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Antagonizing potencies of saturated and unsaturated long-chain free fatty acids to isoflurane in goldfish

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Abstract
Purpose. We have previously reported that myristate, a saturated free fatty acid (FFA) with 14 carbons (C14), antagonizes volatile anesthetics in goldfish. The hydrophobicity and molecular configuration of FFAs may play an important role in the antagonizing effect. To examine their contribution, we investigated the antagonizing potencies of saturated and unsaturated long-chain FFAs in goldfish.
Methods. Saturated and monounsaturated FFAs of C14–18 were tested. We determined the anesthetic concentration producing a 50% effect (EC_{50}) of isoflurane in the absence or presence of FFA by observing the escape reaction of goldfish against an electrical stimulus.
Results. All FFAs increased the EC_{50} of isoflurane dose-dependently compared with reactions in the absence of FFA ($P < 0.05$). For saturated FFAs, the relationship between chain lengths and antagonizing potencies was not linear. C18 was the most effective and C16 was the least effective antagonist ($P < 0.05$). Among unsaturated FFAs, C14 was the most effective antagonist ($P < 0.05$). In a comparison of saturated and unsaturated FFAs, saturated C14 and C18 were more effective antagonists than unsaturated FFAs of the same carbon numbers ($P < 0.05$).
Conclusion. The hydrophobicity of FFAs increases as the chain length increases. Therefore, our findings suggest that the antagonizing effect of long-chain FFAs in goldfish, in terms of their capacity to perturb the lipid membrane structure, may be determined not solely by their hydrophobicity but also by their molecular configuration.

Key words Free fatty acid · Volatile anesthetics · Antagonist

Introduction

Free fatty acids (FFAs) play an important role in cellular signaling by acting as messengers [1]. Long-chain FFAs (chain length between 14 and 18 carbons) directly regulate the activity of specific ion channels and receptors, such as potassium channels, estradiol, and muscarinic receptors [1–3]. In recent years, oleamide, the amide derivative of oleic acid, has attracted attention as an endogenous sleep-inducing substance by potentiating γ-aminobutyric $acid_A$ ($GABA_A$) receptor function [4]. Furthermore, lipid emulsions, oil-in-water formulations clinically used as drug carriers, have been reported to affect N-methyl-D-aspartate (NMDA) receptor activity [5]. Because $GABA_A$ and NMDA receptors are considered to be possible acting sites of general anesthetics [6,7], these findings suggest that FFAs may modulate anesthetic action. However, little work has been performed to explore the effect of FFAs on anesthetic potency in vivo.

Tatara et al. [8] reported that myristate (saturated free fatty acid with 14 carbons, saturated C14) increased the anesthetic concentration producing a 50% effect (EC_{50}) of volatile anesthetics in goldfish. That was the first report to demonstrate the action of an FFA on anesthetic potency in vivo. A previous in vitro study, using firefly luciferase, showed that anesthetics decreased the thermal transition temperature of firefly luciferase by relaxing its molecular conformation, whereas saturated C14 increased the transition temperature by tightening the conformation [9]. This contrasting effect of anesthetics and saturated C14 may explain the antagonizing effect of saturated C14 in goldfish. Furthermore, Matsuki et al. [10] reported that this tightening effect was observed in saturated FFAs with a carbon chain longer than 10. The effects were enhanced as chain length increased, suggesting that the hydrophobicity of FFAs may play an important role in the tightening of molecular conformation and thus anesthetic antagonism.

Based on these earlier findings, we investigated the antagonizing potencies of saturated and monounsaturated C14–18 to isoflurane in goldfish in order to

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examine the contribution of the hydrophobicity and the molecular configuration of FFAs to their antagonizing potency in vivo. The presence of a double bond in the carbon chain may affect antagonizing potencies because it changes the molecular configuration. We used goldfish to determine anesthetic potency because of (1) easy test administration, (2) inexpensiveness, and (3) commercial availability at any season [11].

