

Propofol Inhibits Muscarinic Acetylcholine Receptor-Mediated Signal Transduction in *Xenopus* Oocytes Expressing the Rat M1 Receptor

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ABSTRACT—The effects of propofol, 2,6-diisopropylphenol, an intravenous general anesthetic, on signal transduction mediated by the rat M1 muscarinic acetylcholine (ACh) receptor (M1 receptor) were examined in electrophysiological studies by analyzing receptor-stimulated, Ca^{2+} -activated Cl^- -current responses in the *Xenopus* oocyte expression system. In oocytes expressing the M1 receptor, ACh induced the Ca^{2+} -activated Cl^- current, in a dose-dependent manner ($\text{EC}_{50}=114$ nM). Propofol (5–50 μM) reversibly and dose-dependently inhibited induction of the Ca^{2+} -activated Cl^- current by ACh (100 nM) ($\text{IC}_{50}=5.6$ μM). To determine a possible site affected by propofol in this signal transduction, we tested the effects of this anesthetic (10 μM) on the activation of current by injection of CaCl_2 and aluminum fluoride (AlF_4^-). Propofol did not affect activation of the current by the intracellular injected Ca^{2+} , or activation of the current by the intracellular injected AlF_4^- . These results indicate that propofol does not affect G protein, the inositol phosphate turnover, release of Ca^{2+} from Ca^{2+} store or the Ca^{2+} -activated Cl^- channel. Propofol apparently inhibits the M1 receptor-mediated signal transduction at the receptor site and/or the site of interaction between the receptor and associated G protein.

Keywords: Propofol, Muscarinic receptor, m1 Receptor, *Xenopus* oocyte, G protein

General anesthetics act on ligand-gated ion channels, voltage-gated ion channels, G protein-coupled receptors and second messenger systems (1). The main effect of anesthetics is potentiation or inhibition of synaptic ligand-gated ion channels: γ -aminobutyric acid_A (GABA_A) receptor and glutamate receptor (1). It has been reported that muscarinic receptors, one of the G protein-coupled receptors, which have a significant role in the maintenance of consciousness, are affected by anesthetics (2–4). Recent studies indicate that general anesthetics affect the function of the M1 muscarinic acetylcholine (ACh) receptor (M1 receptor) (5–7).

Propofol, 2,6-diisopropylphenol, a compound structurally unrelated to other general anesthetics, is used to achieve general anesthesia (8). Extensive studies indicate that the sites involving anesthetic action of propofol are the GABA_A receptor in rat cerebral cortex (9) and in bovine adrenomedullary chromaffin cells (10). A relatively high concentration of propofol affected the nicotinic ACh

receptor in mouse tumor cells (11), sodium channel reconstituted from human brain cortical tissue (12), potassium channel in undifferentiated clonal pheochromocytoma (PC 12) cells (13), calcium current in dorsal root ganglionic neurons of chick embryos (14), and protein kinase C purified from rat forebrain (15). Much less is known about the effects of propofol on muscarinic receptors.

We attempted to clarify effects of propofol on the M1 receptor expressed in *Xenopus* oocytes by analyzing receptor-stimulated Ca^{2+} -activated Cl^- current responses. The M1 receptor is linked to a signal transduction pathway: activation of G proteins, activation of phospholipase C, increase of inositol 1,4,5-triphosphate (IP_3), release of intracellular Ca^{2+} and activation of the Ca^{2+} -activated Cl^- currents (16, 17). Independently of receptor activation, we elicited the Ca^{2+} -activated Cl^- currents via G protein by injecting aluminum fluoride (AlF_4^-) (18) to examine effects of propofol on the intracellular signal transduction pathway. AlF_4^- activates the GDP-bound form of G protein (19). In this activation, a release of

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GDP from G protein is not necessary (20). In addition, we directly activated the Ca^{2+} -activated Cl^- currents by injecting CaCl_2 . Our observations using these three independent methods provide insight into the effects of this anesthetic on signal transduction of G protein-coupled receptor.

