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Propofol-induced Depression of Cultured Rat Ventricular Myocytes Is Related to the M_2-acetylcholine Receptor–NO–cGMP Signaling Pathway

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Background: **It is well-known that propofol sometimes causes bradycardia or asystole during anesthesia; however, the direct effect of propofol on the myocardium remains unclear. Previous reports showed the contribution of muscarinic acetylcholine receptors to propofol-induced bradycardia. Conversely, it was suggested recently that nitric oxide (NO) plays an important role in mediating the effect of vagal stimulation in the autonomic regulation of the heart. Therefore, the authors investigated the effects of propofol on spontaneous contraction and NO production in cultured rat ventricular myocytes.**

Methods: **The authors measured chronotropic responses of cultured rat ventricular myocytes induced by propofol stimulation with a sensor, a fiber-optic displacement measurement instrument. The authors also quantitatively analyzed NO metabolite production in cultured myocytes by measuring the levels of nitrite and nitrate in a high-performance liquid chromatography reaction system. The influence of propofol on muscarinic acetylcholine receptors of myocyte membranes was also measured with a competitive binding assay using [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB).**

Results: **Propofol caused negative chronotropy in a dose-dependent manner. Propofol (IC_{50}) also caused the enhancement of nitrite production in cultured myocytes. Eighty percent of the enhancement of nitrite production induced by propofol (IC_{50}) stimulation was abolished by pretreatment with atropine, methoctramine, or N^G-monomethyl-L-arginine acetate (L-NMMA). The negative chronotropy induced by propofol (IC_{50}) stimulation was reduced to 40–50% by pretreatment with atropine, methoctramine, L-NMMA, or 1*H*[1,2,4]oxadiazolo[4,3-*a*]quanoxalin-1-one, a selective inhibitor of guanylyl cyclase. Propofol displaced [^{3}H]QNB binding to the cell membrane of myocytes in a concentration-dependent manner.**

Conclusion: **These results suggest that the negative chronotropy induced by propofol is mediated in part by M_2-acetylcholine receptor activation, which involves the enhancement of NO production in cultured rat ventricular myocytes. (Key words: Anesthetics; beating rate; high-performance liquid chromatography reaction system; M_2-acetylcholine receptor; nitrite; nitrate; sensor.**

PROPOFOL (2,6-diisopropylphenol) is an intravenous anesthetic agent commonly used for general anesthesia. There have been many reports of profound bradycardias associated with its use, including sinus bradycardia[1] and even fetal asystole,[2] in clinical use. Although modulation of the tone of the autonomic nervous system[3] or alteration of baroreflex sensitivity[4] would be included in the mechanisms of bradydysrhythmic effects, the direct effects of propofol on the myocardium remain unclear. Recently, Alphin *et al.*[5] reported that propofol slowed the atrial rate and depressed atrioventricular nodal conduction in the isolated guinea pig heart. The negative dromotropic effect of propofol was shown to be predominantly mediated by muscarinic acetylcholine receptors (mAChRs). Results showed the contribution of mAChRs to propofol-induced bradycardia.

In the autonomic regulation of cardiac contractility, nitric oxide (NO) has been reported to play a significant role.[6–8] We previously demonstrated the enhancement of NO production with negative chronotropy induced by carbachol, a muscarinic receptor agonist, in cultured rat ventricular myocytes.[9,10] These effects of carbachol were abolished by atropine, an mAChR antagonist, methoctramine, an M_2-AChR antagonist and N^G-mono-

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methyl-L-arginine acetate (L-NMMA), an NO synthase (NOS) inhibitor.[11] Our results indicated that M_2-AChR mediated the activation of the NO-signaling pathway in cultured rat ventricular myocytes, which contributed to the depression of myocardial contractility. These results are consistent with those that Balligand *et al.*[6,8] reported for spontaneously beating neonatal rat cardiac myocytes. We hypothesized that the NO signaling pathway mediated by M_2-AChR also plays an important role in the cardiac depressant effect of propofol.

