

Modulation of GABA Receptors Expressed in *Xenopus* Oocytes by 13-L-Hydroxylinoleic Acid and Food Additives

Hitoshi AOSHIMA and Yukihiro TENPAKU

Department of Biology, Physics and Informatics, Faculty of Science, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753, Japan

Received June 2, 1997

To study the effects of 13-L-hydroxylinoleic acid (LOH) and food additives on γ -aminobutyric acid (GABA) receptors, ionotropic GABA receptors were expressed in *Xenopus* oocytes by injecting mRNAs prepared from rat whole brain. LOH, which was prepared by reduction of 13-L-hydroperoxylinoleic acid (LOOH), inhibited the response of GABA receptors in the presence of high concentrations of GABA. LOH also inhibited nicotinic acetylcholine, glycine, and kainate receptors, while it had little effect on NMDA receptors expressed in *Xenopus* oocytes. However, LOH potentiated the response of GABA receptors as well as LOOH in the presence of low concentrations of GABA, possibly increasing the affinity of GABA for the receptors, while linoleic acid did not. Since some modification of the compounds seemed to change their effects on GABA receptors, the responses of GABA receptors elicited by 10 μ M GABA were measured in the presence of compounds with various kinds of functional groups or the structural isomers of pentanol. Potentiation of GABA receptors depended strongly on the species of functional groups and also depended on the structure of the isomers. Then effects of various kinds of food additives on GABA receptors were also examined; perfumes such as alcohols or esters potentiated the responses strongly, while hexylamine, nicotinamide, or caffeine inhibited the responses, mainly in a competitive manner, and vanillin inhibited the responses noncompetitively. These results suggest the possibility that production of LOOH and LOH, or intake of much of some food additives, modulates the neural transmission in the brain, especially through ionotropic GABA receptors and changes the frame of the human mind, as alcohol or tobacco does.

Key words: food additive; GABA receptor; hydroxylinoleic acid; potentiation; *Xenopus* oocyte

Oxidative stress may be a significant factor in aging or carcinogenesis.¹⁾ Lipid hydroperoxide is a reactive oxygen species and is produced by oxygenation of unsaturated fatty acids such as linoleic acid, linolenic acid, and arachidonic acid. The lipid hydroperoxide is very toxic, since its decomposition results in various reactive secondary products of free radicals²⁾ or 4-hydroxy-2-nonenal, which reacts specifically with histidyl, cysteinyl, or lysyl residues in proteins.³⁾ It is also found that exposure to lipid hydroperoxide induces cell death *via* apoptosis.⁴⁾

We have examined the effects of a lipid hydroperoxide, 13-L-hydroperoxylinoleic acid (LOOH), on nicotinic acetylcholine,⁵⁾ glycine,⁶⁾ and glutamate receptors⁷⁾ expressed in *Xenopus* oocytes by injection of electric eel or rat brain mRNA, and have found the inhibition of these receptors by LOOH. Though LOOH also inhibited the responses of ionotropic GABA receptors in the presence of high concentrations of GABA, it potentiated the response in the presence of low concentrations of GABA, while linoleic acid did not cause such potentiation.⁷⁾ These results suggest the possibility that production of lipid hydroperoxides modulate the neural transmission in the brain, especially through GABA receptors. Lipid hydroperoxides are usually reduced to their alcohols *in vivo* by glutathione peroxidase⁸⁾ or peroxiredoxin.⁹⁾ So it is also important to study the effects of the lipid alcohols on these receptors.

The effects of ethanol on neurotransmitter receptors and channels have been studied extensively^{10,11)} because of the

importance of behavior disorders related to alcohol abuse. Though many kinds of compounds are added to processed foods to protect against oxygen and microorganisms, or to give the foods a desirable fragrance and a good taste, little is known about the effects of the food additives on these receptors. In our previous papers, effects of food additives on nicotinic acetylcholine¹²⁾ and glutamate¹³⁾ receptors were examined using a *Xenopus* oocyte expression system and electrophysiological methods. Some food additives inhibited these receptors competitively or non-competitively. So it is important to examine effects of food additives on ionotropic GABA receptors, which are one of the important inhibitory neurotransmitter receptors and are similar in the amino acid sequences of their subunits to nicotinic acetylcholine receptors.¹⁴⁾

For this paper, we prepared 13-L-hydroxylinoleic acid (LOH) by the reduction of LOOH^{15,16)} and examined the effects of LOH on the receptors expressed in *Xenopus* oocytes. LOH potentiated the GABA receptors as well as LOOH did in the presence of low concentrations of GABA, but linoleic acid did not.⁷⁾ To examine the effects of functional groups or structure of the compounds on the potentiation of GABA receptors, the responses elicited by 10 μ M GABA were examined in the presence of various compounds. It was found that potentiation of GABA receptors depended strongly on both the functional groups and structures of the compounds examined. Further we also examined the effects of various kinds of food additives

Abbreviations: GABA, γ -aminobutyric acid; LOH, 13-L-hydroxylinoleic acid; LOOH, 13-L-hydroperoxylinoleic acid; KA, kainate; NMDA, N-methyl-D-aspartate.

on GABA receptors and found their various effects; some perfumes potentiated the GABA responses extensively, caffeine and nicotinamide inhibited the responses mainly in a competitive manner and vanillin inhibited the responses noncompetitively. These results suggest the possibility that intake of much of some food additives or drugs modulates the transmission through GABA receptors during their catabolism in the brain.

