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Ganglioside GM_1 prevents and reverses toluene-induced increases in membrane fluidity and calcium levels in rat brain synaptosomes

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The effects of exposure to ganglioside GM_1 and to toluene in vitro upon synaptosomal integrity have been examined using fluorescence polarization of two probes: 1-[4(trimethylamino)phenyl]-1,3,5-hexatriene (TMA-DPH) and 1,6-diphenyl-1,3,5-hexatriene (DPH) to measure membrane anisotropy, and the fluorescent indicator fura-2 to assay levels of cytosolic calcium ($[Ca^{2+}]_i$). The anisotropy of both TMA-DPH and DPH was decreased by toluene, implying increased membrane fluidity. The decrease in TMA-DPH but not in DPH anisotropy was prevented by pretreatment with GM_1 in concentrations as low as 10 μM. This is not an additive interaction since 10 μM of GM_1 alone did not significantly modulate TMA-DPH anisotropy. When the GM_1 treatment succeeded the addition of toluene the decrease in anisotropy of both probes was reversed. Toluene treatment increased $[Ca^{2+}]_i$ in a dose- and time-dependent manner. This increase could partially be both prevented and reversed by treatment with 50 μM of GM_1. These effects may reflect an additive interaction, since this concentration of GM_1 alone reduced $[Ca^{2+}]_i$. The present results show that toluene increases membrane fluidity and intracellular calcium levels. These effects may be counteracted by the endogenous compound GM_1.

INTRODUCTION

The monosialoganglioside GM_1 is an endogenous constituent of the plasma membrane, and is concentrated in nerve endings[29]. GM_1, in contrast to polysialogangliosides, has been implicated in synaptic transmission[1,3,21,36,44,45] and behavior[23]. Thus, gangliosides have been demonstrated to affect adenylate cyclase[7,33] and protein kinase C[7,24]. Furthermore, GM_1 can bind calcium in vitro[20,35,40], and may thus be involved in the control of calcium flux over the nerve cell membrane[46]. Treatment with GM_1 has by some authors been implicated to decrease membrane fluidity[2,42], while others have found increases of membrane fluidity[31]. GM_1 has frequently been reported to increase neural regenerative capacity[22,28,32,41]. More specifically, this ganglioside can decrease synaptosomal vulnerability to chemical injury[4].

The organic solvent toluene has been suggested to induce its effects on chemical neurotransmission in the nervous system by altering membrane fluidity[13,15] and calcium flux[9,10]. In view of the above, GM_1 may thus interfere with the actions of toluene. In line with this hypothesis, treatment with GM_1 antagonizes the effects of toluene on central dopamine D_2 receptors[11].

Therefore, we have investigated the effects of GM_1 in vitro on toluene-induced changes in membrane fluidity parameters and changes in synaptosomal calcium levels.

MATERIALS AND METHODS

Adult male rats (CR1CD), 3–4 months old, weighing 290–340 g were used.

Synaptosomal preparation

Brains were rapidly removed and the forebrain was dissected out on ice, weighed and homogenized in 10 vols. of 0.32 M sucrose at 0 °C. The homogenate was centrifuged (1500 *g*, 10 min) to give a post nuclear supernatant which was layered over 1.2 M sucrose and centrifuged for 25 min (250,000 *g*). The interphase band was removed and layered over 0.8 M sucrose and centrifuged again for 25 min at 250,000 *g*[8,16]. The purified synaptosomes were resuspended in HEPES buffer (pH 7.4) at 0.15 g-equiv/ml corresponding to a final concentration of 120–140 μg protein/ml[5]. The HEPES buffer was composed of (in mM): NaCl 125, KCl 5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 5, Glucose 6, $CaCl_2$ 1, HEPES 25; and adjusted with NaOH to pH 7.4.

Membrane order

Synaptosomal membrane order was evaluated by fluorescence polarization studies using two probes. 1,6-Diphenyl-1,3,5-hexatriene (DPH) is a no-polar lipophilic molecule capable of penetrating into and through inner lipid-rich membrane layers[38]. 1-[4(trimethylamino)phenyl]-1,3,5-hexatriene (TMA-DPH) is a related compound with a polar-constituent that causes the molecule to be aligned at the outer surface of limiting membranes with the polar head at the hydrophilic surface, while the non-polar body penetrates the lipid interior[26,34].

