

# Lipid interaction of Tetanus neurotoxin

## A calorimetric and fluorescence spectroscopy study

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Received 13 July 1992

The interaction of Tetanus toxin with phospholipid vesicles containing gangliosides (GD1a, GD1b or GT1b) or phosphatidic acid has been investigated at neutral or acidic pH. Change in the thermotropic properties of the vesicles occurred only after addition of the toxin at acidic pH, and led to surface binding or membrane insertion of the protein, dependent on the physical state of the membrane. Most remarkably, toxin addition at acidic pH to dipalmitoyl-phosphatidylcholine vesicles containing GT1b ganglioside, caused formation of ganglioside microdomains on the vesicle surface.

Tetanus toxin: Ganglioside microdomain

## 1. INTRODUCTION

A large group of bacterial protein toxins with intracellular targets, including Tetanus toxin (TeNT), are characterized by a binding that occurs on the cell surface followed by a translocation across the membrane to reach and modify a specific target [1]. In the case of TeNT, lipids have been implicated in both steps [2], also because, after binding, the toxin has to cross the lipid bilayer to reach the cytosol. The binding of the toxin and its insertion into the membrane have been shown to be potentiated by acidic lipids [2,3]. Among those, the gangliosides of the 'b' series, the high content of which in the nervous tissue [4,5] would account for TeNT neurospecificity, have been addressed as the cellular receptors of the toxin. Other evidence indicates a sialoglycoprotein as the neuronal receptor for the toxin [6]. Moreover, it is known that TeNT increases its binding and insertion into the lipid bilayer [2,7-10] at acidic pH, suggesting that TeNT can penetrate into cells via a low-pH intracellular compartment [7-10].

In order to gain more insight in these problems we

have studied the interactions of TeNT with model membranes (phospholipid vesicles) containing gangliosides or phosphatidic acid at neutral and acidic pH, using differential scanning calorimetry (DSC) and fluorescence spectroscopy techniques.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC); dimyristoylphosphatidylcholine (DMPC); 1,6-diphenyl-1,3,5-hexatriene (DPH), pyrenedecanoylphosphatidylcholine (Pyrene-PC), dipalmitoylphosphatidic acid (DPPA), dimyristoyl-phosphatidic acid (DMPA) were from Sigma Chem. Co. (St. Louis, MO, USA). Gangliosides GD1a, GD1b and GT1b (the latter two belonging to the 'b' series) were prepared from calf brain and structurally characterized as described [11]. Tetanus toxin (TeNT) was prepared as previously described [12].

### 2.2. Differential scanning calorimetry

Calorimetric experiments were performed with a Microcal MC2D differential scanning calorimeter (Amherst, MS, USA). For the calorimetric experiments the lipid concentration was 2 mM and the scan rate was 20°C/h.

### 2.3. Preparation of phospholipid vesicles

DMPC or DPPC mixed or not with 5% (molar) ganglioside or phosphatidic acid in chloroform/methanol (1:1, v/v), were dried with a N<sub>2</sub> flow and freeze-dried. The lipids were resuspended in 5 mM Tris-acetate buffer, pH 7.4, containing 50 mM KCl, at a temperature above the gel to liquid crystalline temperature transition (*T<sub>m</sub>*) of the lipid mixture and vortexed. Large unilamellar vesicles were prepared by ten successive extrusions of the lipids through 100 nm pore filters (Nucleopore, Pleasanton, CA, USA), using a N<sub>2</sub> pressure-operated extruder (Lipoprep, Ottawa, Canada). Vesicles were used within the same day of preparation. Size and homogeneity of the vesicle preparation were checked by laser-light scattering [13].

**Abbreviations:** TeNT, Tetanus toxin; DPPC, Dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; Pyrene-PC, pyrenedecanoylphosphatidylcholine; DPPA, dipalmitoylphosphatidic acid; DMPA, dimyristoyl-phosphatidic acid; *T<sub>m</sub>*, Gel to liquid crystalline temperature transition; DSC, Differential Scanning Calorimetry.