Materials and methods

This study was approved by the Animal Experimentation Committee of Kyorin University. Goldfish, 4 to 6 cm in body length, were purchased from Edogawa Fish Farm (Tokyo, Japan) and acclimated at 28°C in aerated tap water for at least 1 week before the experiment. All chemicals were purchased from Sigma (St. Louis, MO, USA). Isoflurane was obtained from Abbott (North Chicago, IL, USA). The following FFAs were studied (structural formulas shown in Fig. 1); Na-myristate (saturated C14), myristoleic acid (unsaturated C14), Na-palmitate (saturated C16), palmitoleic acid (unsaturated C16), Na-stearate (saturated C18), and oleic acid (unsaturated C18). Because long-chain FFAs are sparingly soluble in water, we made stock solutions in methanol. The final concentration of methanol was adjusted to 5 mM. Control studies without FFA were performed in water with the same concentration of methanol. Because the final concentration of methanol in water was much lower than the EC_{50} of methanol (590 mM) [12], the effect of dissolved methanol on the EC_{50} of isoflurane was considered to be negligible.

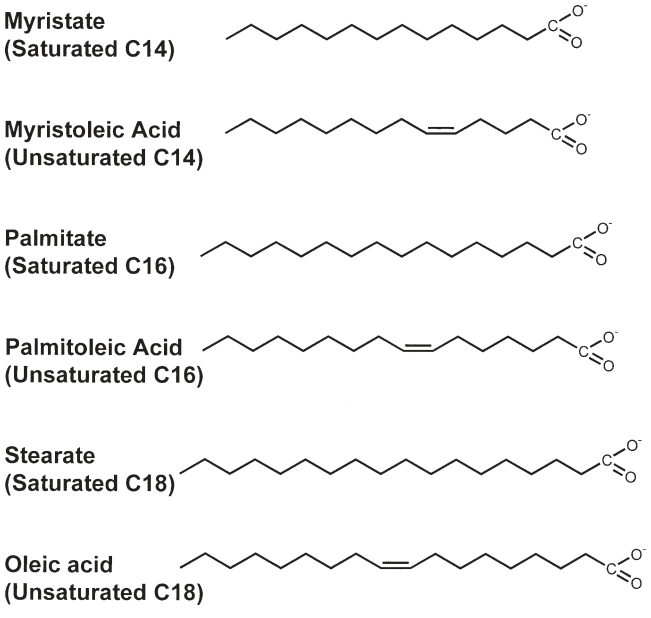

Fig. 1. Structural formulas of tested free fatty acids (FFAs)

We used a glass tank 200 mm in diameter and 90 mm in depth for the experiment. A pair of circular stainless steel screens 180 mm in diameter were placed on the surface and the bottom to apply electrical stimuli by a constant current generator (Tokushu Keisoku, Yokohama, Japan). The threshold of electrical current intensity at which the goldfish showed escape motion was determined. Because all goldfish responded to an electrical current of 8.0 mA and no adverse residual effect was observed after stimulation, we adopted 8.0 mA of square-wave current for the electric stimulation throughout the experiments.

Before each experiment, goldfish were placed in a bucket with distilled water containing 5, 10, or 20 μM of each FFA for 2 h. A suspension micelle was formed at a high concentration of FFA. Saturated C18, the most hydrophobic FFA, became muddy at over 20 μM, so the concentration of each FFA was maintained at less than 20 μM. The glass tank was placed on a hot stirrer (AS ONE, Osaka, Japan), and the temperature of the water was maintained at 21 ± 1°C throughout the experiment. Isoflurane was bubbled for 30 min and stirred in the glass tank, which contained 3200 ml of distilled water with the same concentration of each FFA as in the bucket. Ten goldfish were placed in the test tank and exposed to the anesthetic for 30 min. Then, they were electrically stimulated at 8.0 mA for 200 ms four times, with a 10-s interval between stimulations. Each goldfish was evaluated as unanesthetized when it showed an escape motion at least once. The movements of each goldfish were recorded with a digital video camera, and the number of goldfish in an unanesthetized state was rechecked. We used 20 goldfish for each concentration of isoflurane.

The concentration-response curves of isoflurane with or without FFA were obtained according to the following equation:

$$y = 1/(1 + (p/EC_{50})^n)$$

where y is the fraction of goldfish responding to the stimulus, p is the concentration of isoflurane (%), and n is the slope of the curve [13]. The EC_{50} was calculated by logistic regression with GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). The EC_{50} values in the presence of FFA were compared with that in the control using the extra sum-of-squares F test [14]. $P < 0.05$ was considered statistically significant. Data are presented as the EC_{50} ± SE.

