline determination of the minimum alveolar concentration (MAC), a selective cGMP-dependent protein kinase Iα inhibitor, Rp-8-p-CPT-cGMPS, or an NO donor, (NOC-12), were injected intrathecally. Ten minutes later, MAC measurement was repeated. The rats also were evaluated for the presence of locomotor dysfunction by intrathecal administration of Rp-8-p-CPT-cGMPS and NOC-12 in conscious rats.**

Results: **Rp-8-p-CPT-cGMPS at 25, 50, 100, and 200 μg/10 μl produced a significant decrease from isoflurane control MAC of −4 ± 3.1%, 16 ± 4.5%, 30 ± 5.0%, and 21 ± 2.2%, respectively, which was not accompanied by significant changes in either blood pressure or heart rate. In contrast, NOC-12 at 100 μg/10 μl caused an increase from isoflurane control MAC of 23 ± 5.8%, which was accompanied by significant decrease in blood pressure but not in heart rate. Rp-8-p-CPT-cGMPS (100 μg/10 μl) produced a significant reversal of isoflurane MAC increase induced by NOC-12 (100 μg/10 μl), which was accompanied by significant reversal of the reduction of blood pressure induced by NOC-12. Locomotor activity was not changed.**

Conclusions: **The results indicate that cGMP-dependent protein kinase Iα inhibitor not only markedly reduces MAC for isoflurane, but also completely blocks the NO-induced increase in isoflurane MAC, which suggests that cGMP-dependent protein kinase Iα may mediate the action for the NO–cGMP pathway in anesthetic mechanisms at the spinal cord level. (Key words: Cyclic guanosine monophosphate; locomotor activity; minimum alveolar concentration; nitric oxide.)**

IT has been shown that the nitric oxide (NO)–cyclic guanosine 3′,5′-monophosphate (cGMP) signaling pathway is present in the neurons of the spinal cord and contributes to the development of spinal hyperalgesia in models of acute and chronic pain.[1,2] Noxious stimulation increases NO synthase (NOS) expression and cGMP content in the spinal cord.[3–6] Administration of inhibitors of NOS and soluble guanylate cyclase (sGC) causes analgesic effects[7–12] and enhances antinociception mediated by opioid receptors.[13–15] Moreover, the NO donors and cGMP analogs applied intrathecally cause a reduction in tail flick or paw withdrawal latency.[16–19] These data indicate that the NO–cGMP signaling pathway plays an important role in the processing of noxious stimulation at the spinal cord level.

Several studies have shown that inhalational anesthetics depressed cGMP content and modified synaptic transmission in the central nervous system (CNS).[20,21] Previous data from our laboratory indicated that the inhibition of NOS and soluble guanylate cyclase dose-dependently and significantly decreased the minimum alveolar concentration (MAC) of halothane or isoflurane.[22–24] These observations indicate that the NO–cGMP pathway is involved in mechanisms of anesthesia, analgesia, and consciousness in the CNS.

Cyclic GMP–dependent protein kinases (PKGs) have been found to serve as major effectors for cGMP in the vascular and nervous systems.[2,25] We hypothesized that inhibition of PKGs in the spinal cord might result in

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analgesic and anesthetic effects, which may mediate the role of the NO-cGMP pathway in the anesthetic state. To test this hypothesis, we first investigated the effect of a selective PKGIα inhibitor, Rp-8-p-CPT-cGMPS, on isoflurane MAC at the spinal cord level. Second, the effect of Rp-8-p-CPT-cGMPS on NO donor (NOC-12)-induced change in isoflurane MAC was observed. Third, the effect of Rp-8-p-CPT-cGMPS on the locomotor activity was evaluated in conscious rats.

Materials and Methods

This study protocol was approved by the Animal Care Committee at the University of Virginia. Male Sprague-Dawley rats (250-300 g) were housed in different cages on a standard 12 h-12 h light-dark cycle. Water and food were available *ad libitum* until rats were transported to the laboratory approximately 1 h before the experiments. We performed all experiments under normal room light and temperature.

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (45 mg/kg). Long-term intrathecal catheters were inserted by passing a polyethylene-10 (PE-10) catheter through an incision in the atlantooccipital membrane to a position 8 cm caudal to the cisterna at the level of the lumbar subarachnoid space, according to the methods of Yaksh and Rudy.[26] The animals were allowed to recover for a week before experiments were initiated. Rats that showed neurologic deficits postoperatively were removed from the study.