Materials and Methods

Materials. 13-L-Hydroperoxylinoleic acid (LOOH) was prepared from linoleic acid (Sigma) with soybean lipoxygenase (type I; Sigma), and purified on a silica gel dry column (2 × 30 cm), eluting with diethyl ether and petroleum ether, as described previously.^{15,17} After the eluent was evaporated, LOOH was dissolved in ethanol. 13-L-Hydroxylinoleic acid (LOH) was prepared by reduction of LOOH in ethanol with sodium borohydride at low temperature on ice.¹⁵ The concentration of LOH was calculated using a molar absorption coefficient of $24,000\text{ M}^{-1}\text{ cm}^{-1}$ at 234.5 nm .¹⁶

GABA, 1-pentanol, 2-methyl-1-butanol, 2-methyl-2-butanol, 2,2-dimethyl-1-propanol, 1-hexanol, hexanal, hexanoic acid, propylene glycol, D-sorbitol, vanillin, nicotinamide, sodium benzoate, chondroitin sulfate C sodium salt, *myo*-inositol, L-menthol, geraniol, and 1,6-hexanediamine were purchased from Nacalai Tesque, Inc., Kyoto. 3-Methyl-1-butanol and caffeine were purchased from Katayama Chemical Co., Osaka. 2,5-Hexanedione was purchased from Kanto Chemical Co., Tokyo. Butyl acetate, isoamyl acetate, cinnamyl acetate, isopropyl acetate, isovaleric acid methyl ester, isobutyric acid ethyl ester, and di-*tert*-butyl-hydroxy-toluene were purchased from Tokyo Kasei Co., Tokyo. 1-Hexylamine and 1,2,6-hexanetriol were purchased from Wako Pure Chemical Ind., Osaka. Acetylcholine (ACh) bromide, *N*-methyl-D-aspartate (NMDA), kainate (KA), saccharin sodium salt, and (3*Z*)-hexen-1-ol (leaf alcohol) were purchased from Sigma Chemical Co., St. Louis. All chemicals were of guaranteed reagent quality.

Preparation of poly(A)⁺ RNA and *Xenopus* oocytes. An electric eel, *Electrophorus electricus*, was purchased from Shimonoseki Pet Center, Shimonoseki. The eel was killed and dissected in ice and the electroplax was stored in a deep freeze at -85°C . The whole brains were obtained from male adult Wistar rats (weighing about 100 g) after they were anesthetized with diethyl ether. Poly(A)⁺ RNA was prepared from the electroplax or brains by the procedure described by Maniatis *et al.*¹⁸

Adult female frogs (*Xenopus laevis*) were purchased from Hamamatsu Seibutu Kyozai (Hamamatsu, Japan). The oocytes were dissected from the ovaries of adult female frogs kept in ice for 1 h. They were detached manually from the inner ovarian epithelium and follicular envelope after incubation in collagenase (type I, 1 mg/ml; Sigma) solution for 1 h by the procedure of Kusano *et al.*¹⁹ Oocytes were microinjected with about 50 ng of the poly(A)⁺ RNA in sterilized water and incubated in modified Barth solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.33 mM Ca(NO₃)₂, and 0.41 mM CaCl₂ in 5 mM Tris, pH 7.6) containing 25 mg/liter of penicillin and 50 mg/liter of streptomycin, at $15\text{--}18^{\circ}\text{C}$ for 2–7 days before electrophysiological measurements.

Electrophysiological measurements. The membrane current of the receptors evoked by agonists was measured by the voltage clamping method with a voltage clamp amplifier (CEZ-1100; Nihon Kohden Kogyo, Tokyo, Japan). An oocyte was placed on a net of a small chamber (about 0.3 ml) and impaled with two microelectrodes filled with 3 M KCl, one for monitoring the membrane potential and the other for flowing current for clamping the membrane potential usually at -80 mV . The electrical response of the current was filtered by a P-84 filter (NF Electronic Instruments, Kanagawa). The *Xenopus* oocyte placed on a net was continuously perfused from the bottom with normal frog Ringer solution (115 mM NaCl, 1 mM KCl, and 1.8 mM CaCl₂ in 5 mM Tris, pH 7.2) by a gravity feed system, usually at a flow rate of about 2 ml/min.²⁰

Measurement of receptor response. Agonist and the compound such as LOH or food additives were dissolved in normal frog Ringer solution. The solution was changed to another one by changing a cock of the flow system. The control response was obtained by perfusing the agonist solution without any other compound and taken to be 100%. The effect of the compound on the response of the receptors was measured by the

use of a mixture of agonist and the compound; in some cases, the compound was added for 1 min before co-application with agonist when desensitization of the receptors was induced significantly before the attainment of equilibrium of the inhibitor binding.²¹ The measurement was repeated several times with the same oocyte, and the control values were obtained every two or three measurements. To eliminate desensitization of the receptors, the oocyte was washed for more than 10 min in normal frog Ringer solution before the next measurement, since the desensitization of ionotropic GABA receptors is a reversible process and the receptors usually recover from the desensitization in about 10 min of washing.²²

A Student's *t* test was used to evaluate the significance in the mean values, compared with the control.

Results

Figure 1 shows the electrical responses of GABA receptors expressed in *Xenopus* oocytes by injecting rat whole brain mRNA. These responses are thought to be induced by ionotropic GABA receptors (GABA_A receptors), since metabotropic ones (GABA_B receptors) are not expressed in *Xenopus* oocyte.²³ Addition of $20\text{ }\mu\text{M}$ LOH to a solution of 1 mM GABA inhibited the response of the receptor in the oocyte, when $20\text{ }\mu\text{M}$ LOH was applied for 1 min before co-application with 1 mM GABA. This protocol was used

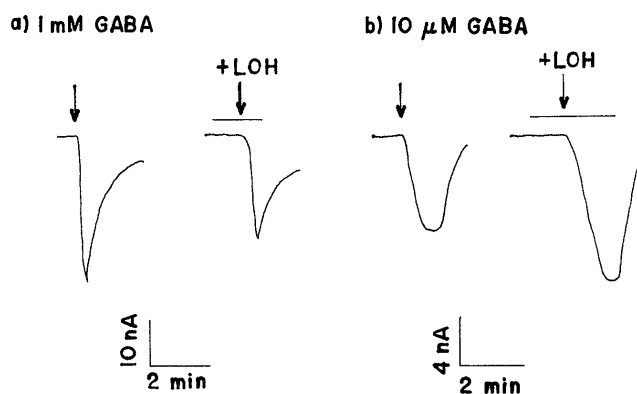


Fig. 1. Examples of the Inhibition or the Potentiation of GABA-Mediated Current by $20\text{ }\mu\text{M}$ LOH in the Presence of 1 mM or $10\text{ }\mu\text{M}$ GABA.