Synaptosomes were prepared as described above and were incubated at 37 °C in the presence of TMA-DPH (30 min) or DPH

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(15 min) added in tetrahydrofuran (Fisher Scientific, Pittsburgh, PA, U.S.A). The final concentrations of the probes were 10 μM. The synaptosomes were reprecipitated (13,000 g, 2 min) using a microfuge (235B, Fisher Scientific), resuspended in HEPES buffer and allowed to equilibrate for 10 min at 37 °C prior to measuring of fluorescence intensity and polarization in a water-jacketed cuvette holder maintained at 37 °C (Aminco SPF-500 spectrofluorometer, American Instrument Co., Urbana, IL, U.S.A.). An excitation wavelength of 360 nm (bandwidth 10 nm) was used with a determination of emission at 430 nm (bandwidth 10 nM). Corrections for light scattering (membrane suspension minus probe) and for fluorescence in the ambient medium (after pelleting membranes) were made.

Fluorescence anisotropy (r) was determined by the formula $r = I_{VV}-I_{VH}/[I_{VV} + 2(I_{VH})]$. I_{VV} is the fluorescence intensity with excitation and emitted light polarized vertically and I_{VH} is the intensity obtained with a vertical orientation of the exciting polarizer with the emitted fluorescence passing through a horizontal polarizer. Total fluorescence intensity $F = I_{VV} + 2(I_{VH})$. Relative microviscosity is proportional to $r_o/r-1$, where r_o is the maximal limiting anisotropy of the probe; 0.362 for DPH[37] and 0.39 for TMA-DPH[34]. A correction factor (G) for instrument asymmetry was also made using $G = I_{HV}/I_{HH}$ where I_{HV} is fluorescence intensity with horizontal excitation light and emitted light read vertically, and I_{HH} is the corresponding value with the entire light path horizontally aligned. This compensates for the sensitivity of the detection system toward vertically and horizontally polarized light. All values of I_{VH} were multiplied by G in the calculation of r. All corrections made amounted to less than 6% of the original unmodified readings.

Intrasynaptosomal calcium levels

Synaptosomes were prepared as described above and were incubated at 37 °C in the presence of fura-2 dissolved in DMSO for 10 min[25]. They were then diluted 10 times in HEPES buffer and incubated for another 5 min. The final concentration of fura-2 was 5 μM. The synaptosomes were centrifuged for 8 min at 3000 g and the pellet was resuspended in HEPES buffer.

For each assay, 0.5 ml of synaptosomes was rapidly centrifuged (2 min, 13,000 g) and the resulting pellet was resuspended in 1 ml HEPES buffer at 37 °C. The buffer was as described above without $NaHCO_3$ and NaH_2PO_4 to prevent the precipitation of calcium at elevated pH (required during the determination of minimal fluorescence). The tube was rinsed with another 1 ml of HEPES buffer and the total 2 ml sample was placed in a quartz cuvette at 37 °C and left to equilibrate for 10 min. Excitation of fura-2 was at 340 and 380 nm (bandwidth 3 nm) and emission determinations were made at 510 nm (bandwidth 20 nm). Corrections for light scattering (membrane suspension minus probe) and fluorescence in the ambient medium (after pelleting membranes) were made.

Extrasynaptosomal fura-2 was quenched by 4 μM $MnCl_2$[17]. The ratio (R) between the fluorescence excitation at 340 and 380 nm was used. $[Ca^{2+}]_i$ was calculated using the formula $[Ca^{2+}]_i = K_d (R - R_{min})/(R_{max} - R) \times [S_{f2}/S_{b2}]$, where K_d is the dissociation constant of the fura-2–Ca^{2+} complex, taken to be 224 nM[17], R_{min} is the ratio in the presence of excess amounts of EGTA (10 mM) and R_{max} the ratio in excess amounts of calcium (18 mM). S_{f2} and S_{b2} denote fluorescence of fura-2 at zero calcium concentration and full calcium saturation, respectively, at an excitation wavelength of 380 nm.

Dye leakage into the extracellular compartment was calculated after the addition of $MnCl_2$ to a final concentration of 4 μM. The resulting depression in emitted fluorescence when excitation was at 340 nm, was expressed as a percentage of the difference between the corresponding value prior to $MnCl_2$ addition and the value at zero calcium conditions (EGTA and 0.1% sodium dodecyl sulfate being present).

