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Effects of Toluene Exposure on Signal Transduction: Toluene Reduced the Signaling via Stimulation of Human Muscarinic Acetylcholine Receptor m2 Subtypes in CHO Cells

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ABSTRACT—The organic solvent toluene is used widely in industry and is toxic to the central nervous system (CNS). To clarify the mechanisms of CNS toxicity following toluene exposure, especially with respect to the G protein-coupling of receptors, we determined the effects of toluene on the activation of G_i by stimulating human muscarinic acetylcholine receptor m2 subtypes (hm2 receptors) expressed in Chinese hamster ovary (CHO) cells. We first examined whether toluene affects the inhibition of adenylyl cyclase by G_i. The attenuation of forskolin-stimulated cAMP formation by the stimulation of hm2 receptors was reduced in a medium containing toluene. Next, we determined the effects of toluene on carbamylcholine-stimulated [^{35}S]GTPγS binding using membrane fractions of CHO cells expressing hm2 receptors. Carbamylcholine-stimulated [^{35}S]GTPγS binding activity was markedly reduced when assayed using reaction buffers containing toluene. However, carbamylcholine-stimulated [^{35}S]GTPγS binding activity was essentially unchanged following pretreatment of the cells with a toluene-saturated medium prior to membrane isolation. Toluene pretreatment and the toluene itself did not alter the characteristics of the binding of carbamylcholine and [^{3}H]*N*-methylscopolamine to hm2 receptors. On the contrary of the effect of toluene for [^{35}S]GTPγS binding, the effect of toluene for attenuation of forskolin-stimulated cAMP formation by the stimulation of hm2 receptors was irreversible. These observations indicate that toluene acts as an inhibitor of the signal transduction via hm2 receptor stimulation in CHO cells, and at least two mechanisms exist in the inhibition mechanisms by toluene.

Keywords: Adenylyl cyclase, G protein, [^{35}S]GTPγS binding, Muscarinic acetylcholine receptor m2 subtype, Toluene

Toluene is one of the most widely used organic solvents in industry. Toluene inhalation results in various symptoms such as fatigue, headache, vertigo, and ataxia (1). These symptoms are thought to be due to brain dysfunction, because the effects of toluene on peripheral system, other than the central nervous system (CNS) are very weak (2). The long-term inhalation of toluene leads to addiction, anorexia, and memory disorders. Inhalation of high concentrations of toluene may induce dementia (3). Many reports of poisoning and animal experiments indicate that toluene is toxic to the CNS. To clarify the toxicity mechanism of toluene, behavioral, electrophysiological, and biochemical approaches have been used. Brain function is maintained by impulse flow inside nerve cells and impulse flow from nerve cells to the adjacent cells at synapses. Therefore, changes in the events at synapses are in many cases critical to brain function. In the field of neurotoxicity of chemicals, mechanistic studies have focused on synaptic neurotransmission (4 – 6). Of the many organic chemical substances that possess neurotoxicity, ethanol has been the

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most intensively studied (for review, see ref. 7). The toxicity mechanism of toluene has not been established. Since many incidents of toluene intoxication in the workplaces continue to be reported, and because "glue sniffing" among young people has lately become a concern, toluene toxicity should be studied much more intensively.

G protein-coupled receptors mediate signals from outside to the inside of the cell by the activation of heterotrimeric G proteins. Activated G proteins regulate the activity of enzymes to alter the cytosolic second messengers such as adenylyl cyclase and phospholipase-Cβ. The effects of ethanol on signal transduction systems via G protein-coupled receptors and G proteins have been also well studied (for review, see ref. 8). Ethanol activates the adenylyl cyclase via G protein G_s in cultured cells (9). Alcohol inhibits the function of 5-hydoxytryptamine (serotonin) receptors (10, 11). The in vivo effects of ethanol on the characteristics of the binding of agonists to β-adrenergic receptors and the activity of adenylyl cyclase in the rat brain were reported (12).

Toluene exposure has also been found to induce changes in the characteristics of the binding of agonists to G protein-coupled receptors in the rat brain (13 – 16). Recently, we determined the effects of toluene exposure on inhibition of adenylyl cyclase by stimulating human muscarinic acetylcholine receptor m2 subtypes (hm2 receptors) expressed in Chinese hamster ovary (CHO) cells (17). The inhibition of adenylyl cyclase by 10 mM of carbamylcholine stimulation of hm2 receptors was attenuated in the presence of toluene. In the present study, we further investigated the effects of toluene on the activation of the G protein G_i by stimulating hm2 receptors expressed in CHO cells. In our recent study of animal exposure to toluene vapor, changes in the binding affinity of the muscarinic acetylcholine receptor agonist carbamylcholine were determined in membranes isolated from the brains of rats exposed to toluene (18).

MATERIALS AND METHODS

Materials

[^{3}H]*N*-Methyl scopolamine (NMS, specific activity of 71.3 Ci/mmol) and [^{35}S]GTPγS (specific activity of 30 – 40 cpm/fmol) were purchased from Du Pont-New England Nuclear (Boston, MA, USA). The Chinese hamster ovary CHO-K1 cells were donated from the Health Science Research Resources Bank (Tokyo), and the cDNA encoding hm2 receptors was a gift from Dr. W. Sadee (University of California at San Francisco, San Francisco, CA, USA).