All traces were obtained with a voltage clamp. An inward current is shown as a downward curve. The arrows show when 1 mM (a) or $10\text{ }\mu\text{M}$ (b) GABA was added and removed later after the peak current. The lines above the traces show when $20\text{ }\mu\text{M}$ LOH was present. Each of the two responses was obtained from the same oocyte, but the responses in (a) and (b) were from different one.

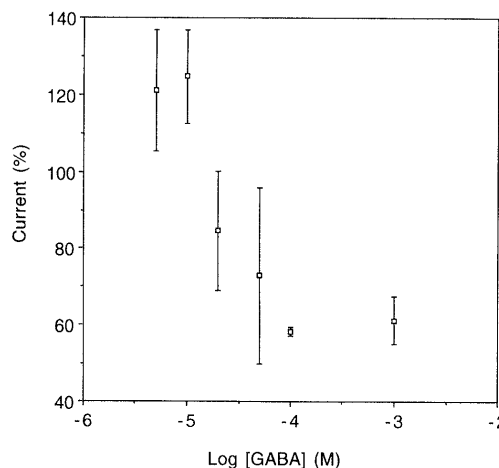


Fig. 2. Effect of GABA Concentrations on the Current of GABA Receptors in the Presence of $20\text{ }\mu\text{M}$ LOH, Where $20\text{ }\mu\text{M}$ LOH Was Applied for 1 min before Co-application with GABA.

Data are mean \pm SD (bars) values from three or four experiments.

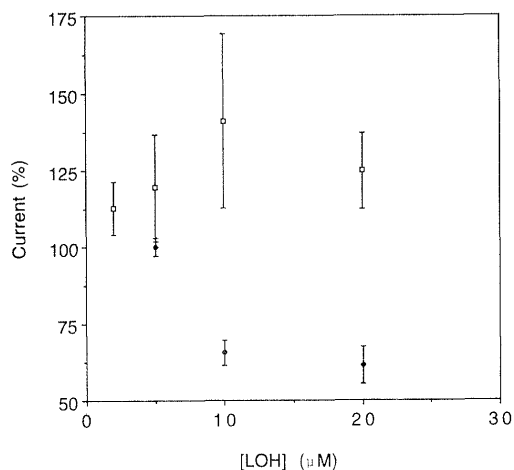


Fig. 3. Dose-inhibition or -potentiation Relationship of LOH in the Presence of 1 mM (circles) or 10 μM (squares) GABA.

LOH was applied simultaneously with 10 μM (squares) GABA, but it was applied for 1 min before co-application with 1 mM GABA as 1 mM GABA induced fast desensitization of GABA receptors before attainment to the equilibrium of LOH binding. Data are mean ± SD (bars) values from three to six experiments.

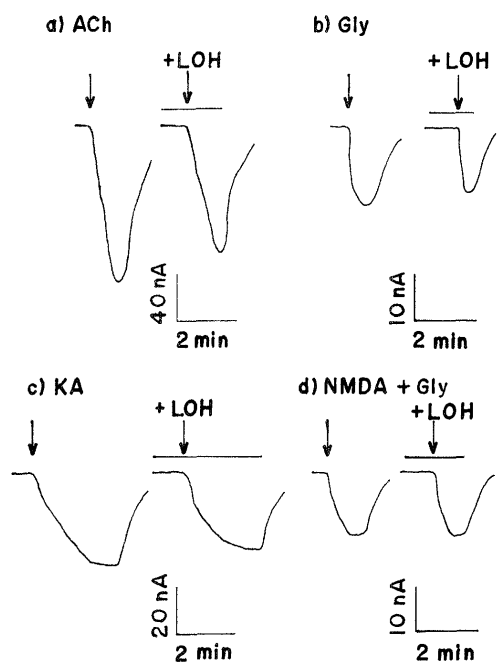


Fig. 4. Effects of 20 μM LOH on the Response Induced by a) 30 μM Acetylcholine (ACh), b) 1 mM Gly, c) 10 μM Kainate (KA), and d) the Mixture of 1 mM NMDA and 25 μM Gly.

The arrows show when agonist was added and removed later. The lines above the traces show 20 μM LOH was present. Each pair of the responses was obtained from the same oocyte, but the responses in a) d) were from different injected oocytes.

Table I. Effect of 20 μM LOH on the Response of the Ionotropic Neurotransmitter Receptors

The solutions of 20 μM LOH was added for 1 min before co-application with the agonist. Response is mean ± SD value from *n* experiments when the response without LOH was taken to be 100%.

| Agonist | Response (%) | <i>n</i> | Significance |
|---------------------|--------------|----------|-----------------|
| 30 μM Acetylcholine | 88.4 ± 9.5 | 3 | <i>p</i> < 0.1 |
| 1 mM GABA | 61.3 ± 6.1 | 4 | <i>p</i> < 0.01 |
| 1 mM Glycine | 85.2 ± 5.7 | 4 | <i>p</i> < 0.02 |
| 10 μM Kainate | 88.4 ± 7.6 | 3 | <i>p</i> < 0.1 |
| 1 mM NMDA | 100.3 ± 4.9 | 4 | <i>p</i> > 0.9 |

because 1 mM GABA induced fast desensitization of GABA receptors before attainment of the equilibrium of LOH binding.^{21,22} On the other hand, addition of 20 μM LOH to 10 μM GABA solution potentiated the response of GABA receptors. Since the rate of desensitization of GABA receptors induced by 10 μM GABA was slow, preliminary treatment of the oocyte with 20 μM LOH had no significant effect on the potentiation by 20 μM LOH (data not shown). Figure 2 shows the effects of GABA concentration on the inhibition or potentiation of GABA receptors caused by 20 μM LOH. Effect of 20 μM LOH on GABA receptors changed from inhibition to potentiation with the decrease of GABA concentration (1 mM–5 μM). Figure 3 shows the dose dependence of LOH on the response of GABA receptors in the presence of 10 μM or 1 mM GABA. LOH inhibited the response caused by 1 mM GABA in a dose-dependent manner, while it potentiated the response caused by 10 μM GABA. Thus, the effects of LOH on the GABA receptors depended strongly on the concentrations of both LOH and GABA.