Incubations

After initial determinations, toluene (0.06–6 μl) or ganglioside GM_1 (10–50 μl) were added to the synaptosomal suspension and values were measured after a 10-min incubation for calcium determinations, or after a 30-min equilibration for fluorescence polarization assays. Toluene was mixed with the synaptosomes by vortexing (5 s) in 10 ml polypropylene test tubes. Following this, any further additions were made and the fluorescence was read again following a further 10 or 30 min, respectively, of incubation.

TABLE I

Anisotropy (r) of TMA-DPH and DPH in synaptosomal membranes incubated for 30 min with or without various concentrations of ganglioside GM_1

Data represent mean ± S.E.M. of 5–7 individual determinations.

Treatment	*Anisotropy (r)*	
	TMA-DPH	*DPH*
Control	0.288 ± 0.003	0.239 ± 0.003
GM_1 (10 μM)	0.284 ± 0.003	0.232 ± 0.005
GM_1 (50 μM)	0.297 ± 0.002*	0.246 ± 0.002*

* $P < 0.05$ that value differs from that of untreated controls.

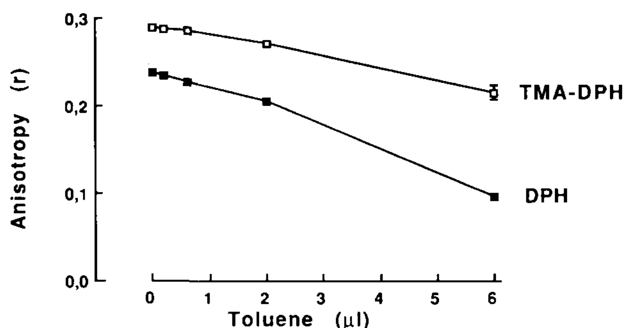

Fig. 1. The effect of toluene on TMA-DPH and DPH anisotropy in synaptosomal membranes. The synaptosomes, preloaded with the respective fluorescent indicator, were incubated for 30 min at 37 °C with varying amounts of toluene. Data represents mean ± S.E.M. of 4 individual determinations.

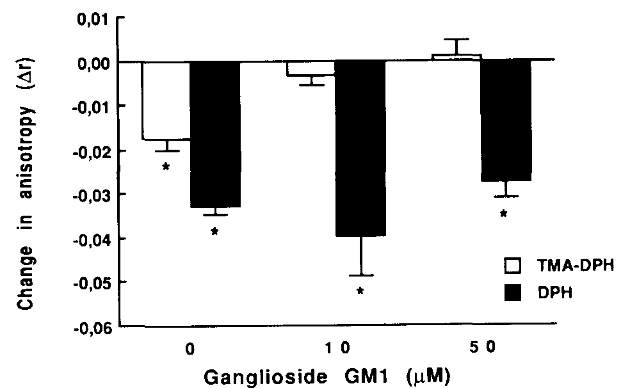

Fig. 2. Anisotropy changes of TMA-DPH and DPH within synaptosomal membranes exposed to 2 μl toluene for 30 min at 37 °C. The synaptosomes were pretreated for 30 min with varying amounts of ganglioside GM_1 before exposure to toluene. Data represent mean ± S.E.M. of 5–7 individual experiments. * $P < 0.05$ that value differs from that of untreated controls.

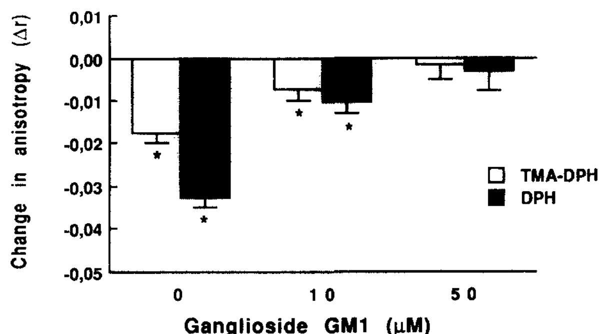

Fig. 3. Anisotropy changes of TMA-DPH and DPH within brain synaptosomal membranes exposed to 2 μl toluene for 30 min at 37 °C. After this treatment, synaptosomes were incubated together with varying amounts of ganglioside GM_1 for a further 30 min. Data represent mean ± S.E.M. of 4–5 individual experiments. *$P < 0.05$ that value differs from that of untreated controls.