Construction of stable transfectants expressing hm2 receptors

The construction of the mammalian expression vectors for c-Myc epitope-tagged hm2 receptor (CHO-hm2) was described previously (19). The cells were cultured in F-12 nutrient mixture (Ham's) (Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 40 units/ml penicillin G (Meiji Seika, Tokyo), 40 μg/ml streptomycin sulfate (Meiji Seika) and 100 μg/ml geneticin at 37°C in 95% air and 5% CO_2.

Adenylyl cyclase assay

CHO-hm2 cells (1×10^4 cells/well) were plated in 12-well culture dishes. Forty to forty-eight hours after plating, the culture medium was changed to serum-free medium with or without 3.5 μM of toluene. In some experiments, cells were pretreated with 3.5 μM of toluene for 15 min at 37°C and washed with 1 ml of culture medium three times. Then various concentrations of carbamylcholine, 1 mM of 3-isobtyl-1-methylxatine, and 50 μM of forskolin were added to the media and incubated for 7 min at 37°C. After this incubation, the media were removed and the cells were incubated in 1 ml of 2.5% perchloric acid for 30 min on ice. Next, the samples were neutralized with 90 ml of 4.2 M KOH and centrifuged at 12,000 rpm for 10 min at 4°C. After centrifugation, the amount of cAMP in the supernatant was measured by use of the Biotrak cAMP enzyme immunoassay system (Amersham Pharmacia Biotech, Ltd., Uppsala, Sweden).

Membrane preparation

Semi-confluent CHO-hm2 cells cultured in 15-cm diameter dishes were treated with or without 3.7 μM of toluene for 15 min, and then washed with 10 ml of ice-cold phosphate-buffered saline (PBS) three times. The washed cells were scraped, suspended in HMEE buffer (20 mM Hepes-KOH, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA, pH 7.4) and homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1,500 rpm for 5 min at 4°C, and the supernatant was centrifuged at $100{,}000 \times g$ for 30 min at 4°C. The pellet was resuspended in HMEE buffer and subjected to [^{35}S]GTPγS and ligand binding assays.

Ligand binding assay

For estimation of the K_D value of carbamylcholine, 50 μg of membrane fractions of CHO-hm2 cells was incubated with 0.6 nM [^{3}H]NMS and different concentrations of carbamylcholine in 1 ml of HEN buffer (20 mM Hepes-KOH, 1 mM EDTA, 160 mM NaCl, pH 7.4) supplemented with 2 mM GTP at 30°C for 60 min. After incubation, the membranes were trapped on Whatman GF-B glass fiber filters and washed with 1 ml of 20 mM K-phosphate buffer (pH 7). Membrane-bound [^{3}H]NMS trapped on the filter was counted with an ACS II liquid scintillation counter

(Amersham Pharmacia Biotech, Ltd.). For the estimation of the B_{max} and K_D values of [^{3}H]NMS, 50 μg of membrane fractions was incubated with 1 pM – 10 nM of [^{3}H]NMS in 1 ml of HEN buffer at 30°C for 60 min, and then the membrane-bound [^{3}H]NMS was measured as described above.

Measurement of the uncoupling of hm2 receptors from G proteins

The function of hm2 receptors was measured as the agonist-stimulated [^{35}S]GTPγS binding activity of the membrane preparation. The [^{35}S]GTPγS binding assay was carried out as described by Lazareno et al. (20) and Tsuga et al. (21). Seventy-five milligrams of membrane fractions of CHO-hm2 cells were incubated in 0.2 ml of HENDM buffer (20 mM Hepes-KOH, 2 mM $MgCl_2$, 1 mM EDTA, 160 mM NaCl, 1 mM dithiothreitol, pH 7.4) supplemented with 1 μM GDP, 0.1 nM [^{35}S]GTPγS, and various concentrations of carbamylcholine at 30°C for 20 min. In some experiments, the HENDM buffer made with toluene dissolved in water was used in place of the normal HENDM buffer. After incubation, 0.8 ml of ice-cold HEN buffer supplemented with 0.1 mM GTP was added to the reaction mixture, and then the membranes were trapped on Whatman GF-B glass fiber filters and washed four times with 1 ml of washing buffer (20 mM Tris, 100 mM NaCl and 25 mM $MgCl_2$, pH 8.0). The radioactivity was then determined.

Measurement of toluene concentration

The actual concentrations of toluene dissolved in the medium and reaction buffers were measured by a gas chromatography system equipped with a flame ionization detector (Type 5880A; Hewlett-Packard, Wilmington, DE, USA) as described by Tsuruta (22).

Data analyses

Dose-response curves of carbamylcholine of Figs. 1 and 3b were fitted to the following equation: $E_{max} \times EC_{50} / ([\text{Carbamylcholine}] + EC_{50}) + (100 - E_{max})$, where the value in the cells that were not stimulated with carbamylcholine was taken as 100%. Dose-response curves of Figs. 2 and 3a were fitted to the following equation: $B_{max} \times [\text{Carbamylcholine}] / (EC_{50} + [\text{Carbamylcholine}])$, where the value in the presence of 10^{-3} M carbamylcholine as the control condition was taken as 100%. Data was analyzed with one-way analysis of variance followed by Dunnett's test.

