Characterization of tetanus toxin binding to rat brain membranes

Evidence for a high-affinity proteinase-sensitive receptor

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Binding of ^{125}I -labelled tetanus toxin to rat brain membranes in 25 mM-Tris/acetate, pH 6.0, was saturable and there was a single class of high-affinity site (K_D 0.26–1.14 nM) present in high abundance ($B_{\max}$ 0.9–1.89 nmol/mg). The sites were largely resistant to proteolysis and heating but were markedly sensitive to neuraminidase. Trisialogangliosides were effective inhibitors of toxin binding (IC_{50} 10 nM) and trisialo-gangliosides inserted into membranes lacking a toxin receptor were able to bind toxin with high affinity (K_D 2.6 nM). The results are consistent with previous studies and the hypothesis that di- and trisialogangliosides act as the primary receptor for tetanus toxin under these conditions. In contrast, when toxin binding was assayed in Krebs–Ringer buffer, pH 7.4, binding was greatly reduced, was non-saturable and competition binding studies showed evidence for a small number of high-affinity sites (K_D 0.42 nM, $B_{\max}$ 0.90 pmol/mg) and a larger number of low-affinity sites (K_D 146 nM, $B_{\max}$ 179 pmol/mg). Treatment of membranes with proteinases, heat, and neuraminidase markedly reduced binding. Trisialogangliosides were poor inhibitors of toxin binding (IC_{50} 11.0 μM), and trisialo-gangliosides inserted into membranes bound toxin with low affinity. The results suggest that in physiological buffers tetanus toxin binds with high affinity to a protein receptor, and that gangliosides represent only a low-affinity site.

INTRODUCTION

Tetanus toxin, a potent neurotoxin secreted by the bacterium *Clostridium tetani*, is a protein composed of two non-identical polypeptide chains of M_r 48000 and 87000 linked by at least one disulphide bridge (Robinson & Hash, 1982). Binding of the toxin to nerve axons in the region of the neuromuscular junction is followed by internalization and transport to the spinal cord and brain stem, where it accumulates presynaptically. The spastic paralysis produced by the toxin is thought to result from inhibition of release of neurotransmitters, presumably those involved in inhibitory pathways (for reviews, see Bizzini, 1979; van Heyningen, 1980; Mellanby & Green, 1981; Wellhoner, 1982), although the exact mode of action of the toxin is poorly understood (Wendon & Gill 1982; Higashida *et al.*, 1983).

Investigations into the chemical nature of the receptor for tetanus toxin have concentrated largely on a role for gangliosides, since the observation that gangliosides neutralize the neurotoxic activity of the toxin (van Heyningen & Mellanby, 1968). Subsequently, tetanus toxin was shown to bind to gangliosides adsorbed to plastic with affinities in the order $G_{T1b} > G_{Q1b} > G_{D1b} \gg G_{M1} > G_{D1a}$ (Holmgren *et al.*, 1980). Limited evidence for a correlation between ganglioside composition and the capacity of cells to bind toxin has been presented (Dimpfel *et al.*, 1977), and binding of toxin to cell lines lacking complex gangliosides was increased by

culturing them in ganglioside-supplemented medium (Yavin, 1984). Studies on the binding of ^{125}I -labelled tetanus toxin to isolated membranes conducted under conditions optimal for binding (i.e. in 25 mM-Tris/acetate, pH 6.0, at 0 °C), are also consistent with a role for gangliosides as the toxin receptor (Ledley *et al.*, 1977; Lee *et al.*, 1979; Rogers & Snyder, 1981). The high affinity (K_D 1.2 nM) binding of tetanus toxin to rat brain membranes was shown to be inhibited by G_{T1b} and G_{D1b} with K_i values of 6 nM and 10 nM respectively (Rogers & Snyder, 1981). Binding of tetanus toxin was not affected by pretreating membranes with trypsin or protein modification agents, but was substantially reduced by neuraminidase. Goldberg *et al.* (1981) calculated that there were a large number of high-affinity sites (2–9 nmol of toxin bound/mg of membrane protein, K_D 2.5–4.1 nM), a level compatible with the reported concentration of ganglioside G_{T1b} and G_{D1b} in rat brain (Ando *et al.*, 1978). Finally, a non-toxic fragment derived from the heavy chain of tetanus toxin by papain cleavage (fragment C), and which competes with intact toxin for binding *in vitro* (Goldberg *et al.*, 1981) and *in vivo* (Simpson, 1984), also retained the ability to bind to gangliosides (Morris *et al.*, 1980). Interestingly, a monoclonal antibody that recognizes fragment C blocked binding of toxin to gangliosides and was also partially neutralizing (Kennimer *et al.*, 1983).