MATERIALS AND METHODS

In vitro transcription of cRNA and injection into Xenopus oocytes

The cDNA for rat m1 muscarinic receptor (m1 receptor) was obtained from Dr. Lester (Caltech, Pasadena, CA, USA). cRNA was synthesized in vitro with T7 polymerase using Ambion's MEGAscript™ kits (Ambion, Austin, TX, USA) from linearized cDNA with Hind III. Stage V–VI *Xenopus* oocytes were isolated and defolliculated by gently shaking them at room temperature (21–23°C) for 60 min in Ca^{2+} -free solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 10 mM HEPES, pH 7.4) containing 0.5 mg/ml collagenase obtained from Yakult (Tokyo) (17). cRNA (5 ng) was injected into the oocytes using Picospritzer™ II (General Valve Co., Fairfield, NJ, USA); then the oocytes were incubated at 19°C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 0.82 mM MgSO_4 , 0.41 mM CaCl_2 , 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 7.4] containing 2.5 mM sodium pyruvate and 20 mg/ml gentamicin for 2–4 days. The Ca^{2+} -free solution and the modified Barth's solution were sterilized prior to use.

Electrophysiological studies and statistical analysis

Electrophysiological measurements were made between 2 and 4 days after cRNA injection, using a two-electrode voltage clamp amplifier (TEV-200; Dagan, Minneapolis, MN, USA) (17). The resting membrane potential of the oocytes injected with cRNA was -51.8 ± 10.7 mV ($n=50$). The oocytes were clamped at -60 mV and continuously superfused with bath solution containing 80 mM NaCl, 2.5 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 and 10 mM HEPES at pH 7.4. The bath had a volume of 150 μl and the flow rate was 2 ml/min. Each concentration of chemical compounds (ACh, pirenzepine, propofol and Intralipid®) was dissolved into the bath solution and superfused at the same flow rate. Reversal potential of induced current, obtained by subtracting the current elicited by a ramp wave before induction from the current elicited by a ramp wave during induction, was measured. The ramp waves were produced using a multifunction synthesizer (NF, Tokyo). The currents induced by the ramp waves were fed into a personal computer (NEC, Tokyo) and analyzed. The experiments

were carried out at room temperature (21–23°C).

To obtain the ACh concentration-response curve, we first gave 10 μM ACh to each oocyte, and then we applied the lower concentrations of ACh at 30-min interval. Each concentration of ACh was superfused for 15 sec. At 30 min after the initial application of 100 nM ACh, the effects of propofol on the activation of current by ACh were observed in oocytes superfused with each concentration of propofol for 10 min. The initial application-induced current served as a control. At 75 min after application of propofol, we checked the induction of a similar magnitude of current by ACh as the initial application. In some oocytes, the effects of different concentrations of propofol were studied at 75-min interval. The effects of vehicle solution alone were studied using the same protocol as used for propofol. The effects of pirenzepine (10 nM–1 μM) on activation of the current by ACh (100 nM) were observed in oocytes superfused with each concentration of pirenzepine for 45 sec. The initial application-induced current served as a control. At 30 min after application of pirenzepine, we checked induction of a similar magnitude of current by ACh as the initial application.

Results were expressed as the mean \pm S.E.M. Comparisons were made by the unpaired Student's *t*-test where appropriate and one-way ANOVA (analysis of variance) complemented by Dunn's procedure as a multiple comparison procedure. IC_{50} and EC_{50} values were determined with a nonlinear regression program, Kaleida Graph™ Ver. 3.08 (Synergy Software, Reading, PA, USA).

Injection of AlF_4^- or CaCl_2

Under two-electrode voltage clamp, oocytes were injected with CaCl_2 or AlF_4^- . The concentration of CaCl_2 was 1 M. Activation of G protein by AlF_4^- is a generally used biochemical procedure. The concentrations of NaF and AlCl_3 used in biochemical studies were 10 mM and 30 μM , respectively (19). On the basis of this procedure, we injected approximately 10 nl of a mixture of NaF/ AlCl_3 (1 M/1 mM) into each oocyte using a pressure injector, Picospritzer™ II (General Valve Co.). We prepared a solution of NaF and AlCl_3 in different polypropylene tubes and mixed them just prior to injection.

During the procedure of injection, we sometimes observed inward currents with reversal potentials above -10 mV, indicating that the induced currents were not specifically Cl^- currents. To avoid this type of current, 1) we used glass pipettes with a small tip, 2) we decreased the volume of injection, and 3) the injection pipette was kept impaled after injection. Since we could not inject a constant volume of solution with the small tipped glass pipette using a mechanical injector, Nanoject (Drummond Scientific Co., Broomall, PA, USA), we injected them with the pressure injector.

