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EFFECT OF TOLUENE ON RAT SYNAPTOSOMAL PHOSPHOLIPID METHYLATION AND MEMBRANE FLUIDITY

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Abstract—This study investigated the effects of toluene (1 g/kg, 1 hr, i.p.) on rat synaptosomal phospholipid methylation (PLM), phospholipid composition, and membrane fluidity. Toluene significantly decreased basal PLM (35%) in studies using [^{3}H]methionine ([^{3}H]Met) as the methyl donor; this was reflected by similar decreases in phosphatidylmonomethylethanolamine (PME) (30%). No effects were observed in either PLM reactions that used [^{3}H]adenosylmethionine ([^{3}H]AdoMet) as methyl donor, or AdoMet synthetase, suggesting that toluene preferentially affects PLM reactions that derive methyl groups from [^{3}H]Met. Also, toluene decreased synaptosomal phosphatidylethanolamine (PE) (24%), the initial substrate for PLM, and the addition of PE back to PE-depleted synaptosomes restored methyltransferase activity. Agonist-stimulated PLM using norepinephrine (NE) demonstrated that agonist–receptor coupling returned PLM to control values in synaptosomes from toluene-treated rats. NE-stimulated PLM was also blocked by propranolol (PRO), suggesting a role for toluene in receptor-mediated events. Membrane fluidity studies demonstrated that *in vivo* administration of toluene increased the outer synaptosomal membrane fluidity, whereas *in vitro* administration of toluene had no effect. Our observations support a positive relationship between increased PLM activity and increased outer, not core, membrane fluidity. These data demonstrate that specific toluene–phospholipid interactions occur in synaptosomes, resulting in altered membrane composition, function and fluidity.

Organic solvent exposures, whether they originate from occupational or recreational sources, pose serious threats to public health. It has been established that the organic solvent toluene, present in commercial paints and adhesives, produces an acute intoxication like that of other classical central nervous system (CNS) depressants [1]. Several mechanisms have been proposed for the toxic effects of toluene, among those being that it disrupts neuromembrane function by a non-specific partitioning within the lipid-bilayer [2], and that it acts by binding at hydrophobic pockets on integral membrane proteins [3, 4].

Toluene inhibits various membrane-associated enzymes [3] and affects neuronal lipid composition [5]. The activity of another integral membrane protein, phospholipid *N*-methyltransferase (EC 2.1.1.17) (PMT), has been proposed to regulate the biophysical, as well as the functional, properties of various cell types [6]. Methylation involved in the conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) has been demonstrated in synaptosomes [7–10]. We were therefore interested in whether toluene, an extremely lipophilic solvent, altered synaptosomal biophysical and functional parameters using PMT as an index.

A relationship between basal and β-adrenergic agonist-stimulated phospholipid methylation (PLM) and membrane fluidity has been proposed [6, 11, 12]. Recently, Fuxe *et al.* [13] reported that subacute doses of toluene increased the number and reduced the affinity of β-adrenergic receptors in rat frontoparietal cortex, possibly mediated by changes in membrane fluidity. These data, taken together, suggest an involvement between toluene-induced CNS depression, synaptosomal neurotransmitter–receptor mechanisms of synaptic function, and membrane fluidity.

In this study, we tested the hypothesis that toluene exposure affects both basal and agonist-stimulated synaptosomal PLM, as well as phospholipid composition, and determined the relationship between the aforementioned events and altered membrane fluidity.

