Affinity-Purified Tetanus Neurotoxin Interaction with Synaptic Membranes: Properties of a Protease-Sensitive Receptor Component†

Philip Lazarovici and Ephraim Yavin*
Department of Neurobiology, The Weizmann Institute of Science, Rehovot 76100, Israel
Received January 13, 1986; Revised Manuscript Received May 6, 1986

ABSTRACT: The pharmacokinetic interaction of an affinity-purified ^{125}I-labeled tetanotoxin fraction with guinea pig brain synaptosomal preparations was investigated. Binding of tetanotoxin was time- and temperature-dependent, was proportional to protein concentration, and was saturable at about 8×10^{-9} M as estimated by a solid-surface binding assay. Binding was optimal at pH 6.5 under low ionic strength buffer and was almost entirely blocked by gangliosides or antitoxin. In analogy to intact nerve cells, binding of toxin to membranes resulted in a tight association operationally defined as sequestration. Binding and sequestration were abolished after membrane pretreatment with sialidase. The enzyme could not dissociate the membrane-bound toxin formed at 4 or 37 °C under low ionic strength conditions, which is in part compatible with internalization as defined in nerve cell cultures. In the latter system the toxin could be removed at 4 °C but not at 37 °C. Binding was significantly reduced upon pretreatment of guinea pig brain membranes by a variety of hydrolytic enzymes. Trypsin and chymotrypsin inhibited binding between 55% and 68% while bacterial protease abolished it by 91–95%. The effect was species-specific as it was not seen in rat or bovine synaptosomes. Collagenase and hyaluronidase had little or no inhibitory effect when applied to synaptosomes (27% and 9%) but inhibited binding to synaptic vesicles by 56% and 49%, respectively. Phospholipases A_2 and C caused 42–43% inhibition of binding in vesicles and less than 22% in synaptosomes. Trypsin (0.1%) had limited or no effect on binding of ^{125}I-labeled cholera toxin to synaptosomes but reduced substantially (61%) binding to sonicated synaptosome preparations. It is proposed that, in addition to a ganglioside, interaction of tetanotoxin with synaptic membranes is facilitated by a protein and may also require an appropriate lipid environment. These latter membrane constituents may play a pivotal role in the sequestration of the toxin.

The 150000-dalton polypeptide secreted by the *Clostridium tetani* microorganism is one of the most powerful known bacterial neurotoxins, which produces in some rodents spastic paralysis and death at doses in the range of nanograms per kilogram body weight (Gill, 1982). It is believed that, after a series of processes including binding to specific peripheral nerve receptors, tetanotoxin is transported retrogradely via the axon to presynaptic terminals in the spinal cord and brain, where it blocks neurotransmitter release [reviewed by Wellhoner (1982)]. Ample evidence implicates polysialogangliosides of the G1b series as putative receptors for the toxin (Van Heyningen, 1974). The existence of additional receptors has been proposed but not documented (Stoeckel et al., 1977).

Cells in tissue culture have been widely used to study the pharmacokinetics of tetanotoxin (Dimpfel et al., 1977; Yavin et al., 1981; Bergery et al., 1983). Using rat primary neuron cultures enriched in polysialogangliosides (Yavin et al., 1981) and somatic neurohybrid cells (Yavin, 1984) and human erythrocytes artificially supplemented with these substances (Lazarovici & Yavin, 1985a,b), we defined a ganglioside-mediated three-step interaction of ^{125}I-labeled tetanotoxin (^{125}I-tetanotoxin) with these cells. The first step involved a reversible, low ionic strength and energy-independent binding, followed by a second, salt-insensitive and detergent-nonextractable toxin–cell association, operationally defined as sequestration. Finally, in living cells, this process resulted in an energy-dependent internalization defined by the criteria of resistance of the toxin–membrane complex to sialidase treatment (Yavin et al., 1983). While all of these steps required the presence of a disialosyl residue, recently an additional trypsin-sensitive component that facilitated binding was also reported (Yavin & Nathan, 1986). In this report we expand these observations to investigate the existence of a similar activity in guinea pig synaptosomes and synaptic membrane vesicle preparations. These preparations avidly bind tetanotoxin (Mellanby et al., 1965), are rich in G1b gangliosides (Morgan et al., 1973), and have been useful for studying structure/function relationships following toxin binding (Ramos et al., 1979; Habermann et al., 1981). Using a ganglioside affinity-purified tetanotoxin fraction (Lazarovici et al., 1984), we demonstrate that binding to guinea pig synaptic preparations is facilitated by protease-sensitive and phospholipase-sensitive components. The validity of the three-step model and the relevance of these results to the in vitro action of tetanotoxin are discussed.

Materials and Methods

Enzymes. Phospholipase A_2 from *Naja mosambica* venom was purified as previously described (Lazarovici et al., 1982). Phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* and phospholipase D from cabbage were kindly provided by Dr. M. Menashe, Makor Chemicals Co., Jerusalem. Nonproteolytic enzymes were essentially free of proteolytic activity as tested by ^{125}I-labeled casein hydrolysis. Purified bovine brain ganglioside mixture was purchased from ICN Pharmaceuticals (Cleveland, OH). Enzymes and other reagents of analytical grade were purchased from regular commercial sources.

Toxins. Tetanotoxin, kindly supplied by Dr. B. Bizzini, Institute Pasteur, Paris, and cholera toxin from Dr. M. Menashe, Makor Chemicals Co., Jerusalem, were iodinated by

† Supported by a grant from the Israel Academy of Sciences and Humanities, Jerusalem.
* Author to whom correspondence should be addressed.

0006-2960/86/0425-7047$01.50/0

the Bolton–Hunter reagent to a specific activity of about 1 mCi/mg (Lazarovici et al., 1984) and 0.1–0.5 mCi/mg, respectively. Labeled tetanotoxin was further purified on a ganglioside affinity column and the "B" fraction (TeToB)[1] eluted by 6% NaCl was employed throughout (Lazarovici et al., 1984). Labeled toxins were diluted in 25 mM Tris buffer (pH 7.4) in either 0.3 M mannitol or 0.25 M sucrose, supplemented with 0.2% gelatin and stored at 4 °C. Tetanus toxin fragments B and C were purchased from Calbiochem. Rabbit tetanus antitoxin was obtained from Dr. R. Mirsky (University College, London).

