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Tetanus toxin receptor

Specific cross-linking of tetanus toxin to a protein of NGF-differentiated PC 12 cells

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A subclone of rat pheochromocytoma cells expresses high affinity receptors for tetanus toxin on differentiation with NGF [Walton, K.M., Sandberg, K., Rogers, T.B. and Schnaar, R.L. (1988) J. Biol. Chem. 263, 2055–2063]. In the presence of protein cross-linking agents, [^{125}I]tetanus toxin, bound to these cells at 0°C, forms a cross-linked product with apparent molecular weight of 120 kDa. The formation of [^{125}I]tetanus toxin conjugate involves the heavy chain of the toxin, is prevented by cold toxin and it is largely reduced by pretreating cells with proteases. The cross-linked product is formed only upon incubation of the toxin with NGF-differentiated cells. These results suggest that a protein with apparent molecular weight of 20 kDa is involved in the neurospecific binding of tetanus toxin.

Tetanus toxin; Protein receptor; Pheochromocytoma cell; Nerve growth factor; Cross-linker

1. INTRODUCTION

Tetanus toxin induces the spastic paralysis of tetanus by blocking neurotransmitter release at the spinal cord inhibitory inteneurons [1]. In most mammals the LD_{50} of tetanus toxin is in the range 1–5 ng/kg and this extreme potency is due, at least in part, to a very specific binding to neuronal cells [1].

Tetanus toxin (150 kDa) is composed of a heavy chain (100 kDa) and a light chain (50 kDa) held together by a single disulfide bridge and by non-covalent forces [1]. There is evidence that the intracellular activity of the toxin resides in the light chain while the heavy chain is responsible for cell binding and membrane translocation [2,3].

Despite efforts begun nearly one hundred years ago, the nature of the neurospecific receptor of tetanus toxin is still unknown [4,5]. The toxin was shown to bind to negatively charged lipids, particularly to polysialogangliosides, and treatment with neuraminidase decreases toxin binding to cells [6–20]. On the other hand, the sensitivity to proteases of tetanus toxin binding to cells and neuronal membranes suggests that a protein component is involved [21–24]. Research on this issue has been hampered by the lack of cell lines sensitive to tetanus toxin suitable for biochemical investigations.

Recently a NGF-differentiated sub-clone of the rat phenochromocytoma cell line (PC 12) with high affinity tetanus toxin binding sites and inhibition of catecholamine release was described [16,25]. Here we present the results of experiments performed with chemical cross-linking agents that provide a first direct evidence of the interaction of tetanus toxin with a receptor protein at the surface of a neuronal cell line.

Abbreviations: PC 12 cells, rat pheochromocytoma cell line; NGF, nerve growth factor; DSS, disuccinimidyl suberate; DSP, dithio*bis*-(succinimidylpropionate); BSOCOES, *Bis*-2- (succinimidooxycarbonyloxy)ethyl sulfone; HBS, Hanks Balanced Solution supplemented with 1.1 g/l glucose; PMSF, phenylmethylsulfonyl fluoride; TPCK, *n*-tosyl-L-phenylalanine chloromethyl ketone.

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2. EXPERIMENTAL

2.1. *Materials*

Tetanus toxin was purified as detailed before [26] and stored at −80°C in 10 mM Hepes-Na, 50 mM sodium chloride, pH 7.2. [^{125}I]Tetanus toxin was prepared with the Bolton-Hunter reagent (Amersham, U.K.) to a specific activity of about 3.5 mCi/mg with a residual toxicity higher than 85%. Disuccinimidyl suberate (DSS), dithio*bis*-(succinimidylpropionate) (DSP) and *bis*-2-(succinimidooxycarbonyloxy)ethyl sulfone (BSOCOES) were from Pierce (Oud Beijerland, The Netherlands). TPCK treated-trypsin, α-chymotrypsin and octyl glucoside were from Boehringer (Mannheim, Germany) and pronase from Calbiochem (San Diego, USA). PMSF, iodoacetamide, benzamidine, TPCK, *o*-phenantroline and *Clostridium perfringens* neuraminidase were from Sigma (St. Louis, MO, USA). Antiserum anti-tetanus toxin was raised by immunizing New Zealand white rabbits with tetanus toxoid (Sclavo, Siena, Italy) following the procedure of Vaitukaitis [27]. Antisera versus the light chain and the heavy chain of tetanus toxin were obtained by immunizing rabbits with the isolated chains purified as described [28]. The immunoglobulin fraction was isolated and affinity purified using the immobilized antigen as described previously [29].