METHODS

Chemicals. Solvents (toluene, chloroform, methanol) and 1-amino-2-naphthol-4-sulfonate (ANSA) were obtained from Fisher Scientific (Medford, MA). Bovine serum albumin, L-α-phosphatidylserine (PS), L-α-phosphatidylinositol (PI), sphingomyelin (SM), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylmonomethylethanolamine (PME), L-α-phosphatidyldimethylethanolamine (PDE), L-α-phosphatidylcholine (PC) (all dipalmitoyl species), L-methionine (Met), and DL-propranolol (PRO) were obtained from the Sigma Chemical Co. (St Louis, MO). *S*-Adenosyl-L-methionine disulfate di-*p*-toluene sulfonate (AdoMet) was donated by Dr Georgio Stramentinoli (Bioresearch Laboratories, Liscata, Italy). The probe 1,6-diphenyl-1,3,5-hexatriene (DPH), and L-norepinephrine HCl (NE) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). The probe 1-(4-trimethylammoniumphenyl) - 6 - phenyl - 1,3,5 - hexatriene

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(TMA-DPH) was obtained from Molecular Probes Inc. (Eugene, OR). Silica gel DC-Fertigplatten Kieselgel 60F-254 plates were obtained from EM Science (Darmstadt, Germany). [L-*methyl*-^{3}H]Methionine ([^{3}H]Met) (15 μCi/nmol) was purchased from New England Nuclear (Boston, MA), and *S*-adenosyl-[L-*methyl*-^{3}H]methionine ([^{3}H]AdoMet) (15 μCi/nmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA).

Animals and solvent administration. Adult male Sprague–Dawley rats (200–225 g) were obtained from the Charles River Breeding Laboratories (Wilmington, MA) and were maintained in the animal facility in clear polypropylene cages with water and food provided *ad lib*. All treatments were administered by intraperitoneal injection; control animals received corn oil (preservative free) while treated animals received toluene in corn oil (4 ml/kg).

Tissue preparation. Animals were killed by decapitation, and the brains were rapidly excised and placed into ice-cold 0.32 M sucrose in 5 mM Tris–HCl (pH 7.4). Brains were homogenized (10%, w/v) in a Potter–Elvehjem teflon–glass vessel. Synaptosomes were prepared according to Cotman and Matthews [14]. Protein was determined by the method of Lowry *et al.* [15].

Basal phospholipid methylation. Studies employing [^{3}H]Met used an incubation buffer that contained 5 mM ATP, 15 mM $MgCl_2$, 25 μM [^{3}H]Met (sp. act. 0.2 μCi/nmol), in 50 mM Tris–HCl, pH 7.4. For studies using [^{3}H]AdoMet, the buffer contained 200 μM [^{3}H]AdoMet (sp. act. 0.0625 μCi/nmol), 10 mM $MgCl_2$ in 50 mM Tris–HCl, pH 7.4. Equal volumes of buffer and synaptosomes (1–2 mg protein/ml) were mixed to start the reaction. At various times (0–30 min), an aliquot was removed and placed into a tube containing 4 ml chloroform: methanol:HCl (2:1:0.02, by vol.) to terminate the reaction. Both tissue blanks (heat-inactivated enzyme) and substrate blanks (Tris–HCl) were included for each experiment. After all reactions were terminated, the organic phase was washed twice with 50% methanol/0.1 M KCl, and centrifuged at 4000 *g* for 10 min; then a 0.1-ml aliquot of the chloroform phase was placed into a 20-ml scintillation vial and allowed to evaporate. The amount of radioactivity in the evaporated lipid extract, a measure of phospholipid methylation, was determined by the addition of 10 ml of Scintiverse E (Fisher Scientific, Medford, MA) and quantitated by scintillation spectrometry (Packard TRI-CARB 4530).

Individual methylated phospholipids. Extractions for individual methylated phospholipids (PME, PDE, PC) from [^{3}H]Met reactions were carried out as outlined above. Separation of these lipids was performed according to Crews *et al.* [10].