Preparation of Synaptosomes and Synaptosomal Membrane Vesicles. Subcellular fractions from guinea pig brain were prepared by a modified method similar to that described by Gill et al. (1981) and Kanner (1978). Pooled brains (25 g wet weight) were homogenized by 10 strokes on a Dounce homogenizer in 100 mL of mannitol–EDTA (0.3 M D-mannitol and 1 mM KEDTA, pH 7.4) at 4 °C, and the homogenate was adjusted to 300-mL final volume. After centrifugation at 3000*g* for 10 min the supernatant was further centrifuged for 30 min at 25000*g*. The resulting pellet was resuspended in 30 mL of mannitol–EDTA, and 5-mL portions were layered over a discontinuous gradient consisting of five layers of 20%, 16%, 12%, 8%, and 2% Ficoll in mannitol–EDTA. After centrifugation at 20000*g* for 90 min, fractions from the Ficoll gradient were collected in the following order: fraction 1, 2–8% layer; fraction 2, interphase 8–12%; fraction 3, 12% layer; fraction 4, interphase 12–16; fraction 5, interphase 16–20%; fraction 6, 20% layer; and fraction 7, residual pellet. The 8–12% interphase enriched with synaptosomes (about 60 mg of protein) was washed once with mannitol–EDTA, and the pellet obtained after centrifugation was lysed by hypotonic shock and gentle homogenization in 10 mM Tris-HCl and 1 mM KEDTA, pH 7.4. After being gently stirred for 30 min at 4 °C, the suspension (40 mL) was centrifuged for 20 min at 30000*g* and the pellet adjusted to 5 mg of protein/mL in 0.32 M sucrose containing 25 mM Tris-HCl, 1 mM $MgSO_4$, and 0.5 mM EDTA, pH 7.4 (Tris–sucrose buffer). All Ficoll fractions, washed with sucrose buffer as above, were stored for up to 3 months at −70 °C for use in binding assays. The 8–12% interface fraction was highly enriched in synaptosomes while its derived osmotically lysed preparation (SMV) contained a high proportion of vesicular membranous structures (about 0.2 μm diameter) devoid of subcellular organelles, as visualized by transmission electron microscopy.

Synaptosomes from either bovine or rat brain prepared by sucrose density gradients by the method of Dodd et al. (1981) were kindly obtained from S. Orgad of our department.

Binding of ^{125}I-Labeled Toxins by the Solid-Surface Technique. Unless otherwise stated, binding of labeled toxins was done by a solid-surface radioreceptor assay. Aliquots of 50 μL of membrane suspension containing nanogram to microgram quantities of protein in Tris–sucrose or Tris–mannitol buffer (pH 6.8) were added to a 96-well microtiter plate. After 2 h at 22 °C (or overnight at 4 °C), the plate was centrifuged at 3000*g* for 10 min and unattached membranes were removed by suction. Blocking buffer (1% ovalbumin and 0.2% gelatin in Tris–sucrose or in Tris–mannitol buffer), added for 30 min at room temperature, was removed by suction. ^{125}I-labeled toxin in 50 μL Tris–sucrose or Tris–mannitol binding buffer (pH 6.8) augmented with 0.04% gelatin and 0.06% ovalbumin was added for specified times and temperature. At the end of incubation, the plate was centrifuged as above and unbound toxin was removed by suction. Wells were then washed twice with 0.2 mL of the nonradioactive binding buffer to which 0.15 M NaCl (high ionic strength) was added. After heat drying, single wells were counted in a Kontron Gammamatic counter at 92% efficiency.

Specific binding of ^{125}I-tetanotoxin was defined as the difference between the total amount of bound toxin (after subtraction of background binding) and that obtained in the presence of 1 mM bovine brain ganglioside mixture. Binding in the presence of the latter was similar to that achieved in the presence of excess (500-fold) unlabeled toxin, and it was less than 0.2% of the added radioactivity. Specific binding of ^{125}I cholera toxin was routinely determined in the presence of 10^{-6} M unlabeled toxin. Experiments were repeated at least twice on different occasions and values are the mean of 2–3 wells.

Enzymatic Treatment of Membranes. (a) Proteolysis. Aliquots of membrane suspension (0.2–1 mg of protein) were treated with varying concentrations of protease, α-chymotrypsin, or trypsin in 0.5 mL of phosphate-buffered saline (P_i/saline), pH 7.4, containing 5 mM $CaCl_2$ for 30 min at 37 °C. Unless otherwise stated, trypsin activity was always terminated by adding soybean trypsin inhibitor (Sigma), and membranes were washed twice with P_i/saline and twice with Tris–sucrose buffer by using high-speed centrifugation (2 min at 20000g) before well coating. (b) Phospholipases. Aliquots of membrane suspension (0.5 mg of protein) were incubated with various phospholipases (0.1% w/v) in 0.5 mL of P_i/saline containing 20 mM $CaCl_2$ for 30 min at 37 °C. Membranes were washed twice with P_i/saline and twice with Tris–sucrose buffer (both without Ca^{2+}) as above, prior to coating. (c) Sialidase. Aliquots of membrane suspension (0.5 mg of protein/mL) or diluted membranes (nanogram–microgram range) after coating to plates were treated with 50–100 units/mL of *Vibrio cholera* neuraminidase (Behringwerke) in Tris–sucrose buffer containing 2 mM $CaCl_2$, pH 6.8, for times designated at 37 °C. The enzyme was removed, and membranes were rinsed with P_i/saline and Tris–sucrose buffer as above. Control membranes consisted of identical preparations in the absence of the enzyme.

Miscellaneous Procedures. Protein was determined by the method of Lowry et al. (1951), using ovalbumin as a standard. Extractions of membrane lipids were performed as previously described (Yavin et al., 1981). Lipid-bound sialic acid was determined by a slight modification of the procedure of Hahn et al. (1974). Membrane-lipid phosphorus was determined by using the procedure of Bartlett (1959).

Ganglioside composition was determined after extraction with chloroform/methanol solution (2:1 v/v) and chromatography of the lipid extract on silica gel thin-layer chromatographic plates (Yavin et al., 1981). Extraction of the salt-resistant, membrane-associated ^{125}I-tetanotoxin by detergents was performed as previously described (Lazarovici & Yavin, 1985b). The neurotoxicity of tetanotoxin was tested in mice as detailed elsewhere (Lazarovici et al., 1984).

RESULTS

Properties of ^{125}I-Labeled Tetanotoxin Binding to Guinea Pig Brain Synaptosomal Preparations by the Solid-Surface Assay. Binding of ^{125}I-tetanotoxin to synaptosomal preparations under low salt conditions exhibits a biphasic, linear relationship with a break in the curve at about 0.5 μg of protein (Figure 1A). Detection of specific binding below 0.1 μg of

[1] Abbreviations: EDTA, ethylenediaminetetraacetate; P_i/saline, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SMV, synaptic membrane vesicles; TeToA and TeToB, affinity-purified tetanotoxin fractions; Tris, tris(hydroxymethyl)aminomethane.

