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Kinetic Analyses of Alcohol-Induced Potentiation of the Response of $GABA_A$ Receptors Composed of α_1 and β_1 Subunits[1]

Hitoshi Aoshima,*[2] Sheikh Julfikar Hossain,* Koutaro Hamamoto,* Terufumi Yokoyama,† Mamoru Yamada,† and Ryuzo Shingai‡

Department of Physics, Biology and Informatics, Faculty of Science, and †Department of Biological Science, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8512; and ‡Department of Welfare Engineering, Faculty of Engineering, Iwate University, 4 Ueda, Morioka 020-8551

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To investigate the kinetics of both the potentiation and desensitization of the response of ionotropic GABA receptors ($GABA_A$ receptors) in the presence of various compounds, we expressed receptors composed of α_1 and β_1 subunits by injecting cells with the cRNAs synthesized from cloned bovine $GABA_A$ receptor cDNAs and measured the electrical responses of the cells electrophysiologically with or without the compounds. The potentiation of the $GABA_A$ receptor-mediated response was quantitatively analyzed using a simple model with the assumption that the receptors have two identical binding sites for GABA molecules with a dissociation constant of K_I, and one potentiation site for the compound with a dissociation constant of K_p, and that the binding of the compound to the potentiation site only increases the affinity of the GABA binding sites, changing K_I to K_{Ip}. The estimated K_p and K_{Ip} were dependent on the functional groups and the chain length of the compounds. These results could be satisfactorily analyzed using this simple model. The potentiation of the $GABA_A$ receptor-mediated response by the components of essential oils used for aromatherapy was also examined. These compounds accelerated the decay of the response, possibly due to desensitization of the receptors, which was also analyzed on the basis of the model.

Key words: aromatherapy, fragrance, $GABA_A$ receptor, potentiation, *Xenopus* oocyte.

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the brain, and is essential for the overall balance between neuronal excitation and inhibition. $GABA_A$ receptors are ligand gated ion channels whose subunits have amino acid sequences similar to those of ionotropic nicotinic acetylcholine, serotonin (type 3), and glycine receptors (*1*). They are thought to be heteropentamers made up of subunits likely to have been derived from a common ancestor. To date, at least 16 human $GABA_A$ receptor proteins have been described. There are six α-subunits, four β-subunits with two splice variants, four γ-subunits with two splice variants, one δ-subunit and one ε-subunit (*2*).

The $GABA_A$ receptors are known as mood-defining receptors and have a complex pharmacology, with binding sites for direct GABA agonists and antagonists together with multiple allosteric sites for benzodiazepine tranquillizers, barbiturate central nervous system depressants, both synthetic and endogenous steroids, general anaesthetics, and ethanol (*2*). These structurally diverse compounds enhance the response of $GABA_A$ receptors in the presence of low concentrations of GABA. The action of GABA is cooperative, suggesting the presence of two GABA binding sites (*1*).

In previous studies (*3–5*), we expressed $GABA_A$ receptors in *Xenopus* oocytes by injecting rat whole brain mRNA and found that their responses were potentiated by various compounds such as fragrant compounds. However, quantitative analysis of those results was difficult, since rat whole brain mRNA will express an enormous number of combinations of pentamers of $GABA_A$ receptors, which will show different and complex pharmacology (*1*).

In the present study, we expressed $GABA_A$ receptors composed of α_1 and β_1 subunits, and examined the effects of various compounds on the electrical response of $GABA_A$ receptors. The data were analyzed quantitatively using a simple model (Fig. 1) with the following assumptions: (i) Two identical binding sites for the GABA molecule with a dissociation constant of K_I (*6, 7*), and one potentiation site for alcohols with a dissociation constant of K_p are present in the receptor. (ii) The binding of a fragrant compound to the potentiation site increases the affinity of only the GABA binding sites (*8*), changing the K_I to K_{Ip}.

MATERIALS AND METHODS

Materials—All chemicals were purchased from Nacalai Tesque (Kyoto) and were of guaranteed reagent quality.

Preparation of cRNA and Xenopus Oocytes—The cDNAs of $GABA_A$ receptors cloned from bovine brain were gifts from Prof. Eric A. Barnard of the MCR Center of the UK. The cRNAs of $GABA_A$ receptors were synthesized from these cloned cDNAs with RNA polymerase according to standard procedures.