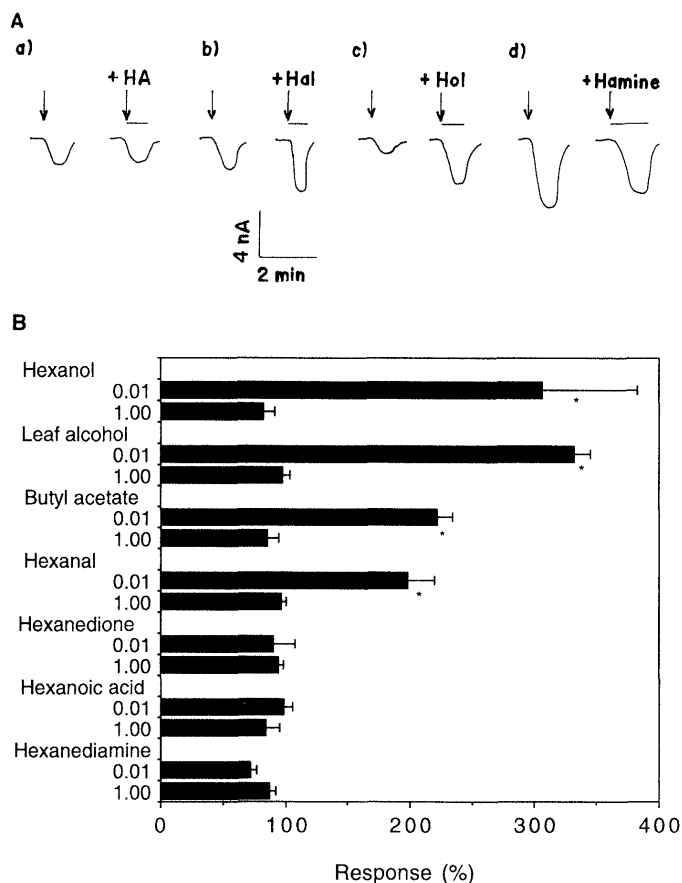


Fig. 5. Effects of Functional Groups on the Response of GABA Receptors.

A) Examples of the potentiation or inhibition of GABA-mediated current by hydrocarbons with a linear chain of six carbon atoms which contain various functional groups. All traces were obtained with a voltage clamp. An inward current is shown as a downward curve. The arrows show when 10 μM GABA was added for about 1 min. The lines above the traces show when 5 mM hydrocarbons with various functional groups were applied. Each pair of the responses was obtained from the same oocyte. However, as the responses in a)–d) were from different injected oocytes, the control responses showed different sizes. a) HA, hexanoic acid; b) Hal, hexanal; c) Hol, 1-hexanol; d) Hamine, 1,6-hexanediamine.

B) Potentiation or inhibition of GABA receptors by 5 mM hydrocarbons with various functional groups of six carbon atoms. Numbers at the left side, 0.01 and 1.00 represent the GABA concentrations in mM when the effects of the hydrocarbons were examined. Values are means of four to ten experiments when the response without any hydrocarbon was taken to be 100%, and error bars represent the standard deviations. **p* < 0.01 in the comparison of the effects when 0.01 or 1 mM GABA was present, by Student's *t* test. Leaf alcohol, (3Z)-hexen-1-ol.

Since LOOH inhibited the responses of nicotinic acetylcholine,¹²⁾ glycine,⁶⁾ and glutamate¹³⁾ receptors expressed in the oocyte by injection of electric eel or rat brain mRNA, effects of LOH on the responses of these receptors were also examined. As shown in Fig. 4 and Table I, LOH also inhibited these receptors, except for NMDA receptors. The responses of these receptors were not potentiated by LOH even in the presence of low concentrations of agonist.

Since peroxidation⁷⁾ and hydroxylation (Figs. 1–3) of linoleic acid changed their effect on the response of GABA receptors, especially at low concentrations of GABA, the effect of the species of functional groups of the compounds with linear chains of six carbon atoms on the response of GABA receptors was examined in the presence of 10 μ M GABA. Figure 5A showed some examples of the effects of the compounds at 5 mM on the response caused by 10 μ M (0.01 mM) GABA. 1-Hexanol and hexanal potentiated the response to different extents, while hexanoic acid had little effect on the receptor, and 1,6-hexanediamine showed a tendency toward inhibition. Figure 5B shows the effects of the compounds of 5 mM in the presence of 0.01 mM and 1 mM GABA, where the control response was obtained by perfusing 0.01 mM or 1 mM GABA solution without any

other compound and taken to be 100%. 1-Hexanol, leaf alcohol, butyl acetate, and hexanal potentiated the response evoked by 0.01 mM GABA significantly, but hexanoic acid, 2,5-hexanedione and 1,6-hexanediamine did not. On the other hand, these compounds had little effect on the response evoked by 1 mM GABA. Figure 6 shows the potentiation of the response by various structural isomers of pentanol in the presence of 10 μ M GABA. Potentiation of GABA receptors also varied with structures of the pentanols.

Figure 7 shows some examples of the effects of food additives on the responses of GABA receptors. Figure 8 shows the dose dependence of GABA on the effect of food additives on GABA receptors, while Fig. 9 shows the dose dependence of food additives on the responses. Table II shows the effects of food additives on the response of GABA receptors induced by 1 mM or 10 μ M GABA. Since

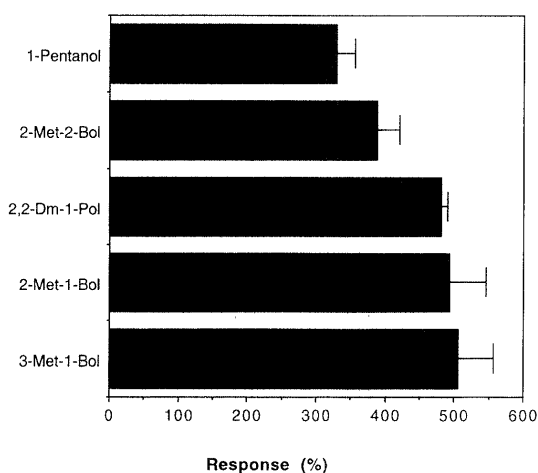


Fig. 6. Potentiation of GABA Receptors by 10 mM Pentanol and Its Isomers in the Presence of 10 μ M GABA.