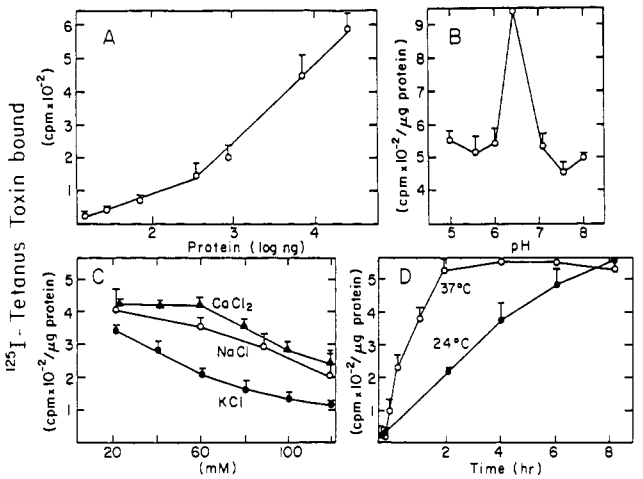

FIGURE 1: Binding of ^{125}I-labeled tetanotoxin to guinea pig brain synaptosomes by a solid-surface assay as a function of membrane protein (A), pH (B), ionic strength (C), and time and incubation temperature (D). Binding of ^{125}I-tetanotoxin (50000 cpm/well) was done for 2 h (apart from A, which was done for 1 h) at 37 °C in Tris–maleate–mannitol buffer as described under Materials and Methods. Values are the mean of triplicate wells + SEM.

Table I: Solid-Surface Binding of ^{125}I-Labeled Tetanotoxin to Synaptosomes and Synaptic Membrane Vesicles (SMV) in Various Buffers[a]

medium composition	synaptosomes (cpm/μg of protein)	SMV (cpm/μg of protein)
0.02 M Tris/0.3 M mannitol	560 ± 35	1410 ± 130
+5 mM EDTA	590 ± 40	1300 ± 85
+10 mM $CaCl_2$	620 ± 40	1260 ± 60
+0.15 M NaCl	90 ± 20	180 ± 45
0.02 M Tris/0.25 M sucrose	870 ± 35	1740 ± 145
+0.15 M NaCl	90 ± 20	150 ± 20
0.02 M Tris	500 ± 40	1000 ± 110

[a] ^{125}I-Tetanotoxin (15000 cpm) in various buffers was added to guinea pig brain membrane preparations (3 μg of protein), and binding was performed at pH 7.4 for 2 h at 37 °C by using the solid-surface assay detailed under Materials and Methods. Values are the mean of triplicate wells ± SE.

protein is notable and illustrates the sensitivity of the solid-surface technique employed. The optimum pH for toxin binding is around pH 6.5 (Figure 1B), while at pH 7.4 binding is considerably lower by about 50% in line with data obtained by others (Lee et al., 1979). Binding of tetanotoxin to synaptosomes and synaptic membrane vesicles is most effective under low ionic strength conditions. This is summarized in Table I and Figure 1C. EDTA (5 mM) or $CaCl_2$ (10 mM) do not increase binding while 0.15 M NaCl reduces binding substantially (Table I). The ionic strength rather than ion specificity is the principal variable that determines the extent of binding of the toxin. Thus increasing the concentration of monovalent cations in the binding buffer from 20 to 100 mM decreases by 2–3-fold the amount of bound toxin (Figure 1C). The effect of Ca^{2+} ions at concentrations higher than 60 mM probably reflects a steric hindrance of sialic acid caused by Ca^{2+} ions (Leskawa & Rosenberg, 1981). This is also in accord with the reported inhibition of tetanotoxin by calcium in synaptic membranes (Mellanby & Pope, 1976). A typical time course of the salt-resistant association of tetanotoxin with guinea pig brain membranes at 24 and 37 °C, is depicted in Figure 1D. Binding is greater at 37 °C than at 24 °C and is proportional with time for up to 2 h, after which time it reaches a plateau, which is maintained for at least 6 h. Similar results are obtained when nanogram quantities of synaptosomes are used (data not shown).

Table II: Successive Release of Membrane-Bound ^{125}I-Labeled Tetanotoxin by Various Reagents[a]

treatment	^{125}I-tetanotoxin bound (cpm/μg of protein): synaptosomes	SMV	biotoxicity
(1) Tris–mannitol (low ionic strength)	443	1722	600
(2) Tris–mannitol + 0.15 M NaCl (high ionic strength)	291	1360	600
(3) 1% Triton X-100	120	320	800
(4) 1% SDS	25	24	1500

[a] Binding to membranes in suspension was performed for 2 h at 37 °C with ^{125}I-tetanotoxin (70000 cpm/tube) in 20 mM Tris–0.3 M mannitol buffer. Excess toxin was removed by three rinses with Tris–mannitol buffer by use of high-speed centrifugation (2 min at 20000*g*). Membrane pellets were counted and subjected to 0.15 M NaCl for 10 min at 4 °C, and after centrifugation pellets were recounted. Further extraction of membrane-bound toxin was done by consecutive treatment with Triton X-100 (15 min at 4 °C) and SDS. Values represent the amount of membrane-associated toxin remaining after the respective treatment. Biotoxicity (Lazarovici et al., 1984) is expressed as the cpm equivalent to one minimum lethal dose.

Additional properties of the toxin-synaptosome interaction are summarized in Table II. Bound toxin is effectively displaced by treatment with high ionic strength buffer. Nearly 34% and 20% of the toxin bound under low ionic strength conditions is removed by 0.15 M NaCl from synaptosomes and synaptic vesicles, respectively. Subsequent treatment with Triton X-100 removes between 40% and 60% of the salt-insensitive, membrane-associated toxin, while SDS solubilizes the remaining (5% or less) pellet-associated toxin. Evidently, the toxin released exhibits a fairly uniform (600–800 cpm) toxicity value as assessed by the mouse bioassay. These data suggest that a substantial portion of the bound toxin is Triton X-100 resistant in line with similar observations concerning toxin sequestration by whole cells (Yavin et al., 1981; Yavin, 1984; Lazarovici & Yavin, 1985b). Binding of tetanotoxin to synaptic membranes is inhibited by excess (500-fold) of unlabeled toxin, toxin fragments, antitoxin, and polysialogangliosides (Table V, first column) but not by several presynaptic toxins including taipoxin, β-bungarotoxin, and pardaxin (data not shown). This indicates a remarkable degree of specificity of the tetanotoxin for the binding sites on the synaptic membranes.

Effect of Sialidase on ^{125}I-Labeled Tetanotoxin Binding to Membranes. Pretreatment of guinea pig synaptosomes with sialidase causes substantial losses of tetanotoxin binding sites (Figure 2). Binding is inhibited whether low or high ionic strength conditions are applied and at 4 and 37 °C. The losses in binding activity are greater than that previously reported for rat membrane preparations (Rogers & Snyder, 1981) and may be attributed to the difference in species or even to the binding technique employed. Removal of bound toxin by sialidase is more complex and depends on the initial binding conditions; under low ionic strength the toxin–membrane complex is stable to sialidase (Figure 2, left panel) while under high ionic strength conditions, sialidase destablizes the complex (Figure 2, right panel). Stability of the complex formed at 37 °C to sialidase is compatible with toxin internalization (Yavin et al., 1983). On the other hand, the remarkable binding at 4 °C and the equally effective stability of the complex to sialidase (left panel) suggests that binding of toxin to synaptosomal membranes is not entirely homologous to uptake and internalization by cultured nerve cells (Yavin et al., 1983).