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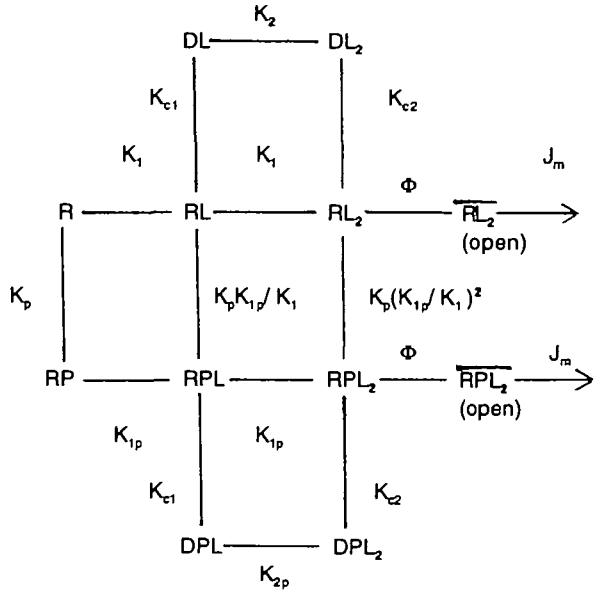
[2] To whom correspondence should be addressed. Tel/Fax: +81-083-933-5762, E-mail: aoshima@po.cc.yamaguchi-u.ac.jp



Adult female frogs (*Xenopus laevis*) were purchased from Hamamatsu Seibutsu Kyozai (Hamamatsu). The oocytes were dissected from the ovaries of adult female frogs that had been kept in ice for 1 h. They were manually detached from the inner ovarian epithelium and follicular envelope after incubation in a collagenase (type I, 1 mg/ml; Sigma) solution for 1 h by the procedure of Kusano *et al.* (*9*). The oocytes were microinjected with cRNAs in sterilized water and then incubated in a modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, and 0.41 mM $CaCl_2$ in 5 mM Tris, pH 7.6] containing 25 mg/liter of penicillin and 50 mg/liter of streptomycin at 15–18°C for 2–7 days before the electrophysiological measurements.

Electrophysiological Measurements—The membrane current of the receptors evoked by GABA was measured by the voltage clamping method with a voltage clamp amplifier (CEZ-1100, Nihon Kohden Kogyo, Tokyo). An oocyte was placed on a net in 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 passing the current for clamping the membrane potential, usually at –40 mV. The oocyte placed on the net was continuously perfused from the bottom with normal frog Ringer's solutions (115 mM NaCl, 1 mM KCl, and 1.8 mM $CaCl_2$ in 5 mM Tris, pH 7.2) by means of a gravity feed system, usually at a flow rate of about 2 ml/min (*10*)

Measurement of the Receptor Response—GABA was dissolved in normal frog Ringer's solution. To examine the effects of such compounds as alcohols on the GABA-elicited response, each compound was added to the Ringer's solution and GABA solution, which were then shaken vigorously for 1 min. One or other of the solutions was selected by switching a cock in the flow system. The control response was obtained by perfusing the GABA solution without any added compound and was taken as 100% The effect of the compound on the response of the receptors was measured by using a mixture of GABA and the compound. In some cases, the compound was applied 1 min before the co-application with GABA when desensitization of the receptors was significantly induced before equilibrium of the binding of the compound was attained (*11*) in the presence of high concentrations of GABA. The measurement was repeated several times with the same oocyte, and control values were obtained after every two or three measurements. To reverse the desensitization of the receptors, the oocyte was washed for more than 10 min in a normal frog Ringer's solution before the next measurement, since desensitization of the $GABA_A$ receptors is a reversible process and the receptors usually recover after about 10 min of washing (*7*).

When the effect of a compound on the rate of desensitization of $GABA_A$ receptors was examined, the compound was

Fig 1 **The minimum schematic model accounting for the effects of various compounds on the potentiation of the $GABA_A$ receptor-mediated response.** The active form (R) and the desensitized form (D) of the receptor bind the ligand (L GABA) more rapidly than the rate of desensitization of the receptor The receptors bind two GABA molecules with a dissociation constant of K_1 The receptors with two bound ligands (RL_2) are converted rapidly to an open channel ($\overline{RL_2}$) with an equilibrium constant for channel opening (1/Φ) (*6, 7*) In the presence of a compound which potentiates the response, the receptor binds the compound with a dissociation constant of K_p The receptor occupied by the compound (RP) binds two GABA molecules with a dissociation constant of K_{1p} Both the receptor occupied by two GABA molecules (RL_2), and the receptor occupied by two GABA molecules and the compound (RPL_2) have the same ability as to channel opening The rate constants of desensitization of the RL and RL_2 forms are assumed to be k_{12} and k_{34}, respectively The rate constants of recovery of the DL and DL_2 forms are assumed to be k_{21} and k_{43}, respectively These constants are assumed to be the same even when the receptors are occupied by the compound. The concentration of the receptors is assumed to be much less than that of the ligand or the compound.