Results

The goldfish showed no remarkable adverse effects, such as toxicity or agitation in response to the FFAs. The application of each FFA before the administration

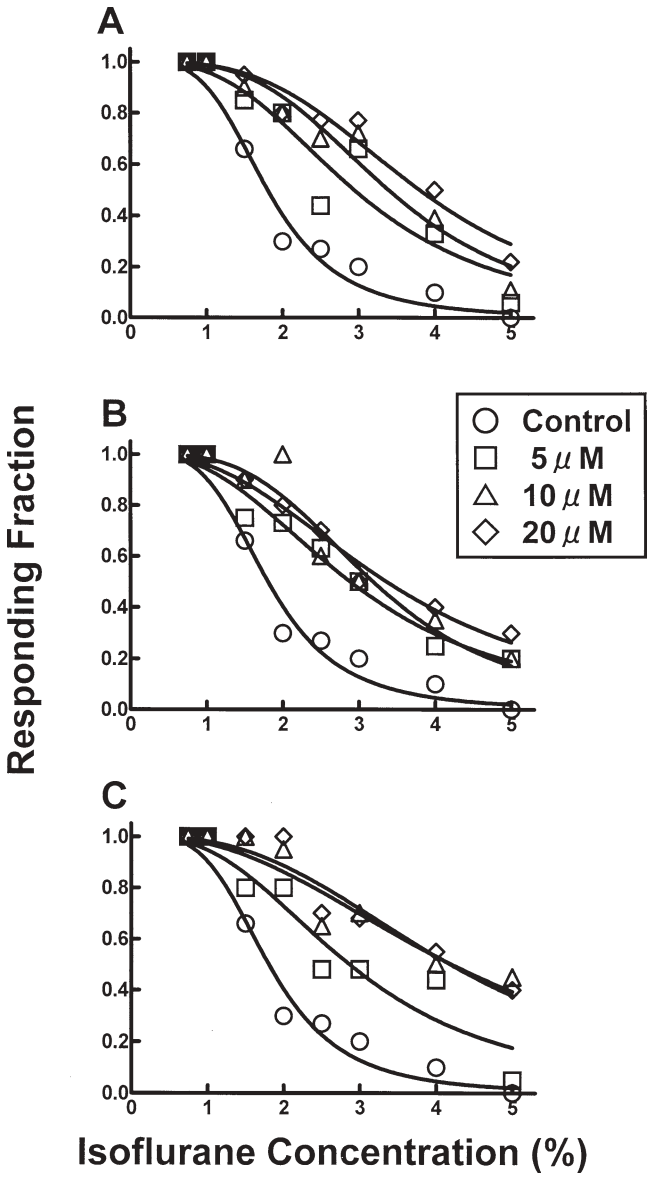

Fig. 2A–C. Concentration-response curves of isoflurane in goldfish in the absence and presence of saturated FFAs (*concentrations shown in box*). **A** Myristate (saturated C14); **B** palmitate (saturated C16); and **C** stearate (saturated C18)