Drugs

Each concentration of propofol was prepared from Diprivan® (Zeneca, Osaka), a compound containing 10 mg/ml propofol, 100 mg/ml soybean oil, 12 mg/ml egg lecithin and 22.5 mg/ml glycerin. Vehicle solution was prepared from Intralipid® (Pharmacia AB, Stockholm, Sweden), which contained 100 mg/ml soybean oil, 12 mg/ml egg lecithin and 25 mg/ml glycerin. The following drugs were used: acetylcholine chloride, NaF, pirenzepine dihydrochloride (Sigma Chemical Co., St. Louis, MO, USA), AlCl_3 (Nacalai Tesque, Kyoto). All solutions were freshly prepared before use.

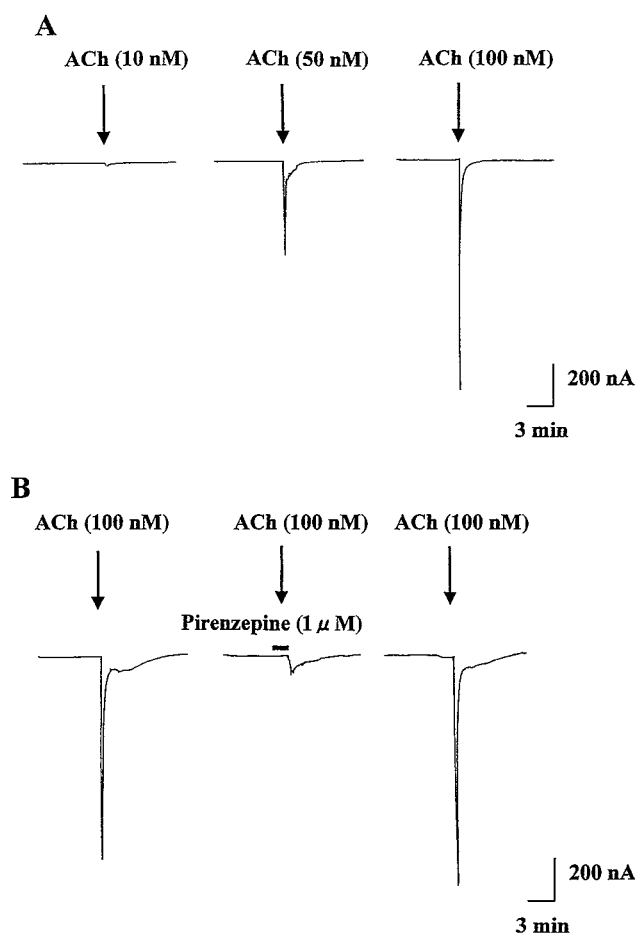


Fig. 1. Effects of acetylcholine (ACh) on oocytes expressing the rat M1 muscarinic acetylcholine receptor. A: Sequential application of ACh (10 nM, 50 nM and 100 nM) at 30 min intervals for 15 sec to a single oocyte injected with cRNA of the rat M1 muscarinic receptor activated inward currents dose-dependently. B: Application of pirenzepine (1 μM) for 45 sec before a 15-sec co-application with ACh (100 nM) reversibly inhibited activation of the current by ACh in an oocyte injected with cRNA of the rat M1 muscarinic receptor.

RESULTS

*Effects of ACh on the M1 receptors expressed in *Xenopus* oocytes*

In defolliculated oocytes not injected with cRNA for the rat M1 receptor, application of ACh (10 μM) did not induce a significant current (data not shown) (3 *Xenopus*, 24 oocytes). In oocytes expressing the M1 receptor, ACh activated the Ca^{2+} -activated Cl^- current (17) in a dose-dependent manner (Fig. 1A). The maximal current was observed at 10 μM (1665 ± 159 nA, $n=5$) and the EC_{50} value for ACh was 114 nM (Fig. 2). Re-application of the same concentration of ACh as the initial application at 30 min after the initial application resulted in induction of a similar magnitude of current (data not shown). Pirenzepine, a relatively selective antagonist of the M1 receptor, inhibited induction of the current by ACh, reversibly (Fig. 1B), with an IC_{50} of 84 nM (data not shown).