In the absence of the compounds, the current (I) is expressed as

$$I = J_m [\overline{RL_2}] = J_m [L]^2/\{(1+\Phi)[L]^2 + 2K_1\Phi[L] + K_1^2\Phi\} \quad (1)$$

where J_m, [L], and $[\overline{RL_2}]$ represent the maximum current when all receptors open their channels, the concentration of GABA, and the fraction of the receptors in the open channel form The first-order rate constant of desensitization, α, is given as

$$\alpha = \frac{k_{43}[L] + 2k_{21}K_2}{[L] + 2K_2} + \frac{\Phi(k_{34}[L]^2 + 2k_{12}K_1[L])}{(1+\Phi)[L]^2 + 2\Phi K_1[L] + \Phi K_1^2} \quad (2)$$

In the presence of the compounds (which are assumed to increase the affinity of GABA binding to the receptors, but not to affect the open channel current or the rate constant of the desensitization of the RPL and RPL_2 forms), the current (I) is expressed as

$$I = J_m \{[\overline{RL_2}] + [\overline{RPL_2}]\}$$
$$= \{J_m([L]^2/K_1^2\Phi + [L]^2[P]/K_{1p}^2K_p\Phi)\}/\{(1+2[L]/K_1 + [L]^2/K_1^2 + [L]^2/K_1^2\Phi) + [P](1+2[L]/K_{1p} + [L]^2/K_{1p}^2 + [L]^2/K_{1p}^2\Phi)/K_p\} \quad (3)$$

where [P] represents the concentration of the compound which potentiates the response When the concentration of the compound is much larger than the dissociation constant of the receptor-compound complex, *i.e.*, $[P] \gg K_p$, the equation above is expressed simply as

$$I_p = J_m [\overline{RPL_2}] = J_m [L]^2/\{(1+\Phi)[L]^2 + 2K_{1p}\Phi[L] + K_{1p}^2\Phi\} \quad (4)$$

The first-order rate constant of desensitization in the presence of a saturating amount of the compound, α_p, is given as

$$\alpha_p = (k_{43}[L] + 2k_{21}K_{2p})/([L] + 2K_{2p}) + \{\Phi(k_{34}[L]^2 + 2k_{12}K_{1p}[L])\}/\{(1+\Phi)[L]^2 + 2\Phi K_{1p}[L] + \Phi K_{1p}^2\} \quad (5)$$

The equilibrium constants pertaining to the model are defined as follows (*6, 7*): $K_1 = 2[R][L]/[RL] = [RL][L]/2[RL_2]$, $K_2 = [DL][L]/2[DL_2]$, $K_{c1} = k_{21}/k_{12} = [RL]/[DL] = [RPL]/[DPL]$, $K_{c2} = k_{43}/k_{34} = [RL_2]/[DL_2] = [RPL_2]/[DPL_2]$, $K_{1p} = 2[RP][L]/[RPL] = [RPL][L]/2[RPL_2]$; $K_{2p} = K_{1p}K_{c2}/K_{c1} = [DPL][L]/2[DPL_2]$, $K_p = [R][P]/[RP]$; $\Phi = [RL_2]/[\overline{RL_2}] = [RPL_2]/[\overline{RPL_2}]$

applied to the oocytes 20 s before the mixture of GABA and the compound was applied to simplify the analysis (*11*). Both the flow rate of the bath application and the chart speed of the recorder were increased in this case.

RESULTS

Effect of the Functional Groups on the Potentiation—Figure 2 shows some examples of the electrical responses of $GABA_A$ receptors expressed in *Xenopus* oocytes on injection of cRNAs synthesized from cloned cDNAs of the receptor subunits The addition of 5 mM 1-hexanol to a 10 μM GABA solution potentiated the response of $GABA_A$ receptors composed of α_1 and β_1 subunits (a). The addition of 5 mM hexanal to a 10 μM GABA solution (b) also potentiated the response of $GABA_A$ receptors, but less efficiently than that of 5 mM 1-hexanol. This potentiation was reversible, since we obtained almost the same response as the control response after washing of the oocytes with normal frog Ringer's solution for several minutes We then examined the effect of the functional group of various six-carbon hydrocarbons on the response of $GABA_A$ receptors in the presence of 10 μM GABA. Since the addition of γ_2 subunit cRNA to the injected cRNAs did not cause any difference in the potentiation of the $GABA_A$ receptor–response (data not shown), we used simple $GABA_A$ receptors composed of only α_1 and β_1 subunits to examine in detail the effects of the compounds on the responses.

We examined the dose-dependence of the compounds with various functional groups on the $GABA_A$ receptor–mediated response evoked by 10 μM GABA. Typical examples of 1-hexanol, butyl acetate, and hexanoic acid are shown in Fig. 3a. Only hexanoic acid inhibited the response, the others potentiating it. For the compound which potentiated the response, its dissociation constant (K_p), and the maximum potentiation of the receptors (V_m) when all the potentiation sites of the receptors were occupied by the compound were estimated from its dose-dependency with the assumption of a simple equilibrium between the compound and the receptor, and are shown in Table Ia. Both K_p and V_m were dependent on the functional groups of the compounds. Alcohol potentiated the response of the $GABA_A$ receptors best.