[^{3}H]Met and [^{3}H]AdoMet analysis. At each corresponding time point in PLM reactions, 100 μl of [^{3}H]Met incubation mixtures was removed and placed into a microcentrifuge tube containing 100 μl of 7% perchloric acid. These samples were frozen in liquid N_2 and stored at −80° until analysis. The samples were centrifuged at 8000 *g* for 5 min, and 25 μl was applied to TLC plates. A standard containing Met and AdoMet was co-chromatographed at the edge of each plate. The mobile phase employed was *n*-butyl alcohol:acetic acid:water (60:30:30, by vol.), and the R_f values were AdoMet = 0.06 and Met = 0.80. Met and AdoMet spots were visualized with chloroplatinate (4 ml of 2 mM chloroplatinate, 0.25 ml of 1 M KI, 0.4 ml of 2 N HCl, in 78 ml acetone) spray, scraped into scintillation vials containing 1 ml water, and extracted at 60° for 1 hr. [^{3}H]Met and [^{3}H]AdoMet levels were quantitated by liquid scintillation spectrometry.

Drug effects on phospholipid methylation. Synaptosomes were preincubated in the presence of 25 μM [^{3}H]Met (sp. act. 0.2 μCi/nmol) in 50 mM Tris–HCl, pH 7.4, containing 15 mM $MgCl_2$ and 5 mM ATP, for 30 min at 37°, prior to the addition of NE (100 μM, final concentration). Methylation reactions were all terminated after 30 min, and isolation and quantitation of PLM were performed as previously outlined herein. For experiments using antagonist, synaptosomes were incubated in the presence of PRO (100 μM) for 10 min prior to the addition of NE.

To inhibit phospholipid methylation, synaptosomes were preincubated in the presence of *S*-adenosyl-L-homocysteine (AdoHcy) (10 μM) for 10 min prior to the addition of [^{3}H]Met incubation buffer. Again, all reactions were terminated after 30 min, and isolation and quantitation of PLM were performed as previously outlined.

Membrane fluidity analysis. The fluidity of synaptosomes was determined by the method of Deliconstantinos *et al.* [16]. Synaptosomes were diluted in 50 mM Tris–HCl (pH 7.4) to a final protein concentration of 50 μg/ml. A 1-ml volume of synaptosomes was mixed with 1 ml of either 2 μM DPH or 2 μM TMA-DPH in 50 mM Tris–HCl (pH 7.4). The DPH and TMA-DPH were diluted from stock solutions of 2 mM in tetrahydrofuran. The mixture was incubated at 37° for 45 min. The excitation wavelength for both proves was 365 nm, and changes in both fluorescence intensity and anisotropy were monitored at an emission wavelength of 430 nm. After incubation, polarization, anisotropy and temperature were recorded. All fluidity determinations were recorded at 31°. Experiments were carried out using an SLM Aminco SPF 500C fluorescence spectrofluorometrer equipped with a polarization accessory. The measured fluorescence signal was collected on-line by an IBM XT-286 which performed both computation of fluorescence anisotropy and correction of emission spectra.

Lipid analysis. Total phospholipid (PL) was measured as inorganic phosphorus (P_i) according to Hess and Derr [17]. The lipids were first extracted as described by Bligh and Dyer [18]. Individual lipids were separated by the method of Magruder *et al.* [19].

Statistical analysis. The statistical difference between control and toluene-treated animals was determined using Student's *t*-test. Agonist-stimulated PLM studies employed analysis of variance (ANOVA) and Duncan's new multiple range test (DNMRT) to determine significant differences between groups.