Effects of propofol on the current activated by ACh in oocytes expressing the M1 receptor

Application of propofol for 1 min did not affect activation of the current by ACh (data not shown). We found that a relatively long period of application of propofol was required to inhibit activation of the current, as shown in Fig. 3A. As shown in Figs. 3A and 4, application of propofol for 10 min reversibly attenuated the effect of ACh on the oocytes expressing the M1 receptor, in a dose-dependent manner. Propofol inhibited the ACh-induced currents by $77.9 \pm 3.9\%$ at the concentration of 10 μM and by $91.0 \pm 3.6\%$ at the concentration of 50 μM . The effects of vehicle solution on activation of the cur-

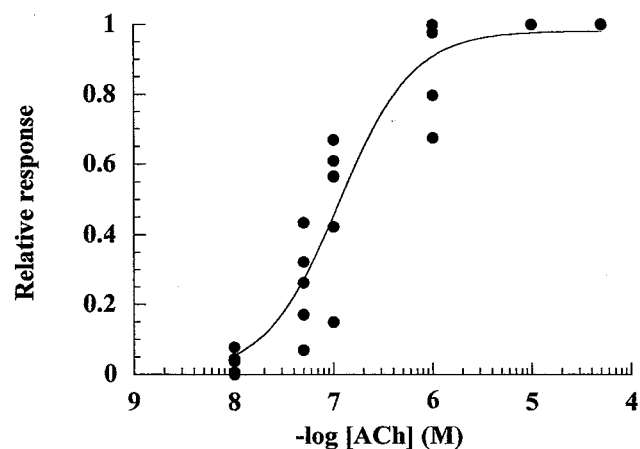


Fig. 2. Relative concentration-response relationships for ACh-induced currents in oocytes injected with cRNA of the rat M1 muscarinic acetylcholine receptor. The EC_{50} value was 114 nM and the Hill slope was 1.2. Maximal currents were obtained at the concentration of 10 μM ($n=5$). Values are given relative to the maximal response elicited by 10 μM ACh ($n=4-5$).

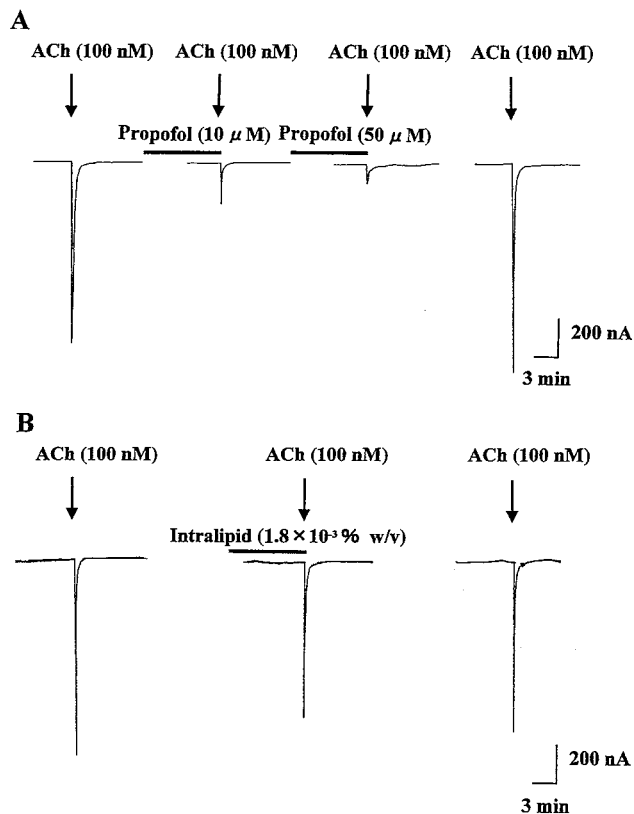


Fig. 3. Effects of propofol on the induction of current by ACh in oocytes expressing the rat M1 muscarinic acetylcholine receptor. **A:** Sequential application of propofol (10 μ M, 50 μ M) for 10 min before a 15-sec co-application with ACh at 75-min intervals inhibited the activation of current by ACh dose-dependently. The fourth application of ACh 75 min after the second application of propofol (50 μ M) induced a similar magnitude of current to the initial application of ACh in an oocyte. **B:** Application of Intralipid[®] ($1.8 \times 10^{-3}\%$ w/v) for 10 min before a 15-sec co-application with ACh (100 nM) slightly inhibited activation of the current by ACh. The third application of ACh at 75 min after the application of Intralipid[®] induced a current similar in size to that seen with the initial application of ACh.