The equilibrium constant between the closed and open forms of the receptor occupied by two GABA molecules, Φ (= $[RL_2]/[\overline{RL_2}]$), in a model (Fig. 1) was assumed to be 0.15 based on the report about a single channel current of a $GABA_A$ receptor channel (*12*). The dissociation constant of the GABA-recptor complex, K_1, was estimated to be 59 μM from the dose-response curve on the basis of the minimal model proposed previously (*6, 7*) (data not shown). The addition of the compound which potentiated the response shifted the GABA-dose-response curve to a lower concentration (Fig. 3b). The dissociation constant of the GABA-receptor complex (K_{1p}) when the potentiation sites of the receptors were occupied by each compound was then estimated according to the following procedure; the response in

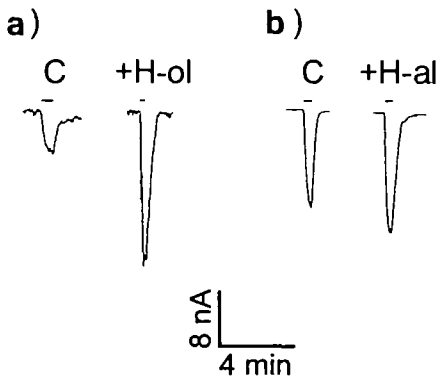


Fig. 2 **Effects of 5 mM 1-hexanol (a) and hexanal (b) on the 10 μM GABA-mediated current of $GABA_A$ receptors expressed in *Xenopus* oocytes.** All traces were obtained with a voltage clamp usually at –40 mV An inward current is shown as a downward curve The upper bars show when GABA or a mixture of GABA and the compound was applied Both responses in a given panel were obtained for the same injected oocyte, but the responses in panels a) and b) are for different oocytes. H-ol 5 mM 1-hexanol, H-al 5 mM hexanal

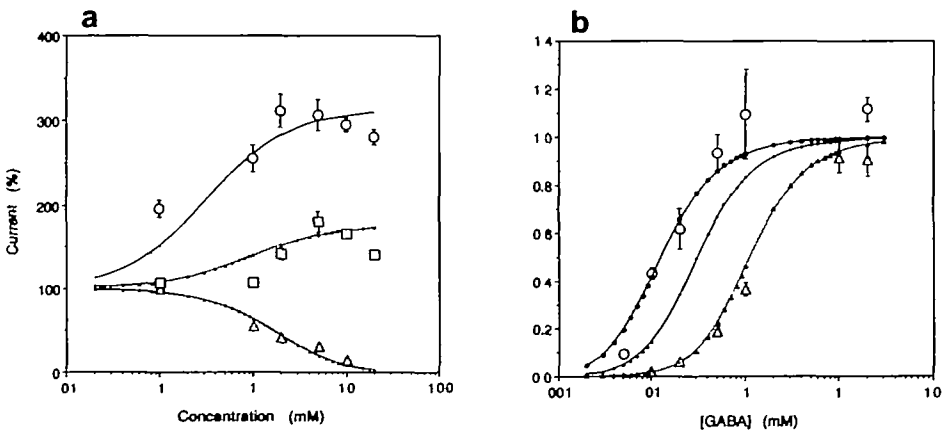


Fig 3 **a) Some examples of the dose–response relationship of compounds with various functional groups in the presence of 10 μM GABA.** Compounds at various concentrations were applied simultaneously with 10 μM GABA. The dissociation constant (K_p) and the maximum potentiation (V_m) for 1-hexanol and butyl acetate were estimated from these data and are shown in Table 1a The competitive inhibition constant (K_i) of hexanoic acid was estimated to be 3 8 mM from the data in a) and b) on the basis of the schematic model proposed previously (*13*). Data are mean ± SD (bars) values for four experiments. 1-hexanol ○, hexanoic acid △, butyl acetate: □ **b) Some examples of the effects of six-carbon hydrocarbons with different functional groups on the GABA-dose-response curve.** Theoretical curves for 1-hexanol were derived by using Eq 3 and the constants estimated in Table Ia. A theoretical curve for hexanoic acid was derived by using the equation described in the previous paper (*13*) and the K_i of 3.8 mM Data are mean ± SD (bars) values for four experiments. The maximum response elicited by a high concentration of GABA without any compound was taken as 1. 5 mM 1-hexanol· ○, 10 mM hexanoic acid: △.

Fig. 3b is expressed as I/I_{max}, where I is expressed by means of Eq. 3 in the legend to Fig. 1, and I_{max} [= $J_m/(1 + \Phi)$] represents the GABA-elicited maximum current without any compound when the concentration of GABA is very large (usually 1 or 2 mM). By substituting the values to the constants (Φ, K_l, and K_p) and the concentrations ([L] and [P]) in I/I_{max}, I/I_{max} is expressed by a quadratic equation of K_{lp}, which can be solved. The estimated values of K_{lp} are also summarized in Table Ia.

