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Comparison of antagonizing potencies of dodecane analogues to isoflurane in goldfish

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Abstract
Purpose. We reported previously that long-chain fatty acids (carbon atoms ≧12) antagonize volatile anesthetics in goldfish. To examine the contribution of the carboxyl group to the antagonizing potency of fatty acids in vivo, we compared antagonizing potencies to isoflurane in goldfish among terminally substituted dodecane analogues.
Methods. Dodecane (carbon atoms = 12) analogues [fatty acid (DoAC), alcohol (DoAL), alkane (DoAK), sulfate (DoSF), trimethylammonium (DoTA)] were examined. We determined the EC_{50} (the anesthetic concentration producing a 50% effect) values of isoflurane in the absence or presence of these chemical compounds in goldfish by observing the escape reaction of goldfish from an electrical stimulus.
Results. DoAC at higher than 10 μM and DoAL at higher than 20 μM increased the EC_{50} values of isoflurane in a concentration-dependent manner compared with the control ($P < 0.05$). DoAC at 50 μM and DoAL at 100 μM increased the EC_{50} 1.7- and 1.6 fold, respectively. DoAK, DoSF, and DoTA showed no significant differences from the control. In the comparison of DoAC and DoAL at the same concentration, DoAC was more effective than DoAL ($P < 0.001$).
Conclusion. DoAC and DoAL showed antagonizing potencies to isoflurane, whereas DoAK, DoSF, and DoTA had no effect. DoAC was more effective than DoAL. The findings suggest that polarity of the chemical compounds may be necessary to exert antagonizing potency to isoflurane. Furthermore, a highly negative charge density of the carboxyl group may be responsible for the effective antagonization of DoAC to isoflurane.

Key words Dodecane · Isoflurane · Antagonist

Introduction

In spite of the wide use of volatile anesthetics, specific antagonists have not been developed. Volatile anesthetics may act on multicomponents in the central nervous system, such as γ-aminobutyric acid type A ($GABA_A$) and *N*-methyl-D-aspartate (NMDA) receptors [1,2]. Tatara et al. [3] reported that myristate, a 14-carbon fatty acid, increased the EC_{50} (the anesthetic concentration producing a 50% effect) of volatile anesthetics two- to threefold in goldfish; that was the first report demonstrating that chemical compounds effectively antagonized volatile anesthetics in vivo. Although the mechanism of antagonism by myristate is not clear, the antagonism may be explained by comparing the effects of myristate and volatile anesthetics on the protein conformation, which reveals that the former tightens the tertiary structure of firefly luciferase (FFL) whereas the latter loosens it [4].

Long-chain fatty acids have no specific structure other than a long alkyl chain and a carboxyl group. The antagonizing effects of fatty acids to halothane have been reported in a 12-carbon fatty acid but disappeared in fatty acids of chain lengths less than 10 carbons [5]. Elongation of the alkyl chain increases the hydrophobicity of fatty acids. Therefore, the finding that fatty acids required chain lengths longer than 12 carbons for antagonization suggests that hydrophobicities of fatty acids may play an important role in their antagonizing potencies.

On the other hand, the contribution of the carboxyl group of fatty acids to antagonizing potency is not clear. In vitro studies, using the K^+ channel of gastric smooth muscle cells in toads [6] and the Ca^{2+}-activated K^+ channel of pulmonary arteries in rabbits [7,8], showed that long-chain fatty acids and negatively charged lipids activate ion channels, whereas positively charged lipids suppress activity and neutral lipids have no effects. These findings suggest that the charge of lipids may determine their effects on the activities of membrane proteins.

To examine the contribution of the carboxyl group to antagonizing potency in vivo, we compared antagonizing potencies to isoflurane in goldfish among dodecane

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analogues. The carboxyl group of fatty acid was substituted for the hydroxyl, methyl, sulfate, or trimethylammonium group.