2.2. *Cells and cell cultures*

The PC 12 subclone described in [16,25] was generously provided by Dr. T.R. Rogers (University of Maryland School of Medicine) and

Dr. L. Rosenthal (Istituto Scientifico San Raffaele, Milan) and was cultured according to the method described by Greene et al. [30]. Briefly, cells were grown at 37°C on plastic dishes (100 or 24 mm diameter) coated with collagen as described [16]. Cells were cultured in Dulbecco's modified Eagle's medium (Flow, UK; supplemented with 44 mM sodium bicarbonate), 7.5% horse serum and 7.5% fetal calf serum, pH 7.4 in a humidified atmosphere of 5% CO_2 in air. Weekly subcultures were performed with initial cell density of 0.5–1 million cells per 100 mm diameter dish. Experiments were performed on cells seeded on 24 mm dishes (20 000 cells per dish) and differentiated with 50 ng/ml of NGF extracted from mouse submammillary glands as described [31].

2.3. *Tetanus toxin cell binding and cross-linking*

Cells, treated or not treated with NGF, were cooled on ice, washed with cold HBS, pH 7.5, and incubated with [^{125}I]tetanus toxin (0.5–1 nM) for 2 h at 0°C. The procedure for cross-linking is adapted from previous work [32–37]. Briefly, after two washings with cold HBS, cells were treated with DSS or BSOCOES or DSP dissolved in HBS (200 μg/ml from 10 mg/ml stock solutions in dry freshly distilled DMSO) at 0°C for 20 min. The medium was removed and replaced with HBS containing 20 mM glycine–NaOH, pH 7.5 for 5 min. Cells were washed two times with HBS and dissolved in 1 ml of HBS containing 0.4% octyl glucoside, 1 mM iodoacetamide, 1 mM benzamidine, 0.1 mM PMSF, 0.1 mM *o*-phenantroline, and 10 μM TPCK. The samples were centrifuged in a refrigerated table centrifuge for 5 min and supernatants were made 6.5% in trichloroacetic acid. Pellets were recovered by centrifugation and dissolved in 8% SDS, 10 mM Tris-acetate, 0.1 mM EDTA, pH 6.8, containing 5% 2-mercaptoethanol. Samples were boiled for 2 min and analyzed by sodium dodecylsulfate gel electrophoresis on a discontinous system (6–12% polyacrylamide gradient) according to [38]. After Coomassie Blue staining, gels were dried and exposed to Kodak X-Omat films at −80°C. The films were scanned with a dual wavelength densitometer (Shimadzu CS-930).

In some experiments, before incubation with [^{125}I]tetanus toxin, cells were treated with pronase (1–10 μg/ml) or with neuraminidase (25–50 mU/ml) in HBS for 30 min at 37°C or with a mixture of trypsin (0.5 mg/ml) and chymotrypsin (0.5 mg/ml) in HBS for 6 min at 37°C. After two washings with 1 ml of cold HBS, cells were incubated with HBS containing protease inhibitors for 5 min at 0°C and then treated with BSOCOES as described above. Cells were counted on lysates by using 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; Boehringer, Mannheim, Germany) [39]. Briefly, 5 μl of cell lysates were mixed with 1.5 ml of 10 mM Tris-HCl, 10 mM EDTA, 100 mM sodium chloride, 0.05% Triton X-100, 100 ng/ml DAPI, pH 7.0, and fluorescence was measured.