Hexanoic acid inhibited the $GABA_A$ receptor–mediated response weakly. The extent of the inhbition was dependent on the GABA concentration (Fig 3b), indicating competitive inhibition. Its inhibition constant was estimated to be 3.8 mM using the results in Fig. 3, a and b, on the basis of the schematic model proposed previously (*13*)

Effect of Chain Length on the Potentiation—The effect of the chain length of 1 mM normal alcohols on the potentiation in the presence of 10 μM GABA was examined. The alcohols showed increasing potentiation of the $GABA_A$ receptor–mediated response as the chain length increased (data not shown). Potentiation of $GABA_A$ receptor–mediated responses by normal alcohols also depended strongly on the concentrations of both the compound and GABA. Some examples of the dose dependence of the potentiation of the $GABA_A$ receptor–mediated responses are shown in Fig. 4, a and b. The dissociation constant of the compound-receptor complex (K_p) and the maximum potentiation (V_m) in the presence of 10 μM GABA were then estimated from this dose dependency, and are shown in Table Ib together with K_{lp}. The estimated values, K_p, V_m, and K_{lp}, varied with the chain length of the alcohols.

Effects of Fragrant Compounds on the Potentiation and Desensitization—The effects of several fragrant compounds that are components of essential oils used for aromatherapy on GABA-elicited responses were also examined. Though they clearly induced potentiation of the response, the potentiation showed much variation, possibly because of the difficulty in solubilizing these compounds in an aqueous solution. Potentiation of $GABA_A$ receptors by these compounds also depended strongly on the concentrations of both the compound and GABA (Fig. 5, a and b). These compounds shifted the GABA-dose-response curve to a lower concentration (Fig. 5b), as general anesthetics do (*8*). All of the examined compounds, which were higher alcohols or aldehyde, potentiated the response even at very low concentrations. In particular, terpinen-4-ol potentiated the response remarkably. Table Ic shows K_p and V_m of these compounds in the presence of 5 μM GABA. To examine the effect of the GABA concentration on K_{lp}, the K_{lp} values of these compounds were estimated in the presence of 5, 10, and 20 μM GABA. The calculated K_{lp} values were independent of the GABA concentration (average standard deviation: 5% of K_{lp} values). The average K_{lp} values are shown in Table Ic.

Figure 6a shows the effect of 0.61 mM terpinen-4-ol on the decay of the $GABA_A$ receptor-mediated response elicited by 5 μM GABA, when the GABA solution with and without terpinen-4-ol was continuously applied. Terpinen-4-ol accelerated the decay of the response, possibly due to desensiti-

TABLE I **Estimated K_p, V_m and K_{lp} of various compounds.**

Compound	K_p (mM)	V_m (%)	K_{lp} (μM)
a) Six-carbon hydrocarbons with various functional groups			
Control	—	—	59
1-Hexanol	0.32	313	23
(3*Z*)-hexen-1-ol	0 67	321	22
Hexanal	3 6	228	31
Butyl acetate	0 91	175	39
Hexylamine	1 2	174	40
b) Normal alcohols with various chain lengths			
Ethanol	40	147	45
1-Propanol	56	189	37
1-Butanol	14	264	27
1-Pentanol	2.2	279	26
1-Hexanol	0 32	312	23
1-Heptanol	0 11	328	22
1-Octanol	0 85	322	22
1-Nonanol	0 24	338	21
1-Decanol	0.18	413	16
c) Components of essential oils used for aromatherapy			
Cineol	0 11	255	34
Citral	0 21	181	41
Eugenol	0 18	284	32
Terpinen-4-ol	0 15	635	16

The constants, K_p, V_m, and K_{lp}, in a) and b) were estimated from the results in the presence of 10 μM GABA. The constants, K_p and V_m, in c) were estimated from the results in the presence of 5 μM GABA The constant, K_{lp}, in c) was the average of those estimated from the results in the presence of 5, 10, and 20 μM GABA.