Few reports indicate that propofol stimulates NO production in any tissues. Petros *et al.*[12] found that propofol stimulated the production and release of NO from cultured porcine aortic endothelial cells. Park *et al.*[13] reported that propofol caused a direct vasodilative effect on distal coronary arteries in rats, which was primarily endothelium-dependent and was mediated by multiple substances, including NO and a vasodilative prostanoid. However, no evidence indicates that propofol stimulates NO production in the myocardium.

In this study, we evaluated propofol-induced direct negative chronotropy in cultured rat ventricular myocytes and enhancement of NO production. We also evaluated M_2-AChRs and other components in the NO signaling pathway that contribute to the negative chronotropy induced by propofol stimulation.

Materials and Methods

Preparation of Cultured Rat Ventricular Myocytes

All experiments were performed during the supervision of the Animal Care Committee of Sapporo Medical University. Primary cultured cardiac myocytes were prepared from ventricles of neonatal Wistar rats (1–3 days old) by collagenase (Wako Chemical, Osaka, Japan) digestion, as described previously.[14] Briefly, neonatal rat ventricles were removed and incubated with collagenase (200 U/ml) in Ca^{2+}- and Mg^{2+}-free Hank salt-balanced solution (HBSS, pH 7.4) during 95% O_2 and 5% CO_2 gas bubbling at 37°C. The isolated ventricular myocytes were seeded at a density of 1.5×10^6 cells/35-mm dish and cultured in the Dulbecco modified Eagle minimum essential medium (DMEM; Dainihon Seiyaku, Osaka, Japan) containing 10% fetal bovine serum (Flow Laboratories, Irvine, CA) in a humidified atmosphere of 95% air and 5% CO_2. Serum-containing medium was replaced by fresh medium every 2 days. After 2 days of culture, myocytes started beating spontaneously, and after 4 to 5 days of culture, they formed a confluent monolayer and beat regularly and synchronously. All experiments were performed at 6 to 7 days of culture.

Measurement of Contraction of Myocytes

Spontaneous contraction of cultured myocytes was measured using a Fotonic Sensor, a fiber-optic displacement measurement instrument (MTI 1000; MTI Co., Latham, NY) in a multigas incubator.[9–11,14–17] The principle of measurement is to detect changes in the distance between the probe and myocytes vertically extruded by contraction. The fiber-optic probe consists of adjacent pairs of light-transmitting and light-receiving fibers. The ratio of reflected light to transmitted light changes in proportion to the distance between the probe and an object at a certain range shown by a calibration curve specific to each probe. We used a needle-type probe (MTI- 3806R; MTI Co.) 0.7-mm in diameter, which could cover approximately 100–150 myocytes. The serum-containing medium was replaced by 2,000 μl serum-free Dulbecco modified Eagle minimum essential medium buffered with HEPES (SFD) 2 h before measurement, and the cells were stabilized in a multigas incubator (Sanyo, Tokyo, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The probe was set perpendicular to the myocytes in the dish. They were analyzed after being agitated on a horizontally rotating shaker after 15 min in an incubator for stabilization. Two hundred microliters of vehicle or a 10-fold concentrated solution of propofol was added to 1,800 μl SFD in the dish and then mixed with the shaker for 15 s at 60 rpm. The resultant concentrations of propofol (Aldrich, Milwaukee, WI) were 100 nM to 100 μM. We measured the changes in spontaneous beating rates of myocytes with the Fotonic Sensor for 5 min after vehicle or propofol addition. The IC_{50} value of propofol was obtained from the dose–response curve. We also measured the effects of pretreatment with 1 μM atropine for 10 min; 1 μM methoctramine (RBI, Natick, MA), an M_2-acetylcholine receptor subtype antagonist, for 10 min; 300 μM L-NMMA (RBI), an NOS inhibitor, for 60 min; or 100 μM 1*H*-[1,2,4]oxadiazolo[4,3-α]quanoxalin-1-one (ODQ; Tocris Cookson, Langford, UK), a selective guanylyl cyclase inhibitor, for 60 min using propofol-induced (IC_{50}) chronotropy.