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#### 2.4. Addition of Tetanus toxin to the samples

TeNT (10 mg/ml) was dialyzed against 50 mM KCl for 4 h at 4°C and added to the vesicle sample at a protein/lipid molar ratio of 1:1500, at a temperature of 30°C. At this temperature DMPC-containing samples were in the fluid state and DPPC-containing samples in the gel state. The pH of the samples was lowered to 4.0 by addition of the appropriate amount of acetic acid (10%, by vol). When required, the proper volume of 2 M Tris was used to return the pH from 4.0 to 7.4.

#### 2.5. Fluorescence spectroscopy studies

The steady-state degree of fluorescence polarization (*p*) of DPH in DMPC vesicles, containing or not GT1b ganglioside, was measured. Fluorophore incorporation in the vesicle preparation and fluorescence determinations were carried out at 37°C as described [14], using a Jasco spectrofluorometer (Jasco, Tokyo, Japan). Fluorescence excimer formation of pyrene-PC embedded (3% molar) in DMPC vesicles, containing or not GT1b ganglioside, was measured as described [15]. The emission intensities of monomer (*M*) and excimer (*E*) were taken at their maxima at 395 and 480 nm, respectively [15].

#### 2.6. Other assays

Phospholipid concentration was determined from phosphate analysis [16]. Ganglioside-bound sialic acid was determined according to Svennerholm [17].

### 3. RESULTS

#### 3.1. Effect of TeNT on the thermotropic properties of vesicles

The thermotropic properties ( $T_m$  and total enthalpy change associated with the transition,  $\Delta H$ ) of DMPC or DPPC vesicles, containing or not 5% (molar) of any ganglioside (GT1b, GD1a, GD1b) or phosphatidic acid (DPPA or DMPA) were not affected by TeNT addition at pH 7.4, and the DSC data are not reported.

At pH 4.0 the calorimetric scan of DMPC vesicles containing 5% GT1b (a putative receptor of TeNT) is characterized by a  $T_m$  of 25.5°C, with a  $\Delta H$  of 5.5 kcal/mol (Fig. 1; Table I). Upon addition of TeNT to the sample, a strong decrease of  $\Delta H$  to 4.2 kcal/mol was observed, with a concomitant small decrease of  $T_m$  to 25.2°C. The decrease of  $\Delta H$  suggests that, after TeNT addition, part of the phospholipids are subtracted from the phase transition [18–20] indicating the occurrence of protein interdigitation with phospholipid fatty acids. Identical results were obtained adding TeNT to the lipid sample at pH 7.4, then lowering the pH to 4.0. This indicates that TeNT does not aggregate in the low pH medium before reaching the lipid bilayer, at least in fashion altering its interaction with the membrane surface.

The calorimetric scan of DPPC vesicles containing 5% GT1b at pH 4.0 shows the presence of a peak centered at 42.3°C with a  $\Delta H$  of 8.2 kcal/mol (Fig. 1). Upon addition of TeNT, the scan showed the presence of a main transition at 42.5°C, and the appearance of a minor, well defined peak, at a higher temperature (46.1°C), indicating occurrence of a lateral phase separation phenomenon [21,22]. The total enthalpy change remained constant. The minor peak, not occurring with pure DPPC vesicles, indicates that in the presence of

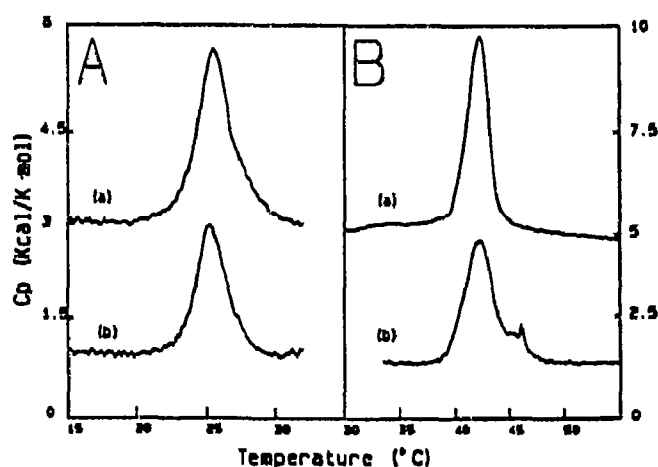


Fig. 1. Heat capacity vs. temperature plot for DMPC (panel A) or DPPC (panel B) vesicles containing 5% GT1b ganglioside before (trace a) and after (trace b) addition of TeNT at pH 4.0.