The assumption implicit in all these studies is that gangliosides also represent the toxin receptor under physiological conditions. In the present study we have

Abbreviations used: BSA, bovine serum albumin; DMEM, Dulbecco-modified Eagle's medium; IC_{50} , concentration of inhibitor that reduces binding by 50%; Krebs–Ringer buffer, pH 7.4, 118 mM-NaCl/4.69 mM- CaCl_2 /1.47 mM- KH_2PO_4 /1.18 mM- MgSO_4 /25 mM- NaHCO_3 , adjusted to pH 7.4 at 4 °C with HCl; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline; ganglioside nomenclature is according to Svennerholm (1963).

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therefore compared the binding characteristics of tetanus toxin to rat brain membranes in 25 mM-Tris/acetate, pH 6.0, with that in Krebs–Ringer buffer, pH 7.4, and have also investigated the affinity of the toxin for gangliosides under both sets of conditions.

MATERIALS AND METHODS

Tetanus toxin was purified to homogeneity as outlined previously (Ledley *et al.*, 1977), and had approx. 2×10^7 mouse lethal doses/mg of protein. The toxin was divided into portions and stored at -70°C as a 6.87 mg/ml solution in 0.05 M-phosphate buffer, pH 6.9. Tetanus toxin was iodinated with Bolton–Hunter reagent (Amersham). Briefly, 110 μg of toxin in 100 μl of 0.1 M-sodium borate buffer, pH 8.5, was incubated with 1 mCi of ^{125}I -labelled Bolton–Hunter reagent for 30 min at room temperature. The reaction was quenched by the addition of 300 μl of 0.2 M-glycine in borate buffer, pH 8.5, and labelled toxin was isolated on a Sephadex G-25 (Pharmacia) column pre-equilibrated with 50 mM-sodium phosphate buffer, pH 6.9, containing 0.1% (w/v) gelatin. Toxin labelled by this procedure had a specific radioactivity in the range of 3–6 $\mu\text{Ci}/\mu\text{g}$ and retained 60–70% of its original neurotoxicity. Approx. 60% of the labelled toxin was able to bind specifically to rat brain membranes and conditions in all binding assays were adjusted such that less than 10% of this was bound at equilibrium. Cholera toxin was obtained from List Biologicals (Campbell, CA, U.S.A.) and iodinated to a specific radioactivity of 20 $\mu\text{Ci}/\mu\text{g}$ by using a chloramine-T procedure as outlined previously (Streuli *et al.*, 1981).

Preparation of rat brain membranes

Membranes were prepared from the brains of adult Sprague–Dawley rats by an adaptation of the method of Young & Snyder (1973). Brains were homogenized in 20 vol. of PBS containing 1 mM-iodoacetamide, 0.1 mM-PMSF and 1 mM-1,10-phenanthroline with ten strokes of a Potter–Elvehjem homogenizer fitted with a Teflon pestle. The proteinase inhibitors were present in all of the buffers unless otherwise stated. The homogenate was centrifuged for 5 min at 1000 g at 4°C and the supernatant fluid (S.1) was retained. The crude nuclear pellet was resuspended in PBS and centrifuged as above. The supernatant fluids were combined and centrifuged for 20 min at 17000 g . The resulting crude mitochondrial pellet (P.2) was resuspended with a single stroke of the homogenizer, osmotically shocked in ice-cold 2% PBS and dilute NaOH was added to maintain a pH of 7.4. After 15 min the suspension was centrifuged at 9000 g for 20 min at 4°C , the supernatant fluid (S.3) was retained and the pellet was rinsed with PBS to collect the upper layer. The combined S.3/upper layer was centrifuged at 48000 g for 20 min at 4°C , the resulting pellet was resuspended to 5–7.5 mg/ml in 50 mM-phosphate buffer, pH 7.4, and the membranes were stored at -70°C for up to 1 month. The protein concentration was determined by the method of Markwell *et al.* (1978) using BSA (fraction V; BDH) as a standard.