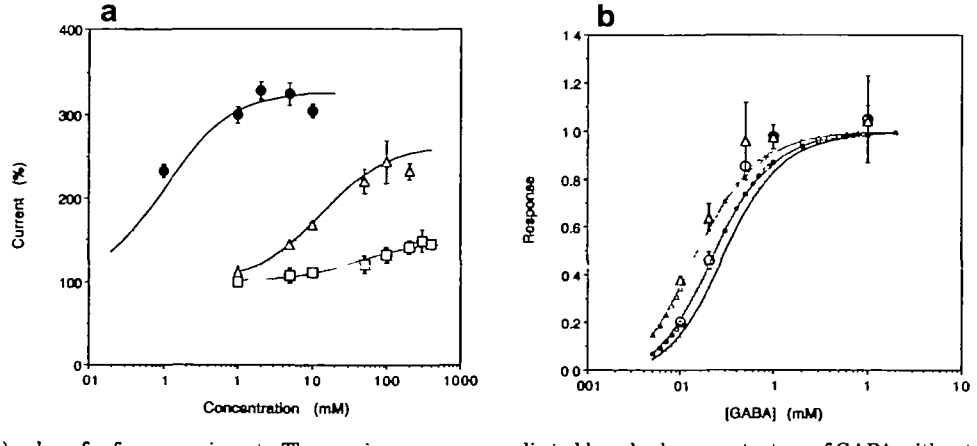


Fig. 4. **a) Some examples of the dose–response relationship of normal alcohols with various chain lengths in the presence of 10 μM GABA.** The dissociation constant (K_p) and the maximum potentiation (V_m) were estimated from these data, and are shown in Table Ib Data are mean ± SD (bars) values for four experiments Ethanol (□), 1-butanol (△), and 1-heptanol (●) **b) Some examples of the effects of normal alcohols with different chain lengths on the GABA-dose-response curve.** Theoretical curves were derived by using Eq. 3 and the constants estimated in Table Ib Data are mean ± SD (bars) values for four experiments. The maximum response elicited by a high concentration of GABA without any compound was taken as 1 400 mM ethanol ○, 10 mM 1-pentanol △

zation of the receptors. Assuming first order kinetics for the desensitization, as shown in Fig. 6b, the rate constants of desensitization caused by 5 μM GABA in the absence and presence of terpinen-4-ol were estimated to be 0.19 m^{-1} and 0.34 m^{-1}, respectively. Figure 7 shows the effect of the GABA concentration on the ratio of the rate of decay with and without terpinen-4-ol. The acceleration of the response-decay of $GABA_A$ receptors was dependent on the GABA concentration. The theoretical curve was drawn using Eqs. 2 and 5 in the legend to Fig. 1 and the rate constants reported previously (7).

Competitive Potentiation by the Compounds—It is important to know whether each compound binds to the same potentiation site or not. We measured the potentiation of the response by the addition of a mixture of two compounds which showed different maximum potentiation (V_m), and compared it with the potentiation on the addition of each compound alone, as shown in Table II. The potentiation by a mixture of terpinen-4-ol and citral, cineol, or butanol was greater than that by citral, cineol, or butanol, but less than that by terpinen-4-ol itself, which suggests the competitive binding of terpinen-4-ol with these three compounds to the potentiation site.

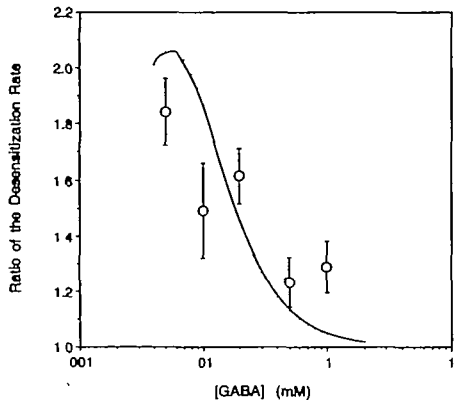


Fig 7 **Effect of the GABA concentration on the ratio of the decay rate of $GABA_A$ receptors with 0.61 mM terpinen-4-ol to that without it.** The theoretical curve of desensitization was drawn by using Eqs 2 and 4, and the rate constants estimated previously (7), *i e.*, k_{12} =0.029 s^{-1}, k_{21} = 0.0061 s^{-1}, k_{34} = 0 36 s^{-1}, and k_{43} = 0 0023 s^{-1}, K_p and K_{1p} of terpinen-4-ol being assumed to be 0 15 mM and 16 μM, respectively Data are mean ± SD (bars) values for four experiments.

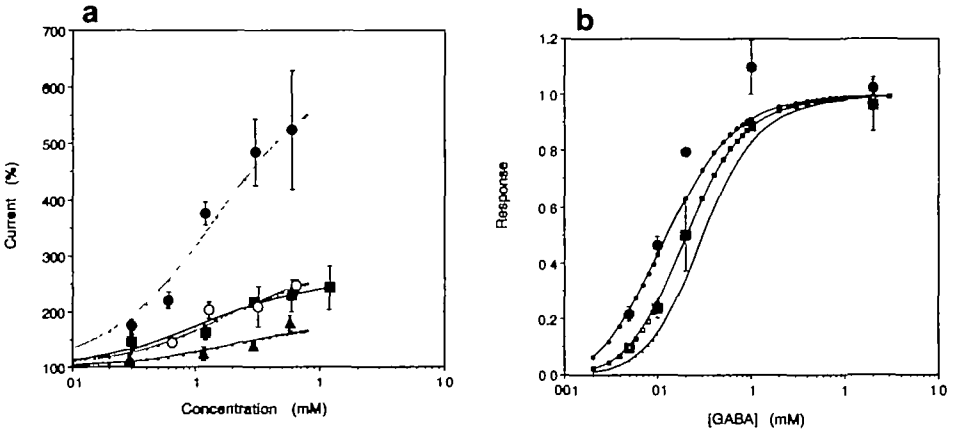


Fig 5 **a) Dose–response relationship of fragrant compounds that are components of essential oils used for aromatherapy in the presence of 5 μM GABA.** The dissociation constant (K_p) and the maximum potentiation (V_m) were estimated from these data, and are shown in Table Ic Data are mean ± SD (bars) values for four experiments. Cineol (■), citral (▲), eugenol (○) and terpinen-4-ol (●) **b) Effects of 0.30 mM cineol (■) and 0.30 mM terpinen-4-ol (●) on the GABA-dose-response curve.** Theoretical curves were derived by using Eq 3 and the constants estimated in Table Ic. Data are mean ± SD (bars) values for four experiments. The maximum response elicited by a high concentration of GABA without any compound was taken as 1.