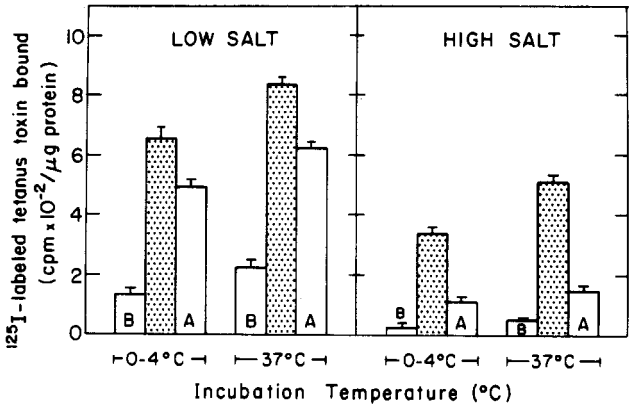

FIGURE 2: Effect of sialidase on binding of tetanotoxin (A) and displacement of bound toxin (B) under various temperatures and high and low ionic strength conditions. Synaptosomes (100 μg of protein) were treated in suspension with sialidase (50 milliunits/mL) for 30 min at 37 °C and subsequently coated (50 ng/well) as described under Materials and Methods. Control (dotted bars) and sialidase treated membranes (B) were incubated with tetanotoxin (47 000 cpm, 2 h at 0–4 or 37 °C) in the presence of Tris–sucrose buffer, pH 7.4 (low salt), or Tris–sucrose–NaCl buffer, pH 7.4 (high salt). After washing, the medium was counted. Sialidase (5 milliunits/0.1 mL) was added to selected wells (A), and the residual membrane-associated radioactivity after 30 min at 37 °C was determined. Values are the mean of triplicate wells + SEM.

Table III: Binding of Tetanotoxin and Sialic Acid Content of Guinea Pig Brain Membrane Fractions Isolated on Ficoll Gradient and Treated with Sialidase

gradient fraction[a]	^{125}I-toxin bound (cpm/μg of protein)[b]	sialic acid (units)[c]	after sialidase treatment[c] inhibition of binding (%)	after sialidase treatment[c] loss of sialic acid (%)
1	290	2.2	18	9
synaptosomes	660	7.1	42	59
5	400	2.6	50	62
6	100	3.7	50	73
7	100	1.4	29	20
SMV	800	5.0	40	36

[a] Isolation of fractions of the Ficoll gradient is detailed under Materials and Methods. [b] Binding was performed by the solid-surface assay in the presence of 70 000 cpm of toxin for 2 h at 37 °C. [c] Lipid-linked sialic acid and treatment of membranes with sialidase is described under Materials and Methods.

The effect of sialidase on binding of the toxin and the corresponding changes in the lipid-associated sialic acid content of various membrane fractions is summarized in Table III. Evidently the synaptosomal fraction and the vesicles derived from it exhibit the highest toxin binding activity and are most abundant in lipid-bound sialic acid. After treatment with sialidase all Ficoll fractions isolated from the gradient exhibit a lower binding activity, which parallels the reduction in the total lipid-bound sialic acid. Analysis of the gangliosides composition reveals an increase of GM1 at the expense of disialo- and trisialogangliosides (data not shown).

Effect of Trypsin on ^{125}I-Labeled Tetanotoxin Binding to Membranes. The effect of trypsin treatment and that of sialidase on binding of tetanotoxin to synaptosomes and synaptic vesicles is shown in Table IV. Under the experimental conditions studied, trypsin appears more potent than sialidase in reducing the number of binding sites when added prior to tetanotoxin (experiment A). Displacement of bound toxin by trypsin treatment (experiment B) is also effective and reaches values of 64% and 57% for synaptosomes and SMV, respectively, compared to control values. Sialidase, on the other hand, is less effective in displacing the bound toxin, the values being 50% and 30% for synaptosomes and synaptic vesicles,

Table IV: Effect of Sialidase and/or Trypsin Treatment on Binding (Experiment A) or Displacement (Experiment B) of ^{125}I-Labeled Tetanotoxin

membrane treatment	% inhibition synaptosomes	% inhibition SMV
experiment A[a]		
trypsin	82	83
sialidase	72	40
sialidase + trypsin	85	89
experiment B[b]		
trypsin	64	57
sialidase	50	30
sialidase + trypsin	84	89

[a] Experiment A: Synaptosomal or SMV membrane preparations (20 μg/well) adhered to plastic wells were treated with either 20 milliunits of sialidase or 0.2% trypsin or with both successively as described under Materials and Methods. Membranes were washed once with P_i/saline and once with Tris–sucrose buffer. ^{125}I-Labeled toxin (75 000 cpm/well) was added for 2 h at 37 °C, and radioactivity remaining after P_i/saline wash was measured. [b] Experiment B: Same as in (A) except that enzymatic treatment was done on membranes incubated first with labeled toxin. Values are average of triplicate wells with a standard error of less than 15%.

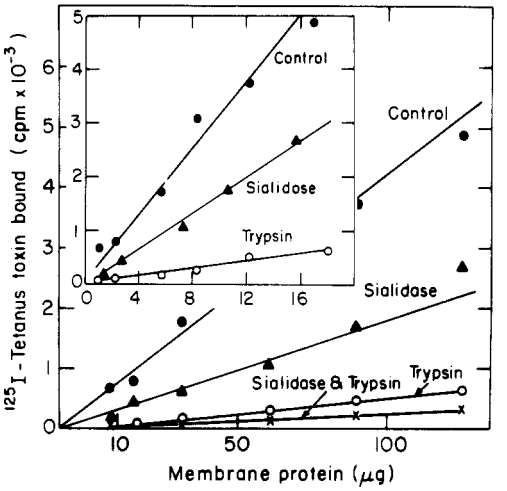

FIGURE 3: Effect of membrane concentration on ^{125}I-tetanotoxin binding to synaptic membranes after treatment with sialidase or trypsin. Membranes treated with trypsin (0.1%) or sialidase (50 milliunits/mL) for 30 min at 37 °C in P_i/saline were collected by high-speed centrifugation and after resuspension were used for well coating. ^{125}I-Tetanotoxin (54 000 cpm/well) was added for 2 h at 37 °C, and the membrane-bound radioactivity after rinse in P_i/saline was determined by the solid-surface technique. (Inset) Same values expressed per nanomole of lipid phosphorus.

respectively. The residual membrane-associated toxin is analogous to the tight interaction reported for nerve cells in culture (Yavin et al., 1983). A combination of both enzymes is effective in removing 84–89% of bound toxin in both membrane preparations.