Binding of ^{125}I -labelled tetanus toxin to rat brain membranes

Membranes and ^{125}I -labelled toxin were incubated with constant mixing at 0 – 4°C in a final volume of 200 μl of

the specified buffer. Unless otherwise stated, all buffers contained 0.1 mM-PMSF, 1 mM-iodoacetamide, 1 mM-1,10-phenanthroline and 0.1% (w/v) BSA. To determine the level of specific binding, membranes were preincubated with unlabelled toxin for 30 min prior to the addition of ^{125}I -labelled toxin. After a further 90 min, incubations were terminated either by filtration or centrifugation. Filtration was performed through 0.5 μm Millipore filters [type EHWP presoaked in buffer containing 1% (w/v) BSA for 12 h], and the filters were rapidly washed with 10 ml of ice-cold buffer before counting in a Beckman Gamma 5500 (counting efficiency 72–74%). The total separation time was < 10 s. Filter blanks were generally less than 1–2% of the input counts. Where incubations were terminated by centrifugation the reaction mixture was diluted by addition of 1 ml of cold buffer, and the tubes centrifuged in a microfuge at 11600 g for 45 s at 20°C . The supernatant fluids were carefully withdrawn and the tubes counted as above. Binding of the toxin to the tubes in the absence of membranes was less than 2% of the input counts. It should be noted that where the K_D of a ligand–receptor interaction is > 10 nm, separation times of > 10 s can lead to underestimation of the K_D (Bennett, 1978).

Analysis of equilibrium binding data by using the LIGAND program

Models of ligand receptor binding consisting of one and two independent classes of site were fitted to equilibrium binding data with the LIGAND weighted non-linear least squares method based on the Marquardt–Levenberg algorithm (Munson & Rodbard, 1980). Using this method the non-specific binding component is adjusted along with the binding constants of association and receptor concentrations until the models fit the data points as closely as possible. No experimentally determined measure of nonspecific binding is required. The fit of the models involving one and two classes of site to the data were compared by using an F test (Munson & Rodbard, 1980), which gave the probability (P) that the one-site model fitted the data as well as the two-site model. The program used was a version of LIGAND adapted for use on a microcomputer by Dr. M. D. Baron (Baron & Sonksen, 1982).

Preparation of enzyme-treated membranes

Membranes (1 mg/ml) in Krebs–Ringer buffer, pH 7.4, in the absence of proteinase inhibitors, were treated with 100 $\mu\text{g}/\text{ml}$ either of trypsin (Sigma, porcine pancreas; 15000 units/mg) or of *Streptomyces griseus* proteinase (Sigma; 5.8 units/mg) at 37°C for 30 min. Membranes were also treated with 0.025 units of neuraminidase/ml (Behring, *Vibrio cholerae*; 1 unit/ml) in 25 mM-Tris/acetate, pH 6.0, containing 4 mM- CaCl_2 at 37°C for 60 min, or with heat (100°C for 5 min). Enzyme activity was inhibited by dilution with either ice-cold Krebs–Ringer buffer, pH 7.4, containing proteinase inhibitors or with cold Ca^{2+} -free 25 mM-Tris/acetate, pH 6.0, buffer as appropriate. Membranes were pelleted at 12000 g for 30 min, and then washed a further three times before resuspension in an appropriate buffer at a nominal protein concentration of 1 mg/ml. Controls consisted of membranes treated as above, but in the absence of enzymes or heat.

Ganglioside purification

Human brain gangliosides were purified by the method of Svennerholm & Fredman (1980) and separated into mono-, di- and tri-sialogangliosides by using DEAE-Sephadex ion exchange chromatography (Fredman *et al.*, 1980). Bovine mixed brain gangliosides were purchased from Sigma.

Insertion of trisialogangliosides into rat brush border membranes

Brush border membranes were isolated from rat intestinal epithelial cells by the method of Miller & Crane (1961) in 10 mM-Tris/HCl/5 mM-EDTA, pH 7.4, containing 0.1 mM-PMSF. Membranes (1.5 mg/ml) were incubated with 70 μ g of trisialoganglioside/ml in the above buffer for 2 h at 37 °C. The membranes were washed twice by centrifugation (11 600 *g* for 45 s at 20 °C) and stored at -20 °C.

Immunofluorescence detection of tetanus toxin binding to ganglioside-treated BHK 21 cells