In this study, we used SFD as a vehicle for drugs. All drugs were dissolved in SFD, except propofol was first applied in Intralipos (20% soybean oil, 2.2% glycerol, 1.2% purified phospholipid; The Green Cross Co., Osaka, Japan) and then diluted with SFD, and ODQ was first dissolved in ethanol and then diluted with SFD, and the

final ethanol concentration was less than 0.1%. We also evaluated whether Intralipos alone had any chronotropic effect on myocytes.

Measurement of Nitric Oxide Metabolite Production

We quantitatively analyzed NO metabolite production in cultured rat ventricular myocytes by measuring the levels of nitrite and nitrate in a high-performance liquid chromatography–Griess reaction system (ENO-11; EICOM, Kyoto, Japan) according to the procedures previously described.[9–11] After collecting samples from SFD in the dish to measure the control nitrite and nitrate (NOx) levels, samples were taken at 5 min after addition of vehicle or propofol (IC_{50}). We also collected samples at 5 min after propofol (IC_{50}) stimulation following pretreatment with 1 μM atropine for 10 min, 1 μM methoctramine for 10 min, or 300 μM L-NMMA for 60 min, and evaluated whether Intralipos alone had any effect on NO production. The NOx production by 1.5×10^6 cells/dish in 5 min was calculated from the changes of the NOx level, taking into account the NOx in SFD used as a vehicle of drugs.

Radioligand Binding Assay

Competitive assays to determine the affinity of propofol for the mAChR were performed with the cell membranes of myocytes using the procedures previously described.[11] Aliquots of cell membranes were preserved at 25°C for 2 h in 50 mM Tris–HCl buffer (pH 7.4) containing atropine (1 nM to 1 mM final concentration), propofol with Intralipos as the vehicle (1 nM to 10 mM final concentration) or Intralipos alone. The concentrations of Intralipos used alone corresponded to each concentration of propofol. The binding reaction was initiated by adding [^{3}H]quinuclidinyl benzilate (QNB; NEN Life Science Products, Inc., Boston, MA) (0.1 nM final concentration), followed by incubation for 45 min at 25 °C. The incubated samples were filtered using a Brandel M-30 cell harvester (Biochemical Research and Development Laboratories, Inc., Gaithersburg, MD) through Whatman GF/B glass fiber filters (Whatman International Ltd., Maidstone, UK) with three 5 ml washes of 50 mM Tris/HCl buffer. Filters were dried at 80°C for 1 h, placed in 8 ml scintillation fluid (ACS II; Amersham, Buckinghamshire, UK), and counted in a liquid-scintillation counter (LS-9000; Beckmann Instrument Inc., Fullerton, CA). Competition analysis data were determined using the computer program Prism (GraphPad Software, Inc., San Diego, CA). Protein was determined by the Coomassie Blue binding method,[18] with bovine serum albumin as a standard.

Data and Statistical Analysis

In the following experiments, beating rates of myocytes were expressed as a percent of the control during each set of conditions (before stimulation). Enhancements of nitrite and nitrate production were measured by subtracting the spontaneous production after SFD addition from the production after propofol stimulation and were expressed as a percent of the control level during each set of conditions (before stimulation). All results were expressed as the mean ± SE. Results from dose–response analysis of chronotropy induced by propofol stimulation were compared nonparametrically using the Mann–Whitney U test. Other results were compared by one-factor analysis of variance followed by the Scheffe F *post hoc* test. A P value < 0.05 was considered to be significant.

Results

Chronotropic Response of Myocytes to Propofol

Myocytes cultured for 6 or 7 days beat regularly at the rate of 131 ± 4 beats/min (n = 160). Propofol caused negative chronotropy in a dose-dependent manner (fig. 1). The beating rate (% of control) at 5 min after SFD administration and 100 nM, 1 μM, 10 μM, 50 μM, and 100 μM propofol addition were 100.2 ± 1.4, 98.5 ± 1.5, 94.6 ± 1.4, 84.9 ± 3.0, 29.2 ± 17.9, and 2.7 ± 2.7, respectively. The IC_{50} value of propofol obtained from the dose–response curve was 39 ± 16 μM (fig. 1C). Pretreatment with 1 μM atropine, 1 μM methoctramine, or 300 μM L-NMMA abolished 44, 49, and 50% of the negative chronotropy induced by propofol (IC_{50}) stimulation, respectively (fig. 2, left and fig. 3, left). Pretreatment with 100 μM ODQ also abolished 54% of the negative chronotropy induced by propofol (IC_{50}) stimulation (fig. 4). Atropine, methoctramine, L-NMMA, and ODQ used for pretreatment had no effect on the spontaneous beating rate of myocytes (data not shown).