Materials and methods

This study was approved by the Animal Experimentation Committee of Kyorin University. Goldfish, 7–9 cm in body length, were purchased at the Edogawa Fish Farm (Tokyo, Japan). They were acclimated at 28°C for at least a week in aerated tap water before the experiment. Isoflurane was obtained from Abbott (North Chicago, IL, USA). The dodecane analogues obtained from Sigma (St. Louis, MO, USA) were as follows: fatty acid (DoAC, sodium dodecanoate; negatively charged), alcohol (DoAL, 1-dodecanol; polar), alkane (DoAK, *n*-dodecane; neutral), sulfate (DoSF, sodium dodecyl sulfate; negatively charged), and trimethylammonium (DoTA, dodecyltrimethylammonium bromide; positively charged). The chemical structures are shown in Fig. 1. In advance, we made a stock solution with each compound dissolved in methanol. The concentration of the solutions was adjusted to equal 5 mM of methanol in water. The effect of dissolved methanol on EC_{50} of isoflurane was considered to be negligible because the final concentration of methanol in water was much smaller than EC_{50} of methanol (590 mM) [9]. Control studies without any compounds were performed in water with the same amount of methanol. The maximum concentration of the compounds was 50 μM for DoAC and DoAK, 100 μM for DoAL, and 200 μM for DoSF, because at higher concentrations the solutions precipitated due to oversaturation. For DoTA, we used a concentration of 10 μM because higher concentrations were found to be extremely toxic to goldfish.

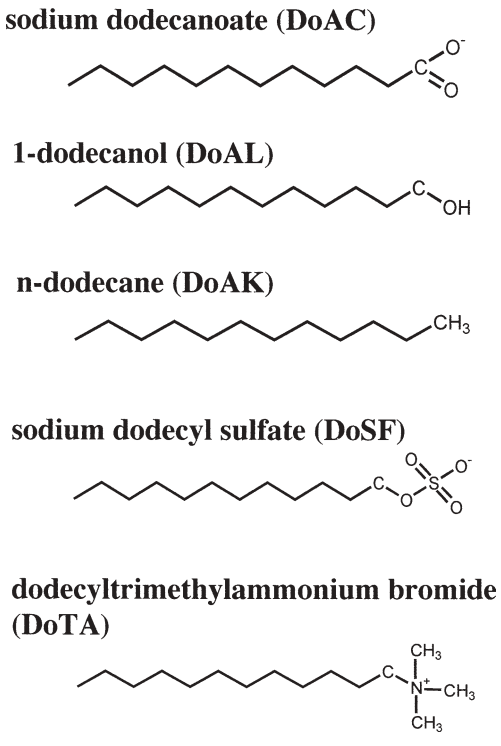

Fig. 1. Structure of dodecane analogues

The anesthetic effect was measured by recording the avoidance response to electrical stimuli in goldfish [10,11]. We used a glass tank 200 mm in diameter and 90 mm in depth for this experiment. A pair of circular stainless steel screens, 180 mm in diameter, was placed on the water surface and the bottom to apply electrical stimuli by a constant current generator (Tokushu Keisoku, Yokohama, Japan), and we determined the threshold of the electrical current intensity where goldfish showed avoidance response to stimuli. Because all goldfish responded at 8.0 mA of electrical current (0.2 s in duration, square wave) and no adverse residual effect was observed after stimulation, we adopted the current intensity at 8.0 mA for electrical stimulation.

Before each experiment, goldfish were placed in a bucket with distilled water containing each concentration of the compounds or the control for 2 h. Isoflurane was vaporized by an anesthesia machine with oxygen flow of 2 l/min, bubbled and stirred in the glass tank for 30 min. The vaporizer was set at 0.5%–5%. The glass tank contained 3200 ml distilled water with the same concentration of each compound and was placed on the stirrer with the temperature control unit. Groups of 10 goldfish were put in the glass tank and exposed to isoflurane for 30 min. Then, they were given electrical stimuli, applied four times at 10-s intervals. Each goldfish was determined to be in a nonanesthetic state if it showed at least one avoidance response to electrical stimulus. Each goldfish was used once. The temperature of the water was controlled at 21° ± 2°C using the temperature control unit of the stirrer. A preliminary experiment had shown that a single application of each compound did not affect the avoidance response of goldfish with electrical stimuli. We used 20 goldfish to decide the anesthetic effect of each concentration of each compound and of the control.