Each rat was placed in a clear plastic cone and anesthetized with 5% isoflurane and oxygen for 3-5 min. The inspired isoflurane concentration was reduced to 2% and the animals breathed spontaneously until cannulation of a carotid artery and a jugular vein with PE-50 tubing was accomplished. The trachea was then intubated with a 16-gauge polyethylene catheter. The isoflurane concentration was decreased further by 1.5%, and ventilation was controlled using a Harvard animal respirator (Harvard Apparatus, South Natick, MA) using measurement of arterial blood gases to maintain normal partial pressure of oxygen (P_{O_2}), partial pressure of carbon dioxide (P_{CO_2}) and *p*H. Electrocardiography and systolic and diastolic blood pressure were monitored using a Grass polygraph (Astromed Grass, Quincy, MA) and Gould pressure transducer (Gould, Cleveland, OH). Temperature was measured using a Yellow Springs thermistor and maintained at normothermia with a heating blanket and warming lights.

A PE-10 catheter was introduced through and beyond the endotracheal tube until obstruction to passage was met and then withdrawn 1 to 2 mm. Gas samples for measuring alveolar anesthetic concentration were obtained by withdrawing 10 ml gas through the catheter into gas-tight glass syringes for more than 3-5 min at the time of tail clamp and then were assayed using gas chromatography with a Varian model 3700 gas chromatograph with a flame ionization detector (Hewlett-Packard, Wilmington, DE). Constant alveolar concentration of isoflurane was verified by analyzing triplicate samples. Control MAC was established according to the methods described by Eger *et al.*[27] using a long hemostat (8-inch Rochester Dean Hemostatic Forceps) clamped to the first ratchet lock on the tail for 1 min. The tail was always stimulated proximal to a previous test site. Gross movement of the head, extremities, or body was taken as a positive test result, whereas grimacing, swallowing, chewing, or tail flick were considered negative results. The isoflurane concentration was reduced in decrements of 0.12 to 0.15% until the negative response became positive, with 12-15 min equilibration allowed after changes in concentration.[28] The MAC was considered to be the concentration midway between the highest concentration that permitted movement in response to the stimulus and the lowest concentration that prevented movement.

The agents administered intrathecally were a selective and potent cell-permeable inhibitor of PKGIα, Rp-8-[(4-chlorophenyl)thio]-cGMPS triethylamine,[29] (Rp-8-p-CPT-cGMPS) (RBI, Natick, MA) and an NO donor, *N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine, (NOC-12) (Calbiochem-Novabiochem Corp., La Jolla, CA). The drugs were dissolved in distilled water before administration. After initial baseline MAC determination, Rp-8-p-CPT-cGMPS at the doses of 25, 50, 100, or 200 μg or NOC-12 at a dose of 100 μg were injected intrathecally in a volume of 10 μl, followed by an injection of 10 μl distilled water to flush the catheter. Seven or eight rats were studied at each of the Rp-8-p-CPT-cGMPS or NOC-12 concentrations. An isoflurane concentration was chosen at which movement did not occur in the last negative response before the positive test response. At this isoflurane concentration, 10 min after the intrathecal injection of Rp-8-p-CPT-cGMPS or NOC-12, the animal was tested again for reactivity to tail clamp. The concentration of isoflurane was reduced or increased, and response to tail clamp was checked every 12-15 min thereafter until a positive or negative response was achieved.

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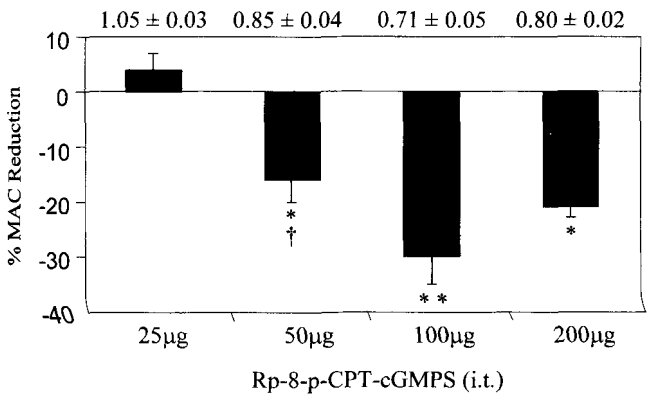

Fig. 1. Isoflurane minimum alveolar concentration (MAC) decreases by increasing the concentration of a cyclic guanosine monophosphate (cGMP)–dependent protein kinases Iα inhibitor, Rp-8-p-CPT-cGMPS. Data are presented as the mean ± SEM. n = 8 animals for each dose, except n = 7 for the 200 μg Rp-8-p-CPT-cGMPS. *$P < 0.05$ *versus* control; **$P < 0.01$ *versus* control; †$P < 0.05$ *versus* preceding concentration.

