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Affinity purified tetanus toxin binds to isolated chromaffin granules and inhibits catecholamine release in digitonin-permeabilized chromaffin cells

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Tetanus toxin, a potent neurotoxin which blocks neurotransmitter release in the CNS, also inhibits Ca^{2+}-induced catecholamine release from digitonin-permeabilized, but not from intact bovine chromaffin cells. In searching for intracellular targets for the toxin we studied the binding of affinity-purified tetanus toxin to bovine adrenal chromaffin granules. Tetanus toxin bound in a neuraminidase-sensitive fashion to intact granules and to isolated granule membranes, as assayed biochemically and visualized by electron microscopic techniques. The binding characteristics of the toxin to chromaffin granule membranes are very similar to the binding of tetanus toxin to brain synaptosomal membranes. We suggest that the toxin-binding site is a glycoconjugate of the G1b type (a polysialoganglioside or a glycoprotein-proteoglycan) which is localized on the cytoplasmic face of the granule membrane and might directly be involved in exocytotic membrane fusion.

Chromaffin granule; Tetanus toxin; Catecholamine secretion; Polysialoglyconjugate; Exocytosis; (Chromaffin cell)

1. INTRODUCTION

Tetanus toxin (TT), the 150 kDa protein secreted by *Clostridium tetani* is a powerful bacterial neurotoxin which produces spastic paralysis and death in rodents and humans, in the range of nanograms per kg body weight [1]. Tetanus toxin is recognized by peripheral neurons, internalized, and transported in a retrograde fashion to the central nervous system, where it blocks neurotransmitter release [2]. Ample evidence implicates GT1b and GD1b polysialogangliosides as receptors for the toxin on neuronal membranes [3]. Despite this evidence, it is not known whether intracellular binding of TT to polysialogangliosides is necessary for axonal transport [4] or for the toxin-evoked inhibition of neurotransmitter release [5]. From a clinical vantage point, despite preventive immunization, tetanus remains a potentially significant health problem due to the inability to reverse the course of the disease once the toxin is internalized. Identifying the intracellular targets of TT might lead to an understanding of how the toxin causes the pathological responses and could help in development of new approaches to inactivate the toxin, even after it has interacted with the central nervous system.

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Abbreviations: GD1b, galactosyl-*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl - *N* - acetylneuraminyl]galactosylglucosylceramide; GT1b, galactosyl-*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl-*N*-acetylneuraminyl-*N*-acetylneuraminyl]galactosylglucosylceramide; TeToB, tetanus toxin 'B' form, affinity purified on a 'G1b' ganglioside gel; TT, tetanus toxin

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Adrenal chromaffin cells are well-characterized cellular models for studying exocytotic neurotransmitter release [6]. While tetanus toxin does not interfere with catecholamine release from intact bovine chromaffin cells [7], microinjection of the toxin into intact cells [8], or addition of TT to digitonin-permeabilized chromaffin cells [9] was shown to inhibit exocytosis, suggesting the existence of an intracellular target site for the toxin action. More recently, Ahnert-Hilger et al. [10] demonstrated that the light chain of tetanus toxin inhibited the release of [^{3}H]noradrenaline from chromaffin cells permeabilized with streptolysin O [10]. Using a homogeneous, G1b ([*N*-acetylneuraminyl-*N*-acetylneuraminyl] galactosylglucosylceramide) affinity purified tetanus toxin probe [11], named tetanus toxin 'B' (TeToB), we studied the inhibitory effect of this toxin on calcium-induced catecholamine secretion from permeabilized chromaffin cells. The same TeToB probe was also used to characterize and, for the first time, to visualize neuraminidase-sensitive tetanus toxin-binding sites on the cytoplasmic face of isolated chromaffin granule membranes. As inferred from their binding characteristics, these sites appear to be polysialoconjugates of the GD1b or GT1b type and might be directly involved in the exocytotic release of neurotransmitters.