rent by ACh in oocytes expressing the M1 receptor were then examined. As shown in Fig. 3B, Intralipid[®] at the concentration of $1.8 \times 10^{-3}\%$ w/v, which was composed of soybean oil, egg lecithin and glycerin at similar concentrations in 10 μ M propofol, attenuated the ACh-induced current by $16.2 \pm 8.1\%$ ($n=3$). Intralipid[®] at $9.0 \times 10^{-3}\%$ w/v inhibited the ACh-induced currents by $24.2 \pm 17.8\%$ ($n=4$). These effects of Intralipid[®], however, were significantly less than the effects of propofol that had the same concentrations of components as the vehicle solution.

Effects of propofol on the activation of current by injected CaCl_2 and AlF_4^-

To search for possible sites of propofol action on the

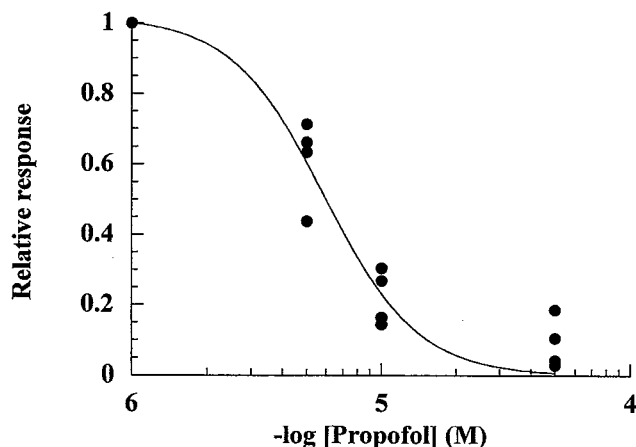


Fig. 4. Propofol inhibited the induction of current by ACh in oocytes expressing the rat M1 muscarinic acetylcholine receptor. The IC_{50} value was 5.6 μ M and the Hill slope was 2.3. The relative current amplitude obtained in the presence of 1 μ M propofol was 1.06 ± 0.05 ($n=4$). Values are given relative to the control response elicited by 10 μ M ACh ($n=4$).

M1 receptor-mediated signal transduction, the effects of propofol on CaCl_2 - and AlF_4^- -induced current were examined. Re-injection of CaCl_2 occasionally induced a larger current than that seen with the initial injection (data not shown). Therefore, each oocyte was given only one CaCl_2 injection. As shown in Fig. 5B, the injection of CaCl_2 induced an inward current. As shown in Fig. 5B, there was no detectable time lag between the injection of CaCl_2 and induction of the current. The reversal potential of the current was -13.0 ± 1.0 mV ($n=3$). In the presence of propofol, the injection of CaCl_2 induced an inward current similar in size to the current obtained in the absence of propofol (propofol(+), 853 ± 25 nA, $n=5$; propofol(-), 893 ± 49 nA, $n=5$) (Fig. 6). Re-injection of AlF_4^- 60 min after the initial injection did not induce a significant current (data not shown). Therefore, each oocyte was given only one AlF_4^- injection. As shown in Fig. 5C, AlF_4^- induced an inward current within 1 min after the injection. There was a significant time lag between the injection of AlF_4^- and induction of the current (mean = 21 sec, $n=5$). The reversal potential of the current was -16.3 ± 2.9 mV ($n=5$). The peak amplitude of AlF_4^- -induced current in the absence of propofol was 745 ± 92 nA ($n=6$), and that in the presence of propofol was 834 ± 118 nA ($n=6$) (Fig. 6). Propofol did not affect induction of the current by AlF_4^- .