Baby hamster kidney (BHK 21) cells were grown in monolayer culture in DMEM containing 5% (v/v) newborn-calf serum (both from Gibco BioCult). Cells grown on glass coverslips were washed with DMEM/25 mM-Hepes, pH 7.4, and incubated in the same buffer with or without bovine mixed brain gangliosides for 3 h at 37 °C. The cells were washed three times with DMEM and incubated with either cholera toxin or tetanus toxin in DMEM/25 mM-Hepes, pH 7.4, with 0.1% BSA for 1 h at 4 °C. Unbound toxin was removed by washing the cells three times in DMEM and the cells were fixed for 1 h at 20 °C with PBS containing 3.8% (v/v) formaldehyde and 5% (w/v) sucrose. Bound cholera toxin was detected by using a 1:50 dilution of rabbit anti-(cholera toxin) serum (Critchley *et al.*, 1979), and affinity purified Rhodamine-labelled goat anti-(rabbit immunoglobulin) (25 μ g/ml) (Tissue Culture Services Ltd., Loughborough, Leicestershire, U.K.). Bound tetanus toxin was detected with a 1:25 dilution of rabbit anti-(tetanus toxoid) serum (generously provided by Dr. R. O. Thompson, Wellcome Biotechnology Ltd., Beckenham, Kent, U.K.), and Rhodamine-labelled goat anti-(rabbit immunoglobulin). Antibodies were diluted in PBS containing 0.1% BSA and incubated with the cells on coverslips for 1 h at 20 °C. The cells were washed extensively with PBS between successive antibodies, mounted in PBS containing 50% (v/v) glycerol, and viewed with a Zeiss standard 16 microscope equipped with epifluorescence and a $\times 100$ oil immersion objective. Photographs were taken using Ilford HP5 35 mm film uprated to 800 ASA during developing.

RESULTS

The binding of ^{125} I-labelled tetanus toxin to rat brain membranes in 25 mM-Tris/acetate, pH 6.0, was saturable, with half-maximal binding occurring at 0.5 nM-toxin (Fig. 1a). Analysis of the results with the LIGAND program showed that a one-site model adequately accounted for the data with a K_D of 0.26 nM and a B_{max} of 0.9 nmol/mg. The Scatchard transformation of the data is shown in Fig. 1(a) inset. In contrast the binding of ^{125} I-labelled toxin to brain membranes in Krebs-Ringer buffer, pH 7.4, was non-saturable and consisted of two

Fig. 1. Binding of ^{125} I-labelled tetanus toxin to brain membranes as a function of toxin concentration

Rat brain membranes were incubated with increasing concentrations of ^{125} I-labelled tetanus toxin (1300 c.p.m./fmol) and binding was assayed by filtration as described in the Materials and methods section. The results have been corrected for binding of ^{125} I-labelled toxin to filters in the absence of membranes. Each point represents a single determination and the experiment was performed twice. Identical results were obtained in small-scale experiments where corrections were made for nonspecific binding to membranes in the presence of a 1000-fold excess of unlabelled toxin. However because of the large amounts of toxin required and the limited amount of pure toxin available, we were unable to carry out such experiments as a matter of routine. (a) Binding of ^{125} I-labelled tetanus toxin to 10 ng of membranes in 25 mM-Tris/acetate, pH 6.0. Comparison of the fit of one- and two-site models to the data by using the LIGAND program gave P (one site as good as two sites) > 0.9 . Scatchard transformation of this data (one-site model) is shown in the inset. (b) Binding of ^{125} I-labelled tetanus toxin to 1 μ g of membranes in Krebs-Ringer buffer, pH 7.4.

Fig. 2. Binding of ^{125}I -labelled tetanus toxin to brain membranes in the presence of increasing concentrations of unlabelled toxin

Binding of ^{125}I -labelled toxin (0.3 nM, 1300 c.p.m./fmol) to membranes with increasing concentrations of unlabelled toxin was assayed by filtration as described in the Materials and methods section. Each point represents the mean of two experiments, where $n = 3$ for each experiment with a standard deviation of $< 15\%$. The displacement curves have been corrected for nonspecific binding determined in the presence of 0.5 μM unlabelled toxin. However, when performing the LIGAND analysis the uncorrected data were used. (a) Binding to 20 ng of membranes in 25 mM-Tris/acetate, pH 6.0. Comparison of the fit of one- and two-site models to the data with the LIGAND program gave P (one site as good as two sites) > 0.45 for both sets of data, with a mean K_D of 1.14 nM, and a B_{max} of 1.89 nmol/mg. (Actual values from the two individual experiments were: K_D 0.59 nM and 1.69 nM, and B_{max} 1.68 and 2.10 nmol/mg.) Scatchard transformation of the data from one experiment (one-site model) is shown in the inset. (b) Binding to 10 μg of membranes in Krebs-Ringer buffer, pH 7.4. Comparison of the fit of one- and two-site models to the data gave P (one site as good as two sites) < 0.001 for both sets of data, with mean K_D values of 0.42 nM and 146 nM and B_{max} values of 0.9 pmol/mg and 179 pmol/mg. (Actual values for the two individual experiments were: K_D 0.48 nM and 0.36 nM, 169 nM and 123 nM, and B_{max} 1.0 and 0.8 pmol/mg, 132 and 225 pmol/mg.) Scatchard transformation of the data from one experiment (two-site model) is shown in the inset.

phases with one component appearing to reach saturation at approx. 1 nM-Toxin (Fig. 1b). The second component was not saturated at concentrations as high as 12 nM (results not shown).