The concentration–response curves of isoflurane at various concentrations of compounds and the control were obtained. We analyzed data using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). EC_{50} was calculated, and the concentration–response curves were fitted according to the following equation:

$$y = 1 \Big/ \left(1 + \left[p/\mathrm{EC}_{50}\right]^{n}\right)$$

where y is the fraction of avoidance response of goldfish (numbers of avoiding goldfish/total numbers of gold-

fish), p is the concentration of isoflurane (%), and n is the Hill slope [12]. The data are presented as $EC_{50} \pm$ SE. EC_{50} in the presence of compounds was compared with that in the control using the extra sum-of-squares F test [13]. Differences were considered to be statistically significant when P was less than 0.05. Furthermore, when the compounds significantly increased EC_{50} compared with the control, the effects were compared between compounds at the same concentration.

Results

The compounds tested did not exert a side effect such as excitation or sedation. All goldfish apparently recovered from the anesthesia after the experiments.

Dose–response curves of isoflurane in goldfish

Figure 2 demonstrates dose–response curves of isoflurane in the presence of DoAC or DoAL. Both compounds shifted the curves to the right compared with the control. In the presence of DoAC above 10 μM and DoAL at 20 μM, more than 20% of tested goldfish responded to the electrical stimuli at as high as 4% isoflurane, whereas the control group did not respond. In contrast, DoAK, DoSF, and DoTA did not change the curves (Fig. 3). The n values were between 3 and 4 in all dose–response curves.

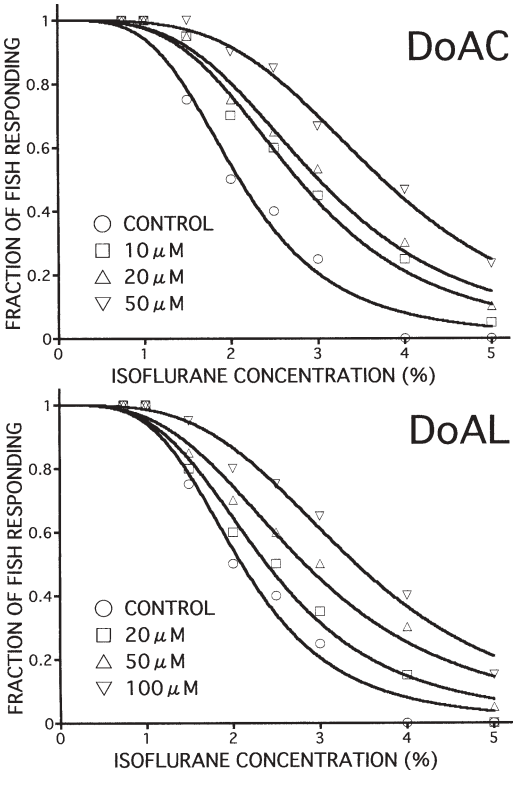

Fig. 2. Dose–response curves of isoflurane in the presence of sodium dodecanoate (*DoAC*) or 1-dodecanol (*DoAL*) in goldfish. *Drawn lines* indicate the fitted curves

EC_{50} of isoflurane in goldfish

Effects of the compounds on the EC_{50} values of isoflurane are shown in Fig. 4. DoAC above 10 μM and DoAL above 20 μM significantly increased the EC_{50} values of isoflurane in a concentration-dependent manner compared with the control. DoAC at 50 μM and DoAL