2. MATERIALS AND METHODS

2.1. *Chromaffin cell cultures and catecholamine release*

Bovine adrenal chromaffin cells were isolated and cultured as previously described [12]. Three-day-old cultures were permeabilized [13] with 20 μM digitonin for 10 min in calcium-free buffer (containing 1 mM EGTA) in the absence or presence of increasing tetanus toxin concentrations. After 10 min, the media were changed and the cells were incubated in the same buffer (basal release) or in buffer containing 20 μM free ionized calcium. After additional 10 min, the supernatants were collected and centrifuged to remove floating cells and cellular debris. Aliquots from the supernatants and the freeze/thawed pellets were assayed for catecholamine contents fluorimetrically, using a modified trihydroxyindole technique [14]. The results were calculated:

$$\text{Net release (\%)} = \frac{Ca^{2+}\text{-stimulated release} - \text{basal release}}{\text{total catecholamine content}}$$

and the data are presented as percent of control (cells without toxin). The experiments, carried out in triplicates, were repeated twice with similar results; the standard error within each experiment was $<5\%$.

2.2. *Tetanus toxin binding to chromaffin granules*

Chromaffin granules were isolated from intact adrenal medulla by sequential centrifugation and further purified over a 1.3 M discontinuous sucrose gradient according to [15]. Binding of ^{125}I-tetanus toxin B form (192 Ci/mmol), purified by the ganglioside-affinity chromatography [9], was performed for 2 h at 37°C with either increasing concentrations or with a fixed concentration of toxin of 4×10^{-9} M/sample in 0.32 M sucrose containing 25 mM Tris-HCl, pH 7.4, using 360 mg granule protein/ml. For some of the experiments, the granules were pretreated with 50 mU/ml neuraminidase for 30 min at 37°C prior to the binding studies. Throughout the experiments we used a highly purified neuraminidase from *Vibrio cholerae* (Behringwerke), which is free of proteolytic activity as assayed with ^{125}I-casein [16]. The pH, saturation, and displacement experiments were performed by a solid-surface radioreceptor assay as previously described [16,17]. Freshly prepared chromaffin granules (3.6 mg protein/ml) pretreated with neuraminidase or untreated were incubated in suspension with the tetanus toxin at 4°C or 37°C. In some experiments, granules were lysed by osmotic shock or repeated freezing and thawing, as previously described [18] to yield right-side-out oriented granule membrane vesicles (ghosts). After 2 h the binding was stopped, the medium removed and the granules washed; neuraminidase (50 mU/ml) was added to selected tubes and the radioactivity associated with the granules after 30 min at 37°C was determined [16]. For the displacement experiments, chromaffin granules were incubated with ^{125}I-labeled tetanus toxin for 2 h at 37°C, and then the granules were washed and the unlabeled toxin or ganglioside was added for an additional 30 min at 37°C. After two rinses, the granule-associated radioactivity was counted. In all experiments nonspecific binding of ^{125}I-TeToB was defined as the amount of radioactivity found in the presence of excess (1 unit of antitoxin) [19]. This value represents less than 15% of the largest value determined and was subtracted from all data presented. The values are expressed as percent of maximal binding. The binding values are the mean of triplicate determinations. The standard errors for all experiments were less than 5%.

2.3. *Electron microscopy*

Freshly prepared suspensions of chromaffin granules (2.5 mg protein/sample) were incubated for 2 h at 37°C with biotinylated tetanus toxin (5×10^{-8} M) purified by chromatofocussing and G1b-ganglioside affinity chromatography [20]. After washing and gentle fixation (5 min, room temperature with 2.5% glutaraldehyde, 5% sucrose in 0.1 M sodium cacodylate buffer, pH 7.3) the granules were incubated with streptavidin-gold (20 nm diameter) for 1 h at room temperature followed by fixation, processing and embedding for electron microscopy by conventional methods. Thin sections, lightly stained with lead acetate, were examined at 80 kV in a Philips 400 electron microscope. To assess the specificity of the TT binding, aliquots of the granule suspensions were pretreated with 50 mU/ml neuraminidase, as described above. In another set of control experiments, the biotinylated toxin was omitted to permit evaluation of the nonspecific binding of streptavidin-gold.