2.4. *Immunoblotting*

After tetanus toxin binding and cross-linking, cells were solubilized as described above and treated with affinity purified rabbit immunoglobulins anti-tetanus toxin. The mixture was incubated for 2 h at 4°C and 200 μl of 10% Protein A-Sepharose (Pharmacia, Uppsala, Sweden) was added and incubation prolonged for additional 2 h. The pellet was collected by centrifugation, washed twice with HBS containing 0.4% octyl glucoside, solubilized and electrophoresed as above. Proteins were transferred onto nitrocellulose, incubated with affinity-purified rabbit immunoglobulins anti either the light chain or the heavy chain of tetanus toxin and stained with goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase (Boehringer, Mannheim, Germany) as described [40].

3. RESULTS AND DISCUSSION

The cellular structures involved in the neurospecific high affinity cell binding of tetanus toxin are still undetermined. Both polysialogangliosides and protein have been proposed to play a receptor role [2–5]. Recently a subclone of the PC-12 cell line was shown to bind tetanus toxin with consequent inhibition of catecholamine release after differentiation with NGF [16,25]. The induction of neurite growth by NGF on this subclone is apparent already after two days and an extensive neuronal network is established within one week (not shown).

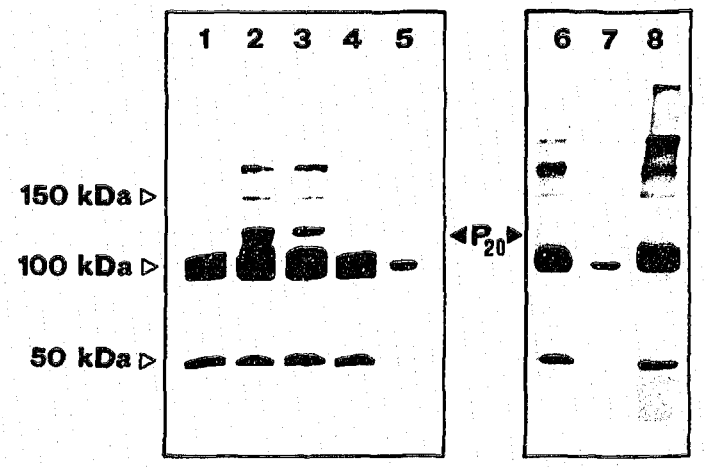

Fig. 1. Cross-linking of [^{125}I]tetanus toxin to PC-12 cells. Cells from a PC-12 subclone [16] (lanes 1–5 and 7) or PC-12 cells [30] (lane 8) were cultured in the presence (lanes 1–5 and 8) or in the absence (lane 7) of NGF (50 ng/ml) for 15 days. After washing, the cells were incubated at 0°C with [^{125}I]tetanus toxin (0.5–1 nM) for 2 h; the sample of lane 5 contained a 300-fold excess of cold toxin. After washing, cross-linkers were added: none in the sample of lane 1, DSS (lane 3); BSOCOES (lanes 2, 5–8), DSP (lane 4). Lane 6 refers to [^{125}I]tetanus toxin incubated in the absence of cells and treated with BSOCOES as above. Proteins were electrophoresed under reducing conditions and, after Coomassie Blue staining, gels were dried and exposed to Kodak X-Omat films at −80°C. The two major radioactive bands represent the heavy (100 kDa) and the light chains (50 kDa) of tetanus toxin and the bands at apparent molecular weight 150 kDa and larger are due to cross-linking of the toxin chains among themselves. P_{20} indicates a protein band with apparent M_w 120 kDa which is formed uniquely in the presence of non-cleavable cross-linkers when the [^{125}I]tetanus toxin is incubated with the PC-12 subclone after differentiation with NGF.

Chemical cross-linkers have been used successfully in the identification of the cell surface protein receptors of hormones, growth factors and diphtheria toxin [32–37]. Here we have used three homofunctional protein cross-linkers: DSP, DSS and BSOCOES (differing for solubility properties and cleavability by reducing agents) to investigate the possible involvement of a protein in the binding of tetanus toxin to the PC-12 subclone described above.