RESULTS

The effects of toluene (1 g/kg, 1 hr) on basal

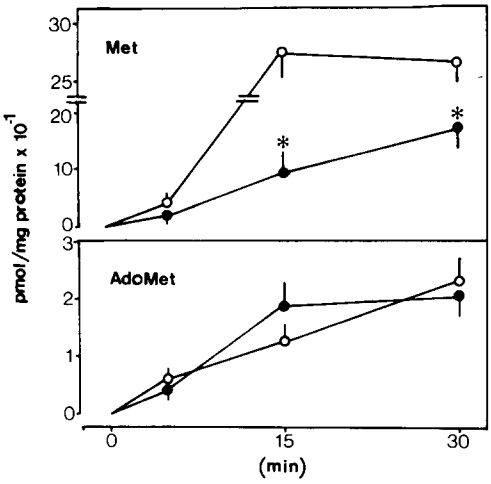

Fig. 1. Effect of toluene (1 g/kg, 1 hr) on rat synaptosomal phospholipid methylation. Key: (○) control synaptosomes, and (●) synaptosomes from toluene-treated rats. Methyl donors employed were Met (sp. act. 0.2 μCi/nmol) and AdoMet (sp. act. 0.0625 μCi/nmol). Data, expressed in pmol/mg protein × 10^{-1}, are means ± SE of five to six rats. Asterisks denote values significantly different from controls at the 0.05 level using Student's *t*-test.

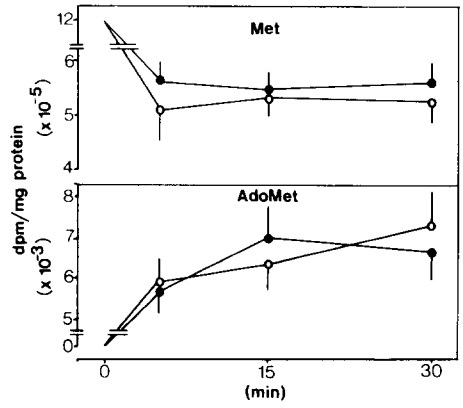

Fig. 2. Effect of toluene (1 g/kg, 1 hr) on rat synaptosomal [^{3}H]Met and [^{3}H]AdoMet levels. Studies employed Met as the methyl donor (sp. act. 0.2 μCi/nmol). Key: (○) control synaptosomes, and (●) synaptosomes isolated from toluene-treated rats. Data, expressed in dpm/mg protein × 10^{-3} (AdoMet) or 10^{-5} (Met), are means ± SE of five to six rats.

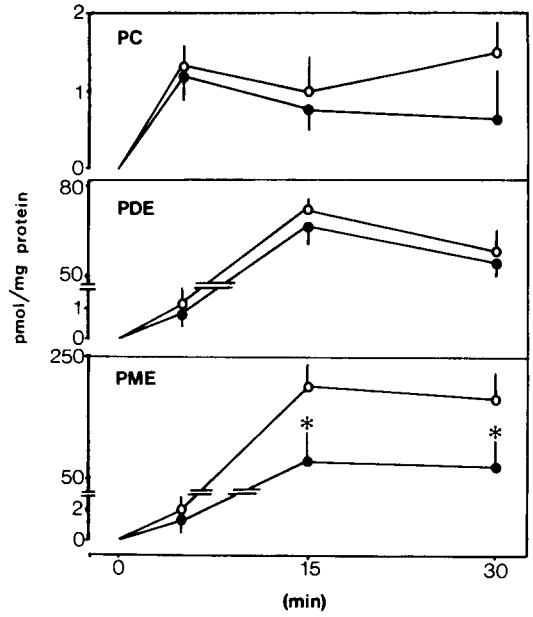

Fig. 3. Effect of toluene (1 g/kg, 1 hr) on rat synaptosomal methylated phospholipids (PME, PDE, PC). Studies employed Met as the methyl donor (sp. act. 0.2 μCi/nmol). Key: (○) control synaptosomes, and (●) synaptosomes isolated from toluene-treated rats. Data, expressed in pmol/mg protein, are means ± SE of five to six rats. Asterisks denote values significantly different from control at the 0.05 level using Student's *t*-test.