The binding of ^{125}I -labelled toxin to rat brain membranes in the presence of increasing amounts of unlabelled toxin is shown in Fig. 2. In 25 mM-Tris/acetate, pH 6.0, binding of ^{125}I -labelled toxin was partially inhibited by concentrations of unlabelled toxin as low as 0.1 nM, with 0.5 μM -toxin reducing binding by over 90%. As this concentration of toxin represented more than a 1000-fold excess over ^{125}I -labelled toxin, the residual binding was considered to be non-specific and was subtracted from all data points prior to plotting the displacement curve shown in Fig. 2(a). Analysis of the uncorrected results with the LIGAND program showed that a one-site model adequately accounted for the data with a K_D of 1.14 nM and a B_{max} of 1.89 nmol/mg. The Scatchard transformation of this data is shown in Fig. 2(a) inset. These results are in reasonable agreement with those obtained from saturation binding studies. The inhibition of ^{125}I -labelled tetanus toxin binding in Krebs-Ringer buffer, pH 7.4, by unlabelled toxin gave rise to a complex curve, quite different from that seen in 25 mM-Tris/acetate, pH 6.0. Inhibition of binding occurred in two distinct phases (Fig. 2b). The first phase occurred within the range 0.1–10 nM unlabelled toxin, and the concentration of toxin giving 50% inhibition of binding (IC_{50}) was about 0.7 nM, which compared with an IC_{50} for the second stage of 100 nM. It is important to note that concentrations of unlabelled toxin as high as 1.0 μM only inhibited binding of ^{125}I -labelled toxin by

Fig. 3. Inhibition of ^{125}I -labelled tetanus toxin binding to brain membranes by trisialogangliosides

^{125}I -labelled tetanus toxin (0.5 nM) was incubated with increasing concentrations of trisialogangliosides for 30 min at 0 °C in either 25 mM-Tris/acetate, pH 6.0, or Krebs-Ringer buffer, pH 7.4, containing 0.1 mM-PMSF followed by the addition of rat brain membranes (20 ng or 5 μg respectively) and further incubation for 90 min. The binding of labelled toxin to membranes was determined by using the filtration assay. Each point represents the mean of triplicate determinations. $\square$, ^{125}I -labelled tetanus toxin binding in 25 mM-Tris/acetate, pH 6.0; $\blacksquare$, ^{125}I -labelled tetanus toxin binding in Krebs-Ringer buffer, pH 7.4.

Fig. 4. Reconstitution of ^{125}I -labelled tetanus toxin binding activity by incorporation of trisialogangliosides into membranes of non-neuronal origin

Trisialogangliosides were inserted into rat brush border membranes as described in the Materials and methods section. ^{125}I -labelled tetanus toxin (0.5 nM, 1230 c.p.m./fmol) was incubated at 0 °C for 90 min with ganglioside-treated or untreated membranes in either 25 mM-Tris/acetate, pH 6.0, or Krebs-Ringer buffer, pH 7.4, each containing 0.1 mM-PMSF. Binding of labelled toxin to membranes (open bars) was determined by the centrifugation assay and nonspecific binding was determined in the presence of 0.1 μM unlabelled toxin (hatched bars). (a) Binding of ^{125}I -labelled tetanus toxin in 25 mM-Tris/acetate, pH 6.0 (1 μg of membranes). (b) Binding of ^{125}I -labelled tetanus toxin in Krebs-Ringer buffer, pH 7.4 (40 μg of membranes). ^{125}I -labelled tetanus toxin (0.5 nM, 1230 c.p.m./fmol) binding to trisialoganglioside-treated membranes with increasing concentrations of unlabelled toxin was determined by centrifugation as described in the Materials and methods section. Each point represents the mean of triplicate determinations with an S.D. of < 15%. The experiment was performed twice. (c) Inhibition by unlabelled toxin of ^{125}I -labelled tetanus toxin binding to treated membranes (200 ng) in 25 mM-Tris/acetate, pH 6.0. Comparison of the fit of one- and two-site models to the data gave P (one site as good as two sites) > 0.16. The K_D (one-site model) was 2.6 nM, and B_{max} was 0.33 nmol/mg. (d) Inhibition by unlabelled toxin of ^{125}I -labelled tetanus toxin binding to treated membranes (18.7 μg) in Krebs-Ringer buffer, pH 7.4.