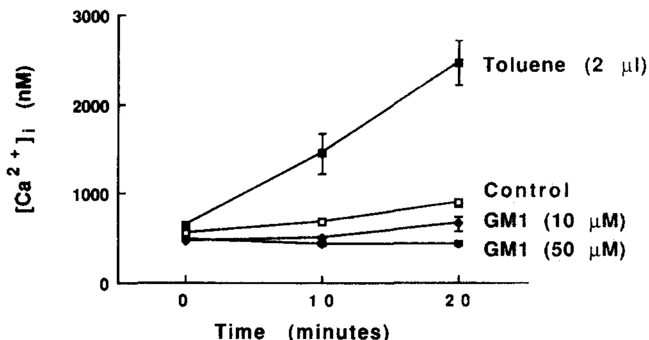

Fig. 5. Levels of synaptosomal cytosolic calcium following incubation for 10 and 20 min with either 2 μl of toluene or with varying concentrations of ganglioside GM_1. Data represent mean ± S.E.M. of 5–10 individual determinations.

Statistical analysis

Differences between groups were assessed by Fisher's Least Significant Difference Test after one-way analysis of variance. The acceptance level of significance was $P < 0.05$ using a two-tailed distribution.

RESULTS

Studies on membrane fluidity

Toluene was found to decrease the anisotropy (r) of TMA-DPH and DPH in a dose-dependent way (Fig. 1). At 2 μl of toluene the change in r-value (Δr) of TMA-DPH and DPH were −0.017 ± 0.003 and −0.033 ± 0.002, respectively. Absence of vortexing did not allow toluene (2 μl) to act on TMA-DPH anisotropy (Δr = 0.001 ± 0.003) or DPH anisotropy (Δr = 0.001 ± 0.003). Ganglioside GM_1 (50 μM but not 10 μM) was found to increase anisotropy of both TMA-DPH and DPH (Table I).

Pretreatment with 10 μM of GM_1 completely abolished the reduction of TMA-DPH anisotropy induced by 2 μl toluene, without affecting the toluene-induced reduction in DPH anisotropy (Fig. 2). Following pretreatment with 50 μM GM_1, the inhibition by toluene on TMA-DPH anisotropy remained, whereas the toluene-induced decrease in DPH anisotropy was reduced by 50%.

The toluene-induced decrease in TMA-DPH and DPH anisotropy was reversed by subsequent treatment with GM_1. Ten μM of GM_1 counteracted the decrease in TMA-DPH and DPH anisotropy by 50%, and 50 μM of GM_1 reversed the decrease completely (Fig. 3). The anisotropy of treated and non-treated synaptosomes did not change over time (last measurement made after 60 min of incubation, data not shown).

Studies on $[Ca^{2+}]_i$

Toluene was found to increase the levels of intrasynaptosomal calcium in a concentration- and time-dependent way without affecting membrane leakage (Figs. 4 and 5). At 2 μl of toluene the $[Ca^{2+}]_i$ was close to the upper limit of the assay capacity of fura-2, and at 6 μl of toluene the synaptosomes clumped together. Absence of vortexing did not allow toluene to act on synaptosomal calcium levels (data not shown). This time-dependent increase in $[Ca^{2+}]_i$ by toluene contrasted with the

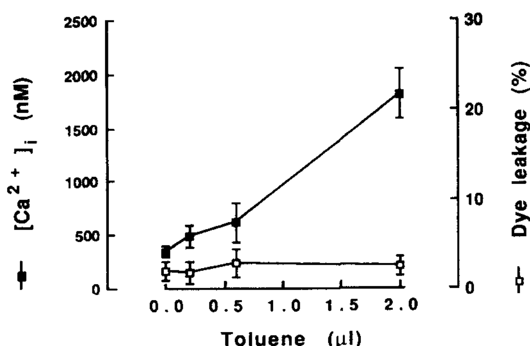

Fig. 4. Levels of synaptosomal cytosolic calcium following a 10 min treatment at 37 °C with varying amounts of toluene. The leakage of fura-2 into the incubation medium is also presented. Data present mean ± S.E.M. of 6 individual determinations.