3. RESULTS AND DISCUSSION

The secretion of catecholamines from intact chromaffin cells is believed to be triggered by a rise in cytosolic [Ca^{2+}], resulting in exocytotic fusion of the secretory vesicular membranes with the plasma membrane [6]. This process can be mimicked in detergent-permeabilized cells by using buffered media containing controlled concentrations of free, ionized calcium in the micromolar range [21]. To access the intracellular machinery which might be involved in the action of tetanus toxin, chromaffin cells were permeabilized by exposure to 20 μM digitonin [13] for 10 min in the absence or presence of 10^{-11}–10^{-6} M of the ganglioside affinity purified tetanus toxin (TeToB). Subsequently, in the continued presence of the toxin or in its absence, the cells were exposed to a medium containing 20 μM ionized calcium. The inhibition of the calcium-induced catecholamine secretion from permeabilized chromaffin cells in the presence of the toxin is dose dependent with an IC_{50} value of approx. 5×10^{-8} M toxin (fig.1). This IC_{50} value is comparable to the half-maximal concentration of TT required to block catecholamine release in digitonin-permeabilized chromaffin cells [9] and also to the efficacy of the holotoxin in blocking exocytosis from streptolysin O-treated cells [10]. Concentrations higher than 10^{-6} M were required to completely block catecholamine secretion in this system (data not shown). By contrast, another tetanus toxin, the G1b ganglioside, low-affinity, previously isolated and characterized (TeToA) [11] was unable to inhibit the calcium-induced catecholamine release when tested under the same experimental conditions, even at concentrations exceeding 10^{-4} M (not shown). These findings underline that binding of TT to its high-affinity receptor is a necessary step to impair exocytosis.

In line with previously published reports [7], catecholamine secretion from intact chromaffin cells, either the basal one or the one stimulated by either 100 μM acetylcholine or 50 mM potassium, could not be inhibited by TeToB at concentrations up to 10^{-5} M and prolonged preincubations up to 48 h (data not shown).

The simplest interpretation of these results is that the site of action of tetanus toxin is an intracellular one and that TT is not internalized by intact bovine adrenal chromaffin cells. However,

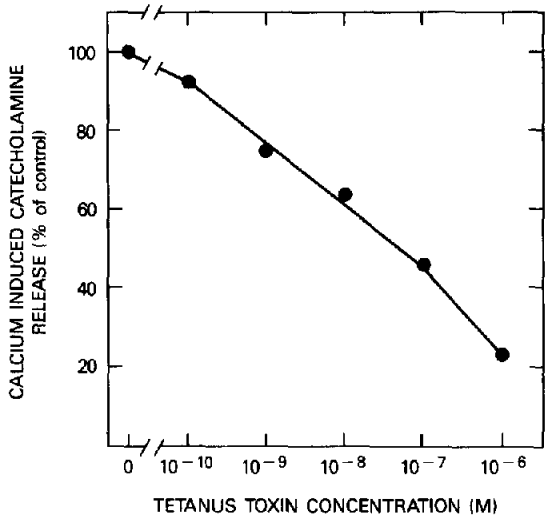

Fig.1. Inhibition of calcium-induced catecholamine release from digitonin-permeabilized chromaffin cells by ganglioside affinity purified tetanus toxin. For details see section 2. In the particular experiment depicted, the basal release was 6.5% and the Ca^{2+}-induced release was 17.1%. The degree of inhibition is thus normalized to the net release in the absence of TT (= 11.6% of the total contents).

once TT gains access to the cytoplasm, as in detergent-permeabilized cells, the calcium-dependent secretory response from these cells is inhibited by the Gd1b-specific, high affinity tetanus toxin (TeToB) at a site distal to calcium entry, presumably by interfering with the site of exocytosis proper. The IC_{50} value obtained in our experiments is in the range of 10^4–10^5 median lethal doses to mice and is comparable to those of another toxin, botulinum type D, required to inhibit Ca^{2+}-induced catecholamine release from electrically permeabilized chromaffin cells [7]. With both toxins, the IC_{50} values are higher than those required to block depolarization-induced release of inhibitory neurotransmitters (glycine, GABA) in neuronal cells, which are in the range of 10^{-8}–10^{-11} M toxin [5]. This result is not surprising given the notion that the secretory machinery in permeabilized cells might lack some essential components that are present in intact cells [22].

