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 Gi 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 Gi. 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 [35S]GTPγS binding using membrane fractions of CHO cells expressing hm2 receptors. Carbamylcholine-stimulated [35S]GTPγS binding activity was markedly reduced when assayed using reaction buffers containing toluene. However, carbamylcholine-stimulated [35S]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 [3H]N-methylscopolamine to hm2 receptors. On the contrary of the effect of toluene for [35S]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, [35S]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
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, in cultured cells (9). Alcohol inhibits the function of 5-hydroxytryptamine (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 μM of carbachol 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 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 carbachol were determined in membranes isolated from the brains of rats exposed to toluene (18).

MATERIALS AND METHODS

Materials

[3H]N-Methyl scopolamine (NMS, specific activity of 71.3 Ci/mmol) and [35S]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% CO2.

Adenylyl cyclase assay

CHO-hm2 cells (1 × 10⁴ 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 carbachol, 1 μM of 3-isobutyl-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 HMEM buffer (20 mM Hepes-KOH, 2 mM MgCl₂, 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 × g for 30 min at 4°C. The pellet was resuspended in HMEM buffer and subjected to [35S]GTPγS and ligand binding assays.

Ligand binding assay

For estimation of the Kᵦ value of carbachol, 50 μg of membrane fractions of CHO-hm2 cells was incubated with 0.6 nM [3H]NMS and different concentrations of carbachol 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 [3H]NMS trapped on the filter was counted with an ACS II liquid scintillation counter.
Measurement of the uncoupling of hm2 receptors from G proteins

The function of hm2 receptors was measured as the agonist-stimulated $[^{35}S]$GTP$\gamma$S binding activity of the membrane preparation. The $[^{35}S]$GTP$\gamma$S 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 $\mu$M GDP, 0.1 nM $[^{35}S]$GTP$\gamma$S, 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 HENDM 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: $E_{\text{max}} \times \text{EC}_{50} \times \frac{[\text{Carbamylcholine}]}{[\text{Carbamylcholine}]} + (100 - E_{\text{max}})$, 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: $B_{\text{max}} \times \frac{[\text{Carbamylcholine}]}{[\text{Carbamylcholine}]}$, 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 adenyl cyclase via the activation of Gi (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 $\mu$M toluene. The $E_{\text{max}}$ 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 $\mu$M) in the presence of toluene. The inhibition of adenyl cyclase by carbamylcholine stimulation of mAChR m2 subtypes was attenuated in the presence of 2.5 $\mu$M or higher concentration of toluene (Fig. 1b). The forskolin-stimulated cAMP formation was also reduced in the presence of 3.5 $\mu$M toluene to 55 ± 15% (mean ± S.D., n = 5) of the control.

Effect of toluene on $[^{35}S]$GTP$\gamma$S 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 $[^{35}S]$GTP$\gamma$S binding assay of membrane preparations of CHO-hm2 cells. We prepared crude membrane fractions from CHO-hm2 cells, and measured the $[^{35}S]$GTP$\gamma$S binding activity in the presence of different concentrations of carbamylcholine, 0.1 nM $[^{35}S]$GTP$\gamma$S, and 1 $\mu$M GDP. As shown in Fig. 2, a and b, the extent of carbamylcholine-stimulated $[^{35}S]$GTP$\gamma$S binding activity was decreased to 76% and 69% in the presence of 2.5 and 3.5 $\mu$M of toluene, respectively. This inhibitory effect of toluene on $[^{35}S]$GTP$\gamma$S binding was seen at the 2.5 $\mu$M 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...
from CHO-hm2 cells that had been treated with 3.5 μM toluene for 15 min, and then we measured the \([\text{[^35S]}GTP_\gamma S]\) binding activity. As shown in Fig. 3a, the carbamylcholine-stimulated \([\text{[^35S]}GTP_\gamma S]\) binding activity was not affected by pretreatment with toluene. Furthermore, as shown in Table 1, the toluene pretreatment also did not alter the \([\text{[^3H]}\text{NMS}]\) binding site or the affinities of GTP-insensitive carbamylcholine binding and \([\text{[^3H]}\text{NMS}]\) 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 \(E_{\text{max}}\) values were reduced to 65% of the control value (55% vs 84%), and the \(E_{50}\) 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 \(\beta_2\)-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 \([\text{[^35S]}\text{GTP_\gamma S}]\) binding activity (Fig. 2). Carbamylcholine stimulates the \([\text{[^35S]}\text{GTP_\gamma S}]\) 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 \([\text{[^35S]}\text{GTP_\gamma S}]\) binding assay. Furthermore,
as shown in Table 1, the [³H]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 adenyl 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 [³S]GTPγS binding, although we have
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 \textsubscript{50} values of carbachol for the attenuation of forskolin-stimulated cAMP formation by a factor of 50, while toluene affected only the E\textsubscript{max} value in the \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding assay. These results suggest that toluene changes the interaction between G\textsubscript{i} and adenylyl cyclase, and this effect seems irreversible. The possible mechanism of the inhibitory effect of toluene on carbachol-stimulated Table 1. The maximal binding (B\textsubscript{max}) and the equilibrium dissociation constant (K\textsubscript{d}) of \[^{3}H\]NMS and low affinity K\textsubscript{d} of carbachol.