Cells were incubated with [^{125}I]tetanus toxin in the cold and, after washing, cross-linker was added under a variety of conditions. Cell proteins were recovered and subjected to SDS-PAGE in the presence of reducing agents. Fig. 1 shows that in the presence of DSS and BSOCOES (lanes 2 and 3) new radioactive bands are formed. A set of bands of apparent M_w 150 kDa and larger derive from cross-linking of the toxin heavy and light chains among themselves since they also occur

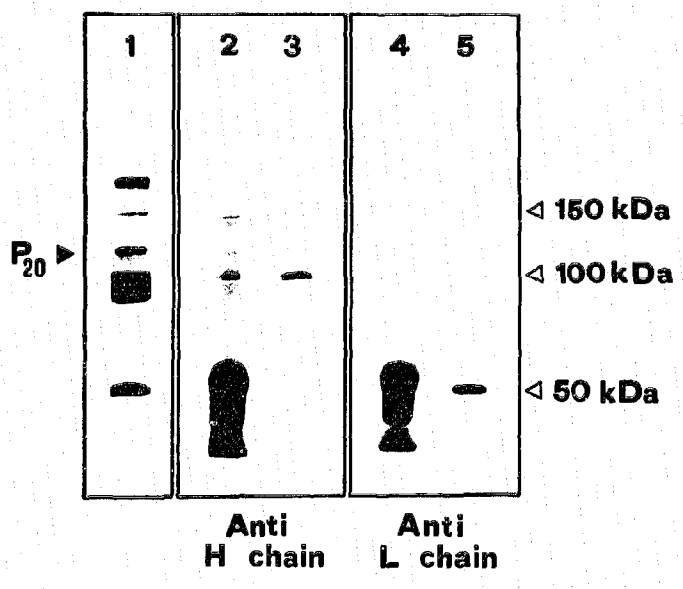

Fig. 2. Immunoblotting of tetanus toxin after binding and cross-linking to PC-12 cells. Lane 1 is an autoradiographic image of a sample treated as in lane 3 of Fig. 1. Lanes 2 and 4 refer to NGF-differentiated PC-12 subclone cells incubated with tetanus toxin and BSOCOES, dissolved and immunoprecipitated with an affinity-purified rabbit anti-tetanus toxin antiserum and Protein A-Sepharose. The sample was electrophoresed, electroblotted on nitrocellulose and immunostained with affinity-purified rabbit anti-tetanus toxin heavy chain antiserum (lane 2; lane 3 is a control of 50 ng of tetanus toxin) or anti light chain (lane 4; lane 5 is a control of 50 ng of tetanus toxin) and goat anti-rabbit IgG antiserum conjugated with alkaline phosphatases. Staining in the lower part of lanes 2 and 4 is related to the rabbit immunoglobulins used for immunoprecipitation.

with the toxin alone (lane 6). The only band which appears uniquely upon cell incubation has an apparent M_w of 120 kDa, as deduced from its migration with respect to protein standards. This band is absent if DSP, a cross-linker cleaved by reduction, is used (lane 4) or in the absence of cross-linker (lane 1) or in the presence of an excess of cold toxin (lane 5). Fig. 1 also shows that the tetanus toxin binding protein is virtually absent in the undifferentiated cells of the PC-12 subclone (lane 7) and also in the NGF-differentiated PC-12 cells established by Greene et al. (lane 8) [30].

The amount of cross-linked conjugate is low and this correlates with the low number of high affinity binding sites evidenced by binding studies [16]. For this reason it is not immediately apparent which of the two toxin subunits is involved in the formation of the cross-linked product. From densitometric scanning, it appears that upon cross-linking there is no decrease of the light chain band, rather there is a decrease of the ratio heavy chain over light chain thus suggesting an involvement of the heavy chain of the toxin. To substantiate this point, tetanus toxin, after binding and cross-linking to NGF-differentiated cells, was immunoprecipitated with affinity purified rabbit anti-tetanus toxin immunoglobulins. Fig. 2 shows that the 120 kDa that appears upon cross-linking is stained by affinity-purified anti-tetanus toxin heavy chain immunoglobulins and not by anti light chain immunoglobulins. These findings are in agreement with studies indicating that the heavy chain of tetanus toxin is involved in cell binding [41–43] in analogy to the closely related botulinum neurotoxins [44–46].