DISCUSSION

Our evidence shows that propofol inhibited the M1-receptor-mediated signal transduction and that this inhi-

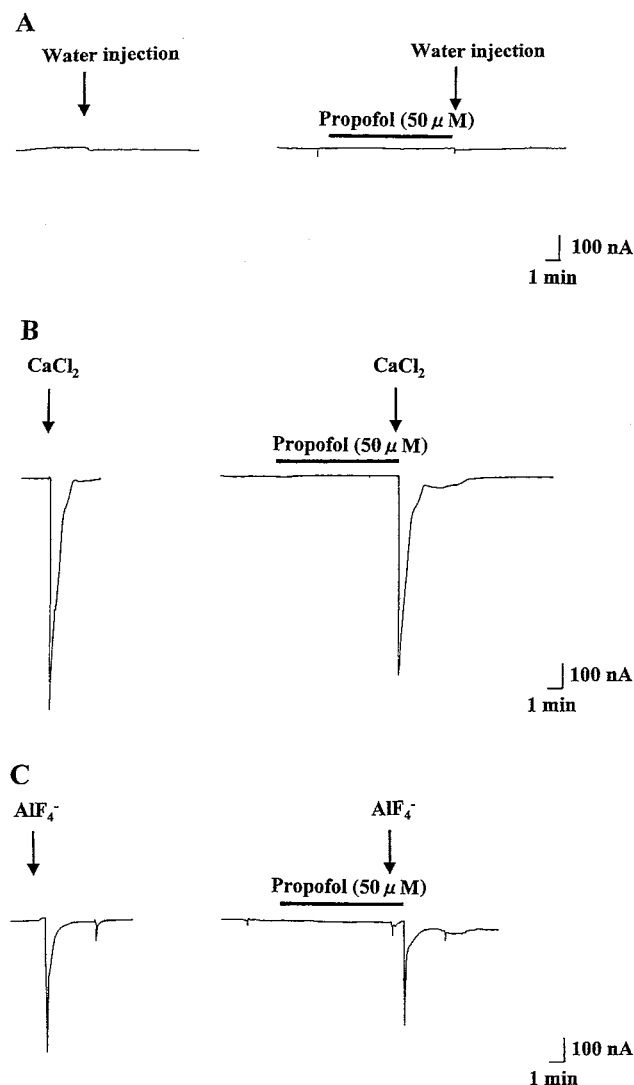


Fig. 5. Effects of propofol on CaCl_2 -induced current and AIF_4^- -induced current in oocytes not injected with cRNA. **A:** Water injection (10–20 nl) did not induce a significant current in the presence or absence of propofol (50 μM) in an oocyte. **B:** Injection of CaCl_2 (approximately 10 nl) activated currents in the absence or presence of propofol (50 μM), in different oocytes. **C:** Injection of AIF_4^- (approximately 10 nl) activated currents in the absence or presence of propofol (50 μM) in different oocytes. In A, B and C, propofol was applied 10 min before injection and was continued for 1 min.

bition was not due to the effect of vehicle solution.

The IC_{50} value of propofol on the M1 receptor-mediated signal transduction was 5.9 μM , a concentration much lower than the effective concentrations of propofol on the nicotinic acetylcholine receptor in BC3H1 mouse tumor cells (IC_{50} = 81 μM) (11), the sodium channel in human brain tissue (IC_{50} = 20 μM) (12), the calcium current in chick sensory neurons in which the inhibition was observed at the concentration of 300 μM (14), and the potassium channel in PC12 cells in which the inhi-

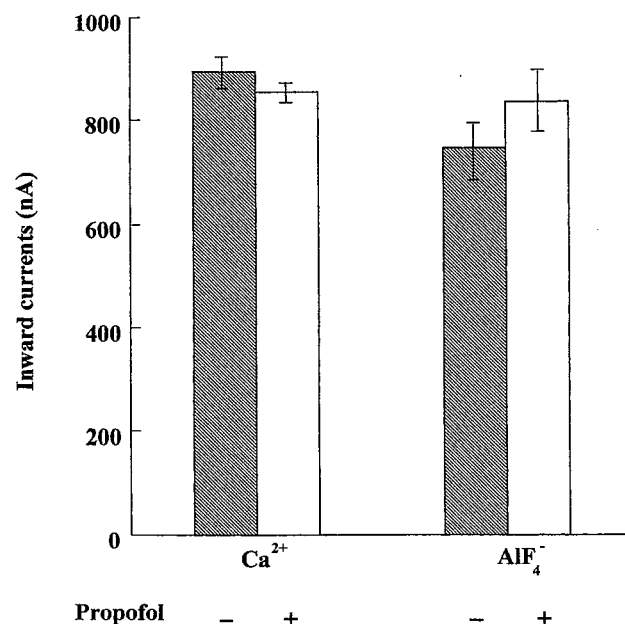


Fig. 6. Propofol did not inhibit CaCl_2 -induced or AIF_4^- -induced current. Data are expressed as the mean \pm S.E.M., n = 5 (CaCl_2) or n = 6 (AIF_4^-).