60–70%. The residual binding at 0.5 μM -toxin was considered to be nonspecific and was subtracted from all data points prior to plotting the displacement curve shown in Fig. 2(b). Analysis of the uncorrected results with the LIGAND program showed that a two-site model best accounted for the data, with K_D values of 0.42 nM and 146 nM and B_{max} values of 0.9 pmol/mg and 179 pmol/mg respectively. The Scatchard transformation of the data is shown in Fig. 2(b) inset. Whether the complexity of the binding observed is due to the presence of two independent classes of receptor or to a single class

of receptor displaying negative co-operativity cannot be determined from these results.

Gangliosides have previously been implicated as the receptor for tetanus toxin based on the finding that nanomolar concentrations of G_{D1b} and G_{T1b} are effective inhibitors of toxin binding in 25 mM-Tris/acetate, pH 6.0 (Rogers & Snyder, 1981). The ability of trisialogangliosides to inhibit toxin binding under these conditions compared with that in Krebs-Ringer buffer, pH 7.4, is shown in Fig. 3. Whereas 10 nM-trisialoganglioside produced 50% inhibition of toxin binding in the low-salt,

Fig. 5. Immunofluorescence detection of cholera and tetanus toxin binding to BHK 21 cells grown in the presence or absence of gangliosides

BHK 21 cells were incubated with or without bovine brain mixed gangliosides for 3 h at 37 °C, washed and incubated with either cholera toxin (1 µg/ml, 12 nM) or tetanus toxin (10 µg/ml, 66 nM). The bound toxin was detected by indirect immunofluorescence as described in the Materials and methods section. The specificity of staining was confirmed in experiments where either the cholera or tetanus toxin was omitted. (a) Cholera toxin binding to BHK 21 cells; (b) cholera toxin binding to BHK 21 cells incubated with 20 µg of mixed brain gangliosides/ml; (c) tetanus toxin binding to BHK 21 cells; (d) tetanus toxin binding to BHK 21 cells incubated with 150 µg of mixed brain gangliosides/ml. The exposure times for (c) and (d) were approximately three times longer than for (a) and (b). Magnification bar = 20 µm.

low-pH buffer, 11 µM-trisialoganglioside was required to produce the same level of inhibition in a physiological salt solution. The result suggests that binding of tetanus toxin to ganglioside under physiological conditions of salt and pH is of low affinity.

To get a more direct measure of the affinity of tetanus toxin for membrane gangliosides, we have inserted trisialogangliosides into membranes lacking complex gangliosides, and compared the affinities of toxin binding under the two conditions. The brush border membrane of rat intestinal epithelial cells was used for this study because it was easy to prepare in substantial quantities, and previous experiments have shown that it lacked any detectable levels of the more complex gangliosides (Critchley *et al.*, 1981). Binding studies performed in 25 mM-Tris/acetate, pH 6.0, confirmed that untreated membranes bound only low levels of ¹²⁵I-labelled toxin. Very little of this binding could be inhibited by preincubation of the membranes with unlabelled toxin and it was therefore presumed to represent non-specific binding (Fig. 4a). In contrast, membranes treated with trisialogangliosides showed a dramatic increase in ¹²⁵I-labelled toxin binding, much of which could be inhibited by unlabelled toxin and was, therefore, considered to represent specific binding. Untreated membrane showed no evidence of ¹²⁵I-labelled toxin

binding in Krebs-Ringer buffer, pH 7.4, but ganglioside-treated membranes were able to bind low levels of ¹²⁵I-labelled toxin although much of the binding could not be inhibited by unlabelled toxin (Fig. 4b). Competition studies with labelled and unlabelled toxin showed that membranes treated with trisialogangliosides were able to bind with high affinity in 25 mM-Tris/acetate, pH 6.0 (Fig. 4c) (K_D 2.6 nM) but with a much lower affinity in Krebs-Ringer buffer, pH 7.4 (Fig. 4d).

That tetanus toxin can however bind to gangliosides in membranes under physiological conditions, albeit with low affinity, was confirmed as follows. BHK21 cells synthesize ganglioside G_{M3} , but little or none of the more complex gangliosides. Immunofluorescence experiments demonstrated that, whereas these cells bind low levels of cholera toxin (Fig. 5a), they were unable to bind tetanus toxin (Fig. 5c). In contrast, cells incubated with 20 µg of mixed brain gangliosides/ml showed a dramatic increase in their ability to bind cholera toxin (1 µg/ml; 12 nM) (Fig. 5b). Similarly, cells treated with gangliosides also showed clear evidence of tetanus toxin binding under physiological conditions of salt and pH (Fig. 5d), although the amounts of gangliosides (50–150 µg/ml) and toxin (5–10 µg/ml; 33–66 nM) required to produce clearly demonstrable binding were comparatively high.