TeNT part of the lipids segregate in a more rigid, higher melting, microdomain, likely a ganglioside cluster [22]. The same result was obtained inverting the order of addition of acetic acid and TeNT.

The addition of TeNT at pH 4.0 to DPPC vesicles containing 5% GD1b, also a putative receptor of TeNT, caused an upward shift of the  $T_m$  (+0.4°C) with no influence upon the enthalpy change associated with the transition (Table I). However, different from GT1b, no lateral phase separation occurred. The addition of the toxin was almost non-influential on the thermotropic properties of DPPC vesicles containing 5% GD1a, which is not a putative receptor of TeNT (Table I).

The above results suggest the possibility that lipid interaction of TeNT at low pH is dictated by electrostatic interactions. To gain further insight in this aspect, DMPC or DPPC vesicles containing 5% phosphatidic acid, carrying the homologous fatty acid, were studied.

Table I

Effect of Tetanus toxin (TeNT) addition at pH 4.0 on the gel-liquid crystalline temperature transition ( $T_m$ ) and on the total associated enthalpy change of phospholipid vesicles containing gangliosides or phosphatidic acid ( $\Delta H$ )

Vesicle composition	- TeNT		+ TeNT	
	$T_m$ (°C)	$\Delta H$ (kcal/mol)	$T_m$ (°C)	$\Delta H$ (kcal/mol)
DMPC	23.9	5.5	23.9	5.5
DMPC GT1b	25.5	5.5	25.2	4.2
DMPC DMPA	25.1	5.5	24.8	3.9
DPPC	41.2	8.2	41.2	8.2
DPPC GT1b	42.3	8.2	42.5 + 46.1	8.2
DPPC DPPA	41.5	8.2	41.9	8.2
DPPC GD1b	41.8	8.2	42.2	8.2
DPPC GD1a	41.8	8.2	41.8	8.2

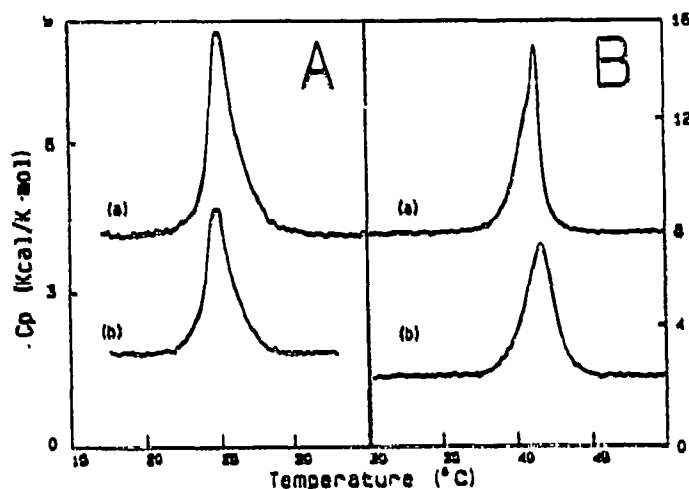


Fig. 2. Heat capacity vs. temperature plot for DMPC (panel A) or DPPC (panel B) vesicles containing 5% of DMPA or DPPA, respectively, before (trace a) and after (trace b) addition of TeNT at pH 4.0.

Upon addition of TeNT at pH 4.0, the  $\Delta H$  of DMPC vesicles containing DMPA decreased from 5.5 to 3.9 kcal/mol (Fig. 2), suggesting interdigitation of the polypeptide chain with the fatty acid chain of phospholipids, as in the case of the DMPC/GT1b vesicles. The  $T_m$  remained virtually unchanged. Upon addition of TeNT at pH 4.0 the  $T_m$  of DPPC/DPPA dispersions increased from 41.5 to 41.9°C, while  $\Delta H$  did not vary. This result indicates a superficial interaction of the toxin, but not followed by lateral phase separation phenomena, as in the case of DPPC/GT1b bilayers (Fig. 2).