RESULTS

The effect of toluene on the attenuation of forskolin-stimulated cAMP formation by stimulation with carbamylcholine in CHO-hm2 cells

To determine whether toluene affects the receptor signaling through G proteins, we examined the effect of toluene on the attenuation of forskolin-stimulated cAMP formation by the stimulation of hm2 receptors in CHO-hm2 cells. The agonist stimulation of hm2 receptors inhibits adenylyl cyclase via the activation of G_i (23). As shown in Fig. 1a, the attenuation of cAMP accumulation by the carbamylcholine stimulation of hm2 receptors was reduced in the presence of 3.5 μM toluene. The E_{max} values were reduced to 60% of the control value (52% vs 84 %), and the EC_{50} values were increased to a 50-fold higher concentration than that of the control (9.2 vs 0.19 μM) in the presence of toluene. The inhibition of adenylyl cyclase by carbamylcholine stimulation of mAChR m2 subtypes was attenuated in the presence of 2.5 μM or higher concentration of toluene (Fig. 1b). The forskolin-stimulated cAMP formation was also reduced in the presence of 3.5 μM toluene to 55 ± 15% (mean ± S.D., n = 5) of the control.

Effect of toluene on [^{35}S]GTPγS binding by the stimulation of hm2 receptors and ligand binding characteristics

We next examined the effect of toluene on the activation of G proteins by hm2 receptor stimulation using a [^{35}S]GTPγS binding assay of membrane preparations of CHO-hm2 cells. We prepared crude membrane fractions from CHO-hm2 cells, and measured the [^{35}S]GTPγS binding activity in the presence of different concentrations of carbamylcholine, 0.1 nM [^{35}S]GTPγS, and 1 μM GDP. As shown in Fig. 2, a and b, the extent of carbamylcholine-stimulated [^{35}S]GTPγS binding activity was decreased to 76% and 69% in the presence of 2.5 and 3.5 μM of toluene, respectively. This inhibitory effect of toluene on [^{35}S]GTPγS binding was seen at the 2.5 μM and higher concentration of toluene. Only a slight effect was seen when the toluene concentrations was reduced to one-fifth (Fig. 2: c and d). We also investigated whether the presence of toluene changes the binding characteristics of hm2 receptors. As shown in Table 1, the [^{3}H]NMS binding sites and affinities of GTP-insensitive carbamylcholine binding and the [^{3}H]NMS binding to hm2 receptors did not change in the presence of toluene.

Effect of toluene pretreatment on the signal transduction via stimulation of hm2 receptors and ligand binding characteristics of hm2 receptors in CHO cells

To determine whether these inhibitory effects of toluene were caused by irreversible changes of receptors and/or G proteins by toluene, we prepared membrane fractions