Effect of Propofol on NOx Production in Cultured Myocytes

We investigated the effect of propofol stimulation on NOx production in cultured myocytes. Because there was no change of the nitrate level in our preparations, we show only the change of nitrite production. The mean value of control levels of nitrite before stimulation was 3,355 ± 147 pmol/dish (n = 87) and cultured myocytes spontaneously produced nitrite (12 ± 2% of control, n = 13) at 5 min after SFD addition. Propofol

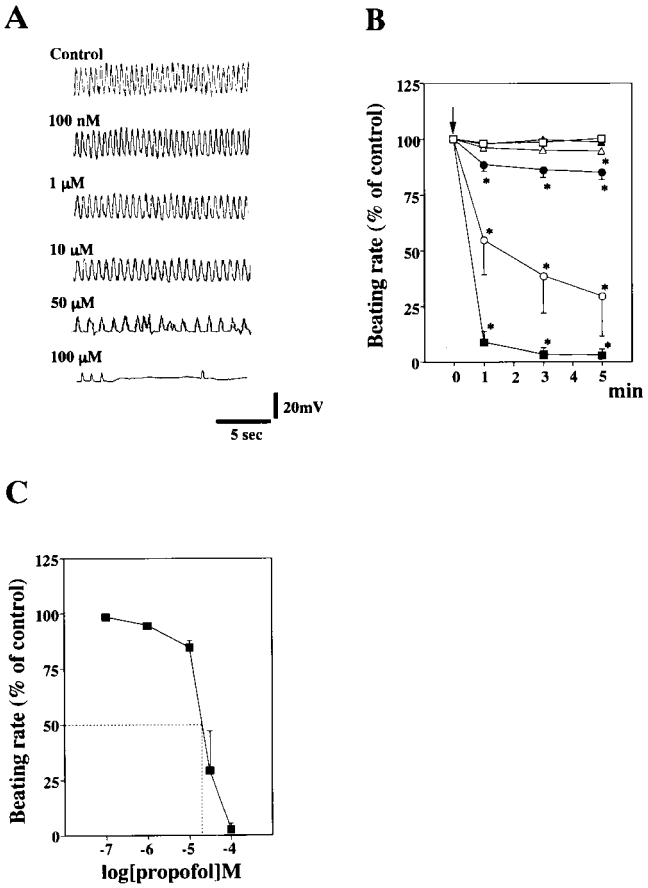

Fig. 1. The negative chronotropy induced by propofol stimulation in cultured rat ventricular myocytes. (*A*) Records of the beating of myocytes obtained with the Fotonic Sensor at each concentration of propofol. (*B*) Beating rates after SFD addition (open squares) and 100 nM (closed triangle), 1 μM (open triangle), 10 μM (closed circle), 50 μM (open circle), and 100 μM (closed squares) propofol stimulation (n = 4–14). (*C*) Dose–response curve of propofol using the data at 5 min after stimulation. The value of IC_{50} is 39 ± 16 μM. Data are expressed as the percent of control (before stimulation) and show the mean ± SE. Propofol stimulation decreased the beating rate in a dose-dependent manner. *$P < 0.05$ *versus* vehicle (SFD).

(IC_{50}) caused an enhancement of nitrite production (13 ± 2%), and the pretreatment with 1 μM atropine, 1 μM methoctramine, or 300 μM L-NMMA abolished 83, 87, and 81% of the enhancement of NO production induced by propofol stimulation (fig. 2, right; fig. 3, right). The enhancement of nitrite production induced by propofol stimulation was measured by subtracting the spontaneous nitrite production of myocytes after SFD addition. Atropine, methoctramine, and L-NMMA used for pretreatment had no effect on spontaneous nitrite production in cultured myocytes (data not shown).