To elucidate the site of action of tetanus toxin we studied its binding to intact chromaffin cells, and to isolated chromaffin granules. Intact chromaffin cells, tested either in suspension or as monolayers, did not express any saturatable, cell

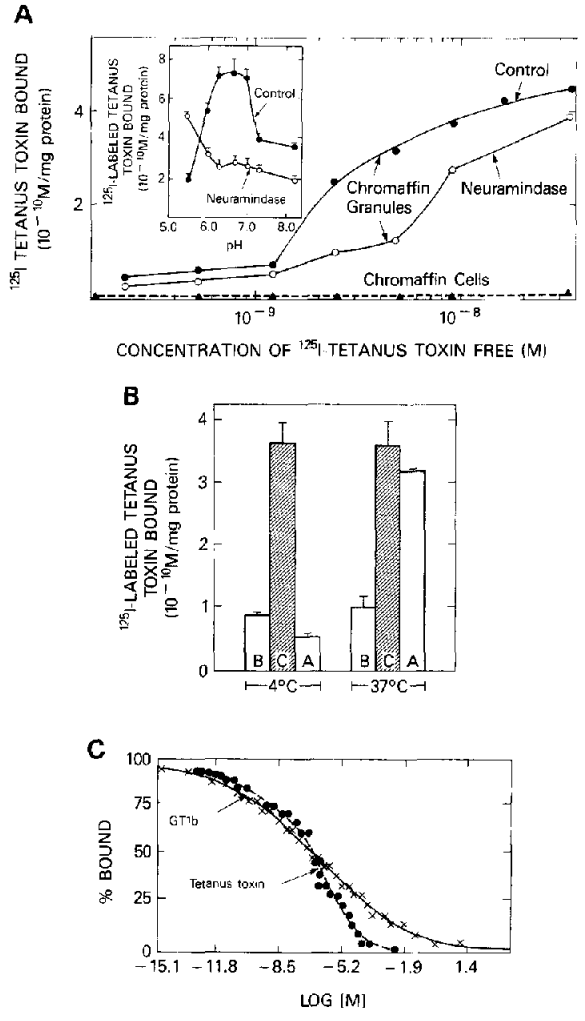

Fig.2. Binding of affinity purified tetanus toxin to control and neuraminidase-treated chromaffin granules as a function of toxin concentration (panel A), pH (inset, panel A), and temperature (panel B); and displacement of ^{125}I-labeled toxin by unlabeled toxin or the GD1b ganglioside (panel C). The graphs in panels A and C are self-explanatory. In panel B, the bars represent ^{125}I-TT binding to untreated (C) and to neuraminidase-pretreated (B) granules, while in A the granules were treated with neuraminidase after ^{125}I-binding at the indicated temperatures. For experimental details see text.

surface specific binding sites for ^{125}I-tetanus toxin (fig.2, panel A), in line with previous findings [23]. In contrast, suspensions of chromaffin granules showed a capacity for binding the labeled toxin, that was saturatable at a concentration of about 3×10^{-8} M free toxin (fig.2, panel A). Scatchard analysis of these data yielded an apparent affinity coefficient (K_d) of about 8×10^{-8} M and an estimated binding capacity of 0.45 nmol/mg granule membrane protein (table 1). These values are comparable in their low affinity and high number of binding sites to the estimates for tetanus toxin binding to synaptosomes derived from guinea pig and bovine brains, respectively [16,17].

To localize the TT-binding sites we also prepared membranes from intact chromaffin granules by osmotic shock and used them either fresh or after storage at −70°C. The isolated membranes exhibited binding characteristics for the toxin similar to those of intact granules: tetanus toxin binding capacity was in the range of 0.2–0.4 nmol/mg protein concomitant with a reduced affinity in the range of 1×10^{-7}–5×10^{-7} M. These results might reflect a loss of membrane proteins and/or their binding properties due to the preparation procedure. The similarity in the binding properties of TT to intact granules and to isolated membranes, before and after rupture by osmotic shock or by freezing and thawing suggests, that the binding site of TT is located exclusively on the cytoplasmic face of the granules. This result is unexpected, since TT has been shown to bind to complex polysialogangliosides [3] and, hitherto, such gangliosides were believed to reside solely on the inner face of the granule membranes [24,25].