bition was observed at concentrations above 50 μM (13). The primary site of action of propofol is apparently on the GABA_A receptor (9, 10). Hales et al. (10) reported that propofol (1.7–16.8 μM) potentiated the amplitude of membrane current elicited by GABA in bovine chromaffin cells; this concentration of propofol is comparable to the effective concentration of propofol on the M1 receptor-mediated signal transduction in our present study.

Application of ACh elicits the Ca^{2+} -activated Cl^- current via a signal transduction pathway through the M1 receptor, G protein, phospholipase C and release of Ca^{2+} . The injection of CaCl_2 into oocytes directly elicited the Ca^{2+} -activated Cl^- current. Propofol did not attenuate induction of the current by the injection of CaCl_2 , indicating that the Ca^{2+} -activated Cl^- was not affected by propofol treatment. AIF_4^- binds to GDP on heterotrimeric G protein, and this GDP- AIF_4^- complex promotes the dissociation of heterotrimeric G protein into subunits, leading to the activation of G protein (20). In our present study, propofol did not affect induction of the current by the injection of AIF_4^- , thereby ruling out the possibility that G protein dissociation, phospholipase C activation, and Ca^{2+} release are responsible sites in the effects of propofol on the M1-receptor-mediated signal transduction.

One possible explanation for the effects of propofol on the M1 receptor-mediated signal transduction is that

binding of ACh to M1 receptor is inhibited by propofol. Another notion is that interaction between M1 receptor and G protein is inhibited by propofol. In *Xenopus* oocytes, the expressed M1 receptor utilizes G proteins of the pertussis toxin-insensitive Gq or G₁₁, and the M1 receptor uses the pertussis toxin-sensitive Gi or Go to couple to phospholipase C (21). Since *Xenopus* Gαq, Gα11 (22), Gαi1, Gαi3 and Gαo (23, 24) are 75–89% identical to their mammalian homologues, the results obtained with the oocyte expression system may mimic the action of propofol in the mammalian system. Hence, the importance of the inhibitory effect of propofol on the M1 receptor-mediated signal transduction in mammalian can be assessed.

Lin et al. (5) reported that enflurane inhibited M1-receptor- and serotonin-receptor-mediated signal transduction in *Xenopus* oocytes; they found that enflurane prevented the activation of G protein by 5'-3-O-(thio)triphosphate (GTPγS), a nonhydrolyzable GTP analog, but did not affect the GTPγS-induced Ca²⁺-activated Cl⁻ current. These findings suggest that enflurane affects GDP-bound heterotrimeric G protein. This mechanism differs from the inhibitory mechanism of propofol on the M1-receptor-mediated signal transduction, which we observed in the present study. The effects of halothane on agonist and antagonist binding to muscarinic receptors in rat brain have been extensively studied (2, 3). They showed that the anesthetic eliminated the potential of guanine nucleotide to lower agonist binding affinity. They speculated that the halothane might interfere with GDP release, GTP binding or the dissociation of receptor-G protein complexes in response to agonist binding. The inhibitory effects of propofol on the M1-receptor-mediated signal transduction could be explained with mechanisms similar to the inhibitory mechanism of halothane.

Rossi et al. (25) reported that propofol did not affect the M1-receptor-mediated signal transduction. Although they used propofol in Intralipid® as we did in the present study, propofol was applied for only one minute. In our present study, a one minute application of propofol failed to inhibit the signal transduction, and we observed the inhibitory effects of propofol applied for 10 min, although the anesthetic effect of propofol is achieved within a few minutes (8). Thus, further observations on this regard are required.

In summary, this study showed that propofol inhibits the M1-receptor-mediated signal transduction at the receptor site and/or the site of interaction between the receptor and associated G protein. Our results are consistent with the proposal of involvement of muscarinic signal transduction processes in actions of anesthetics, although their relationship to anesthetic actions is not

known. Further elucidation of molecular mechanisms underlying the effect of propofol on the M1-receptor-mediated signal transduction would help us to understand the interactions between anesthetics and proteins.

Acknowledgments

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