Effects of Intralipos and Vehicle on Chronotropic Response and NOx Production

We used Intralipos as the vehicle for propofol and ethanol for ODQ in addition to SFD. Intralipos, for which the concentration corresponded to that used as the vehicle for propofol (IC_{50}), did not affect the spontaneous beating rate and nitrite production in cultured myocytes (fig. 2). Ethanol, the final concentration of which was less than 0.1%, did not affect the spontaneous beating rate of myocytes (data not shown).

Influence of Propofol on Muscarinic Acetylcholine Receptors

Affinity of propofol to mAChRs of cell membranes of myocytes was measured with a competitive binding assay using [^{3}H]QNB (fig. 5). Propofol displaced 0.1 nM [^{3}H]QNB binding to cell membranes of myocytes in a concentration-dependent manner. The IC_{50} and dissociation constant (K_i) values of propofol that displaced [^{3}H]QNB binding, obtained by radioligand binding assay, were 320 ± 10 μM and 130 ± 10 μM, respectively.

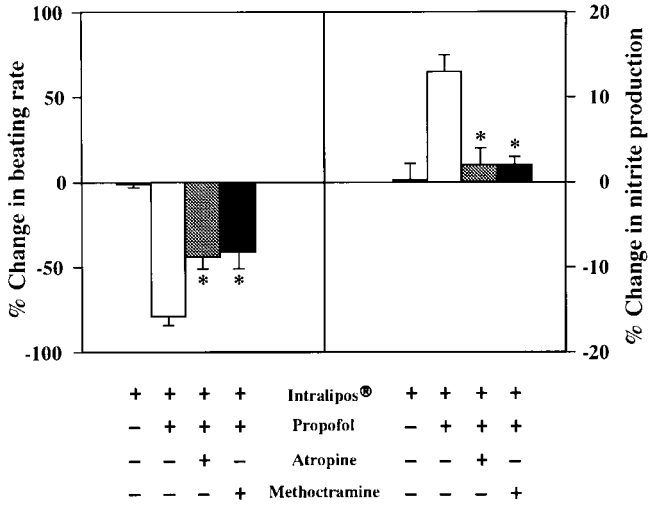

Fig. 2. The effects of muscarinic acetylcholine receptors antagonists on the propofol-induced negative chronotropy and the nitrite production in cultured rat ventricular myocytes. (*Left*) Shows the percent change in beating rate at 5 min after Intralipos addition (n = 8), propofol (IC_{50}) stimulation alone (n = 38), and propofol (IC_{50}) stimulation with 1 μM atropine (n = 23) and 1 μM methoctramine (n = 12). (*Right*) Shows the enhancement of nitrite production at 5 min after Intralipos addition (n = 6), propofol (IC_{50}) stimulation alone (n = 22), and propofol (IC_{50}) stimulation with 1 μM atropine (n = 9) and 1 μM methoctramine (n = 12). Enhancements of nitrite production were measured by subtracting the spontaneous production of nitrite after vehicle (SFD) addition (n = 17) from the production of nitrite after propofol stimulation. Data are expressed as the percent of control (before stimulation) and show the mean ± SE. *$P < 0.05$ *versus* propofol alone.