To clarify further the chemical nature of the tetanus toxin receptor(s) detected under the two sets of conditions, membranes were pretreated with either trypsin, *S. griseus* proteinase, neuraminidase or heat, and subsequently assayed for tetanus toxin binding. To monitor the possible loss of membrane arising from treatment with proteolytic enzymes, membranes were also assayed for cholera toxin binding, a ligand known to interact specifically with a lipid, ganglioside G_{M1} . ¹²⁵I-tetanus toxin binding in 25 mM-Tris/acetate, pH 6.0, showed a limited sensitivity to trypsin, *S. griseus* proteinase and heat (Table 1). In contrast, ¹²⁵I-tetanus toxin binding assayed in Krebs-Ringer buffer, pH 7.4, showed a marked sensitivity to all three treatments. As expected, ¹²⁵I-cholera toxin binding was largely unaffected by any of these procedures. The binding of ¹²⁵I-tetanus toxin under both sets of conditions was reduced considerably by pretreating membranes with neuraminidase, whereas cholera toxin binding was increased due to conversion of di- and tri-sialogangliosides to G_{M1} . The results provide clear evidence that the major receptors for tetanus toxin assayed in Krebs-Ringer buffer, pH 7.4, are protein in nature, possibly sialoglycoproteins, whereas the major determinant of toxin binding in 25 mM-Tris/acetate, pH 6.0, is a proteinase- and heat-resistant component, possibly a sialic acid-containing lipid, i.e. a ganglioside.

DISCUSSION

Binding of ¹²⁵I-labelled tetanus toxin to rat brain membranes under conditions previously shown to be optimal for binding, i.e. in 25 mM-Tris/acetate, pH 6.0, was saturable with evidence for a single site of high affinity (K_D 0.26–1.14 nM) and high abundance (B_{max} 0.9–1.89 nmol of toxin bound/mg of membrane protein). Our results are in good agreement with those of Goldberg *et al.* (1982) and Rogers & Snyder (1981), who concluded that there was a single class of binding site, with a K_D in the range 1–4 nM. Binding was largely resistant to treatment of membranes with proteinases, and heat, but

Table 1. Binding of ^{125}I -labelled tetanus toxin and ^{125}I -labelled cholera toxin to enzyme- and heat-treated brain membranes

Rat brain membranes were treated as described in the Materials and methods section. The binding of ^{125}I -labelled tetanus toxin (0.3 nM, 1300 c.p.m./fmol) in either 25 mM-Tris/acetate, pH 6.0 (a nominal 100 ng of membranes) or Krebs-Ringer Buffer, pH 7.4 (a nominal 20 μg of membranes) was determined by filtration as described in the Materials and methods section. The results have been corrected for nonspecific binding as determined in the presence of 0.3 μM unlabelled toxin. The loss of membranes resulting from the various treatments was estimated from the binding of ^{125}I -labelled cholera toxin (0.1 nM, 12.6 $\mu\text{Ci}/\mu\text{g}$) to a nominal 200 ng of membranes, relative to untreated control membranes. The results on ^{125}I -labelled tetanus toxin binding were then corrected accordingly (values shown in parentheses). Since neuraminidase treatment resulted in an increase in cholera toxin binding, such corrections were inappropriate in this instance. The results are the mean for two experiments $\pm$ S.D., where $n = 6$ and $n = 4$ for the tetanus and cholera toxin data respectively.

Treatment	^{125}I -labelled tetanus toxin binding (% of control) in:		^{125}I -labelled cholera toxin binding (% of control) in Krebs-Ringer buffer, pH 7.4
	25 mM-Tris/acetate, pH 6.0	Krebs-Ringer buffer, pH 7.4	
Control membranes	100 $\pm$ 3 (100)	100 $\pm$ 3 (100)	100 $\pm$ 10
Trypsin	77 $\pm$ 9 (70)	16 $\pm$ 9 (14)	110 $\pm$ 3
<i>S. griseus</i> proteinase	59 $\pm$ 10 (68)	14 $\pm$ 2 (18)	87 $\pm$ 12
Heat	87 $\pm$ 17 (80)	37 $\pm$ 9 (36)	108 $\pm$ 24
Neuraminidase	27 $\pm$ 7 (—)	23 $\pm$ 3 (—)	254 $\pm$ 7

was reduced markedly by neuraminidase. Again these results are in good agreement with those of Rogers & Snyder (1981).