In some experiments, after initial baseline MAC determination, Rp-8-p-CPT-cGMPS at a dose of 100 μg/10 μl was first administered intrathecally. Ten minutes later, NOC-12 at a dose of 100 μg/10 μl was injected intrathecally. The MAC for isoflurane was again determined following the aforementioned procedures.

To control for the possibility that the vehicle (distilled water) may have had an effect on MAC determinations, the MACs of four rats were determined during isoflurane anesthesia, as described previously. Forty-five and 120 min after administering distilled water, the MAC again was determined. The stability of the MAC determinations over time, in the presence of Rp-8-p-CPT-cGMPS or NOC-12, was assessed in the following manner. MAC was determined for 10 animals, as described previously. The MAC was again determined 45 and 120 min after administering Rp-8-p-CPT-cGMPS (100 μg) or NOC-12 (100 μg).

The rats were evaluated for the presence of locomotor dysfunction using the following methods.[30] Rp-8-p-CPT-cGMPS and NOC-12 were injected intrathecally in the manner described previously. The animals were organized randomly into seven groups: distilled water (n = 5); 25 μg Rp-8-p-CPT-cGMPS (n = 5); 50 μg Rp-8-p-CPT-cGMPS (n = 5); 100 μg Rp-8-p-CPT-cGMPS (n = 5); 200 μg Rp-8-p-CPT-cGMPS (n = 5); 100 μg NOC-12 (n = 5); 100 μg Rp-8-p-CPT-cGMPS and NOC-12 (100 μg) (n = 5). The experimenter did not know which group was treated with the drugs, and the following tests were performed: (1) Placing reflex: The rat was held with the hind limbs slightly lower than the forelimbs, and the dorsal surface of the hind paws was brought into contact with the edge of a table. The experimenter recorded whether the hind paws were on the table surface reflexively; (2) Grasping reflex: The rat was placed on a wire grid and the experimenter recorded whether the hind paws grasped the wire on contact; (3) Righting reflex: The rat was placed on its back on a flat surface and the experimenter noted whether it immediately assumed the normal upright position. Scores for placing, grasping, and righting reflexes were based on counts of each normal reflex exhibited in five trials.

The results were assessed statistically by an analysis of variance. Intergroup differences were analyzed using the *Newman-Keuls test. All data are reported as the mean* ± SEM. Significance was set at $P < 0.05$.

Results

The control value for isoflurane MAC was 1.01 ± 0.03 vol%, which is consistent with previous determinations.[31] Intrathecal administration of Rp-8-p-CPT-cGMPS at the doses of 25, 50, 100, and 200 μg produced a decrease from isoflurane control MAC of −4 ± 3.1%, 16 ± 4.5% ($P < 0.05$), 30 ± 5.0% ($P < 0.01$), and 21 ± 2.2% ($P < 0.05$), respectively (fig. 1). Only the concentration of 50 μg was significantly different ($P < 0.05$) from the preceding concentration (fig. 1). No untoward effects were observed at each dose of Rp-8-p-CPT-cGMPS. As shown in figure 2, intrathecal administration of 25, 50, 100, and 200 μg Rp-8-p-CPT-cGMPS resulted in no significant changes in either systolic or diastolic blood pressure. Absolute control blood pressure was 118 ± 2 mmHg systolic and 79 ± 2 mmHg diastolic. As seen in figure 3, the intrathecal administration of 25, 50,

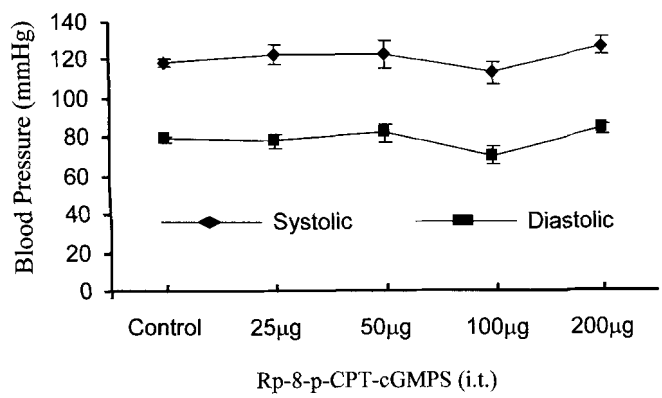

Fig. 2. Effect of Rp-8-p-CPT-cGMPS on systolic and diastolic blood pressure during isoflurane anesthesia. Data are presented as the mean ± SEM. n = 8 animals for each dose, except n = 7 for the 200 μg Rp-8-p-CPT-cGMPS.