To characterize further the nature of the tetanus toxin-binding sites, we pretreated intact chromaffin granules with purified neuraminidase from

Table 1

Binding characteristics of ^{125}I-tetanus toxin to different preparations of chromaffin granules

Granule preparation	K_d (M)	B_{max} (nmol/mg membrane protein
Isolated intact granules	8.0×10^{-8}	0.45
Granules + neuraminidase	2.4×10^{-6}	0.042
Granules + trypsin	5.0×10^{-7}	0.011
Osmotically shocked fresh granules	1.0×10^{-7}	0.40
Osmotically shocked frozen granules	5.0×10^{-7}	0.20

Experimental: ^{125}I-tetanus toxin binding to the various granule preparation was measured as detailed in the legend to fig.1. K_d and B_{max} values were obtained from Scatchard plots of the binding data, each comprising between 15 and 20 experimental points

Vibrio cholerae and with trypsin. Removal of the sialic acid residues by neuraminidase caused a substantial loss (up to 80%) of the tetanus toxin-binding sites at all toxin concentrations (fig.2, panel A) and over the entire pH range investigated (fig.2, panel A, insert). For example, at pH 6.7, neuraminidase treatment reduced tetanus toxin binding by 75%. Scatchard plot analysis of TT binding to neuraminidase-treated granules indicated a more than 400-fold loss in affinity (K_d = 2.4×10^{-6} M). This decrease in TT binding paralleled the reduction in total lipid-bound sialic acid (data not shown). By contrast, extensive trypsinization (1 mg/ml for 30 min at 37°C), which removes most of the proteins on the granule surface but leaves the sialoglycolipids intact, reduced the affinity for TT binding by a factor of 20 (K_d = 5×10^{-7} M).

The optimum pH for tetanus toxin binding to chromaffin granules was found to be around 6.7, while at pH 5.5 and at pH > 7.4 binding was decreased by more than 50% (fig.2, panel A, inset). These results were similar to the bell shaped pH dependence observed for tetanus toxin binding to neuronal membranes [16,17].

Pretreatment of intact chromaffin granules with neuraminidase, prior to adding the toxin, caused a substantial loss of tetanus toxin-binding sites, independent of the temperature (4°C or 37°C) at which the subsequent experiments were carried out (fig.2, panel B). However, the results were more difficult to interpret when neuraminidase was added after the ^{125}I-TT had bound to the granules at different temperatures. When the toxin was first allowed to bind to chromaffin granules at 4°C, a significant fraction of the bound toxin could be removed by neuraminidase (open bars, B). By contrast, the toxin-binding site complex formed at 37°C was stable and refractive to neuraminidase treatment (open bars, A). This finding might reflect a process similar to TT sequestration at 37°C that has previously been reported with cultures of neuronal cells [19].

Displacement of bound TT from chromaffin granules (fig.2, panel C) by unlabeled, ganglioside affinity purified tetanus toxin or by the ganglioside GD1b was observed with IC_{50} values of 1.7×10^{-7} and 2.2×10^{-7} M, respectively. These values are about ten times higher than the K_d values for the actual binding of the toxin to the granules and are consistent with our notion of some irreversible interaction between the bound toxin and its sites, similar to that previously described for toxin sequestration in neuronal systems [16,17,19].

To provide visual evidence for the specificity and the location of tetanus toxin-binding sites, we used a biotinylated TT derivative which had been purified by chromatofocussing and ganglioside-affinity chromatography [20]. After incubation of intact chromaffin granules with biotinylated-TT, streptavidin-gold (20 nm diameter) was added to the preparations. In control experiments, granules were pretreated with neuraminidase or incubated with streptavidin-gold alone. The preparations were then processed for transmission electron microscopy. The electron micrographs reveal specific tetanus toxin-binding sites on the cytoplasmic face of vesicles or vesicular membranes (fig.3, panels a–d). Although our preparations contain a fraction of lysed vesicles with partially open membranes, the gold labeling is invariably limited to the outer surface of the chromaffin vesicle membranes. In view of the controversy over the topography of sialic acid residues on the chromaffin granule membranes [24,25], these electron microscopic images strengthen the notion that the sugar-sialic acid conformation recognized by the 'G1b'-tetanus toxin is strictly localized on the external surface of chromaffin granules. When the granules were treated with neuraminidase prior to binding of the toxin, practically no biotinylated-toxin-streptavidin gold complexes were found (fig.3, panel e). In addition, the specific binding of the biotinylated toxin to chromaffin granules was significantly decreased by competition with unlabeled toxin (data not shown). The nonspecific binding to the granules of the streptavidin-gold probe alone, in the absence of the toxin as mediator, was found to be insignificant (fig.3, panel f).