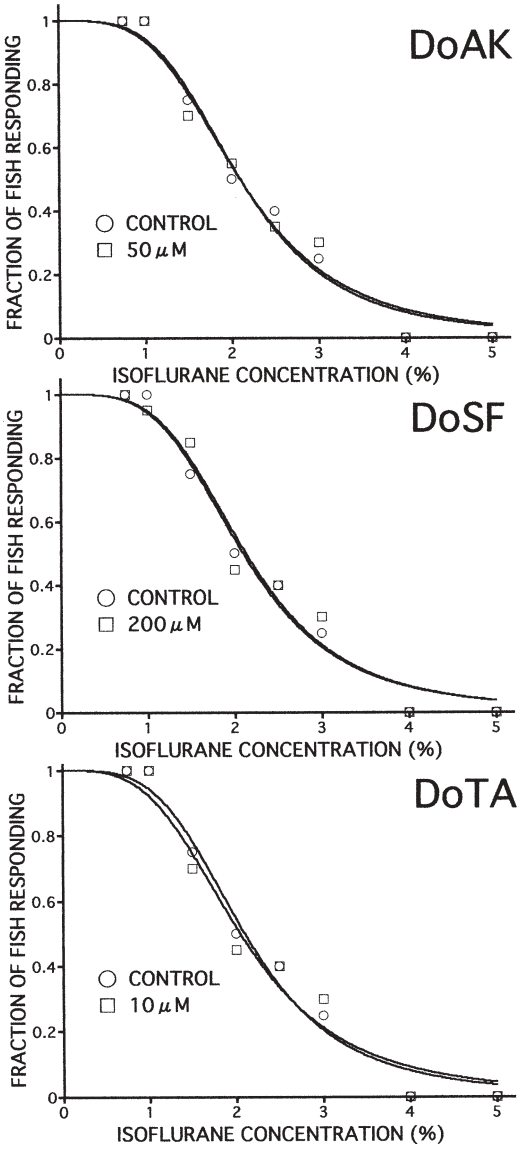

Fig. 3. Dose–response curves of isoflurane in the presence of *n*-dodecane (*DoAK*), sodium dodecyl sulfate (*DoSF*), or dodecyltrimethylammonium bromide (*DoTA*) in goldfish. *Drawn lines* indicate the fitted curves

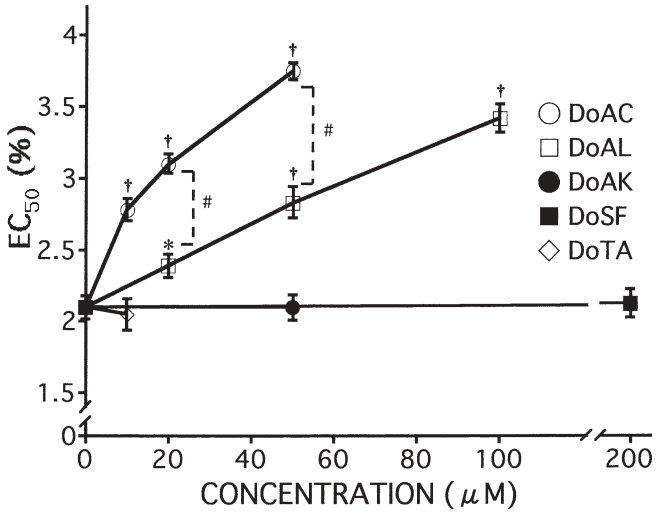

Fig. 4. Effects of dodecane analogues on the 50% effective concentration (EC_{50}) values of isoflurane. Data are $EC_{50} \pm$ SE. Significant differences in comparison with the control ($^{*}P < 0.05$, $^{\dagger}P < 0.001$) and between compounds at the same concentration ($^{\#}P < 0.001$). *DoAC*, sodium dodecanoate; *DoAL*, 1-dodecanol; *DoAK*, *n*-dodecane; *DoSF*, sodium dodecyl sulfate; *DoTA*, dodecyltrimethylammonium bromide

at 100 μM increased the EC_{50} 1.7- and 1.6 fold, respectively. DoAK, DoSF, and DoTA showed no significant difference from the control. In the comparison of DoAC and DoAL at the same concentration, the former was significantly more effective than the latter at 20 μM and 50 μM.