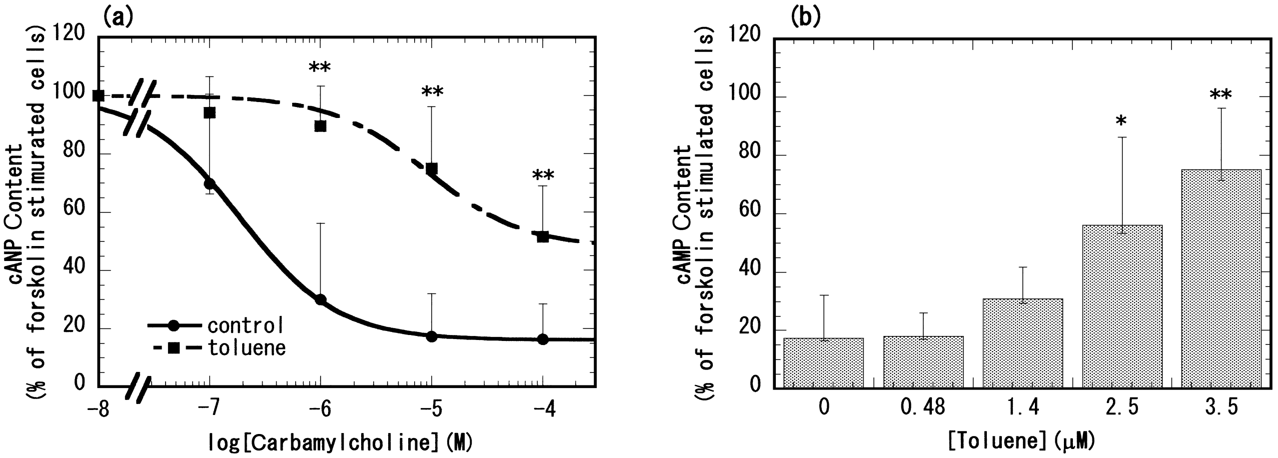

Fig. 1. The effects of toluene on the attenuation of forskolin-stimulated cAMP formation by the stimulation of hm2 receptors in CHO cells. a: CHO-hm2 cells were incubated with various concentrations of carbamylcholine, 1 mM 3-isobtyl-1-methylxatine, and 50 μM forskolin, and in the presence or absence of 3.5 μM of toluene for 7 min at 37°C. After incubation, the amount of cAMP in the cells was measured as described under Materials and Methods. The results shown are the means ± S.D. of five independent experiments. Dose-response curves of carbamylcholine were fitted to the following equation: $E_{max} \times EC_{50}$ / ([Carbamylcholine] + EC_{50}) + (100 – E_{max}), where the value in the cells that were not stimulated with carbamylcholine was taken as 100%. The E_{max} values were estimated to be 52% and 84% for cells treated or not treated with 3.5 μM toluene, respectively. The EC_{50} values were estimated to be 9.2 and 0.19 μM for cells treated or not treated with 3.5 μM toluene, respectively. The absolute values (means ± S.D.) for 0% and 100% are 150 ± 140 fmol/10^4 cells and 3600 ± 1100 fmol/10^4 cells for the control and 220 ± 200 fmol/10^4 cells cpm and 2000 ± 640 fmol/10^4 cells for 3.5 μM toluene, respectively. b: CHO-hm2 cells were treated with 10 μM carbamylcholine, 1 mM 3-isobtyl-1-methylxatine, 50 μM forskolin, and various concentrations of toluene for 7 min at 37°C. After treatment, the amount of cAMP in the cells was measured as described in Matrials and Methods. The results shown are the means ± S.D. of four to seven independent experiments. Data was analyzed with one-way analysis of variance followed by Dunnett's test. The symbols * and ** denote the significant differences compared with control cells at P<0.05 and P<0.01, respectively.

from CHO-hm2 cells that had been treated with 3.5 μM toluene for 15 min, and then we measured the [^{35}S]GTPγS binding activity. As shown in Fig. 3a, the carbamylcholine-stimulated [^{35}S]GTPγS binding activity was not affected by pretreatment with toluene. Furthermore, as shown in Table 1, the toluene pretreatment also did not alter the [^{3}H]NMS binding site or the affinities of GTP-insensitive carbamylcholine binding and [^{3}H]NMS binding to hm2 receptors. However, toluene pretreatment affects the attenuation of forskolin-stimulated cAMP formation by the stimulation of hm2 receptors in CHO-hm2 cells in the same way as in the presence of 3.5 μM toluene. As shown in Fig. 3b, the attenuation of cAMP accumulation by the carbamylcholine stimulation of hm2 receptors was reduced by pretreatment with 3.5 μM toluene as with the presence of toluene. The E_{max} values were reduced to 65% of the control value (55% vs 84 %), and the EC_{50} values were increased to a 25-fold higher concentration than that of the control (4.7 vs 0.19 μM) in the presence of toluene.

DISCUSSION

Neurochemical studies have been performed regarding the neurotoxicity of toluene (24 – 27). In our previous study, short-term exposure of rats to high concentrations of toluene gas altered the acetylcholine metabolism in the brain (28). We also elucidated, in a microdialysis study, that the suppression of acetylcholine release from the cholinergic nerve terminals in the rat brain was induced by toluene (29). These findings suggest that toluene may affect the cholinergic neurons in the brain rather than the other neurotransmission systems. Recently, we found that toluene attenuates the inhibition of adenylyl cyclase by stimulating hm2 receptors and does not affect activation of adenylyl cyclase via β_2-adrenergic receptor stimulation (17).

The agonist-stimulated hm2 receptors activated the G protein G_i, then inhibited the adenylyl cyclase in the CHO cells (23). Toluene also inhibited the carbamylcholine-stimulated [^{35}S]GTPγS binding activity (Fig. 2). Carbamylcholine stimulates the [^{35}S]GTPγS binding to G proteins G_i and G_o of membrane preparations in the presence of unlabeled GDP, because carbamylcholine facilitates the dissociation of GDP from G proteins and lowers the affinity for GDP of G proteins (20, 30). Therefore, we can estimate the first response in the signal transduction cascade following receptor-stimulation by an agonist by using a [^{35}S]GTPγS binding assay. Furthermore,