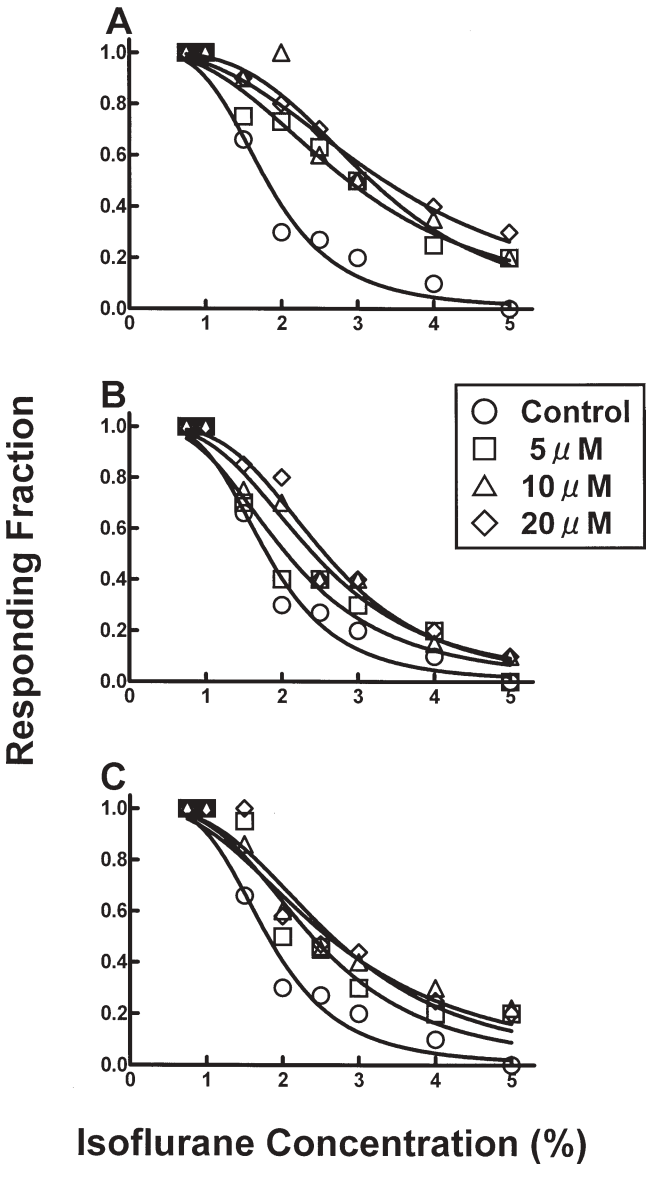

Fig. 3A–C. Concentration-response curves of isoflurane in goldfish in the absence and presence of unsaturated FFAs. **A** Myristoleic acid (unsaturated C14); **B** palmitoleic acid (unsaturated C16); and **C** oleic acid (unsaturated C18)

of isoflurane did not affect the escape reaction of goldfish to an electrical stimulus. All goldfish apparently recovered after the experiments.

Concentration-response curves of isoflurane

The concentration-response curves of isoflurane with saturated or unsaturated FFAs are shown in Figs. 2 and 3. As the concentration of isoflurane increased, the fraction of goldfish responding to the stimulation decreased. The concentration-response curves thus were sigmoid. All FFAs shifted the curve in the control to the right in a concentration-dependent manner. In the presence of saturated C18 at a concentration higher than 10 μM, one-third of the goldfish showed an escape reaction, even at 5% isoflurane, whereas no goldfish in the control exhibited an escape reaction at the same isoflurane concentration.

Effects of FFA on the EC_{50} of isoflurane

The changes of EC_{50} values in the presence of 5, 10, or 20 μM FFAs are shown in Table 1. All FFAs significantly increased the EC_{50} of isoflurane, except for 5 μM

Table 1. EC_{50} (%) of isoflurane in the presence of FFAs

	FFA (μM)			
	0	5	10	20
Control	1.81 ± 0.09			
Saturated C14		2.94 ± 0.24**	3.40 ± 0.16***	3.81 ± 0.17***
Unsaturated C14		2.88 ± 0.12***	3.18 ± 0.18***	3.36 ± 0.11***
Saturated C16		2.20 ± 0.08*	2.50 ± 0.09**	2.75 ± 0.11***
Unsaturated C16		2.06 ± 0.13	2.42 ± 0.10**	2.57 ± 0.11**
Saturated C18		2.86 ± 0.22**	4.20 ± 0.33***	4.19 ± 0.27***
Unsaturated C18		2.39 ± 0.16*	2.60 ± 0.14***	2.65 ± 0.18***

Significant differences in comparison with the control (*$P < 0.05$, **$P < 0.001$, and ***$P < 0.0001$), among saturated FFAs ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$, and $^{\dagger\dagger\dagger}P < 0.0001$), among unsaturated FFAs ($^{\ddagger}P < 0.05$, and $^{\ddagger\ddagger}P < 0.001$), and between saturated and unsaturated FFAs of the same carbon numbers ($^{\S}P < 0.05$, and $^{\S\S}P < 0.001$)

Values are given as the EC_{50} ± SE

EC_{50}, the anesthetic concentration producing a 50% effect; FFA, free fatty acid; saturated C14, myristate; unsaturated C14, myristoleic acid; saturated C16, palmitate; unsaturated C16, palmitoleic acid; saturated C18, stearate; unsaturated C18, oleic acid

unsaturated C16. For the saturated FFAs, it was found that the relationship between the chain length of the FFA and the antagonizing effect was not linear. C18 increased the EC_{50} most effectively, to about 2.5 times that of the control, at concentrations higher than 10 μM. C16, the weakest in the saturated group, increased the EC_{50} about 1.5-fold. For the unsaturated FFAs, C14 increased the EC_{50} of isoflurane most effectively, to about twice that of the control, at 20 μM. Unsaturated C16 and C18, which were weaker than C14, increased the EC_{50} of isoflurane about 1.5-fold. In a comparison of the EC_{50} values between saturated and unsaturated FFAs, we found that saturated C14 and C18 were significantly more effective than the unsaturated group of the same carbon numbers.