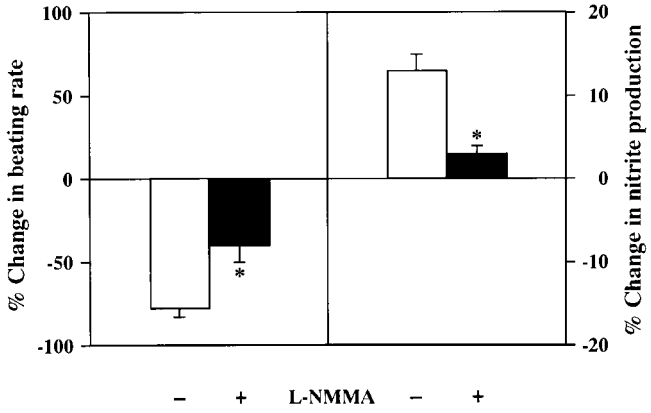

Fig. 3. The effect of L-NMMA on propofol-induced negative chronotropy and nitrite production in cultured rat ventricular myocytes. (*Left*) Shows the percent change in beating rate at 5 min after propofol (IC_{50}) stimulation alone (n = 38) and propofol (IC_{50}) stimulation with 300 μM L-NMMA (n = 12). (*Right*) Shows the enhancement of nitrite production at 5 min after propofol (IC_{50}) stimulation alone (n = 22) and propofol (IC_{50}) stimulation with 300 μM L-NMMA (n = 12). Data are expressed as the percent of control (before stimulation) and show the mean ± SE. *$P < 0.05$ *versus* propofol alone.

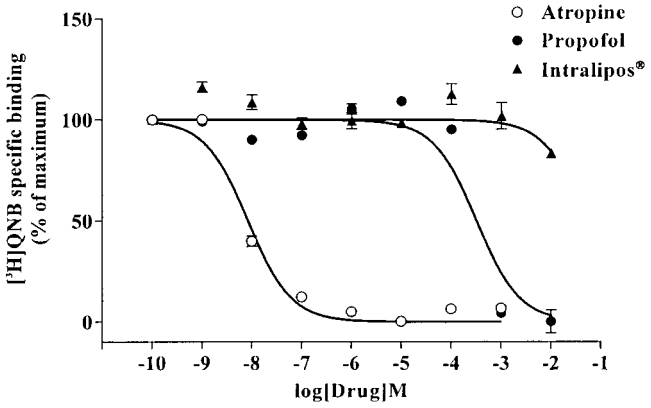

Fig. 5. Displacement analysis of [^{3}H]QNB binding to cell membranes of myocytes by propofol. [^{3}H]QNB (0.1 nM), cell membranes of myocytes, and competing unlabeled drugs (atropine, propofol, and Intralipos) were incubated together for 45 min at room temperature. The values of IC_{50} and dissociation constants (K_i) for atropine and propofol displacement of [^{3}H]QNB binding were 8.5 ± 0.6 nM and 3.4 ± 0.2 nM, and 320 ± 10 μM and 130 ± 10 μM, respectively. Intralipos did not displace [^{3}H]QNB binding.

Atropine also displaced [^{3}H]QNB binding to cell membranes of myocytes (IC_{50} = 8.5 ± 0.6 nM; K_i = 3.4 ± 0.2 nM), whereas Intralipos did not displace [^{3}H]QNB binding even at 1 mM.

Discussion

Many observations revealed that endogenous NO regulates physiologic functions of mammalian tissues, including blood vessels, neurons, and immune systems, and sometimes acts as a toxic agent.[19,20] The human myocardium also contains constitutive NOS (cNOS) and has the capacity to express inducible NOS.[21] Recently, it was reported that propofol stimulated NO production in porcine aortic endothelial cells and rat distal coronary arteries.[12,13] However, there is no evidence that propofol stimulates NO production in myocardium. In this study, we demonstrated that propofol caused the enhancement of NO production in cultured rat ventricular myocytes. We quantified the NO production by directly measuring the NO metabolites, NOx, *i.e.*, nitrite and nitrate, in an high-performance liquid chromatography–Griess reaction system. There was no significant change in nitrate caused by propofol stimulation in our cell preparations. Nitrite is the principle spontaneous product of NO in aqueous solutions that contain no heme protein,[22] such as the solution used in this study. We also found that L-NMMA abolished the enhancement of NO production and the negative chronotropy induced by propofol stimulation. These results indicated that NO production participated in part of the negative chronotropy induced by propofol stimulation in cultured rat ventricular myocytes.