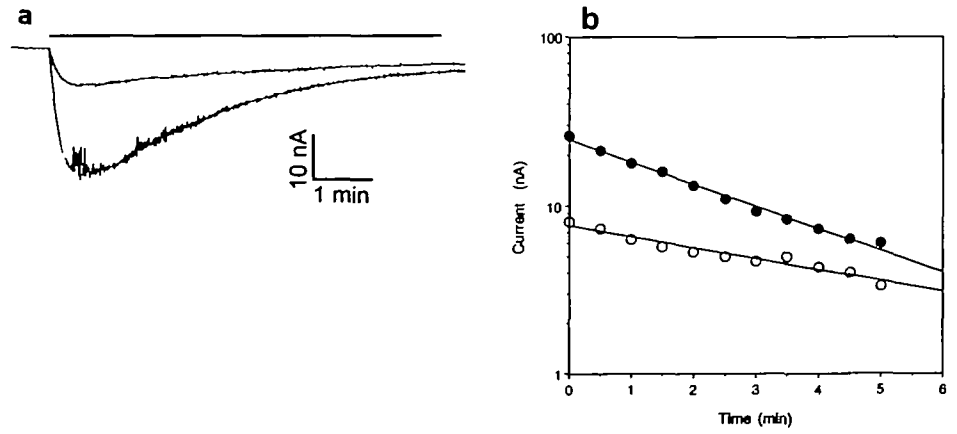


Fig 6 **a) Example of the acceleration of the decay of the $GABA_A$ receptor response when 0.61 mM terpinen-4-ol was added.** To examine the effect of terpinen-4-ol on the decay of the response, 0 61 mM terpinen-4-ol was applied for 20 s, and then a mixture of 5 μM GABA and 0.61 mM terpinen-4-ol was applied continuously to an oocyte in which $GABA_A$ receptors were expressed. The upper bar shows when 5 μM GABA with or without 0 61 mM terpinen-4-ol was present in the bath **b) Evaluation of the rate constants from the plots of logarithmic current versus time.** The plots started just after the peak current in Fig. 6a The rate constants of the decay of the $GABA_A$ receptor response in the absence (○) and presence (●) of 0 61 mM terpinen-4-ol were estimated to be 0.19 m^{-1} and 0 34 m^{-1}, respectively, where the decay was assumed to follow first order kinetics. The acceleration was expressed as the ratio of the rate constant of decay with and without terpinen-4-ol, *i e.* 0 34/0.19 = 1.8.

TABLE II **Comparison of the potentiation by terpinen-4-ol, citral, cineol, or butanol with that of a mixture of terpinen-4-ol and the other compound.**

Compound(s)	Response (%)
a) Terpinen-4-ol (0 61 mM) and citral (0 58 mM)	
Control	100
+ Terpinen-4-ol	575 ± 93
+ Citral	218 ± 22
+ Terpinen-4-ol and citral	400 ± 80
b) Terpinen-4-ol (0.61 mM) and cineol (0 60 mM)	
Control	100
+ Terpinen-4-ol	697 ± 62
+ Cineol	370 ± 21
+ Terpinen-4-ol and cineol	457 ± 42
c) Terpinen-4-ol (0.61 mM) and 1-butanol (11 mM)	
Control	100
+ Terpinen-4-ol	656 ± 53
+ 1-Butanol	316 ± 22
+ Terpinen-4-ol and 1-butanol	609 ± 21

The response elicited by 1 μM GABA was taken as the control (100%) Data are mean ± SD values for four experiments.

DISCUSSION

A number of structurally diverse compounds enhance the action of GABA on $GABA_A$ receptors; these compounds include benzodiazepine, barbiturates, pregnane steroids, general anesthetics, and ethanol (*2*). In animal models, these compounds exhibit anxiolytic, anticonvulsant and sedative activity (*14, 15*).