In contrast, when binding was assayed under more physiological conditions, i.e. in Krebs-Ringer buffer, pH 7.4, binding was non-saturable (up to 12 nM-toxin) and was much lower than that obtained in Tris/acetate, pH 6.0. Salt, calcium and pH effects are thought to contribute to the reduced binding (Ledley *et al.*, 1977; Lee *et al.*, 1979). Competition studies provided evidence for a small number of high-affinity sites (K_D 0.42 nM, B_{max} 0.9 pmol/mg) and a larger number of low-affinity sites (K_D 146 nM, B_{max} 179 pmol/mg). We are unable to conclude whether binding is to more than one class of non-interacting site or to one class of site displaying negative co-operativity. The demonstration that there is a high-affinity receptor for tetanus toxin in neural tissue under physiological conditions is in agreement with the known potency of the toxin. Approx. 2 ng of toxin/kg body wt. is lethal for mice. Binding of ^{125}I -labelled toxin was markedly reduced by pretreating the membranes with proteinases or heat. In addition, the binding was sensitive to pretreatment of membranes with neuraminidase, in agreement with previous results on binding of toxin to neuronal cells and membranes assayed under physiological conditions (Dimpfel & Habermann, 1977; Habermann *et al.*, 1981). The result suggests that sialoglycoconjugates are likely to be involved in toxin binding under both sets of conditions. The pronounced differences in binding of ^{125}I -labelled toxin to brain membranes in 25 mM-Tris/acetate, pH 6.0, and in more physiological buffers have been confirmed by measuring binding of unlabelled toxin with a neurotoxicity assay (D. R. Critchley, W. H. Habig & P. H. Fishman, unpublished work). The results described here are therefore not due to any aberrant behaviour of the iodinated toxin.

The characteristics of tetanus toxin binding to rat brain membranes in 25 mM-Tris/acetate, pH 6.0, are consistent with gangliosides acting as the primary toxin receptor. Under these conditions the toxin receptor was largely resistant to proteolysis and heat but was sensitive to

neuraminidase, which quantitatively converts brain membrane gangliosides G_{D1b} and G_{T1b} to G_{M1} , a ganglioside which binds toxin with only low affinity (Rogers & Snyder, 1981). The K_D for binding of toxin to brain membranes is similar to that for toxin binding to gangliosides incorporated into membranes. The large number of binding sites for toxin is also in agreement with the high level of ganglioside G_{D1b} and G_{T1b} in rat brain (Ando *et al.*, 1978).

It is clear however, that gangliosides cannot represent the high-affinity toxin receptor detected under physiological conditions of salt and pH. This site is sensitive to neuraminidase, proteinases and heat, features that are indicative of a protein (probably a sialoglycoprotein) receptor. Neither do gangliosides bind toxin with high affinity under these conditions, although it remains possible that gangliosides represent a lower affinity receptor for tetanus toxin. Indeed, there is no doubt that gangliosides do bind tetanus toxin in physiological buffers, as shown by experiments in which gangliosides were shown to promote toxin binding by BHK cells. Similar experiments have previously been reported by others (Dimpfel *et al.*, 1977; Yavin, 1984).

The idea that the tetanus toxin receptor might be other than ganglioside has been suggested previously, although based on little firm evidence (Kohn, 1978; Habermann *et al.*, 1981). It is interesting to note, however, that the data presented here on tetanus toxin binding in physiological buffers are very similar to those published by Williams *et al.* (1983) on binding of botulinum neurotoxin type A to a rat synaptosomal preparation. Thus Scatchard analysis of botulinum toxin binding produced a biphasic plot which could be resolved into a small number of high-affinity sites (K_D 0.6 nM, 60 pmol/mg of membrane protein) and a larger number of lower affinity sites ($K_D > 25$ nM). Binding of botulinum toxin was reduced markedly by pretreating the membranes with trypsin, heat and neuraminidase, and was partially inhibited by preincubating the toxin with high concentrations of gangliosides. Interestingly, the two toxins have a number of other features in common. Both

toxins are produced by *Clostridia*, each has an M_r of approx. 150000 and is composed of two non-identical polypeptide chains linked by disulphide bridges (reviewed by Eidels *et al.*, 1983). Both toxins act presynaptically and block the release of neurotransmitters at the neuromuscular junction and spinal cord, although their effectiveness at these two sites differs (Habermann, 1981). Tetanus toxin does not inhibit botulinum toxin type A binding except at high concentrations ($1 \mu\text{M}$), providing clear evidence that the receptors are different (Williams *et al.*, 1983).

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