Discussion

To elucidate the contribution of the carboxyl group to antagonization to volatile anesthetics in vivo, we have examined antagonizing potencies to isoflurane in goldfish by changing the terminal substitution of dodecane analogues. The EC_{50} and *n* values of isoflurane in the absence of dodecane analogues were 2.10% and 3–4, respectively. The considerable differences of these values from MAC and *n* values (6–20) [14] may be in part attributable to the difference in stimulating method. Because multiple contributing systems are considered to increase *n* values [14], less complex pathways may be associated with avoidance response to electrical stimulus in goldfish.

Both DoAC (carboxyl group), and DoAL (hydroxyl group) antagonized isoflurane in a concentration-dependent manner. In contrast, DoAK (methyl group), DoSF (sulfate group), and DoTA (trimethylammonium group) did not show significant effects on anesthetic potencies of isoflurane. Our findings suggest that the physical properties of the terminal groups of dodecane analogues may play an important role in the antagonizing potency to isoflurane. The phenomenon of antagonization cannot be attributed to changes of physical factors induced by the compounds. First, in the preliminary study it was found that the presence of compounds did not significantly affect the electrical threshold of goldfish to electrical stimulus. Therefore, the change of electrical threshold due to the absorption of compounds on the skin of goldfish cannot be a cause of antagonization. Second, the depletion of isoflurane in water by partitioning into the compounds is unlikely because isoflurane was constantly bubbled into water during the experiments. Instead, the antagonization may be explained by the effects of compounds absorbed into the interior of the body of goldfish on the central nervous system of goldfish. Dose-dependent antagonizing effects of DoAC and DoAL lend support to this conclusion. Additionally, it was reported that diffusion across the blood–brain barrier with rapid incorporation is simply dependent on the physicochemical properties of the fatty acids, and even long-chain fatty acids (palmitic acid) injected intravenously may enter the brain about 0.8% of the amount injected [15]. Therefore, it is conceivable that charged or large molecular weight compounds absorbed into the interior of the body of the goldfish, probably through the gill or gastrointestinal tract, may pass the blood–brain barrier only minimally and enter the goldfish brain.

The antagonism of DoAC and DoAL in goldfish thus suggests that these compounds may affect the active site or mode of action of isoflurane, resulting in decreased anesthetic potency. The structural perturbation of lipid bilayers of cell membranes caused by the partitioning of dodecane analogues is not central to the mechanism of antagonization, because compounds with the same alkyl chain may similarly partition into lipid bilayers. Instead, the antagonization may be explained by the interaction between the terminal groups of dodecane analogues and the interface with membrane protein, which is considered to be the main target for volatile anesthetics [16]. For DoAC, it is well recognized that fatty acids are positioned to act as messenger molecules that regulate specific ion channels and receptors such as potassium channels and $GABA_A$ and NMDA receptors, which are candidates for anesthetic sites of action [17–19]. It is unlikely that DoAC antagonizes isoflurane in a competitive manner, because DoAC is negatively charged and isoflurane is noncharged. Therefore, it is reasonable to assume that fatty acids act on these membrane proteins in a noncompetitive manner with isoflurane and thus change their conformation opposite to the action of isoflurane. This scenario is supported by the contrasting effects of myristate and volatile anesthetics on firefly luciferase (FFL), which are that volatile anesthetics decreased the phase transition temperature from the solid

gel to the liquid-crystal state by loosening protein structure, whereas myristate increased it by tightening protein structure [4].