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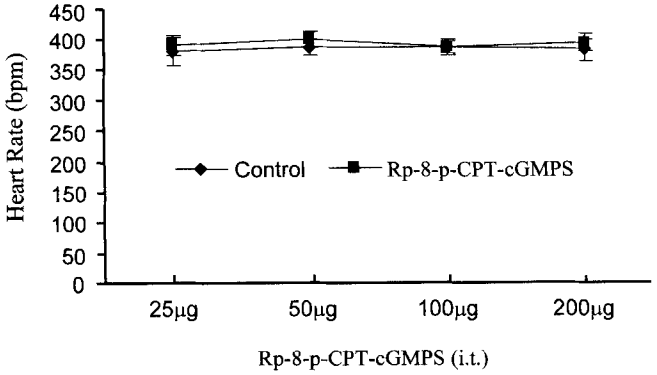

Fig. 3. Effect of Rp-8-p-CPT-cGMPS on heart rate during isoflurane anesthesia. Data are presented as the mean ± SEM. n = 8 animals for each dose, except n = 7 for the 200 μg Rp-8-p-CPT-cGMPS.

100, and 200 μg Rp-8-p-CPT-cGMPS also did not produce significant changes in heart rate. Absolute control heart rate was 384 ± 8 beats/min.

NOC-12 at a dose of 100 μg caused an increase from isoflurane control MAC of 23 ± 5.8 vol% ($P < 0.01$; fig. 4). The NOC-12-induced change in isoflurane MAC was accompanied by a significant decrease in systolic and diastolic blood pressure (93 ± 6 mmHg and 63 ± 4 mmHg, respectively, $P < 0.01$ *vs.* control). However, NOC-12 at a dose of 100 μg had no effect on heart rate (388 ± 6 beats/min). Rp-8-p-CPT-cGMPS at a dose of 100 μg produced a significant reversal of isoflurane MAC increase induced by NOC-12 at a dose of 100 μg ($P < 0.05$; fig. 4), which also was accompanied by significant reversal of the reduction of blood pressure induced by NOC-12 (systolic: 120 ± 5 mmHg; diastolic: 77 ± 3 mmHg).

The effect of the vehicle (distilled water) used to administer Rp-8-p-CPT-cGMPS or NOC-12 on MAC was assessed over time. The control MAC for these four rats was 1.05 ± 0.02 vol%. The MAC after 45 and 120 min was 1.04 ± 0.04 vol% and 1.05 ± 0.03 vol%, respectively. Neither the 45- nor the 120-min time points were significantly different from the control value. The stability of the MAC determinations, in the presence of Rp-8-p-CPT-cGMPS or NOC-12, was assessed 45 and 120 min after their administration. The control MAC for these 10 rats was 1.03 ± 0.02 vol%. In the presence of Rp-8-p-CPT-cGMPS (100 μg), the MAC after 45 and 120 min was 0.73 ± 0.05 vol% and 0.74 ± 0.04 vol%, respectively. In the presence of NOC-12 (100 μg), the MAC after 45 and 120 min was 1.27 ± 0.05 vol% and 1.25 ± 0.03 vol%, respectively. In the presence of either Rp-8-p-CPT-cGMPS or NOC-12, the 45- and 120-min time points were not significantly different from each other.

As shown in table 1, there were no differences between groups of the effects of the agents on locomotor function. Convulsions and hypermobility were not observed in association with Rp-8-p-CPT-cGMPS and NOC-12.

Table 1. Mean (SEM) Changes in Locomotor Test

Drugs	Placing	Grasping	Righting
Distilled water	5 (0)	5 (0)	5 (0)
Rp-8-p-CPT-cGMPS 25 μg	5 (0)	5 (0)	5 (0)
Rp-8-p-CPT-cGMPS 50 μg	5 (0)	5 (0)	5 (0)
Rp-8-p-CPT-cGMPS 100 μg	5 (0)	5 (0)	5 (0)
Rp-8-p-CPT-cGMPS 200 μg	4.8 (0.1)	4.8 (0.1)	5 (0)
NOC-12 100 μg	4.8 (0.1)	4.8 (0.1)	5 (0)
Rp-8-p-CPT-cGMPS 100 μg + NOC-12 100 μg	4.8 (0.1)	5 (0)	5 (0)

n = 5.5 trials.