Our tentative identification of tetanus toxin-binding sites as polysialoconjugates is supported by several lines of evidence: the specific G1b ganglioside affinity of the tetanus toxin probe [4,11], the high binding capacity of toxin sites and their low affinity and, in particular, their sensitivity to neuraminidase. In addition, the pH optimum for binding, the resistance of the tetanus toxin bound at 37°C to neuraminidase treatment and the displacement behavior are entirely consistent with

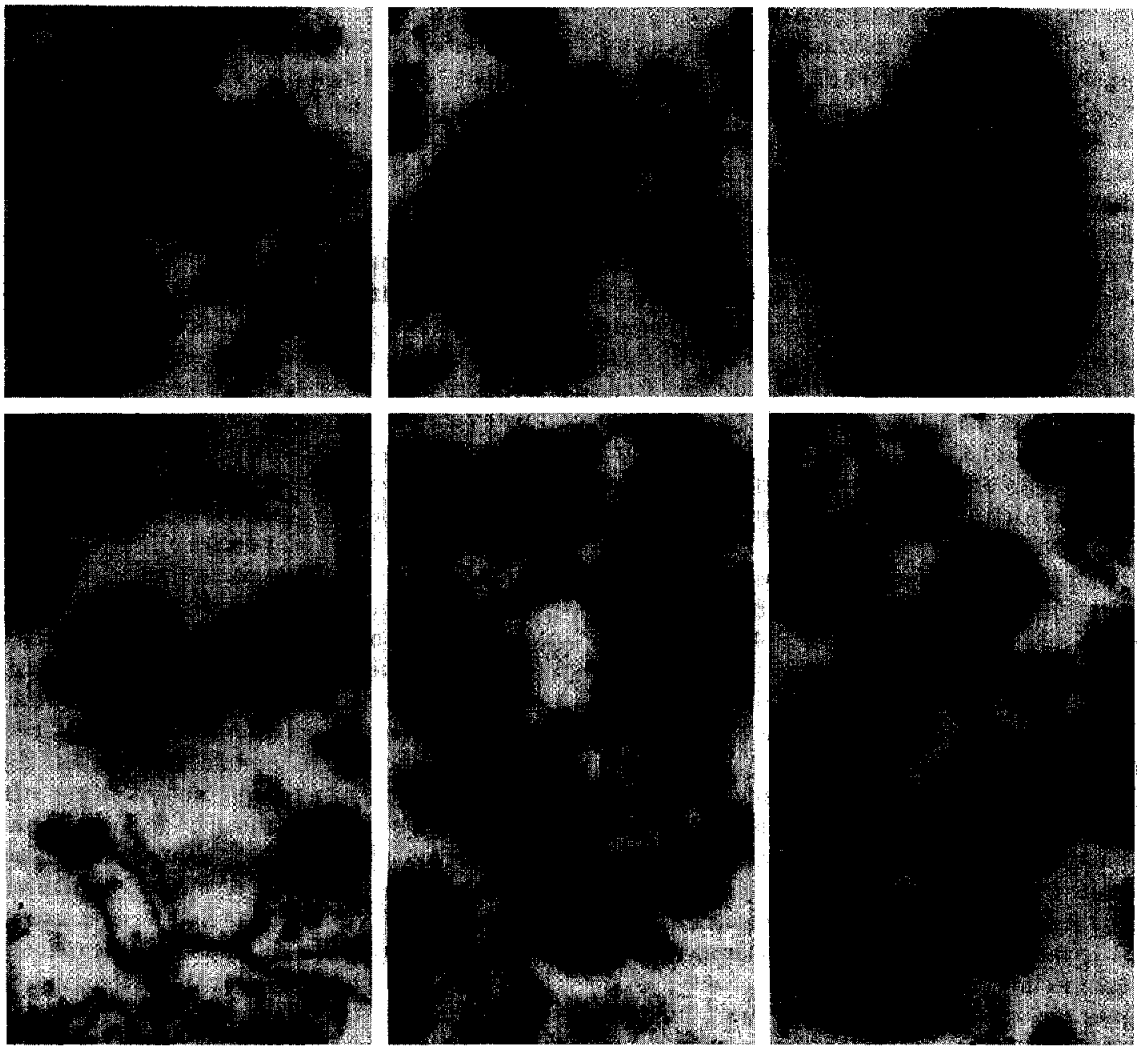

Fig.3. Localization of affinity purified biotinylated tetanus toxin-binding sites, visualized with streptavidin-gold on isolated chromaffin granules. (Panels a–d) Untreated chromaffin granules; (panel e) chromaffin granules after neuraminidase treatment; (panel f) the biotinylated toxin was omitted to permit evaluation of the nonspecific binding of streptavidin-gold. Bar equals 167 nm in panels a,d,e,f, 130 nm in panel b, and 79 nm in panel c. The original magnifications were 60000 × in panels a,d,e,f; 77000 × in panel b; and 127000 × in panel c, respectively.

the binding properties of tetanus toxin to neuronal membranes, where the TT receptors have been identified as G1b type polysialogangliosides [16,17].

Chemical analyses of ganglioside composition of bovine chromaffin granule membranes have failed to detect the presence of polysialoglycolipids of the G1b type [26]. GM_3 gangliosides, which are not recognized by tetanus toxin [27], were found to account for >95% of the total ganglioside species of the chromaffin granules [24,25]. Therefore, the G1b structure recognized by tetanus toxin appears to be a related glycoconjugate on the cytoplasmic face of chromaffin granule membranes that represents either a very minor Gd1-like polyialoganglioside, which hitherto has not been identified [24–26], or a glycoprotein-proteoglycan [28] with a similar sugar-sialic acid structure.

From our data on tetanus toxin binding to granules and toxin inhibition of catecholamine