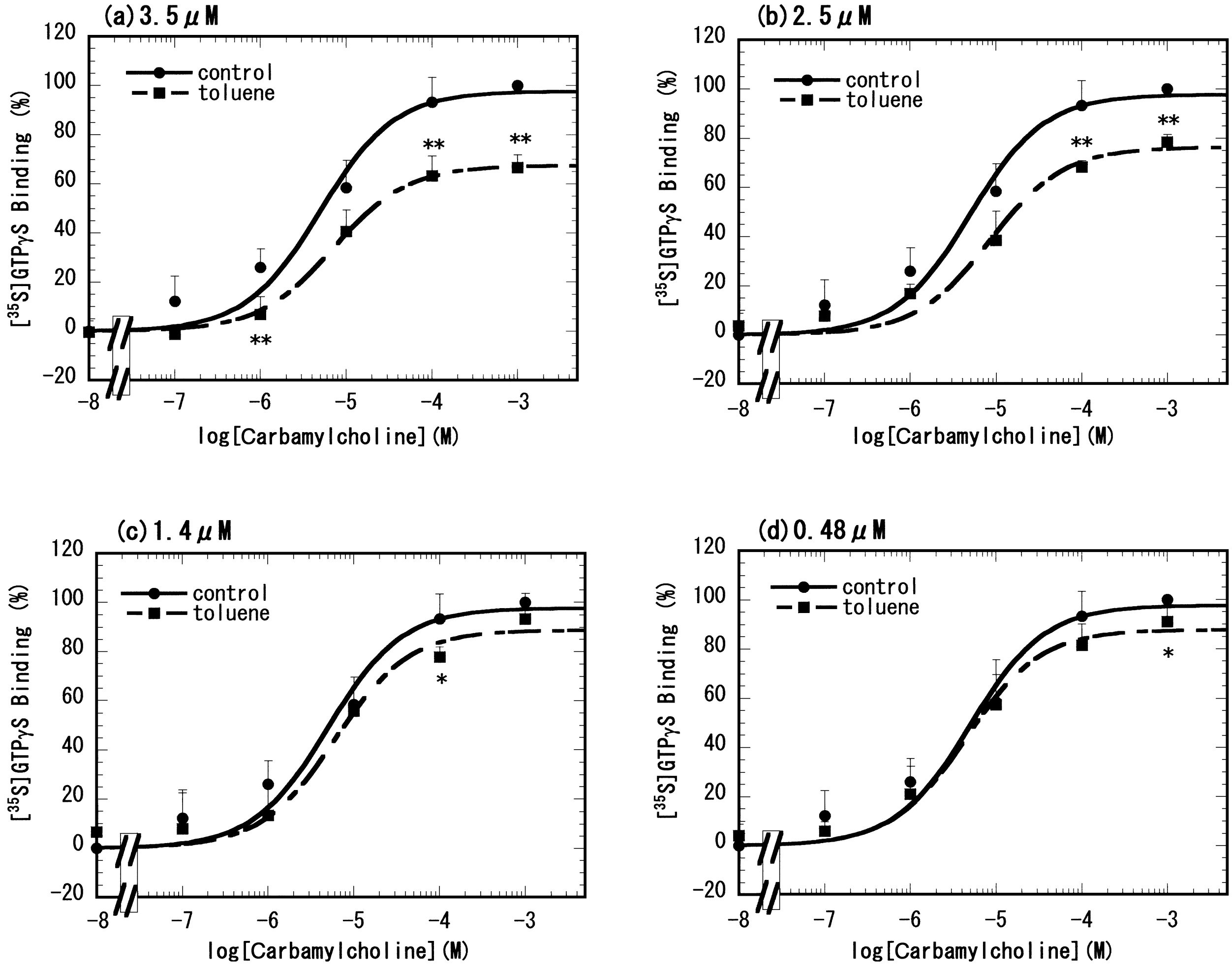

Fig. 2. The effects of toluene on carbamylcholine-stimulated [^{35}S]GTPγS binding. Membrane preparations of CHO-hm2 cells containing 75 mg of protein were subjected to the [^{35}S]GTPγS binding assay with or without 3.5 μM (a), 2.5 μM (b), 1.4 μM (c) and 0.48 μM (d) of toluene, as described under Materials and Methods. The results shown are the means ± S.D. of three independent experiments. Dose-response curves were fitted to the following equation: B_{max} × [Carbamylcholine] / (EC_{50} + [Carbamylcholine]), where the value in the presence of 1 mM carbamylcholine as the control condition was taken as 100%. The B_{max} values were estimated to be 98% for the control, 69% in the presence of 3.5 μM toluene, 76% for 2.5 μM toluene, 89% for 1.4 μM toluene and 88% for 0.48 μM toluene. The EC_{50} values for carbamylcholine were estimated to be 4.9 μM for the control, 7.4 μM in the presence of 3.5 μM toluene, 8.4 μM for 2.5 μM toluene, 6.0 μM for 1.4 μM toluene and 4.5 μM for 0.48 μM toluene. Data were analyzed with one-way analysis of variance followed by Dunnett's test. The significant differences compared with the control cells are denoted *P<0.05 or **P<0.01. The absolute values for 0% and 100% are 470 – 1140 cpm and 930 – 3000 cpm, respectively.

as shown in Table 1, the [^{3}H]NMS binding sites to hm2 receptors and the affinities of carbamylcholine did not change in the presence of or pretreatment with toluene. Thus, toluene itself does not interfere with the agonist-receptor interaction, and toluene does not denature receptors. We did not clearly observe GTP-sensitive carbamylcholine binding in the membrane fraction of CHO-hm2 cells. This may have been due to an excess of receptors that were expressed by transfection. The pretreatment with toluene also did not have an effect. These observations suggest that the inhibitory effect of toluene on the attenuation of cAMP accumulation induced by the carbamylcholine stimulation of hm2 receptors is partially caused by the inhibition of G_i activation by hm2 receptor stimulation.