Binding of tetanotoxin to membrane preparations subjected to sialidase or trypsin depends on the amount of protein added and is linear up to 0.1 mg of protein (Figure 3). It is completely inhibited by trypsin and only partially (about 50%) by sialidase treatments. A similar picture emerges when binding of the toxin to untreated or treated synaptosomes is normalized per lipid phosphorus (Figure 3, inset). The remarkable loss of binding activity after membrane treatment with trypsin or sialidase is also well illustrated as a function of toxin concentration (Figure 4). After sialidase or trypsin treatments, lack of binding at high toxin concentrations is more pronounced than at low toxin concentrations. This effect is less evident when the amount of membranes is reduced 30-fold as shown in the inset of Figure 4. At this membrane concentration,

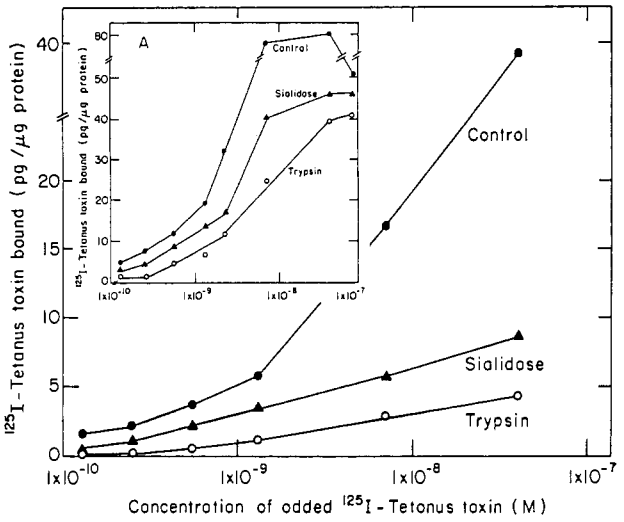

FIGURE 4: Binding of tetanotoxin to control and enzyme-treated synaptic membranes as a function of toxin concentration. Enzyme-treated (as in Figure 3) and control membranes at 300 μg of protein (or 10 μg of protein in the inset) were incubated for 2 h at 37 °C with increasing concentrations of ^{125}I-labeled toxin. Each point represents the mean of 2–3 wells.

binding is saturable at about 8×10^{-9} M toxin. Extrapolation from this curve gives rise to an apparent half-saturation value of about 3 nM and an estimated binding capacity of 80 pg of toxin/μg of protein, which closely corresponds to 0.55 nmol/mg of protein. The number of binding sites estimated on the guinea pig synaptic membrane are in good agreement with the average value of bovine brain membranes (0.23 nmol/mg of protein) reported by Rogers & Snyder (1981) but different from the 1.5–9 nmol/mg of protein value reported by Goldberg et al. (1981). Another notable property characteristic of low membrane concentrations is the smaller effect of sialidase and trypsin particularly upon raising toxin concentrations (Figure 4, inset). For instance, at 1×10^{-7} M inhibition of binding of toxin to trypsin or sialidase treated membranes is less than 20%, while at 8×10^{-9} M binding of toxin to the respective membranes is reduced by 68% and 46%. A lower binding activity was observed in the presence of excess membranes. Under these conditions the effect of trypsin and sialidase was more pronounced at toxin concentrations $>8 \times 10^{-9}$ M in comparison to diluted membrane concentrations.

Displacement of bound tetanotoxin from sialidase- and trypsin-treated synaptosomes by a variety of reagents is summarized in Table V. Evidently sera raised in rabbits against the toxoid or a mixture of GD1b/GT1b gangliosides are the most potent agents in displacing the bound toxin. Native toxin and fragments B and C, each at micromolar concentration, appear more effective in displacing the toxin from controls rather than from enzyme-treated membranes. Treated membranes obviously exhibit a much lower binding capacity.

Effect of Hydrolytic Enzymes. The specificity of the proteoltyic compared to other enzymatic treatments is shown in Table VI. Phospholipases A_2 and C but not phospholipase D effectively reduced binding by about 42% to synaptosome derived vesicle preparations. While phospholipase A_2 stimulated about 60% binding of tetanotoxin to synaptosomes phospholipase C was inhibitory (22%). Hyaluronidase- and collagenase-treated synaptic vesicles exhibited 49–56% less binding activity while α-chymotrypsin inhibited both synaptic preparations in the range of 55–68%. Bacterial protease was most effective (91–95%) in abolishing binding activity.

To further clarify the nature of the sensitivity of the receptors for tetanotoxin to proteolytic treatment, we have

Table V: Displacement of ^{125}I-Labeled Tetanotoxin from Sialidase- or Trypsin-Pretreated Synaptosomes by Various Compounds[a]

compound	% of maximal binding: untreated	after sialidase	after trypsin
tetanotoxin (1 μM)	15	25	50
fragment B (1 μM)	18	43	60
fragment C (1 μM)	5	47	50
rabbit antitoxin (50 μg/mL)	10	0	0
GT1b/GD1b ganglioside mixture (0.1 mg/mL)	16	0	0
max binding (cpm/μg of protein)	550	260	200

[a] Synaptosomal membranes (30 μg of protein) pretreated with sialidase (50 milliunits/mL) or 0.2% trypsin as described under Materials and Methods were subjected to ^{125}I-labeled tetanotoxin (75 000 cpm/well). After 2 h at 37 °C membranes were rinsed with 0.15 M NaCl in Tris–mannitol buffer and the various compounds were added for 30 min at 37 °C. After two rinses with 0.15 M NaCl in Tris–mannitol the residual radioactivity was counted. Values are averages of triplicate wells and are expressed as percent of maximal binding.

Table VI: Binding of ^{125}I-Labeled Tetanotoxin to Synaptosomes and Synaptic Membrane Vesicles Treated with Different Enzymes

treatment	synaptosomes: sp act. (cpm/μg)	synaptosomes: inhibition (%)	SMV: sp act. (cpm/μg)	SMV: inhibition %
control	550 ± 65	0	1000 ± 90	0
phospholipase A_2	880 ± 85	0	570 ± 35	43
phospholipase C	430 ± 40	22	580 ± 50	42
phospholipase D	540 ± 35	2	915 ± 35	8
collagenase	400 ± 65	27	435 ± 15	56
hyaluronidase	500 ± 20	9	510 ± 25	49
protease	50 ± 10	91	50 ± 25	95
α-chymotrypsin	250 ± 45	55	315 ± 55	68
trypsin	205 ± 65	63	405 ± 15	60

[a] Phospholipase treatment was done as described under Materials and Methods. Collagenase, hyaluronidase, protease, and α-chymotrypsin, each at a concentration of 0.1%, were added to membranes in P_i/saline, pH 7.4, for 30 min at 37 °C. Treated membranes were collected by high-speed centrifugation, and after resuspension aliquots (30 μg of protein) were added to plastic wells. Tetanotoxin (76 000 cpm/well) was added for 2 h at 37 °C, and membrane-associated radioactivity after washing with P_i/saline was determined. Values are averages ± SEM of triplicate wells.

studied binding of cholera toxin to the same membrane preparations. Both toxins are known for their affinity to gangliosides and could in principle share similar properties with respect to binding to membranes after proteolytic digestion. As seen in Figure 5, there was only a slight effect on binding of cholera toxin to trypsin-treated membranes up to 0.1 mg of protein. An increase in binding to sialidase-treated membranes may be due to generation of GM1, the putative receptor for cholera toxin (King & Van Heyningen, 1963; Cuatrecasas, 1973). In contrast, a marked decrease in binding of tetanotoxin to treated membranes was noticed. Trypsin concentration greater than 0.1% reduced by 50% binding of both toxins (Figure 5, inset). At trypsin concentrations below 0.1%, there was no activity recovered in the supernatant collected after pelleting the treated membranes suggesting that loss of receptors is due to proteolytic digestion of a component involved in binding rather than to solubilization of the receptor. Some binding activity, however, was recovered in the supernatant of membranes treated with 1% trypsin.