secretion we can also estimate the binding capacity of permeabilized chromaffin cells. 1 mg of cellular protein corresponds to approx. 3×10^6 chromaffin cells, each containing some 20000 granules [29]. Assuming that the granule content of a chromaffin cell represents 15% of total cellular proteins, then 1 mg granule protein is equivalent to 4×10^{11} granules. Estimating an apparent tetanus toxin binding capacity of 0.45 nmol/mg granule protein (fig.2), then one chromaffin granule binds approx. 1000 tetanus toxin molecules. Taking into consideration the known chromaffin cell volume, which is 7.5×10^{-13} l [29], and that at maximum 2×10^7 molecules of toxin could bind to the 2×10^4 granules available per chromaffin cell, it is estimated that the maximal concentration of tetanus toxin required to saturate all the chromaffin granules in a permeabilized cell would be 4×10^{-5} M. This estimation nicely coincides with the concentration of tetanus toxin required to inhibit catecholamine release, experimentally obtained in the present study (fig.1).

Tetanus toxin does not impair catecholamine secretion from intact bovine chromaffin cells, primarily because of the absence of its putative glycolipid receptors on the cell surface [23,30]. However, in neuronal cells which avidly bind this toxin, the toxin affects the neurotransmitter release following internalization and retrograde transport [4,11]. Once the toxin molecules reach their site of action on the granule membranes or, alternatively, on the cytoplasmic face of the neuronal cell membrane [19], they might interfere directly with the fusion of the secretory granule with the neuronal membrane. While the chemical nature of tetanus toxin-binding sites on chromaffin granule membranes requires further elucidation, the present report provides an explanation for the inhibitory potency of tetanus toxin and of botulinum D toxin (which recognizes the same type of gangliosides) to block exocytosis [5,7–9]. We suggest that the polysialoganglioside(s) or glycoprotein(s) on the cytoplasmic face of the chromaffin granule membrane which serve as TT receptor are essential for either the docking of the granule membrane to the plasma membrane [31] or for the fusion process per se.

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