Table I

Effect of proteases and neuraminidase treatment on the amount of 120 kDa [^{125}I]tetanus toxin-conjugate and on the total [^{125}I]tetanus toxin bound to PC-12 cells

	A	B
PC-12 subclone cells	100%	100%
Cells + pronase	35–40%	40–50%
Cells + trypsin/chymotrypsin	65–70%	55–65%
Cells + neuraminidase	95–105%	75–85%

The amount of cross-linked product (column A) and the total radioactivity bound to cells (column B) are expressed as percentage of the corresponding amount found in the absence of pretreatments taken as 100%.

These results indicate that, upon binding to the cell surface of this neuronal cell line, tetanus toxin interacts specifically and closely with a protein via its heavy chain. If a cross-linker is present, a covalent conjugate of about 120 kDa is formed. The apparent molecular weight of the toxic binding protein is 20 kDa, deduced from the difference between the migration in SDS-PAGE of the conjugate and that of the toxin heavy chain.

Previous studies have shown that the binding of tetanus toxin to neuronal cells in culture and to neuronal membranes is sensitive to trypsin, chymotrypsin and pronase and also to neuraminidase [10,22–24]. It is not clear, however, if the effect of these enzymes is due to a direct effect on a proteinic sialylated receptor or to a indirect perturbative effect on other membrane components. Table I shows that proteases largely decrease the amount of tetanus toxin cross-linked product thus indicating that a binding component of tetanus toxin on NGF-differentiated PC-12 cells is indeed a protein. Treatment with neuraminidase does not influence the amount of tetanus toxin conjugate while it does reduce the total amount of toxin bound to cells. This result suggests that at least part of the toxin binding to these cells is mediated by polysialogangliosides.

In conclusion, this study presents compelling evidence that a 20 kDa protein is involved in the binding of tetanus toxin via its heavy chain to a neuronal cell line in culture differentiated with NGF. Together with the demonstration that tetanus toxin binds to negatively charged lipids [6–20], the present findings are in favour of a double receptor model for the binding of tetanus toxin to neuronal cells [2,3,5] and correlate with the results obtained from physiological studies on *Aplysia* neurons [41,45,46].