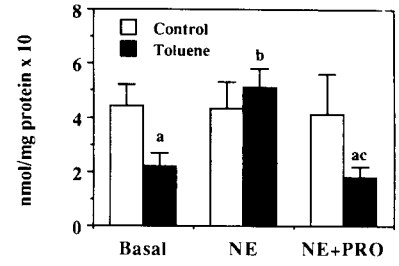

Fig. 4. Effect of toluene on agonist-stimulated synaptosomal phospholipid methylation. Studies employed Met as the methyl donor (sp. act. 0.2 μCi/nmol). Data, expressed in nmol/mg protein/30 min × 10 are means ± SE of five to six rats. Key: (a) values significantly different from controls at the 0.05 level using Student's *t*-test; (b) values significantly different from basal values at the 0.05 level using DNMRT; and (c) values significantly different from NE values at the 0.05 level using DNMRT.

synaptosomal PLM using [^{3}H]Met or [^{3}H]AdoMet as methyl donors are shown in Fig. 1. Toluene did not alter the amount of label incorporated into lipids in studies that used [^{3}H]AdoMet as the methyl donor. However, in [^{3}H]Met methyl donor experiments, chloroform extractable methylated products were decreased significantly at 15 and 30 min in synaptosomes from toluene-treated rats.

To determine whether the differences in methyl group incorporation derived from [^{3}H]Met after toluene exposure were a result of an inhibition of AdoMet synthetase, [^{3}H]Met and [^{3}H]AdoMet levels were quantitated. Figure 2 demonstrates that the [^{3}H]Met pool decreased rapidly and reached steady-state levels by 5 min, whereas the [^{3}H]AdoMet pool steadily increased. No differences were observed between synaptosomes isolated from control or toluene-treated rats at any of the time points studied.

Since a toluene-induced decrease in basal PLM was observed when [^{3}H]Met was used as methyl donor, individual methylated phospholipids were isolated and quantitated. Toluene significantly decreased [^{3}H]PME formation (30–35%, 15–30 min), whereas that of [^{3}H]PDE and [^{3}H]PC remained unchanged (Fig. 3).

The effects of toluene on agonist-stimulated synaptosomal PLM are shown in Fig. 4. NE (100 μM) had no effect on PLM in control synaptosomes,

Table 1. Effect of toluene on rat synaptosomal phospholipid composition

Treatment	P_i (nmol/mg protein) PE	PC	PS ± PI	SM	Total*
Control	116 ± 13	121 ± 14	41 ± 6	27 ± 10	437 ± 19
Toluene	91 ± 6†	108 ± 9	44 ± 7	34 ± 11	332 ± 46†

Rats were administered toluene (1 g/kg) intraperitoneally or corn oil (4 ml/kg) 1 hr prior to being killed. Data are means ± SE of five to six animals.

* Total inorganic phosphorus also includes ethanolamine plasmalogens that were not separated by TLC.

† Significantly different from corresponding controls at the 0.05 level using Student's *t*-test.

Table 2. Effects of exogenous PE and AdoHcy on phospholipid methylation in synaptosomes

Treatment	PLM (nmol/mg protein/30 min × 10^{-1}) Basal	+ PE	AdoHcy
Control	0.316 ± 0.023	0.300 ± 0.040	0.235 ± 0.039*
Toluene	0.228 ± 0.032†	0.261 ± 0.030	0.108 ± 0.35*†

Two hundred micrograms of PE was added per tube and evaporated under N_2; synaptosomes were added and sonicated for 5 min at 37°. PLM was then performed as described in Methods. For inhibition of PLM using AdoHcy (10 μM), see Methods. For animal treatment see Table 1. All values are means ± SE of five to six animals.

* Significantly different from basal values at the 0.05 level using Student's *t*-test.

† Significantly different from control value at the 0.05 level using Student's *t*-test.

compared to a 132% stimulation in synaptosomes from toluene-treated rats. In fact, addition of NE restored PLM activity to that of controls. Furthermore, NE-stimulated PLM was blocked completely by PRO, a β-adrenergic antagonist.

Synaptosomal total phospholipid (PL) was also measured after toluene exposure. Toluene significantly decreased synaptosomal PE (24%), PC was modestly decreased (10%), whereas PS + PI and SM remained unchanged (Table 1).

Addition of exogenous PE to synaptosomes isolated from toluene-treated rats reversed the decrease in PLM returning synaptosomal PMT activity to control levels, whereas addition of exogenous PE did not alter PLM in control synaptosomes (Table 2).