However, the inhibitory effect of toluene on attenuation of forskolin-stimulated cAMP formation via hm2 receptor stimulation is mainly caused by interference of G_i and adenylyl cyclase by toluene. The attenuation of cAMP accumulation by the carbamylcholine stimulation of hm2 receptors was reduced by pretreatment with toluene, the same as with the presence of toluene. Moreover, this inhibitory effect of toluene is greater than that on carbamylcholine-stimulated [^{35}S]GTPγS binding, although we have

Table 1. The maximal binding (B_{max}) and the equilibrium dissociation constant (K_d) of [^{3}H]NMS and low affinity K_d of carbamylcholine

	[^{3}H]NMS		Carbamylcholine
	B_{max} (pmol/mg protein)	K_d (nM)	K_d (low affinity) (μM)
Control	0.95 ± 0.14	0.30 ± 0.069	15 ± 4.5
With toluene	0.91 ± 0.23	0.30 ± 0.055	24 ± 9.8
Toluene-pretreated cells	1.1 ± 0.090	0.32 ± 0.085	23 ± 8.3

The maximal binding (B_{max}) and the equilibrium dissociation constant (K_d) of [^{3}H]NMS and K_d of carbamylcholine with 2 mM GTP (low affinity) in the membrane fraction from CHO-hm2 cells with or without 3.5 μM toluene, and the B_{max} and K_d of [^{3}H]NMS and K_d of carbamylcholine with 2 mM GTP in the membrane fraction of CHO-hm2 cells pretreated with 3.5 μM toluene at 37°C for 15 min. The [^{3}H]NMS binding to muscarinic receptors in membrane fractions of cells was assayed as described under Materials and Methods. The results shown are the means ± S.D. of three to four independent experiments.

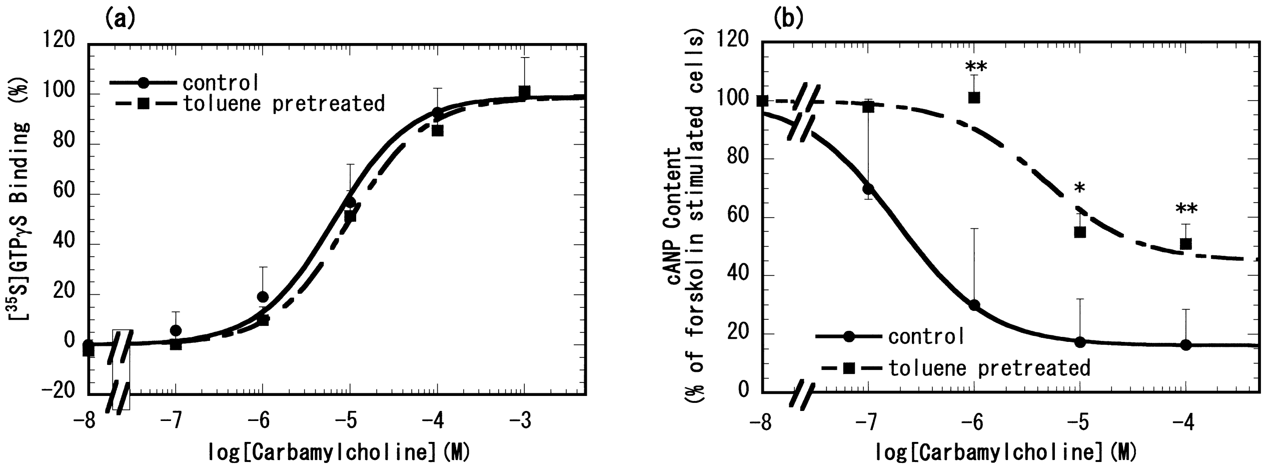

Fig. 3. The effects of toluene-pretreatment on carbamylcholine-stimulated [^{35}S]GTPγS binding, and the effects of toluene-pretreatment on the attenuation of forskolin-stimulated cAMP formation by the stimulation of hm2 receptors in CHO cells. a: The effects of toluene-pretreatment on carbamylcholine-stimulated [^{35}S]GTPγS binding. The experimental procedures were the same as those described in the legend to Fig. 2, except that the CHO-hm2 cells were incubated with or without 3.5 μM toluene for 15 min at 37°C before the preparation of the membrane fractions. The results shown are the means ± S.D. of three independent experiments. Dose-response curves were fitted to the following equation: B_{max} × [Carbamylcholine] / (EC_{50} + [Carbamylcholine]), where the value in the presence of 1 mM carbamylcholine for cells treated without toluene was taken as 100%. The B_{max} values were estimated to be 99% for the cells, regardless of whether the cells were pretreated with 3.5 μM toluene or not. The EC_{50} values were estimated to be 9.7 and 6.5 μM for the cells treated or not treated with 3.5 μM toluene, respectively. The absolute values for 0% and 100% are 600 – 810 cpm and 1280 – 1630 cpm, respectively. b: The effects of toluene-pretreatment on the attenuation of forskolin-stimulated cAMP formation by the stimulation of hm2 receptors in CHO cells. The experimental procedures were the same as those described in the legend to Fig. 1, except that the CHO-hm2 cells were incubated with or without 3.5 μM toluene for 15 min at 37°C before stimulation with carbamylcholine. The results shown are the means ± S.D. of three to five independent experiments. Dose-response curves were fitted to the following equation: $E_{max} \times EC_{50}$ / ([Carbamylcholine] + EC_{50}) + (100 – E_{max}), where the value in the cells that were not stimulated with carbamylcholine was taken as 100%. The E_{max} and EC_{50} values were estimated to be 55% and 4.7 μM for cells pretreated with 3.5 μM toluene. Data were analyzed with one-way analysis of variance followed by Dunnett's test. The symbols * and * denote significant differences compared with control cells at $P<0.05$ and $P<0.01$, respectively. The absolute values (means ± S.D.) for 0% and 100% are 68 ± 7 fmol/10^4 cells cpm and 2000 ± 170 fmol/10^4 cells for toluene pretreated cells, respectively.