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REFERENCES

[1] Simpson, L.L. (ed.) (1989) Botulinum Neurotoxin and Tetanus Toxin, Academic Press, New York.
[2] Niemann, H. (1991) in: Bacterial Protein Toxins: a Source Book (Alouf, J.E. and Freer, J.H. eds.) Academic Press, London, in press.
[3] Montecucco, C. (1989) in: Eight International Conference on Tetanus, (Nisticò, G., Bytchenko, B., Bizzini, B. and Triau, R. eds.) pp. 71–91, Pythagora Press, Rome.
[4] Middlebrook, J.L. (1989) in: Botulinum Neurotoxin and Tetanus Toxin. (Simpson, L.L. ed.) pp. 95–119. Academic Press, New York.
[5] Montecucco, C. (1986) Trends Biochem. Sci. 11, 314–317.
[6] Van Heyningen, W.E. and Miller, P.A. (1961) J. Gen. Microbiol. 24, 107–119.
[7] Mellanby, J., Mellanby, H., Pope, D. and Van Heyningen, W.E. (1968) J. Gen. Microbiol. 54, 161–168.
[8] Helting, T.B., Zwisler, O. and Wiegandt, H. (1977) J. Biol. Chem. 252, 194–196.
[9] Holmgren, J., Elwing, H., Fredman, P. and Svennerholm, L. (1980) Eur. J. Biochem. 106, 371–379.
[10] Yavin, E. (1984) Arch. Biochem. Biophys. 230, 129–137.
[11] Habermann, E. and Tayot, J.L. (1985) Toxicon 23, 913–920.
[12] Critchley, D.R., Nelson, P.G., Habig, W.H. and Fishman, P.H. (1985) J. Cell Biol. 100, 1499–1507.
[13] Habermann, E., Bigalke, H. and Heller, I. (1981) Naunyn-Schmiedeberg's Arch. Pharmacol. 316, 135–142.
[14] Lazarovici, P., Yanai, P. and Yavin, E. (1987) J. Biol. Chem. 262, 2645–2651.
[15] Montecucco, C., Schiavo, G., Gao, Z., Bauerlein, E., Boquet, P. and DasGupta, B.R. (1988) Biochem. J. 251, 919–924.
[16] Walton, K.M., Sandberg, K., Rogers, T.G. and Schnaar, R.L. (1988) J. Biol. Chem. 263, 2055–2063.
[17] Nathan, A. and Yavin, E. (1989) J. Neurochem. 53, 88–94.
[18] Marxen, P., Fuhrmann, U. and Bigalke, H. (1989) Toxicon 27, 849–859.
[19] Fujita, K., Guroff, G., Yavin, E., Goping, G., Orenberg, R. and Lazarovici, P. (1990) Neurochem. Res. 15, 373–383.
[20] Schiavo, G., Demel, R. and Montecucco, C. (1991) Eur. J. Biochem. (in press).
[21] Critchley, D.R., Habig, W.H. and Fishman, P.H. (1986) J. Neurochem. 47, 213–221.
[22] Yavin, E. and Nathan, A. (1986) Eur. J. Biochem. 154, 403–407.
[23] Pierce, E.J., Davison, M.D., Parton, R.G., Habig, W.H. and Critchley, D.R. (1986) Biochem. J. 236, 845–852.
[24] Lazarovici, P. and Yavin, E. (1986) Biochemistry 25, 7047–7054.
[25] Sandberg, K., Berry, C.J. and Rogers, T.R. (1989) J. Biol. Chem. 264, 5679–5686.
[26] Schiavo, G., Papini, E., Genna, G. and Montecucco, C. (1990) Infect. Immun. 58, 4136–4141.
[27] Vaitukaitis, J.L. (1981) Methods Enzymol. 73, 46–52.
[28] Matsuda, M. and Yoneda, M. (1975) Infect. Immun. 12, 1147–1153.
[29] Bisson, R. and Schiavo, G. (1986) J. Biol. Chem. 261, 4373–4376.
[30] Greene, L.A., Aletta, J.M., Rukenstein, A. and Green, S.H. (1987) Methods Enzymol. 147, 207–216.
[31] Mobley, W.C., Schenker, A. and Shooter, E.M. (1976) Biochemistry 15, 5533–5552.
[32] Howard, A.D. (1985) J. Biol. Chem. 260, 10833–10839.
[33] Park, L.S. (1986) J. Biol. Chem. 261, 205–210.
[34] Laburthe, M., Breant, B. and Rouyer-Fessard, C. (1984) Eur. J. Biochem. 139, 181–187.
[35] Ioannides, C.G. (1987) Proc. Natl. Acad. Sci. USA 84, 4244–4248.
[36] Dukovich, M., Wano, Y., Bich Thuy, L., Katz, P., Cullen, B.R., Kehrl, J.H. and Greene, W.C. (1987) Nature 327, 518–522.
[37] Cieplak, W., Gaudin, H.M. and Eidels, L. (1987) J. Biol. Chem. 262, 13246–13253.
[38] Laemmli, U.K. (1970) Nature 227, 680–685.
[39] Russell, W.C., Newman, C. and Williamson, D.H. (1975) Nature 253, 461–462.
[40] Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[41] Poulain, B., Mochida, S., Weller, U., Hogy, B., Habermann, E., Wadsworth, J.D.F., Shone, C., Dolly, J.O. and Tauc, L. (1991) J. Biol. Chem. 266, 9580–9585.
[42] Fishman, P.S. and Carrigan, D.R. (1987) Brain Res. 406, 275–279.
[43] Johnstone, S.R., Morrice, L.M. and van Heyningen, S. (1990) FEBS Lett. 265, 101–103.
[44] Bandyopadhyay, S., Clark, A.W., DasGupta, B.R. and Sathyamorthy (1987) J. Biol. Chem. 262, 2660–2663.
[45] Poulain, B., Mochida, S., Wadsworth, J.D.F., Weller, U., Habermann, E., Dolly, J.O. and Tauc, L. (1990) J. Physiol. (Paris) 84, 247–261.
[46] Poulain, B., Wadsworth, J.D.F., Shone, C.C., Mochida, S., Lande, S., Melling, J., Dolly, J.O. and Tauc, L. (1989) J. Biol. Chem. 264, 21928–21933.