S-Adenosylhomocysteine (AdoHcy), an inhibitor of transmethylation reactions [20, 21], was used to ensure that toluene was affecting a methylation reaction. In the presence of [^{3}H]Met, AdoHcy (10 μM) inhibited PLM in synaptosomes from control and toluene-treated rats 26 and 53% respectively (Table 2).

Membrane fluidity studies demonstrated that toluene did not alter DPH fluorescence polarization or anisotropy values of synaptosomal membranes: however, TMA-DPH fluorescence polarization and anisotropy values were decreased significantly (Table 3). To investigate whether a relationship exists between synaptosomal PLM and membrane fluidity, the *in vitro* effects of toluene, and benzaldehyde (a metabolite of toluene with suggested membrane peroxidative properties) were studied. Toluene and benzaldehyde (1 mM) stimulated synaptosomal PLM

Table 3. Effect of *in vivo* toluene administration on rat synaptosomal membrane fluidity

Treatment	Polarization	Anisotropy
	DPH	
Control	0.277 ± 0.002	0.203 ± 0.001
Toluene	0.278 ± 0.002	0.204 ± 0.002
	TMA-DPH	
Control	0.345 ± 0.002	0.260 ± 0.002
Toluene	0.338 ± 0.001*	0.254 ± 0.001*

Animal treatment is described in Table 1. Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; and TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene. Data are means ± SE with six samples per group.

* Significantly different from corresponding controls at the 0.05 level using Student's *t*-test.

148 and 330% respectively. Further, while neither solvent, *in vitro*, had any statistically significant effect on DPH or TMA-DPH polarization values compared to methanol, there did appear to be a trend towards a decrease in TMA-DPH polarization values (Table 4).

DISCUSSION

The observation that toluene decreased synaptosomal PLM using [^{3}H]Met-derived methyl groups (Fig. 1) and decreased [^{3}H]PME formation (Fig. 3), but was without effect using [^{3}H]AdoMet methyl donors (Fig. 1) suggests that AdoMet formation may be decreased. However, [^{3}H]AdoMet formation was

Table 4. Relationship between synaptosomal phospholipid methylation and membrane fluidity after *in vitro* solvent administration

Treatment	Polarization* DPH	Polarization* TMA-DPH	PLM† (nmol/mg protein/30 min)
Control	0.282 ± 0.002	0.345 ± 0.004	0.321 ± 0.023
0.1% MeOH	0.284 ± 0.006	0.336 ± 0.006	0.310 ± 0.040
Toluene, 1 mM	0.281 ± 0.001	0.332 ± 0.005‡	0.773 ± 0.125‡
Benzaldehyde, 1 mm	0.293 ± 0.005	0.329 ± 0.005‡	1.014 ± 0.222‡

Synaptosomes were incubated with either methanol (0.1%), toluene, or benzaldehyde in methanol in the presence of either DPH or TMA-DPH for 45 min.

* Data are expressed as mean polarization ± SE in six samples per group.

† Data are expressed as mean nmol/mg protein/30 min ± SE in six samples per group.

‡ Significantly different from corresponding control at the 0.05 level using Student's *t*-test.

unaltered after toluene exposure (Fig. 2). Since AdoMet synthetase activity was not altered, a likely possibility exists that selective methyl donor pools are present that have variable access to different PE pools. This has been demonstrated in lipoprotein secretion [22] and with intraventricular injection of [^{3}H]Met in rat brain [23, 24]. Additionally, metabolically distinct pools of Met and AdoMet designated for PLM have been isolated [25, 26]. Therefore, these data suggest that toluene affects a specific phospholipid pool that preferentially utilizes newly synthesized [^{3}H]AdoMet.

The initial lipid substrate for PMT is PE. This study reports that toluene significantly decreased synaptosomal PE (Table 1). Furthermore, when exogenous PE was added back to synaptosomes, PLM activity in synaptosomes from toluene-treated rats returned to that of control (Table 2). Smith [27] has also demonstrated that the addition of exogenous PE to PE-depleted protozoa restores PMT activity.