Values are means of four or five experiments when the response without any alcohols was taken to be 100%, and error bars represent the standard deviations. * $p < 0.05$ between 1-pentanol and other isomers, by Student's t test. 2-Met-2-Bol, 2-methyl-2-butanol; 2,2-Dm-1-Pol, 2,2-dimethyl-1-propanol; 2-Met-1-Bol, 2-methyl-1-butanol; 3-Met-1-Bol, 3-methyl-1-butanol.

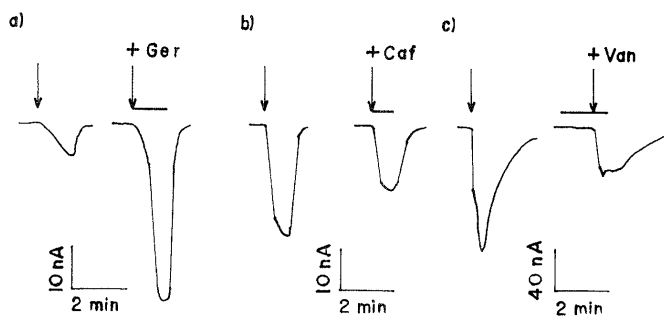


Fig. 7. Examples of the Potentiation or the Inhibition of GABA-induced Current by Food Additives.

All traces were obtained with a voltage clamp. An inward current is shown as a downward curve. The arrows show when GABA (a and b, 10 μ M; c, 1 mM) was added and removed later after the peak current. The lines above the traces show when (a) 1 mM geraniol (Ger), (b) 2 mM caffeine (Caf), or (c) 10 mM vanillin (Van) was present. Each pair of the responses was obtained from the same oocyte, but the responses in (a), (b), and (c) were from different one.

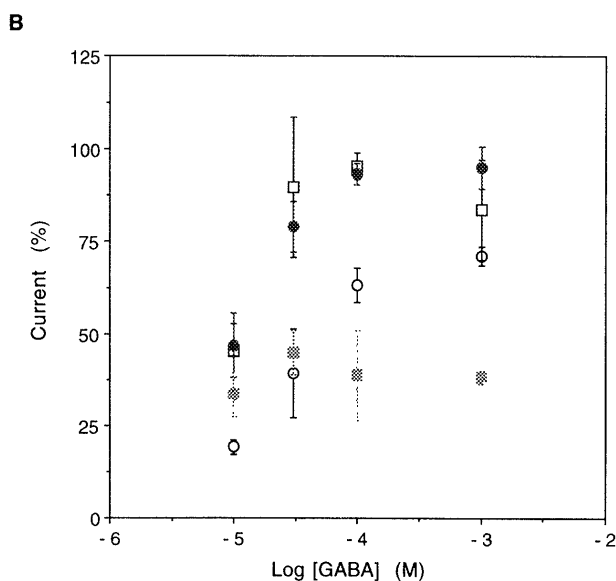
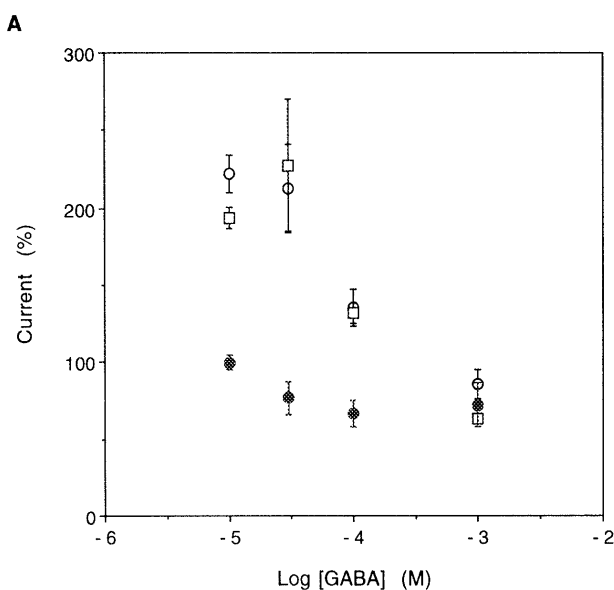


Fig. 8. Effects of GABA Concentration on the Current of GABA Receptors in the Presence of Various Kinds of Food Additives.

Data are mean \pm SD (bars) values from three to seven experiments. A) 5 mM Butyl acetate (○); 10 mM saccharin (□); 50 mM propylene glycol (●). B) 5 mM Hexyl amine (○); 1 mM caffeine (□); 5 mM nicotinamide (●); 10 mM vanillin (■).

some higher esters or alcohols are hard to dissolve in aqueous solution, their real concentrations in the solution may be less than those shown in Table II. Alcohols, esters, or saccharin potentiated the response caused by $10\ \mu\text{M}$ GABA and their potentiation increased with their concentration. The data for 1-hexanol, 1,2,6-hexanetriol, and *myo*-inositol in Table II suggested that the potentiation of the response by alcohols decreased with the increase of their hydroxyl residue number. As shown in Fig. 8B, 1-hexylamine, nicotinamide, and caffeine inhibited the GABA response mainly in a competitive manner, while vanillin inhibited the response noncompetitively.

Discussion

Most anesthetics are very effective at potentiating responses to GABA.²⁴⁾ They are thought to increase the affinity of GABA for its receptors, shifting the GABA dose-response curve to lower concentrations.²⁵⁾ The GABA receptors have been established as a prime anesthetic target by recent studies,^{24,26)} as potentiation of postsynaptic in-

hibitory channel activity best fitted the pharmacological profile observed in general anesthetics. Potentiation of GABA receptors by ethanol and other alcohols is also found in *Xenopus* oocyte¹⁰⁾ or primary cultures of rat dorsal root ganglion neurons²⁷⁾ expressing GABA receptors.