Additional characterization of the reactivity of tetanus and cholera toxins binding toward membranes was performed by comparing binding of untreated and trypsin-treated synaptosomal membranes following sonication or osmotic shock (Table VII). Each of the treatments increased by about 2.5- and

Table VII: Binding of ^{125}I-Labeled Tetanotoxin or ^{125}I-Labeled Cholera Toxin to Sonicated or Osmotically Shocked Synaptosomes Treated with Trypsin[a]

membrane preparation	^{125}I-tetanotoxin			^{125}I cholera toxin		
	control (cpm/μg of protein)	trypsin (cpm/μg of protein)	inhibition (%)	control (cpm/μg of protein)	trypsin (cpm/μg of protein)	inhibition (%)
synaptosomes						
intact	530 ± 45	295 ± 55	44.3	1360 ± 90	1260 ± 120	7.4
after sonication	1310 ± 150	545 ± 75	58.4	5735 ± 135	2230 ± 205	61.1
after osmotic shock	900 ± 70	180 ± 40	80.0	4640 ± 285	4310 ± 155	7.1

[a] Synaptosomes after sonication (20 s at 4 °C) or after osmotic shock (20 mM Tris buffer, pH 7.4, 5 min at 4 °C) were centrifuged for 30 min at 90000*g*, and the resulting pellets were resuspended in Tris–mannitol buffer. Trypsin (0.05%) was added for 30 min at 37 °C, and the treated membranes were applied to 96 well plates. Binding of tetanotoxin and cholera toxin was performed as described under Figure 5. Values are the mean of 3–4 wells ± SEM.

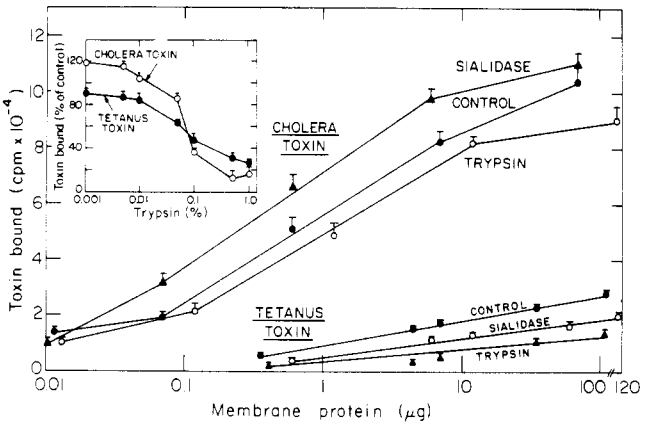

FIGURE 5: Binding of ^{125}I-tetanotoxin and ^{125}I cholera toxin to sialidase- or trypsin-treated synaptic membranes. Synaptosomes (1.2 mg of protein) treated with trypsin or sialidase as in Figure 3 were coated at different concentrations and subjected for 2 h at 37 °C to tetanotoxin (1×10^5 cpm/well) or cholera toxin (2×10^5 cpm/well). Membrane-associated radioactivity was measured by the solid-surface technique. Values are averages + SEM of triplicate wells.

1.9-fold binding of tetanotoxin and 4.2- and 3.4-fold of cholera toxin, respectively. Sonication of trypsin treated membranes raised by almost a factor of 2 the binding capacity of both toxins. In contrast, hypotonic shock of membranes seems to decrease binding of tetanotoxin while increasing by over 3-fold binding of cholera toxin. These results suggest that both toxins are more accessible to their targets perhaps because sonication reveals spare binding sites which escaped proteolytic digestion.

The specificity of the toxins to the synaptic membranes after treatment with trypsin or sialidase is shown in Table VIII. Unlabeled cholera or tetanotoxin at 1 μM concentration prevent 95% and 85% binding of the ^{125}I-labeled counterparts, respectively. After treatment with sialidase the residual binding activity is prevented only 65% and 70% by the unlabeled cholera and tetanotoxin, respectively. Trypsin-treated membranes are inhibited 35% by excess unlabeled tetanotoxin and 85% by unlabeled cholera toxin. Although the absolute values are low in the treated membranes, the inability of the excess unlabeled toxin to prevent binding may indicate a nonspecific binding component.

Finally, it is interesting to note that loss of binding sites by proteolytic treatment of the synaptic membranes exhibits a remarkable specificity with respect to the animal species employed. As illustrated in Figure 6, guinea pig synaptic preparations are most sensitive to proteolytic treatment compared to rat or bovine brain.

Discussion

The thrust of this work has been to characterize and reevaluate the kinetic properties of ganglioside affinity-purified

Table VIII: Prevention of ^{125}I-Labeled Tetanotoxin and ^{125}I-Labeled Cholera Toxin Binding by Unlabeled Tetanus and Cholera Toxins[a]

	^{125}I-labeled toxin (% of maximum)		
	control	sialidase	trypsin
tetanotoxin (1 μM)	15	30	65
cholera toxin (1 μM)	5	35	15

[a] Cholera or tetanus toxins were each (1 μM) added to sialidase- or trypsin-treated membranes for 30 min at 37 °C. ^{125}I-Labeled toxin (0.05 μM) was added for 2 h and membrane-bound toxin was determined by the solid-surface assay. Values are average of triplicates with less than 5% error.

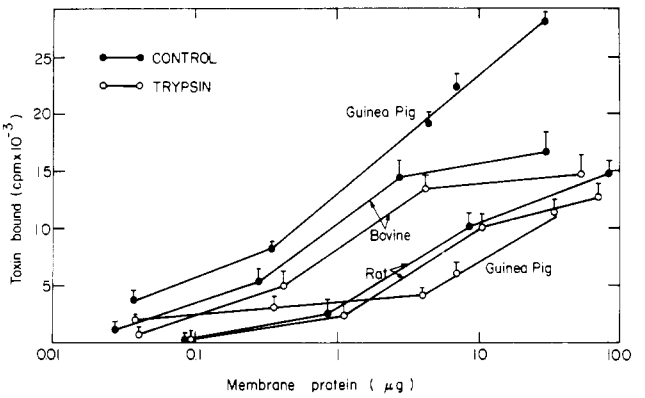

FIGURE 6: Binding of tetanotoxin to trypsin-treated synaptosome preparations from various sources. Trypsinized synaptosome preparations from guinea pig, bovine, and rat brain were subjected to ^{125}I-tetanotoxin under conditions similar to those described in Figure 5. Values are the average of triplicate wells ± SE.

tetanotoxin binding to synaptic membranes and to further identify the receptors that participate in this interaction.