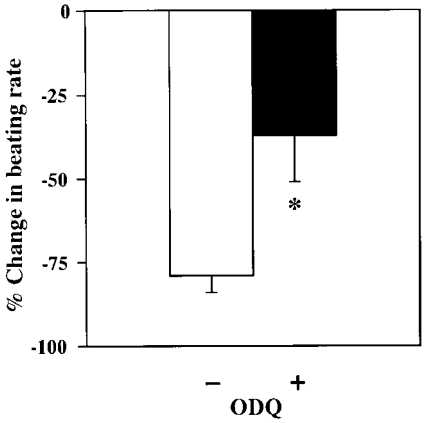

Fig. 4. The effect of ODQ on propofol-induced negative chronotropy in cultured rat ventricular myocytes. The figure shows the percent change in beating rate at 5 min after propofol (IC_{50}) stimulation with (n = 9) and without (n = 38) 1 μM ODQ. Data are expressed as the percent of control (before stimulation) and show the mean ± SE. *$P < 0.05$ *versus* propofol alone.

The activation of mAChRs triggers many different signal transduction pathways, including NO signaling pathways.[6,8,23] We previously demonstrated that the activation of mAChRs by carbachol stimulation caused the enhancement of NO production and negative chronot-

ropy in a dose-dependent manner in cultured rat ventricular myocytes, and these effects of carbachol were completely abolished by pretreatment with atropine.[10] Therefore, we next evaluated the participation of mAChRs in the effects of propofol in this study. Atropine, a potent muscarinic receptor antagonist, abolished the enhancement of NO production and part of the negative chronotropy induced by propofol stimulation. The results of competitive binding assays strongly supported the idea that propofol had an affinity to mAChRs. A 10-fold higher dose of propofol was necessary to inhibit binding by 50% compared with its effect on beating rate in this study (fig. 5), which may be caused by changes in sensitivity of mAChR to propofol or interaction between them caused by the procedures to prepare aliquots of the cell membrane. These results suggested that the enhancement of NO production and the negative chronotropy induced by propofol stimulation were mediated in part by mAChR activation in cultured rat ventricular myocytes. This is in agreement with a previous study,[5] which reported that propofol slowed the atrial rate and atrioventricular nodal conduction and that the negative dromotropic effect of propofol was predominantly mediated by mAChR.

There are five different known subpopulations of mAChR, M_1 through M_5, which can be distinguished based on pharmacologic classification, and five genes for receptors, *m1* through *m5,* have been identified.[24] Some reports suggested that M_1-, M_2- and M_3-AChR subtypes might be present in myocytes in several species,[25–27] and it has been established that the negative inotropic and chronotropic effects in mammalian heart caused by the activation of mAChRs are mainly mediated by the M_2-AChR subtype.[6,7,28] Recently, we found that the enhancement of NO production and negative chronotropy induced by carbachol stimulation were also abolished by methoctramine, an M_2-AChR subtype antagonist. However, pirenzepine, an M_1-AChR subtype antagonist, and HHSiD, an M_3-AChR subtype antagonist, did not affect these effects of carbachol.[11] In addition, we found *m2* and *m3* AChR gene expression with reverse transcription polymerase chain reaction analysis of reverse-transcribed cultured rat ventricular myocytes. However, *m3* AChR gene expression appeared to be considerably weaker than *m2*-cholinergic gene expression.[11] Our previous results indicated that the effects of carbachol in cultured rat ventricular myocytes were mediated by the M_2-AChR subtype. The enhancement of NO production and part of the negative chronotropy induced by propofol stimulation were abolished by pretreatment with methoctramine in the current study, suggesting that these effects of propofol were also mediated by the M_2-AChR subtype.