Discussion

Both saturated and monounsaturated FFAs of C14–18 antagonized isoflurane in goldfish. Our results suggest that the antagonizing effect to volatile anesthetics in goldfish may be a nonspecific characteristic of long-chain FFAs. It is unlikely that isoflurane in water is depleted by partitioning into FFAs, because isoflurane was constantly bubbled into water during the experiments. Therefore, FFAs may affect the active site or mode of action of isoflurane in goldfish and thus decrease its potency.

FFAs bind some kinds of proteins in living bodies, such as albumin [15] and fatty acid binding proteins [16]. There are many hydrophobic cavities at the α-helices in the subdomain of serum albumin, and myristate binds these pockets [17]. NMDA regions have regions similar to fatty acid binding proteins in their known amino acid sequences, and these regions may be involved in the binding of FFAs and the modulation of the receptor activity by FFAs [18]. These studies suggest that membrane proteins may be the targets of FFAs in their modulation of the action of volatile anesthetics in goldfish, although we could not identify the exact binding site of FFAs in the present study.

On the assumption that the targets of FFAs are membrane proteins, two possible mechanisms can explain the antagonizing potency of FFAs in goldfish. First, FFAs may compete for a specific binding site of anesthetics on channel proteins [19]. However, it is unlikely that negatively charged FFAs bind the same site as uncharged isoflurane. Second, FFAs may change the conformation of membrane proteins and modulate the action of isoflurane. This scenario may be supported by the contrasting actions on the structural change of firefly luciferase between volatile anesthetics and FFAs in vitro [9,10].

Saturated C18 was the most effective antagonist among the saturated FFAs tested in the present study. If hydrophobicity alone determines the antagonizing potencies of FFAs, the order of tightening effects on molecular conformation should be C14 < C16 < C18, as observed in the luciferase study, because FFA becomes more hydrophobic as the carbon chain length increases [10]. However, saturated C16 showed less effectiveness than saturated C14 in goldfish. This discrepancy may be explained by the difference between luciferase and membrane proteins. Luciferase is a water-soluble protein, and membrane proteins are bound with hydrophobic lipids. Modification of fatty acid levels in the membrane changes the function of membrane-associated proteins, including transporters, receptors, enzymes, and signaling molecules [20]. The activities of membrane proteins are regulated by the carbon chain length of the lipid bilayer [21], and there may exist a specific lipid thickness for optimum activity. Assuming that not only a direct action on membrane proteins but

also partitioning into the lipid bilayer participate in the antagonism exerted by FFAs, it is not unreasonable that the order of antagonism may not rigidly correspond to the hydrophobicity level of the FFAs.

Saturated C14 and C18 were more effective antagonists than were the monounsaturated types. Reports on the anesthetic actions on $GABA_A$ receptors have shown different effects between saturated and unsaturated FFAs. The binding of diazepam or muscimol to $GABA_A$ receptors was more greatly enhanced by unsaturated FFAs than by saturated types [22]. These effects depend on the length of the carbon chain, and FFAs with a medium chain length were the most effective [23]. In the present study, all the unsaturated FFAs were *cis* type. The *cis* double bond placed centrally in the carbon chain bends the molecular configuration of FFAs. The double-bonded FFAs may perturb the packing of lipid bilayer structures [24] and decrease the activity of membrane proteins. This indirect action of unsaturated FFAs via the lipid bilayer may weaken the tightening effect of FFAs and may be the cause of the weaker antagonism than that of the saturated FFAs.

At present, we have no clinical antagonists of volatile anesthetics. FFAs are contained in our food in the form of glyceryl esters and essential nutrients. Our result suggests that FFAs are a safe, new type of antagonist of volatile anesthetics. To investigate their antagonizing potency and the relevant concentration of FFAs, further studies are needed, using mammalian subjects.

In conclusion, we demonstrated that saturated and monounsaturated long-chain FFAs of C14–18 antagonized isoflurane in goldfish. For saturated FFAs, the relationship between chain lengths and antagonizing potencies was not linear. C18 was the most effective and C16 was the least so. Among unsaturated FFAs, C14 was the most effective antagonist. Saturated C14 and C18 were more effective antagonists than unsaturated FFAs of the same carbon numbers. These findings suggest that the antagonizing effect of long-chain FFAs, in terms of their ability to perturb the lipid membrane structure, was determined not solely by their hydrophobicity but also by their molecular configuration.

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