to consider the difference in the experimental conditions; the former experiment used whole cells and the latter used a membrane preparation. Toluene increased the EC_{50} values of carbamylcholine for the attenuation of folskolin-stimulated cAMP formation by a factor of 50, while toluene affected only the E_{max} value in the [^{35}S]GTPγS binding assay. These results suggest that toluene changes the interaction between G_i and adenylyl cyclase, and this effect seems irreversible. The possible mechanism of the inhibitory effect of toluene on carbamylcholine-stimulated signal transduction is a lipid-mediated mechanism. Mitchell et al. (31) investigated the effect of *n*-alcohols on the formation of metarhodopsin II, which is a photoactivated form of rhodopsin and can activate transduction. Those authors reported a correlation between the enhancement or inhibition of metarhodopsin II formation and the increase or decrease of phospholipid acyl chain free volume, respectively. Engelke et al. (32) reported that toluene increased the synaptosomal membrane fluidity and at the same time inhibited the integral enzymes acetylcholine esterase and ATPase in vitro. Thus, it is likely that toluene changed the property of the membrane of CHO cells, and carbamylcholine-stimulated G protein activation and interaction between G_i and adenylyl cyclase were thereby inhibited. However, this effect of toluene was observed in signal transduction via hm2 receptor stimulation, but was not observed in that via β_2-adrenergic receptor stimulation (17).

In this study, we demonstrated that toluene inhibits signal transduction via the stimulation of hm2 receptors. These findings are compatible with our recent report of the inhalation study (18). Following the inhalation exposure of animals to toluene vapor, changes in the binding affinity of the muscarinic acetylcholine receptor agonist carbamylcholine were determined in membranes isolated from the brains of rats exposed to toluene at concentration of 500 – 2000 ppm for 6 h. In the frontal cortex and hippocampus, the high-affinity carbamylcholine binding was reduced or increased following exposure to 1000 ppm or higher concentration of toluene. The present report is the first step toward clarifying the mechanisms of the CNS toxicity of toluene, focusing on signal transduction via G protein-coupled receptors at the molecular level.

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REFERENCES

1 von Oettingen WF, Neal PA and Donahue DD: The toxicity and potential dangers of toluene. Preliminary report. JAMA **118**, 579 – 584 (1942)

2 Knox JW and Nelson JR: Pretreatment encephalopathy from toluene inhalation. N Engl J Med **275**, 1494 – 1496 (1966)

3 Hogstedt C: Has the Scandinavian solvent syndrome controversy been solved? Scand J Work Environ Health **20**, 59 – 64 (1994)

4 McKenna MJ and DiStefano V: A proposed mechanism of action of carbon disulfide on dopamine-beta-hydroxylase. Toxicol Appl Pharmacol **33**, 137 (1975)

5 Chandra SV and Shulka S: Concentrations of striatal cathechol amines in rats given manganese chloride through drinking water. J Neurochem **36**, 683 – 687 (1981)

6 Honma T, Miyagawa M and Sato M: Inhibition of tyrosine hydroxylase activity by methyl bromide exposure. Neurotoxicol Teratol **13**, 1 – 14 (1991)

7 Deitrich RA, Dunwiddie TV, Harris RA and Erwin VG: Mechanism of action of ethanol: initial central nervous system actions. Pharmacol Rev **41**, 489 – 535 (1989)

8 Hoffman PL and Tabakoff B: Ethanol and guanine nucleotide binding proteins: a selective interaction. FASEB J **4**, 2612 – 2622 (1990)

9 Bode DC and Molinoff PB: Effects of ethanol in vitro on the beta adrenergic receptor-coupled adenylate cyclase system. J Pharmacol Exp Ther **246**, 1040 – 1047 (1998)

10 Sanna E, Dildy-Mayfield JE and Harris RA: Ethanol inhibits the function of 5-hydroxytryptamine type 1c and muscarinic M1 G protein-linked receptors in *Xenopus* oocytes expressing brain mRNA: role of protein kinase C. Mol Pharmacol **5**, 1004 – 1012 (1994)

11 Minami K, Minami M and Harris RA: Inhibition of 5-hydroxytryptamine Type 2A receptor-induced currents by n-alchols and anesthetics. J Pharmacol Exp Ther **281**, 1136 – 1143 (1997)