In the case of DoAL, it is becoming clear that alcohols can affect both the structure and function of a wide variety of cellular proteins such as neurotransmitter receptors and signaling molecules by direct binding [20,21]. It is not surprising that DoAL did not show an anesthetic action in goldfish, because the lack of anesthetic potency of alcohol beyond a certain chain length (about 8–12 carbon atoms) is well known as the "cutoff phenomenon" [9,21]. The alcohol binding site is thought to consist of hydrogen bond acceptor in a turn or loop region that is often situated at the N-terminal end of an α-helix. Binding of alcohols at these sites may alter the local protein structure and perturb the function of key proteins [21]. The formation of hydrogen bonding between the hydroxyl group of DoAL and membrane proteins may therefore play a role in the antagonization to isoflurane, considering that neutral DoAK did not show any significant effects on anesthetic potencies of isoflurane in goldfish.

The antagonizing effect was larger in DoAC than in DoAL. The difference between DoAC and DoAL in their antagonizing potencies may be explained by the higher ability of electrostatic interaction to change protein conformation than hydrogen bonding. In addition, Takehara et al. [22] investigated the binding properties of dodecane analogues to FFL by using the fluorescence characteristics of 2-(*p*-toluidino)naphthalene-6-sulfate (TNS) and the bioluminescence of luciferin–luciferase mixture. Both DoAC and DoAL inhibited the bioluminescence of the luciferin by FFL, but the former entirely inhibited the binding of TNS to FFL, whereas the latter inhibited only about 25% of initially bound TNS. These results suggest that DoAC may bind to FFL in competition with substrate luciferin and stabilize the native state of FFL. In contrast, DoAL binds to FFL in a noncompetitive manner and may slightly change the FFL conformation. These differences between DoAC and DoAL in their binding sites and abilities to change protein conformations may account for the difference in antagonizing potencies of these compounds.

On the other hand, DoSF and DoTA did not show any significant effects on anesthetic potencies of isoflurane, even though they are also charged, which suggests that not all charged molecules antagonize isoflurane. DoTA is a strong detergent and perturbs membrane structures, resulting in toxic effects at the higher concentration in vitro [6–8]. Consistent with this, goldfish died at higher concentrations of DoTA than those used in the present study. In the case of DoSF, the negative charge density is low due to the large size of the sulfate group (OSO_3^-, molecular weight = 96) compared with the carboxyl group (COO^-, molecular weight = 44). Because the electrostatic interaction between charged groups of chemical compounds and membrane proteins grows stronger as the charge densities of terminal groups increase, the lack of an antagonization property of DoSF may be attributable to a weak ability of DoSF to change protein conformation. However, we cannot rule out the possibility that DoSF may require longer time to equilibrate within the central nervous system and higher concentrations to exert some effects on membrane proteins.

The current study has a limitation in the comparison of antagonizing potencies of dodecane analogues because we did not measure the concentrations of these compounds in the brain. However, it is expected that the concentrations of DoAL and DoAK in the brain are higher than those of other dodecane analogues because lipid-soluble substances permeate the blood–brain barrier more easily than charged substances [23]. On the other hand, it is unlikely that the concentrations of DoAC, DoSF, and DoTA in the brain differ markedly because they are all charged and have similar molecular weights. The lack of antagonizing potency of DoAK therefore suggests that charge or hydrogen bonding of compounds may be necessary for exerting antagonizing efficacy to isoflurane. At present, we have no clinical antagonist specific to volatile anesthetics. The development of an antagonist to volatile anesthetics may help accelerate recovery from anesthesia, such as in ambulatory anesthesia. Our findings that high polarity (i.e., highly negative charge density or hydrogen bonding) may increase antagonizing efficacy to isoflurane may be helpful in designing such an antagonist.

In summary, we compared antagonizing potencies to isoflurane in goldfish among dodecane analogues. Both DoAC and DoAL antagonized isoflurane, but DoAC was more effective than DoAL. In contrast, DoAK, DoSF, and DoTA showed no antagonizing potencies at the concentrations tested. The results suggest that polarity of chemical compounds may be necessary to exert an antagonizing potency to isoflurane. Furthermore, a highly negative charge density of the carboxyl group may be responsible for the effective antagonization of DoAC to isoflurane.

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