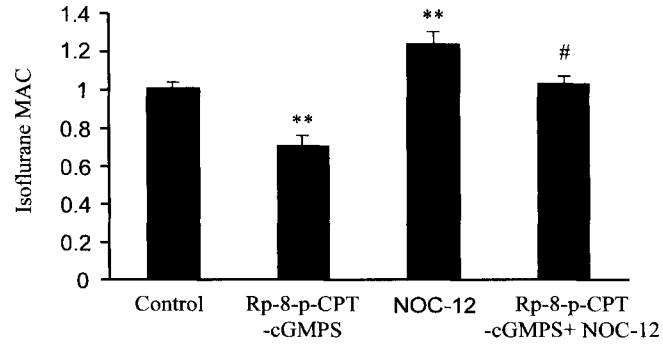

Fig. 4. Effects of Rp-8-p-CPT-cGMPS (100 μg; n = 8), NOC-12 (100 μg; n = 8), and Rp-8-p-CPT-cGMPS (100 μg) + NOC-12 (100 μg; n = 10) on isoflurane minimum alveolar concentration (MAC). Data are presented as the mean ± SEM. **$P < 0.01$ *versus* control. #$P < 0.05$ *versus* NOC-12–treated group.

Discussion

Cyclic GMP-dependent protein kinases are serine-threonine protein kinases and belong to the large family of protein kinases.[32] Two isoenzymes of PKG have been recognized in mammals: cytosolic PKGI and membrane-bound PKGII. Furthermore, PKGI has been shown to exist in two isoforms, designated Iα and Iβ.[32] Several lines of previous evidence have indicated that PKGs serve as major effectors for cGMP in the vascular and nervous system.[2,25] Cyclic GMP has been found to play an important role in the development of hyperalgesia. Intrathecal injection of 8-bromo-cGMP (a cGMP analog) resulted in thermal hyperalgesia[16] and increased neuropathic pain-related autotomy.[17] Iontophoretic applica-

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tion of 8-bromo-cGMP onto dorsal horn neurons preferentially enhanced responses to noxious stimuli.[33] Moreover, an elevated level of immunoreactive cGMP was found in the dorsal horn when hyperalgesia developed after carrageenan injection.[3] These data indicate that PKGs might be involved in hyperalgesia. Indeed, immunohistochemical study showed that PKGI was localized mainly in small-diameter cells in the dorsal root ganglia.[34] Sluka and Willis[35] reported that the mechanical allodynia induced by capsaicin could be reversed by KT5823, a selective PKG but not a selective PKG isoform inhibitor. Rp-8-p-CPT-cGMPS is a novel and selective PKGIα isoform inhibitor without effects on cAMP-dependent protein kinase or cGMP-regulated phosphodiesterases.[29] We recently found that abundant PKGIα, but not PKGIβ, was detected in the superficial laminae of the spinal cord and that intrathecal administration of Rp-8-p-CPT-cGMPS significantly and dose-dependently reduced pain responses evoked by formalin (data not shown). This evidence indicates that PKGIα participates in the processing of pain and that Rp-8-p-CPT-cGMPS has analgesic action at the spinal cord level, which is consistent with our current report of the effect of Rp-8-p-CPT-cGMPS on MAC. This also suggests the possibility that at least part of the effect of inhibition of the NO-cGMP signaling pathway causing the reduction in MAC may be related to effects on analgesia. In the current experiment, we chose doses of Rp-8-p-CPT-cGMPS that did not cause motor dysfunction when administered intrathecally in rats. We believe that intrathecally administered Rp-8-p-CPT-cGMPS may act by inhibiting the activity of PKGIα to disturb the transmission of noxious stimulation in the superficial laminae and result in a decrease in the MAC of isoflurane. However, whether Rp-8-p-CPT-cGMPS produces a sedative effect or decrease consciousness is unknown, although an effect on righting reflex was not observed in the current study. The possibility of this action of PKGIα could not be ruled out from the current results.