12 Valverius P, Hoffman PL and Tabakoff B: Hippocampus and cerebeller beta-adrenergic receptors and adenylate cyclase are differentially altered by chronic ethanol ingestion. J Neurochem **52**, 492 – 497 (1989)

13 Yamawaki S, Segawa T and Sarai K: Effects of acute and chronic toluene inhalation on behavior and [^{3}H]-serotonin binding in rat. Life Sci **30**, 1997 – 2002 (1982)

14 Celani MF, Fuxe K, Agnati LF, Andersson K, Hansson T, Gustafsson J-A, Battistini N and Eneroth P: Effects of subacute treatment with toluene on central monoamine receptors in the rat. Reduced affinity in [^{3}H]5-hydroxytryptamine binding sites and in [^{3}H]spiperone binding sites linked to dopanine receptors. Toxicol Lett **17**, 275 – 281 (1983)

15 von Euler G, Fuxe K, Benfenati F, Hansson T, Agnati LF and Gustafsson J: Neurotensin modulates the binding characteristics of dopanine D2 receptors in rat striatal membranes also following treatment with toluene. Acta Physiol Scand **135**, 443 – 448 (1989)

16 Hillefors-Berglund M, Liu Y and von Eluer G: Persistent, specific and dose-dependent effects of toluene exposure on dopanine D2 agonist binding in the rat caudate-putamen. Toxicology **100**, 185 – 194 (1995)

17 Tsuga H, Wang R-S and Honma T: Effects of toluene on regulation of adenylyl cyclase by stimulation of G protein coupled receptors expressed in CHO cells. Jpn J Pharmacol **81**, 305 – 308 (1999)

18 Tsuga H and Honma T: Effects of short-term toluene exposure on ligand binding to muscarinic acetylcholine receptors in the rat frontal cortex and hippocampus. Neurotoxicol Teratol **22**, 603 – 606 (2000)

19 Tsuga H, Kameyama K, Haga T, Honma T, Lameh J and Sadee W: Internalization and down-regulation of human muscarinic acetylcholine receptor hm2 subtypes: role of third intracellular hm2 loop and G protein-coupled receptor kinase 2. J Biol Chem **273**, 5323 – 5330 (1998)

20 Lazareno S, Farries T and Birdsall NJM: Pharmacological characterization of guanine nucleotide exchange reactions in membranes from CHO cells stably transfected with human muscarinic receptors m1-m4. Life Sci **52**, 449 – 456 (1993)

21 Tsuga H, Kameyama K and Haga T: Desensitization of human

muscarinic acetylcholine receptor m2 subtypes is caused by their sequestration/internalization. J Biochem **124**, 863 – 868 (1998)

22 Tsuruta H: Skin absorption of solvent mixture: effect of vehicles on skin absorption of toluene. Ind Health **34**, 369 – 378 (1996)

23 Hulme EC, Birdsall NJM and Buckley NJ: Muscarinic receptor subtypes. Annual Rev Pharmacol Toxicol **30**, 663 – 673 (1990)

24 Fuxe K, von Andersson K, Nilsen OG, Tofgard R, Eneroth P and Gustafsson J-A: Toluene and telencephalic dopamine: selective reduction of amine turnover in discrete DA nerve terminal systems of the anterior caudate nucleus by low concentrations of toluene. Toxicol Lett **12**, 115 – 123 (1982)

25 Honma T, Sudo A, Miyagawa M, Sato M and Hasegawa H: Significant changes in the amounts of neurotransmitter and related substances in rat brain induced by subacute exposure to low levels of toluene and xylene. Ind Health **21**, 143 – 151 (1983)

26 Rea TM, Nash JF, Zabik JE, Born GS and Kessler V: Effects of toluene inhalation on brain biogenic amines in rat. Toxicology **31**, 143 – 150 (1984)

27 Ikeda M, Koizumi A, Kasahara M and Fujita H: Combined effects of *n*-hexane and toluene on norepinephrine and dopamine levels in rat brain tissues after long-term exposures. Bull Environ Contam Toxcol **36**, 510 – 517 (1986)

28 Honma T: Changes in acetylcholine metabolism in rat brain after short-term exposure to toluene and *n*-hexane. Toxicol Lett **16**, 17 – 22 (1983)

29 Honma T: Microdialysis study of effects of organic solvents on biogenic amines in the brain. Jpn J Ind Health **35**, s390 (1993)

30 Shiozaki K and Haga T: Effects of magnesium ion on the interaction of atrial muscarinic acetylcholine receptors and GTP-binding regulatory proteins. Biochemistry **31**, 10634 – 10642 (1992)

31 Mitchell DC, Lawrence JTR and Litman BJ: Primary alcohols modulate the activation of the G protein-coupled receptor rhodopsin by lipid-mediated mechanism. J Biol Chem **271**, 19033 – 19036 (1996)

32 Engelke M, Diehl H and Tahti H: Effects of toluene and *n*-hexane on rat synaptosomal membrane fluidity and integral enzyme activities. Pharmacol Toxicol **71**, 343 – 347 (1992)