### 3.2. Fluorescence spectroscopy experiments

Fig. 3, panel A, reports the values of the anisotropy parameter  $p$  of DMPC vesicles containing 5% GT1b. No change in the anisotropy value was observed upon addition of TeNT at neutral pH. On the contrary, the addition of the toxin at acidic pH caused a small but significant increase of the order parameter from 0.2 to 0.22 indicating a decrease of membrane fluidity. In the absence of gangliosides, at either pH, the addition of the toxin was non-influential. Fig. 3, panel B, reports the values of the excimer/monomer ratio (E/M) of pyrene-PC embedded in DMPC vesicles containing GT1b. The toxin addition caused a decrease of the E/M ratio at pH 4.0, consistent with a restriction in the lateral mobility of the probe [15], while it was uninfluential at pH 7.4. In the absence of GT1b ganglioside the addition of the toxin was uninfluential on the E/M intensity ratio at either pH.

## 4. DISCUSSION

The data herein reported show that TeNT is almost uninfluential on the thermotropic properties,  $T_m$  and  $\Delta H$ , of the phosphatidylcholine zwitterionic phospho-

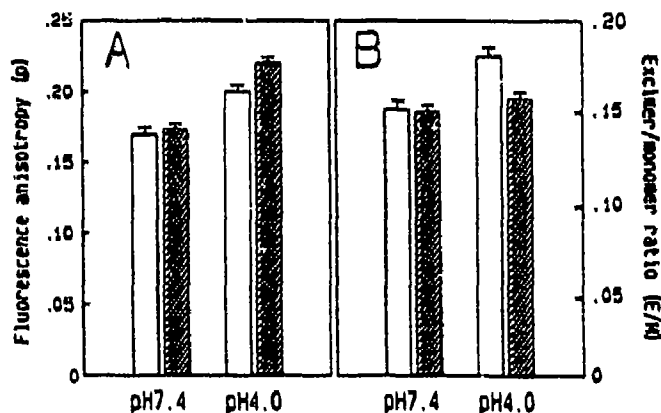


Fig. 3. DPH fluorescence anisotropy (panel A) and pyrene-PC excimer/monomer ratio (panel B) of DMPC vesicles containing 5% GT1b, before (open bars) or after (slash bars) the addition of TeNT at pH 7.4 or 4.0.

lipid bilayers both at neutral or acidic pH, suggesting the occurrence of a weak interaction, if any, under these conditions. When acidic lipids, phosphatidic acids or gangliosides, are embedded in the vesicles, the membrane thermotropic properties are modified by TeNT addition at acidic pH. This responsiveness is clearly shown by changes of  $T_m$  (indicating binding of TeNT to the membrane surface) or by a decrease of the total enthalpy change associated with the lipid phase transition (indicating membrane insertion of the protein). The type of interaction is strongly dependent on the physical state of the bilayer: it is likely more confined at the membrane surface in the case of vesicles that are in the gel state (DPPC) at the temperature of toxin addition; it is extended to the hydrophobic portion of the membrane when the bilayer is in the fluid state (DMPC). This result correlates well with previous investigations and provides further evidence of this phenomenon [9,10,23]. Membrane insertion of the toxin is likely also to be responsible for the fluidity decrease recorded by the fluorescent probe DPH and by the decrease of collisions among pyrene-PC molecules present in the membrane.

However, the most relevant finding of the present investigation is the formation of GT1b microdomains, induced by TeNT addition to DPPC/GT1b vesicles at acidic pH. It is known that the binding of a protein to the membrane can lead to lateral phase separation of lipid components [21,22]. The membrane zones in contact with the protein become enriched in a particular lipid component, while the rest becomes depleted, in a fashion dependent on the specificity of the interaction. In the case of absolute specificity, only those lipids responsible for the specific protein binding will be segregated. This peculiar behavior is displayed by GT1b but not by non-putative receptors (GD1a, phosphatidic acid) and by GD1b (also a putative receptor). This suggests that different molecular mechanisms of interaction

can occur between TeNT and gangliosides belonging to the 'b' series. The lateral segregation of GT1b, induced by TeNT, correlates with the reported ability of this protein to form a physically defined complex with micellar gangliosides [23].

It has been suggested that after binding to the presynaptic membrane, TeNT may penetrate into cells through an acidic intracellular compartment, like diphtheria toxin [24]. The present findings do not support a key role played by gangliosides in the binding of TeNT but suggest their participation to the subsequent, and important, steps of membrane insertion and translocation occurring at low pH. This possibility may be realized via GT1b ganglioside clusters and, due to its possible biological relevance, deserves to be investigated in more detail.

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