Previously, we reported that LOOH even at very low concentrations of a few μM potentiated the responses of GABA receptors in the presence of low concentrations (5–50 μM) of GABA, while linoleic acid did not.⁷⁾ Since LOOH is thought to be reduced *in vivo* to LOH by glutathione peroxidase,⁸⁾ it is important to clarify whether LOH potentiates or inhibits the response of GABA receptors. Our results showed that LOH also potentiated GABA receptors as strongly as LOOH in the presence of low concentrations of GABA. These results suggest that peroxidation of unsaturated fatty acids induces the anesthetic effect in the brain through the potentiation of GABA receptors, even after its reduction to LOH by glutathione peroxidase.

Since addition of hydroperoxyl or hydroxyl group to linoleic acid changed its effect on GABA receptors dramatically,⁷⁾ dependence of functional groups on the potentiation of GABA receptors were examined in the presence of $10\ \mu\text{M}$ GABA for Fig. 5, which showed that 1-hexanol, leaf alcohol, hexanal, and butyl acetate with six carbon atoms induced the potentiation of GABA receptors to different extents, while hexanedione and hexanoic acid had little effect on the receptor and hexanediamine inhibited the receptor. Previously, we examined the effects of food additives on excitatory ionotropic neurotransmitter receptors expressed in *Xenopus* oocytes and found that some hydrophobic food additives such as vanillin or caffeine inhibited their responses,^{12,13)} since many kinds of compounds are used as food additives. So we also examined their effects on GABA receptors expressed in the oocyte. As shown in Figs. 7 and 8 and Table II, food additives induced various complex effects on GABA receptors. Alcohols and esters such as geraniol and butyl acetate, which are often used as perfumes potentiated the response elicited by low concentrations of GABA. Structural isomers of pentanol also

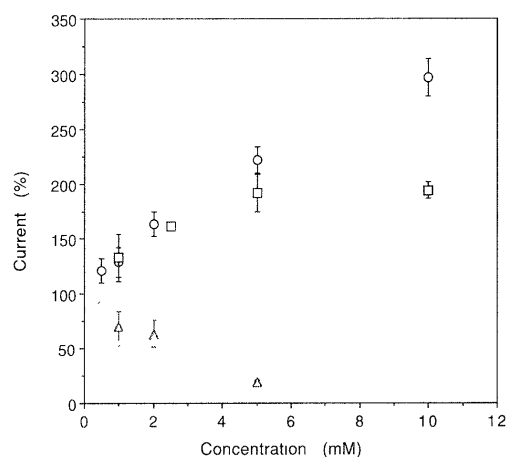


Fig. 9. Dose-inhibition or -potentiation of Food Additives.

Data are mean \pm SD (bars) values from three to seven experiments. Butyl acetate (○), Saccharin (□), Hexyl amine (△), Caffeine (●), Vanillin (■) in the presence of $10\ \mu\text{M}$ GABA.

Table II. Effects of Food Additives on GABA Receptors

Data are mean \pm SD values for the response when the response without any food additives was taken to be 100%. *n*, number of experiments.

| Compounds | [GABA] (mM) | Response (%) | <i>n</i> | Significance |
|-----------------------------------|-------------|-----------------|----------|--------------|
| 50 mM D-Sorbitol | 1 | 92.2 \pm 3.2 | 5 | $p < 0.07$ |
| | 0.01 | 105 \pm 7 | 6 | $p > 0.2$ |
| 1 mg/ml Chondroitin | 0.01 | 99.4 \pm 5.0 | 5 | $p > 0.8$ |
| 50 mM Ethylene glycol | 1 | 75.2 \pm 10.1 | 5 | $p < 0.01$ |
| 50 mM Diethylene glycol | 1 | 75.1 \pm 10.6 | 3 | $p < 0.01$ |
| 50 mM Glycerol | 1 | 78.1 \pm 13.2 | 4 | $p < 0.02$ |
| 5 mM 1-Hexanol | 0.01 | 306 \pm 77 | 4 | $p < 0.01$ |
| 5 mM 1,2,6-Hexanetriol | 0.01 | 115 \pm 5 | 4 | $p < 0.01$ |
| 5 mM <i>myo</i> -Inositol | 0.01 | 106 \pm 2 | 4 | $p > 0.1$ |
| 10 mM Sodium benzoate | 0.01 | 120 \pm 8 | 3 | $p > 0.1$ |
| 1 mM Di-butyl-hydroxytoluene | 0.01 | 156 \pm 32 | 3 | $p < 0.01$ |
| 1 mM L-Menthol | 0.01 | 136 \pm 2 | 3 | $p < 0.01$ |
| 1 mM Geraniol | 0.01 | 531 \pm 65 | 5 | $p < 0.01$ |
| 1 mM Cinnamyl alcohol | 0.01 | 150 \pm 9 | 4 | $p < 0.01$ |
| 5 mM Isoamyl acetate | 0.01 | 210 \pm 7 | 3 | $p < 0.01$ |
| 5 mM Cinnamyl acetate | 0.01 | 238 \pm 46 | 4 | $p < 0.01$ |
| 5 mM Isopropyl acetate | 0.01 | 303 \pm 19 | 4 | $p < 0.01$ |
| 5 mM Isobutyric acid methyl ester | 0.01 | 240 \pm 18 | 4 | $p < 0.01$ |
| 5 mM Isobutyric acid ethyl ester | 0.01 | 257 \pm 28 | 4 | $p < 0.01$ |

potentiated GABA receptors to different extents. Branched chain alcohols with hydroxyl residues far from the branch potentiated the response more strongly. In the effects of stereoisomers of pentobarbital, they showed high stereoselectivity in their potency on general anesthesia.²⁸⁾ These results of selectivity of the compounds in the potentiation suggest that they act directly on the protein of the receptor rather than on lipids.²⁹⁾ On the other hand, hexylamine, caffeine, and nicotinamide inhibited the responses mainly in a competitive manner (Fig. 8B), possibly because they would bind to the GABA binding site(s) in GABA receptors. However, these compounds may also bind to a noncompetitive inhibition site, especially when their concentrations are very high. It is known that caffeine at high millimolar concentrations activates ryanodine receptors and releases intracellular Ca^{2+} , which is thought to be a main physiological activity of caffeine.³⁰⁾ So it cannot be excluded that caffeine changes the Ca^{2+} concentration in the oocyte and decreases the response of GABA receptors indirectly, *via e.g.*, protein kinase C.³⁰⁾ Further experiments are necessary to conclude its direct effect on GABA receptors. Vanillin inhibited them noncompetitively, though its inhibition was not so strong. D-Sorbitol, *myo*-inositol or chondroitin, which have many hydroxyl groups, induced little effect on the responses of GABA receptors. Thus GABA receptors have at least three kinds of binding sites (Fig. 10), *i.e.*, agonist-binding site(s) (G), noncompetitive inhibition site (I), and potentiation site (P) as proposed.³⁰⁾