Although the mechanism of general anesthesia remains largely unknown, several studies show that anesthetics depress neuronal transmission by acting at presynaptic or postsynaptic sites rather than by inhibiting axonal impulse conduction.[36] This suggests that anesthetic effects may be mediated through their action on specific molecular signaling pathways in the CNS. Inhalational anesthetics have been shown to inhibit the NO-cGMP signaling pathway in the CNS. Halothane decreases the level of cGMP in specific regions of the rat brain.[20,21] Halothane and isoflurane both attenuated *N*-methyl-D-aspartate (NMDA)-stimulated increase of cGMP level in the cerebellum.[37] Furthermore, previous data from our laboratory showed that the MAC of halothane or isoflurane in rats was reduced by intravenous administration of the NOS inhibitors L-N^G-nitro-L-arginine methyl ester (L-NAME) or 7-nitroindazole.[22,23] Recently, we also observed that intraperitoneal administration of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a selective soluble guanylate cyclase inhibitor, dose-dependently decreased the threshold for isoflurane anesthesia in the rat.[24] It is suggested that there is an interaction of inhalational anesthetics with the NO-cGMP signaling pathway in the CNS. The inhibition of the NO-cGMP signaling pathway decreases the level of consciousness and augments sedation, analgesia, and anesthesia. The current results indicated a marked reduction with the use of Rp-8-p-CPT-cGMPS and a significant increase with the use of NOC-12, an NO donor, in the MAC for isoflurane anesthesia in rats. With PKGs serving as major effectors for the NO-cGMP signaling pathway,[2,25] these results further support a role for the NO-cGMP signaling pathway in the mechanisms of anesthesia, analgesia, or consciousness. In addition, Rp-8-p-CPT-cGMPS significantly blocked NOC-12-induced increase in isoflurane MAC in this study. It seems that PKGIα mediates the roles for the NO-cGMP signaling pathway in the mechanisms of anesthesia.

In our experiments, no untoward effects were observed with the intrathecal administration of Rp-8-p-CPT-cGMPS and NOC-12, even at the highest doses, during isoflurane anesthesia. However, hemodynamic effects of these two agents differed. The administration of Rp-8-p-CPT-cGMPS resulted in no changes in either blood pressure (systolic and diastolic) or heart rate. The administration of NOC-12 resulted in significant decreases in systolic and diastolic blood pressure, which were not accompanied by significant changes in heart rate. In the previous studies, ODQ injected intraperitoneally did not cause the changes in either heart rate or blood pressure during isoflurane anesthesia.[24] The intravenous administration of L-NAME caused a marked increase in systolic and diastolic blood pressure but not in heart rate during halothane or isoflurane anesthesia.[22,23] L-arginine, an NOS substrate, significantly reversed the increase in blood pressure caused by L-NAME during isoflurane anesthesia.[23] The mechanism of the hemodynamic effects of these agents is not understood fully. Isoflurane alone produces a dose-dependent decrease in blood pressure, primarily through a decrease in myocardial contractility and secondarily through a decrease in systemic vascular resistance. NOC-12 is a newly developed NO-releasing compound, which needs no cofactor to release NO.[38] NO as a signal molecule in the nervous system

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is well-known to play an important role in the central regulation of cardiovascular function. Microinjections of L-arginine or NO donors (*S*-nitrosoglutathione [SNP or SNAP]) into central autonomic sites in the brain led to a decrease in arterial pressure.[38,39] When NOS inhibitors (L-NAME or L-N^G-monomethyl-L-arginine) were applied intracisternally or infused intracerebroventricularly, there were increases in sympathetic activity and arterial pressure.[40,41] It is likely that NO released by NOC-12 administered intrathecally in this study acts on the central autonomic sites and potentiates the decrease in blood pressure caused by a high concentration of isoflurane because of the MAC increase, although a direct vascular action of NO cannot be ruled out. That the action of NO on cardiovascular function is mediated through PKG has been reported.[42,43] The current study showed that Rp-8-p-CPT-cGMPS significantly reversed the decrease in blood pressure induced by NOC-12, which is consistent with the previous reports.[42,43]

In conclusion, we showed that a PKGIα inhibitor not only markedly reduces MAC for isoflurane, but also completely blocks the newly observed NO-induced increase in isoflurane MAC. This is not accompanied by changes in either blood pressure or heart rate. With PKGs serving as major effectors for the NO-cGMP signaling pathway, it is suggested that PKGIα may mediate the role for the NO-cGMP signaling pathway in the mechanisms of anesthesia, analgesia, or consciousness. Our data present the first indication that an interaction of inhalational anesthetics with the NO-cGMP signaling pathway occurs at least partially at the spinal cord level. Our results also suggest a new possibility that PKGIα inhibitors may be clinically useful to decrease the need for inhalational anesthetics and reduce postoperative pain. Further investigation is necessary before PKGIα inhibition is accepted for clinical use.

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