It remains unclear how propofol induced negative chronotropy through the enhancement of NO. Some studies[29–31] suggest that propofol may inhibit transmembrane Ca^{2+} influx through voltage-activated Ca^{2+} channels ($I_{Ca(L)}$), perhaps by interacting with the dihydropyridine binding site.[31] It is well-known that stimulation of the M_2-AChR subtype inhibits adenylyl cyclase by a G_i-protein–mediated mechanism, and, consequently, decreases the cyclic adenosine monophosphate (cAMP) level and inhibits $I_{Ca(L)}$.[32] It was reported that stimulation of the M_2-AChR subtype also activates constitutive NOS and increases NO.[33] NO activates guanylyl cyclase and then increases the cellular level of cyclic guanine monophosphate (cGMP), which interacts with several intracellular receptor proteins, including protein kinases, phosphodiesterases, and ion channels.[34] Some studies indicated that cGMP inhibited $I_{Ca(L)}$ by (1) activation of cGMP-dependent protein kinase (PKG), which is presumed to phosphorylate the L-type Ca^{2+} channel or some regulatory protein,[35] and (2) activation of cGMP-stimulated phosphodiesterase, which leads to a lower cAMP level.[36] Therefore, propofol may affect $I_{Ca(L)}$ *via* a mechanism mediated by the M_2-AChR--NO– cGMP signaling pathway. In fact, the negative chronotropy induced by propofol stimulation was also abolished by ODQ, a selective guanylyl cyclase inhibitor, in addition to atropine, methoctramine, and L-NMMA, in our preparation. Han *et al.*[37,38] reported that, in isolated primary pacemaker cells from the rabbit sinoatrial node in the presence of β-adrenergic stimulation, NO-mediated cholinergic inhibition of $I_{Ca(L)}$ is caused by a cGMP-stimulated cAMP-specific phosphodiesterase that hydrolyzes cAMP. We investigated whether propofol caused the negative chronotropy mediated by NO production in the absence of β-adrenergic stimulation, which may indicate that cGMP-dependent protein kinase is more potently related to its effect in our preparation. Considering that the primary cultured cardiac myocytes used in this study were prepared from the ventricles of the neonatal rats and the fact that some of the receptor and channel functions had not fully developed,[39] the automaticity of our preparations may differ from that of the pacemaker cells. These differences in histology and maturity may be related to the various responses to propofol in our preparation and the sinoatrial nodal cells.

The NO-independent mechanism that participates in the propofol-induced negative chronotropy remains un-

clear. In the pacemaker cells of the heart, the autonomic nervous system controls spontaneous electrophysiologic activity by interaction of different ionic currents, including I_{Ca}, I_f, and $I_{K(ACh)}$. Because propofol has lipid solubility similar to other anesthetics,[40] it might act on the lipid bilayer of membrane or membrane protein, such as ion channels, and modulate these ionic currents directly or indirectly. For instance, it was reported that carbachol activated $I_{K(ACh)}$ by an NO-independent mechanism in isolated primary pacemaker cells from the rabbit sinoatrial node.[37,38] Thus, propofol might also activate $I_{K(ACh)}$. Further study will be needed to elucidate the NO-independent mechanism that participates in the propofol-induced negative chronotropy.

Plasma concentrations of propofol during clinical use range from 3 μM to 90 μM, and a typical plasma concentration of propofol during general anesthesia is estimated to be 35 μM.[41] The concentrations of propofol used in this study ranged from 100 nM to 100 μM, and the value of IC_{50} was 39 μM. These are roughly consistent with the range in clinical use. However, because the protein binding of propofol has been estimated to be 97 to 99%,[42] the effective free-propofol concentration in clinical use is probably less than 1 μM. Our experiments were performed with cultured myocytes in serum-free Dulbecco modified Eagle minimum essential medium, and the greater fraction of propofol administered to the medium probably did not bind to protein. Therefore, the free-propofol concentrations in our experiments must have been higher than those in clinical use. In our study, 1 μM propofol caused only a 5% decrease in the beating rate; therefore, the direct effect of the dosage of propofol in clinical use on the heart may be slight *in vivo*. However, there are differences in animal species and types of preparation between our study and other studies.[3,4,43,44] These differences may affect the response and sensitivity of preparations to drugs. In fact, we previously reported that the activation of mAChRs with a high dosage (1 mM) of carbachol stimulation caused only a 25% decrease in beating rate in our preparations.[9,10] Therefore, we consider that it would be difficult to apply the dosage of propofol in clinical use to our experiments.

In combination, our results showed that propofol caused negative chronotropy in cultured rat ventricular myocytes, and the enhancement of NO production mediated by M_2-AChR participated in this effect of propofol. Our results suggest that propofol may cause the negative chronotropy in part *via* an M_2-AChR–NO–cGMP signaling pathway in ventricular myocytes.

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