Wafford *et al.*¹¹⁾ reported that the degree of ethanol or anesthetic potentiation of GABA receptors depended on their subunit compositions, and that the $\gamma 2\text{L}$ subunit was essential to the potentiation of GABA responses, though Harrison *et al.*³¹⁾ observed the potentiation without γ subunit. For example, potentiation of GABA-activated currents of some subunit composition including $\gamma 2\text{L}$ by enflurane was several times greater than that of GABA receptors expressed by total mRNA injection.³²⁾ GABA receptors formed ρ_1 subunits expressed in *Xenopus* oocytes were inhibited competitively by alcohols and volatile anesthetics.³³⁾ Further, Wisden *et al.*³⁴⁾ reported that the distribution of subunits throughout the central nervous system varied greatly. Since it is likely that potentiation of GABA responses by LOH or food additives also depends on their subunit composition, the potentiation by LOH or food additives must be greater in some brain regions than that observed in our experiments where total mRNAs from whole brain were injected into the oocytes. Further experiments using cRNAs prepared from cloned cDNA of various subunits will be necessary to clarify these problems in the future.

LOH inhibited nicotinic acetylcholine receptors expressed by injecting electric eel mRNA, GABA, and glycine receptors expressed by injecting rat whole brain mRNA (Fig. 1–4, and Table I). These ionotropic receptors were similar in the amino acid sequences of their subunits and form a genetically related family.^{14,35)} They also followed a similar scheme of kinetics.^{6,22,36)} So it is not strange that all of them have similar noncompetitive inhibition site (I in Fig. 10) for LOOH or LOH, which are numerous and lipid dependent, probably at the interface of the receptors with membrane lipids.²⁹⁾ Then some subunits of GABA receptors such as $\gamma 2\text{L}$ subunit must have developed another

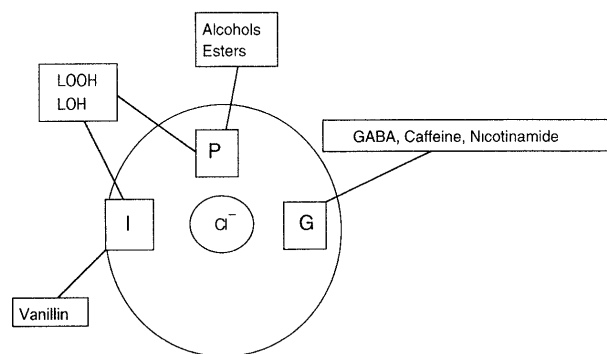


Fig. 10. Scheme of Binding Sites Affecting GABA Receptors.

G, P, and I represent the GABA-binding, potentiation, and noncompetitive inhibition sites, respectively in GABA receptors. Only one GABA-binding site was drawn here because of uncertainty, though GABA may bind to two sites in α subunits from the analogy with the nicotinic acetylcholine receptor and the dose-response relationship of GABA.³⁰⁾ Alcohols and esters bind to potentiation site and increase the affinity of GABA-binding sites to GABA. Hexylamine, caffeine and nicotinamide bind mainly to the GABA-binding site(s) and inhibit the response competitively, though they might also bind to the inhibition site when they are at high concentrations. Vanillin binds mainly to the inhibition site and inhibits the response non-competitively. LOOH and LOH bind to both potentiation and inhibition sites and potentiate the response in the presence of low concentrations of GABA, while they inhibit the response in the presence of high concentrations of GABA.

binding site (P in Fig. 10) with high affinity where anesthetics bind and potentiate the affinity of GABA binding.¹¹⁾ Since LOH and LOOH inhibited GABA receptors in the presence of 1 mM GABA, but potentiated them in the presence of 10 μM GABA, they possibly bind not only at the potentiation site (P) with high affinity, but also at the inhibition site (I) in GABA receptors including $\gamma 2\text{L}$ subunit (Fig. 10). Vanillin must bind mainly only to this inhibition site (I).

Previously, we proposed the possibility that lipid hydroperoxide inhibited or potentiated the ionotropic neurotransmitter receptors, and affected neuronal transmission in the brain,⁷⁾ especially under special conditions such as ischemia and excitotoxicity³⁷⁾ or neurodegenerative diseases such as Alzheimer disease.³⁸⁾ Our experiments in this paper showed that neural transmission might be affected even after reduction of lipid hydroperoxide to their alcohols and also showed that compounds with different functional groups had different or even opposite effects on GABA receptors. These results suggest the risk that some drugs may modulate GABA receptors during their catabolism in the brain and modify the neural transmission. We also found that many food additives caused complex effects, potentiation or inhibition, on GABA receptors expressed in the oocyte. Since these effects were induced by the food additives at more than about 1 mM, it is unlikely that food additives taken with processed food interrupt signal transmission critically under physiological conditions and cause a significant problem in the brain. However, it cannot be denied that intake of much of food additives such as caffeine or perfume may modulate ionotropic neurotransmitter receptors, especially GABA receptors, and change the frame of the human mind as alcohol or tobacco does. Uptake of the compounds into our brains are controlled by the blood-brain barrier. Lipophilic compounds in general go through this barrier easily and cause various kinds of effects on the receptors as reported in this paper. So we should be specially careful when lipophilic compounds are added to food.

Acknowledgments. This work was supported in part by the San-Ei Gen Foundation for Food Chemical Research in Japan.