<table>
<thead>
<tr>
<th></th>
<th>[^{3}H]NMS</th>
<th>Carbamylcholine</th>
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<tbody>
<tr>
<td></td>
<td>B\textsubscript{max} (pmol/mg protein)</td>
<td>K\textsubscript{d} (nM)</td>
</tr>
<tr>
<td>Control</td>
<td>0.95 ± 0.14</td>
<td>0.30 ± 0.069</td>
</tr>
<tr>
<td>With toluene</td>
<td>0.91 ± 0.23</td>
<td>0.30 ± 0.055</td>
</tr>
<tr>
<td>Toluene-pretreated cells</td>
<td>1.1 ± 0.090</td>
<td>0.32 ± 0.085</td>
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The maximal binding (B\textsubscript{max}) and the equilibrium dissociation constant (K\textsubscript{d}) of \[^{3}H\]NMS and K\textsubscript{d} of carbachol with 2 mM GTP (low affinity) in the membrane fraction from CHO-hm2 cells with or without 3.5 \mu M toluene, and the B\textsubscript{max} and K\textsubscript{d} of \[^{3}H\]NMS and K\textsubscript{d} of carbachol with 2 mM GTP in the membrane fraction of CHO-hm2 cells pretreated with 3.5 \mu M 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 carbachol-stimulated \[^{35}\text{S}]\text{GTP} \gamma \text{S} 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 carbachol-stimulated \[^{35}\text{S}]\text{GTP} \gamma \text{S} 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 \mu M 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\textsubscript{max} \times \text{[Carbachol]} / (EC\textsubscript{50} + \text{[Carbachol]}), where the value in the presence of 1 mM carbachol for cells treated without toluene was taken as 100%. The B\textsubscript{max} values were estimated to be 99% for the cells, regardless of whether the cells were pretreated with 3.5 \mu M toluene or not. The EC\textsubscript{50} values were estimated to be 9.7 and 6.5 \mu M for the cells treated or not treated with 3.5 \mu M 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 \mu M toluene for 15 min at 37°C before stimulation with carbachol. The results shown are the means ± S.D. of three to five independent experiments. Dose-response curves were fitted to the following equation: E\textsubscript{max} \times \text{EC\textsubscript{50}} / ([\text{Carbachol}] + \text{EC\textsubscript{50}} + (100 – E\textsubscript{max})), where the value in the cells that were not stimulated with carbachol was taken as 100%. The E\textsubscript{max} and EC\textsubscript{50} values were estimated to be 55% and 4.7 \mu M for cells pretreated with 3.5 \mu M toluene. Data were analyzed with one-way analysis of variance followed by Dunn’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.
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, and adenylyl cyclase were thereby inhibited. However, this effect of toluene was observed in signal transduction via h2 receptor stimulation, but was not observed in that via β2-adrenergic receptor stimulation (17).

In this study, we demonstrated that toluene inhibits signal transduction via the stimulation of h2 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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