The first question we addressed concerned the three-step mechanism of toxin internalization identified in nerve cells (Yavin et al., 1983) and its validity in other nerve membrane preparations such as synaptosomes and synaptic vesicles. This problem appeared particularly relevant in light of our recent discovery concerning the existence of two nearly identical and equally toxic fractions of tetanotoxin after a ganglioside affinity column purification (Lazarovici et al., 1984). One fraction, TeToB, exhibited a strong affinity for gangliosides and reacted avidly with neuronal membranes. The second fraction, TeToA, bound poorly to nerve cells, synaptic membranes, or purified gangliosides. The existence of TeToA in pure tetanotoxin preparations raised for the first time the possibility that binding and biotoxicity may be entirely different phenomena and reopened the question of ganglioside-dependent and -independent interaction of tetanotoxin with the nerve membrane. The legitimacy of such a reevaluation is demonstrated in this work through the analysis of receptor sensitivity to proteolytic

and other hydrolytic digestions.

In this paper we first examine the binding properties of TeToB to isolated guinea pig synaptic membranes and conclude that essentially these preparations are analogous to those reported in other systems (Mellanby & Whittaker, 1968; Rogers & Snyder, 1981; Schmitt et al., 1981).

Binding of TeToB was found proportional to the amount of membrane protein added and like that previously shown (Lee et al., 1979) was greater under low ionic strength than high ionic strength conditions. It exhibited an optimum pH close to pH 6.5 and was prevented by excess unlabeled toxin or agents such as gangliosides, toxin fragments, or antibodies indicating a highly specific interaction. Binding was also dependent on the presence of sialic acid residues, reaffirming the presumed role of gangliosides in the binding process. In analogy to nerve cells there was a salt-dependent release of toxin. A substantial amount of toxin retained after detergent extraction was indicative of a tight association defined as sequestration (Yavin et al., 1983). On the basis of these properties we concluded that synaptic membranes interact with tetanotoxin very similarly to intact nerve cells or erythrocytes artificially supplemented with gangliosides (Lazarovici & Yavin, 1985b). However, unlike nerve cells, the stability of the toxin–membrane complex towards sialidase (Figure 2, left panel) did not depend on the temperature but exhibited a strong dependency on the ionic strength at which the complex was formed. Thus, electrostatic interactions appeared to play a more critical role than temperature in internalization of the toxin as defined by stability to sialidase (Yavin et al., 1983). The presence of low salt concentration may facilitate the transfer of the toxin from a sialo- to an asialo-receptor component or even alter the intrinsic properties of the polypeptide and thus render the complex cryptic to sialidase. Formation of macromolecular toxin–toxin and toxin–membrane complexes are in line with the latter possibility (Lazarovici et al., 1984, 1986). The effect of sialidase originally reported by Kryzhanovsky et al. (1973) and confirmed in the present study under high ionic strength (Figure 2) is also compatible with a shift in the polypeptide physical state (i.e., monomeric) to enable the dissociation of the toxin–membrane complex.

The second subject we questioned in this study concerned the exclusive ganglioside nature of the receptor that binds the toxin. Previous studies have shown that proteolytic treatment of rat brain synaptic membranes had no effect on binding, suggesting that the receptor for tetanotoxin is not a protein (Rogers & Synder, 1981). More recently, we have demonstrated that, in addition to a ganglioside component, binding of tetanotoxin to cultured nerve cells appears to be partially facilitated by a trypsin-sensitive component (Yavin & Nathan, 1986). In extending these studies we now demonstrate that guinea pig synaptic membranes, like cultured nerve cells, seem to possess additional components that participate by yet unclear mechanisms in binding of tetanotoxin. Treatment of membranes by various proteases is particularly effective in removing these constituents, but also phospholipase C or collagenase treatments are rather effective. It is striking that guinea pig and not rat or bovine synaptosomes are sensitive to proteolysis, a finding that could in part explain the failure of other investigators to show this effect. Sensitivity to proteolysis is by no means an argument in favor of the existence of a discrete protein receptor. Gangliosides are still the prime target for tetanotoxin, as shown previously in model membrane systems (Lee et al., 1978) or with pure gangliosides (Holmgren et al., 1980). The novel aspect of this study is that in the native membrane, additional components facilitate this interaction. Cerebrosides in conjunction with gangliosides have been shown to facilitate binding (Van Heyningen & Mellanby, 1968). Artificial lipid bilayers can interact with the toxin B fragment at acidic pH in the absence of gangliosides (Bouquet & Duflot, 1982). Although proteins have not been directly implicated in binding of tetanotoxin (Haberman & Albus, 1986; Yavin & Nathan, 1986), several associations between gangliosides and proteins have been characterized (Sonino et al., 1979; Wong et al., 1984). For example, GM1 and specific membrane proteins are believed to participate in the translocation of cholera toxin (Kellie et al., 1983; Fiani et al., 1984) whereas gangliosides and glycoproteins may interact with each other during the assembly of the extracellular matrix (Sharom & Grant, 1978).

In the context of the current experiments we would like to postulate that the role of certain surface proteins and lipids is to facilitate the irreversible transfer of the toxin from the oligosaccharide recognition site on the ganglioside receptor into a deeper hydrophobic lipid bilayer domain. This mechanism of toxin translocation fits well with the three-step model proposed in nerve cells and is also compatible with the model suggested by others (Schmitt et al., 1981). The integrity of the glycocalyx proteins and the appropriate lipid environment in the biological membrane may be as essential as the receptor itself in providing optimal conditions for furthering the interaction of the ligand with the cell surface.

ACKNOWLEDGMENTS

We thank Riva Chester for excellent technical assistance.

REFERENCES

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.
Bergery, G. K., MacDonald, R. L., Habig, W. H., Hardegree, M. C., & Nelson, P. G. (1983) *J. Neurosci. 3*, 2310–2323.
Boquet, P., & Duflot, E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7614–7618.
Cuatrecasas, P. (1973) *Biochemistry 12*, 3547–3558.
Dimpfel, W., & Habermann, E. (1977) *J. Neurochem. 29*, 1111–1120.
Dodd, P., Hardy, J., Oakley, A., Edwardson, J., Perry, E., & Delanoy, J. (1981) *Brain Res. 226*, 107–118.
Fiani, M. L., Macioce, P., Gallina, A., & Tomasi, M. (1984) *J. Neurosci. Res. 12*, 325–334.
Gill, D. L., Grollman, E. F., & Kohn, L. D. (1981) *J. Biol. Chem. 256*, 184–192.
Gill, D. M. (1982) *Microbiol. Rev. 46*, 86–94.
Goldberg, R. L., Costa, T., Habig, W. H., Kohn, L. D., & Hardegree, M. C. (1981) *Mol. Pharmacol. 20*, 565–570.
Habermann, E., & Albus, U. (1986) *J. Neurochem. 46*, 1219–1226.
Habermann, E., Bigalke, H., & Heller, I. (1981) *Naunyn-Schmiedeberg's Arch. Pharmacol. 316*, 135–142.
Hahn, H. J., Hellman, B., Lernmark, A., Sehlin, J., & Taljedal, I. B. (1974) *J. Biol. Chem. 249*, 5275–5284.
Holmgren, J., Elwing, H., Fredman, P., & Svennerholm, L. (1980) *Eur. J. Biochem. 106*, 371–379.
Kanner, B. I. (1978) *Biochemistry 17*, 1207–1211.
Kellie, S., Patel, B., Pierce, E. J., & Critchley, D. R. (1983) *J. Cell Biol. 97*, 447–454.
King, C. A., & Van Heyningen, W. E. (1973) *J. Infect. Dis. 127*, 639–647.
Kryzhanovsky, G. M., Rozanov, A. J. A., & Bondartchuk, G. N. (1973) *Bull. Exp. Biol. Med. (Engl. Transl.) 76*, 1404–1406.
Lazarovici, P., & Yavin, E. (1985a) *Biochim. Biophys. Acta 812*, 523–531.