In this study, we expressed $GABA_A$ receptors composed of both α_1 and β_1 subunits, and quantitatively analyzed the potentiation of the $GABA_A$ receptor–response by various compounds. It has been reported that in single-channel kinetics of the $GABA_A$ receptor current, alcohols increase the frequency of opening, the mean open time, the percentage of open time, the frequency of bursts, and the mean burst duration of the main single-channel conductance (*16*), as anesthetics do. An allosteric model has been proposed for analyzing the potentiation, in which the binding of an alcohol or anesthetic to the receptor is assumed to only increase the affinity of GABA binding sites (*8*). Though the potentiation of the current has been thoroughly analyzed using this model, the increase in the rate of desensitization by alcohols has not been thoroughly analyzed quantitatively. Therefore, we extended the minimal model to explain the response and desensitization of ionotropic neurotransmitter receptors (*6, 7*) to analyze both the potentiation and desensitization of the $GABA_A$ receptor–mediated response systematically in the presence of various compounds with different functional groups and chain lengths, as shown in Fig. 1.

Table I shows the dissociation constant (K_p) between a compound and its receptor, the maximum potentiation (V_m), and the dissociation constant of GABA (K_{1p}) when the potentiation site of the receptor is fully occupied with the compound. These values were estimated from the experimental results on the basis of the model in Fig. 1. These constants were dependent on the functional group and the carbon chain length of the compounds. The following conclusions can be drawn from our results and analyses:

1) The potentiation site for alcohol is present in $GABA_A$ receptors composed of only α_1 and β_1 subunits (*17, 18*), though the γ subunit is essential for the potentiation of the $GABA_A$ receptor-mediated response by benzodiazepine (*19, 20*).

2) The potentiation site of the receptors appears to have both hydrophilic and hydrophobic group binding regions. The hydrophilic group binding region, which recognizes the functional group, binds best with a hydroxyl group. Phenol derivatives also potentiated the $GABA_A$ receptor–mediated response strongly (*21*). The hydrophobic group binding region is large enough to bind a hydrocarbon of at least up to 10 carbon atoms.

3) The potentiation site is likely to be located at some specific site in the receptors, since the potentiation by the compounds is competitive rather than additive (Table II). This is supported by the report that mutation of two specific amino acid residues in the receptor abolished the potentiation by alcohols, but not the response elicited by GABA (*17*).

The model in Fig. 1 predicts that the compounds which increase the affinity of the receptors to GABA will also accelerate the rate of desensitization, because such compounds increase the fraction of receptors bound by one or two GABA molecules. In fact, Fig. 6 shows the acceleration of the decay of the GABA-elicited response by terpinen-4-ol. Figure 7 shows the effect of the GABA concentration on the ratio between the decay in the presence and absence of the compound. A theoretical curve drawn using Eqs. 2 and 5 in the legend to Fig. 1 and constants reported previously (*7*) could explain the dependence of this ratio on the GABA concentration relatively well. Since oocytes are rather big cells and the flow rate of the bath application system is not fast enough, it is difficult to discuss the exact rate of desensitization quantitatively Therefore we simply showed the semi-quantitative results here.

Recently, both partial agonistic and allosteric models were proposed for the actions of 1-octanol on $GABA_A$ receptors which were expressed in human embryonic kidney 293 cells transfected with α_1, β_2 and γ_{2s} subunit cDNAs of the rat brain receptors, since 1-octanol evoked both a small current and desensitization (*22*). However, we observed neither a current nor desensitization induced by higher alcohols (1-octanol, 1-nonanol and 1-decanol) for $GABA_A$ receptors which were expressed in *Xenopus* oocytes injected with α_1 and β_1 subunit cRNAs. Further experiments are necessary to resolve this discrepancy, since the experiments were performed on different cells and with different combinations of the subunits from different species (rats and cattle).

We examined the potentiation of $GABA_A$ receptors by fragrant compounds that are components of essential oils used for aromatherapy. Even very small amounts (several ppm) of these compounds potentiated the response of the receptors. Higher alcohols have been reported to be several thousand times as potent as ethanol in the potentiation of the $GABA_A$ receptor–mediated response (*23*). As mentioned in a previous report (*5*), these fragrant compounds may be absorbed into the blood and carried to the brain through the blood-brain barrier, and then potentiate the $GABA_A$ receptor-mediated response, which will induce anxiolytic, anticonvulsant and sedative activity in the human brain. The accumulation of essential oil components in the mouse brain when they were given by means of percutaneous absorption was also reported recently (*24*). The tranquillizing effect of aromatherapy or phytoncids (fragrant compounds emanating from plants and trees in the forest) may be due to the direct potentiation of $GABA_A$ receptors by the

fragrant compounds. The direct effect of fragrant compounds on $GABA_A$ receptors was suggested by a report showing that the inhalation of chamomile and lemon oil vapor decreased restriction-stress-induced increases in the plasma adrenocorticotropic hormone (ACTH) level in ovariectomized rats, as did diazepam, a benzodiazepine derivative (*25*). It has also been reported that anti-conflict effects of rose oil and its components were observed in a mouse behavior test (*26, 27*). Further experiments are necessary to prove the relationship between the effects of aromatherapy or phytoncids and the potentiation of the $GABA_A$ receptor-mediated response by fragrant compounds

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