TABLE II

Increase in cytosolic calcium levels in synaptosomes incubated for 10 min with GM_1 or toluene and then for another 10 min after addition of toluene or GM_1, respectively

Results represent the increase (mean ± S.E.M.) of 5–10 individual determinations corrected for baseline values.

First addition	*Second addition*	$\Delta[Ca^{2+}]_i$ *(nM)*
None	Toluene (2 μl)	801 ± 201*
GM_1 (10 μM)	Toluene (2 μl)	628 ± 83
GM_1 (50 μM)	Toluene (2 μl)	179 ± 136
Toluene (2 μl)	None	1463 ± 256*
Toluene (2 μl)	GM_1 (10 μM)	1546 ± 552*
Toluene (2 μl)	GM_1 (50 μM)	642 ± 213

*$P < 0.05$ that value differs from that of untreated controls.

GM_1-induced reduction in calcium levels (Fig. 5). Under control conditions, the $[Ca^{2+}]_i$ increased slowly.

Pretreatment with GM_1 was found to prevent the toluene-induced increase in calcium levels (Table II). The effect was most pronounced at 50 μM of GM_1, at which concentration GM_1 almost completely blocked the toluene effects. Addition of GM_1 (50 μM but not 10 μM) subsequent to toluene treatment was found to reverse the toluene-induced increase of $[Ca^{2+}]_i$ by 50% (Table II).

DISCUSSION

It was found that toluene in vitro reduces the anisotropy of both TMA-DPH and DPH in synaptosomal membranes. Our study directly shows that toluene affects and increases membrane fluidity as earlier postulated[15]. These actions may have neurotoxic consequences in vivo, and are paralleled by effects of other organic solvents[19,30,39]. Although statistically significant, the observed changes in anisotropy are relatively small in absolute terms. However, as is clear from enzyme studies, even minor variations in the physical form of proteins can have major functional effects. Thus changes in biological activity often have a greater magnitude than the structural alterations of the molecules that underlie such changes[43]. However, intraperitoneal injections with toluene in vivo had no effect on membrane fluidity as measured by fluorescence polarization[27].

The present results confirm that GM_1 increases the rigidity of the cell membrane[2,42] rather than decreases it[31]. Already at 10 μM, a concentration at which GM_1 by itself has no effect on membrane fluidity, GM_1 is able to completely antagonize the toluene-induced reduction in TMA-DPH anisotropy. However, GM_1 is unable to prevent the actions of toluene on DPH anisotropy, perhaps due to the exclusive localization of GM_1 to the external surface of the cell membrane[18]. However, GM_1 reversed the toluene-induced reductions of both TMA-DPH and DPH anisotropy.

This may be the first example where treatment with GM_1 after a neurotoxic insult can block potentially deleterious changes in nerve tissue. The ability of GM_1 to reverse the DPH effects may be due to a damage to the membrane integrity by toluene, allowing GM_1 to gain access to the inner, hydrophobic part of the cell membrane.

Toluene increased intracellular Ca^{2+} levels, but interestingly, membrane leakage was not affected by toluene. Other studies have shown that toluene increases calcium uptake in synaptosomes[9,10] and affects calcium regulated protein phosphorylation[12,14]. Thus, the increase in calcium levels may be another mechanism underlying toluene neurotoxicity. GM_1, on the other hand, stabilized intracellular calcium levels, in contrast to the slow increase in $[Ca^{2+}]_i$ seen under control conditions. Thus, both the preventive and the reversal effects of GM_1 on the toluene-induced increases in calcium levels may represent an additive interaction.

The importance of vortexing for attaining the effects of toluene established in the present study emphasizes the need for standardization of vortexing protocols. Thus, it is difficult to quantitate the relation between the amount of toluene added and the resulting concentration in the membrane bilayers. The same problem is also valid for the discussion on whether the results can be transferred to in vivo situations.

In conclusion, the present study shows protective and reversal effects of ganglioside GM_1 on toluene-induced increases in membrane fluidity and intrasynaptosomal levels of free ionic calcium. It may be that treatment with GM_1 may counteract neurotoxic effects induced by toluene and other organic solvents. This suggests a potential therapeutic use of this ganglioside.

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