References

- 1) W. A. Pryor, Ed., "Free Radicals in Biology," Vols. I–VI, Academic Press, New York, 1976–1984; R. S. Sohal and R. Weindruch, *Science*, **273**, 59–63 (1996).
- 2) H. W. Gardner, *J. Agric. Food Chem.*, **23**, 129–136 (1975).
- 3) K. Uchida and E. R. Stadtman, *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 4544–4548 (1992).
- 4) P. A. Sandstrom, P. W. Tebbey, S. V. Cleave, and T. M. Buttke, *J. Biol. Chem.*, **269**, 798–801 (1994); H. Aoshima, T. Satoh, N. Sakai, M. Yamada, Y. Enokido, T. Ikeuchi, and H. Hatanaka, *Biochim. Biophys. Acta*, **1345**, 35–42 (1997).
- 5) H. Aoshima, M. Anan, and H. Ishii, *Arch. Biochem. Biophys.*, **258**, 324–331 (1987).
- 6) H. Aoshima, Y. Inoue, E. Ueda, M. Kitagawa, and T. Nishino, *J. Biochem.*, **111**, 523–528 (1992).
- 7) H. Aoshima, *J. Neurochem.*, **66**, 1300–1305 (1996).
- 8) B. O. Christophersen, *Biochem. J.*, **106**, 515–522 (1968).
- 9) F. S. Jacobson, R. W. Morgan, M. F. Christman, and B. N. Ames, *J. Biol. Chem.*, **264**, 1488–1496 (1989).
- 10) D. M. Lovinger, G. White, and F. Weight, *Science*, **243**, 1721–1724 (1989); K. A. Wafford, D. M. Burnett, T. V. Dunwiddie, and R. A. Harris, *Science*, **249**, 291–293 (1990).
- 11) K. A. Wafford, D. M. Burnett, N. J. Leidenheimer, D. R. Burt, J. B. Wang, P. Kofuji, T. V. Dunwiddie, R. A. Harris, and J. M. Sikela, *Neuron*, **7**, 27–33 (1991).
- 12) H. Aoshima, N. Mitsusada, and T. Nishino, *Biosci. Biotech. Biochem.*, **58**, 1776–1779 (1994).
- 13) H. Aoshima, *Biosci. Biotech. Biochem.*, **60**, 434–438 (1996).
- 14) M. Noda, H. Takahashi, T. Tanabe, M. Toyosato, S. Kikuyotani, Y. Furutani, T. Hirose, H. Takashima, S. Inayama, T. Miyata, and S. Numa, *Nature*, **302**, 528–532 (1983); P. R. Schofield, M. G. Darlison, N. Fujita, D. R. Burt, F. A. Stephenson, H. Rodriguez, L. M. Rhee, J. Ramachandran, V. Reale, T. A. Glencorse, P. H. Seeburg, and E. A. Barnard, *Nature*, **328**, 221–227 (1987).
- 15) H. Aoshima, *Anal. Biochem.*, **87**, 49–55 (1978).
- 16) G. Graff, L. A. Anderson, and L. W. Jaques, *Anal. Biochem.*, **188**, 38–47 (1990).
- 17) H. W.-S. Chan and G. Levett, *Lipids*, **12**, 99–104 (1977).
- 18) T. Maniatis, E. F. Fritsch, and L. Sambrook, "Molecular Cloning. A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1982, pp. 196–198.
- 19) K. Kusano, R. Miledi, and J. Stinnarkre, *J. Physiol.*, **328**, 143–170 (1982).
- 20) S. Kobayashi and H. Aoshima, *Dev. Brain Res.*, **24**, 211–216 (1986).
- 21) H. Aoshima, Y. Inoue, and K. Hori, *J. Biochem.*, **112**, 495–502 (1992).
- 22) H. Aoshima, M. Anan, H. Ishii, and S. Kobayashi, *Biochemistry*, **26**, 4811–4816 (1987).
- 23) K. Kaupmann, K. Huggel, J. Heid, P. J. Flor, S. Bischoff, S. J. Mickel, G. McMaster, C. August, H. Bittiger, W. Froestl, and B. Bettler, *Nature*, **386**, 229–246 (1997).
- 24) N. P. Franks and W. R. Lieb, *Nature*, **367**, 607–614 (1994).
- 25) L. Parker, C. B. Gundersen, and R. Miledi, *J. Neurosci.*, **6**, 2290–2297 (1986).
- 26) M. Y. Jones, P. A. Brooks, and N. L. Harrison, *J. Physiol.*, **449**, 279–293 (1992).
- 27) M. Nakahiro, O. Arakawa, and T. Narahashi, *J. Pharmacol. Exp. Ther.*, **259**, 235–240 (1991).
- 28) L.-Y. M. Huang and J. L. Barker, *Science*, **207**, 195–197 (1980).
- 29) J. P. Changeux, A. Devillers-Thiery, and P. Chemouilli, *Science*, **225**, 1335–1345 (1984).
- 30) D. G. Nicholls, Amino Acids as Neurotransmitters, in "Proteins, Transmitters and Synapses," Blackwell Scientific Publications, Oxford, 1994, pp. 155–185.
- 31) N. L. Harrison, J. O. Kugler, M. V. Jones, E. P. Greenblatt, and D. B. Pritchett, *Mol. Pharmacol.*, **44**, 628–632 (1993).
- 32) L. H. Lin, P. Whiting, and A. Harris, *J. Neurochem.*, **60**, 1548–1553 (1993).
- 33) S. J. Mihic and R. A. Harris, *J. Pharmacol. Exp. Ther.*, **277**, 411–416 (1996).
- 34) W. Wisden, D. J. Laurie, H. Monyer, and P. H. Seeburg, *J. Neurosci.*, **12**, 1040–1062 (1992).
- 35) G. Grenningloh, A. Rienitz, B. Schmitt, C. Methfessel, M. Zensen, K. Beyreuther, E. D. Gundelfinger, and H. Betz, *Nature*, **326**, 215–220 (1987).
- 36) G. P. Hess, D. J. Cash, and H. Aoshima, *Annu. Rev. Biophys. Bioeng.*, **12**, 443–473 (1983).
- 37) R. A. Flovd and J. M. Carney, *Ann. Neurol.*, **32**, S22–27 (1992).
- 38) C. Behl, J. B. Davis, R. Lesley, and D. Schubert, *Cell*, **77**, 817–827 (1994).