The effects of toluene appear to be restricted to phospholipids since synaptosomal cholesterol (CL) content is not affected by solvent treatment [28]. Similarly, PL/CL and PC/PE ratios were not altered by toluene treatment. These indirect membrane fluidity indices appear to be good markers since DPH polarization values are also unaffected by toluene treatment [28].

That this inhibition of PMT is not a direct solvent effect is supported by the fact that *in vitro* administration of toluene stimulated synaptosomal PLM (Table 4), buttressing the previous observation that toluene-induced decreases in synaptosomal PLM are a result of reduced substrate (PE) availability.

The methylation of PE to PC has been shown to play an important role in β-adrenergic receptor–adenylate cyclase coupling events [6, 11, 12]. When synaptosomes were preincubated with [^{3}H]Met, NE stimulated PLM only in synaptosomes from toluene-treated rats (Fig. 4). In addition, this effect was blocked by PRO, suggesting that a receptor-mediated event may be involved.

Further evidence for the involvement of toluene with receptor events is the recent report that toluene increases the density of β-adrenergic receptors in rat cerebral cortex, an effect linked to membrane fluidity alterations [13]. The proposed relationship between β-receptors and PLM is one in which unoccupied β-receptors suppress PMT activity [6]. This suppression is relieved by binding of agonist to the receptor. Therefore, it is possible that increased receptor number could offset suppression to PLM to such an extent that it compensates for the decrease in PE. Alternatively, the possibility exists for the involvement of direct phosphorylation of the PMT enzyme. Although the role of direct phosphorylation of PMT remains unclear [29–31], *in vivo* exposure to toluene may stimulate kinase activity. Indeed, toluene has been show to stimulate protein kinase C in rabbit platelets [32].

Several groups have investigated the relationship between PLM and membrane fluidity [6, 11, 33, 34]. In some cases, both events appear directly related [6, 11], while in others no association was demonstrated [33, 34]. Results from these *in vivo* membrane fluidity studies reported that, while toluene did not affect DPH fluorescence polarization or anisotropy, which reflects the dynamics of the central acyl-side chain region of the membrane [35], it increased the fluidity of the outer membrane region reflected by decreased TMA-DPH polarization and anisotropy values. These results are in agreement with Korpela and Tahti [2, 3] who demonstrated that toluene decreases synaptosomal acetylcholinesterase, an enzyme that is anchored on the outer synaptosomal membrane layer, suggesting an increase in the outer membrane layer fluidity.

The present study also noted differential effects on membrane fluidity after *in vivo* and *in vitro* toluene administrations (Tables 3 and 4). As mentioned, toluene (1 mM), *in vitro*, stimulated PLM, and showed a trend towards increases in outer membrane fluidity (Table 4). Interestingly, preliminary studies investigating the time course effects of toluene (1 g/kg) on PLM demonstrated that toluene rapidly stimulated PLM (15 min), but was not different from controls at 30 min post-dose (data not shown). Since toluene appeared to increase outer synaptosomal membrane fluidity (Table 4), and rapidly stimulated PLM, a positive relationship exists between increased PLM and increased outer, but not core, membrane fluidity in synaptosomes. Conversely, toluene (1 g/kg, 1 hr) increased outer synaptosomal membrane fluidity (Table 3), while PLM was decreased (Fig. 1), presumably due to decreased substrate (PE) availability.

In conclusion, this is the first study to show that *in vivo* exposure to toluene, a classical CNS depressant

with anesthetic properties, caused differential effects on outer synaptosomal membrane fluidity. Thus, we have demonstrated that toluene altered synaptosomal membrane composition and function, and may be linked to alterations in other synaptic functions such as biogenic amine turnover [36–39], electrical conduction [40], and Ca^{2+} flux as reported for toluene and related organic solvents [41–43].

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