Lazarovici, P., & Yavin, E. (1985b) *Biochim. Biophys. Acta 812*, 532-542.
Lazarovici, P., Hochman, J., Menashe, M., Primor, N., & Zlotkin, E. (1982) *Arch. Toxicol. 51*, 167-173.
Lazarovici, P., Tayot, J. L., & Yavin, E. (1984) *Toxicon 22*, 401-413.
Lazarovici, P., Yanai, P., & Yavin, E. (1986) *J. Biol. Chem.* (in press).
Lee, G., Consiglio, E., Habig, H., Dyer, S., Hardegree, C., & Kohn, L. D. (1978) *Biochem. Biophys. Res. Commun. 83*, 313-320.
Lee, G., Grollman, E. F., Dyer, S., Beguinot, F., Kohn, L. D., Habig, W. H., & Hardegree, M. C. (1979) *J. Biol. Chem. 254*, 3826-3832.
Leskawa, K. C., & Rosenberg, A. (1981) *Cell. Mol. Neurobiol. 1*, 373-391.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Mellanby, J., & Whittaker, V. P. (1968) *J. Neurochem. 15*, 205-208.
Mellanby, J., & Pope, D. (1976) *Adv. Exp. Med. Biol. 71*, 215-229.
Mellanby, J., Van Heyningen, W. E., & Whittaker, V. P. (1965) *J. Neurochem. 12*, 77-79.
Morgan, I. G., Tettamanti, G., & Gombos, G. (1976) *Adv. Exp. Med. Biol. 71*, 137-150.
Ramos, S., Grollman, E. F., Lazo, P. S., Dyer, S. A., Habig, W. H., Hardegree, M. C., Kaback, H. R., & Kohn, L. D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4783-4787.
Rogers, T. B., & Snyder, S. H. (1981) *J. Biol. Chem. 256*, 2402-2407.
Schmitt, A., Dreyer, F., & John, C. (1981) *Naunyn-Schmiedeberg's Arch. Pharmacol. 317*, 326-330.
Sharom, F. J., & Grant, C. W. M. (1978) *Biochim. Biophys. Acta 507*, 280-293.
Soninno, S., Ghidoni, R., Marchesini, S., & Tettamanti, G. (1979) *J. Neurochem. 33*, 117-121.
Stoeckel, K., Schwab, M., & Thoenen, H. (1977) *Brain Res. 132*, 273-285.
Van Heyningen, W. E. (1974) *Nature (London) 249*, 415-417.
Van Heyningen, W. E., & Mellanby, J. H. (1968) *J. Gen. Microbiol. 52*, 447-454.
Wellhoner, H. H. (1982) *Rev. Physiol., Biochem. Pharmacol. 93*, 1-68.
Wong, M., Brown, R. E., Barenholz, Y., & Thompson, T. E. (1984) *Biochemistry 23*, 6498-6505.
Yavin, E. (1984) *Arch. Biochem. Biophys. 230*, 129-137.
Yavin, E., & Nathan, A. (1986) *Eur. J. Biochem. 154*, 403-407.
Yavin, E., Yavin, Z., Habig, W. H., Hardegree, M. C., & Kohn, L. D. (1981) *J. Biol. Chem. 256*, 7014-7022.
Yavin, E., Yavin, Z., & Kohn, L. D. (1983) *J. Neurochem. 40*, 1212-1219.

Direct Incorporation of Guanosine 5′-Diphosphate into Microtubules without Guanosine 5′-Triphosphate Hydrolysis

Ernest Hamel,* Janendra K. Batra, and Chii M. Lin

Laboratory of Pharmacology and Experimental Therapeutics, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received January 28, 1986; Revised Manuscript Received July 17, 1986

ABSTRACT: Using highly purified calf brain tubulin bearing [8-^{14}C]guanosine 5′-diphosphate (GDP) in the exchangeable nucleotide site and heat-treated microtubule-associated proteins (both components containing negligible amounts of nucleoside diphosphate kinase and nonspecific phosphatase activities), we have found that a significant proportion of exchangeable-site GDP in microtubules can be incorporated directly during guanosine 5′-triphosphate (GTP) dependent polymerization of tubulin, without an initial exchange of GDP for GTP and subsequent GTP hydrolysis during assembly. The precise amount of GDP incorporated directly into microtubules is highly dependent on specific reaction conditions, being favored by high tubulin concentrations, low GTP and Mg^{2+} concentrations, and exogenous GDP in the reaction mixture. Minimum effects were observed with changes in reaction pH or temperature, changes in concentration of microtubule-associated proteins, alteration of the sulfonate buffer, or the presence of a calcium chelator in the reaction mixture. Under conditions most favorable for direct GDP incorporation, about one-third of the GDP in microtubules is incorporated directly (without GTP hydrolysis) and two-thirds is incorporated hydrolytically (as a consequence of GTP hydrolysis). Direct incorporation of GDP occurs in a constant proportion throughout elongation, and the amount of direct incorporation probably reflects the rapid equilibration of GDP and GTP at the exchangeable site that occurs before the onset of assembly.

Tubulin polymerization, both with and without MAPs,[1] generally requires GTP bound at the exchangeable nucleotide site (Weisenberg et al., 1968) of the protein. In the course of the reaction this GTP is hydrolyzed to GDP (Kobayashi, 1975). These observations have generated much experimental work and a number of intriguing models to elucidate the role of GTP hydrolysis in the formation and stability of microtubules [e.g., Kirschner (1980), Weisenberg (1980), Cote & Borisy (1981), Bonne & Pantaloni (1982), Carlier & Pantaloni (1982), Hill & Chen (1984), and Caplow & Reid (1985)].

GDP, on the other hand, is generally viewed as an inhibitor

[1] Abbreviations: MAPs, microtubule-associated proteins; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; p(CH_2)ppG, guanosine 5′-(β,γ-methylenetriphosphate); ATP, adenosine 5′-triphosphate; UTP, uridine 5′-triphosphate; Mes, 2-(*N*-morpholino)-ethanesulfonate; Pipes, 1,4-piperazinediethanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; EDTA, ethylenediaminetetraacetate; DEAE, diethylaminoethyl.