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TETANUS TOXIN AFFECTS THE K^+-STIMULATED RELEASE OF CATECHOLAMINES FROM NERVE GROWTH FACTOR-TREATED PC12 CELLS

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Summary. Tetanus toxin specifically binds to neuronal surfaces and interferes with the release of transmitters. The effect of tetanus toxin pretreatment of PC12 cell line, taken as a model of neuronal cells in culture, was studied and found that it depresses depolarization-dependent catecholamines secretion. This effect is limited to PC12 cells fully differentiated by the action of Nerve Growth Factor (NGF) and is indicative of the expression of specific binding sites for tetanus toxin during transition from the undifferentiated state. Specific binding of [^{125}I] tetanus toxin to NGF-treated PC12 was demonstrable. The toxin has no effect on the ^{45}Ca accumulation coupled with the depolarization dependent release of catecholamines.

When the cells in culture of the line PC12 (1) derived from a rat pheochromocytoma are grown with nanomolar concentrations of NGF arrest division, sprout electrically excitable neurites and transform into cells possessing several biochemical and immunological properties of a mature neurone. Both NGF-treated and -untreated PC12 cells have been found to release catecholamines in a Ca^{2+} dependent manner when the membrane is depolarized by a high concentration of external K^+ or when the acetylcholine receptor is activated by the cholinergic agonist charbamilcholine (2,3). Therefore, PC12 cells constitute an useful neurone-like cell system in which to examine secretory events induced

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Abbreviations: DA, Dopamine; FUDR, 5-Fluoro-2' deoxyuridine; HEPES, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid; NGF, Nerve Growth Factor; α-LTx, α-Latrotoxin; NE, Norepinephrine; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis.

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by physiological means. In addition this cell line has been used to investigate the ionophore properties of a naturally occurring presynaptic neurotoxin α-LTx, by studying toxin dependent catecholamine release and ^{45}Ca uptake (4).

Tetanus toxin (5) is another natural toxin considered to act presynaptically by interfering with transmitter release: the toxin is a large molecular weight protein which specifically binds to neuronal surfaces (6) and depresses some presynaptic functions (7,8). It was initially considered to be a potentially useful agent capable of modifying α-LTx action on PC12 , but found in the present study, to affect primarily if not exclusively depolarization-coupled release of catecholamines. The ability of tetanus toxin to reduce Ca^{2+}-dependent K^+ stimulated release of [^{3}H] NE or [^{3}H] DA is limited to PC12 cells grown with NGF and is not consequence of a reduced calcium uptake. Binding studies using [^{125}I] labelled tetanus toxin indicate that the toxin displays indeed nanomolar affinity for NGF-treated PC12.

MATERIALS AND METHODS

General. Tetanus toxin was a generous gift of Dr. B. Bizzini (Institute Pasteur, Paris).The liophylized toxin was equilibrated in 50 mM HEPES buffer pH 7.4 by extensive dialysis. Aliquots of the resulting solution having LD50 10 ng Kg^{-1} in mice s.c. were kept frozen at -30°C. In all the experiments reported the concentration of tetanus toxin is expressed on a molar basis, taking 150.000 dalton its molecular weight. Mouse NGF was kindly supplied by Dr. C. Cozzari of this Institute. Veratridine was purchased from Sigma and FUDR from Serva. Bolton-Hunter reagent was purchased from Amersham at 2000 Ci/mmol as well as [^{3}H] NE (30 Ci/mmol) or [^{3}H] DA (40 Ci/mmol) and $^{45}CaCl_2$ (30 mCi/mg). All other reagents were of the highest purity available.

Cell culture. PC12 cells were kindly supplied by Dr. Calissano of this Institute. These cells were subcloned from the PC12 cells (clonal rat pheochromocytoma) established by Dr. Greene and Tishler (1). PC12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 5% heat-inactivated horse serum in a humidified atmosphere of 8% CO_2 and 92% air. One week before experiment the cells were subcultured on a polylisine-coated Falcon dish (35 mm) at a density of 1.5×10^6 cells/dish. NGF in nmolar concentration (2.5 nM) was present in the medium and maintained for the all lenght of the culture. NGF-untreated cells were plated at the same density 48 hrs before experiment.

Assay of transmitter release. Tetanus toxin-treated or untreated cells were washed and incubated at 37°C in 1 ml of the assay medium pH 7.3 (126 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM K_2HPO_4,

30 mM HEPES, 5 mM glucose and 1 mM Ascorbic acid) supplemented with 10^{-7} M [^{3}H] DA or [^{3}H] NE in presence of 0.2 mM Pargyline (3). Following incubation the cells were washed three times with 1 ml aliquots of incubation medium or high K^+ medium. High potassium media were prepared by reducing sodium chloride molarity accordingly with the desired molarity of potassium chloride. The releasing medium was collected from the dishes directly into scintillation vials, by careful suction. At the end of the experiment cells were solubilized with 1 ml of 0.5% SDS and assayed for radioactivity in five volumes of Pico-Fluor 30 (U.T. Packard). The radioactivity released was expressed as percentage of total radioactivity: radioactivity released plus that remaining in the cells. The cells remain firmly attached to the substrate during the whole experiment. Parallel experiments performed centrifuging the sucked media indicated that the total of radioactivity was found in the fluid phase.

Assay of calcium accumulation. Experiments of ^{45}Ca uptake were performed essentially as described (4).

Labelling tetanus toxin with ^{125}I. Tetanus toxin was labelled with ^{125}I according to the method of (6) using the Bolton and Hunter reagent. The reaction mixture was then chromatographed on a Sephadex G25 column, eluting with 30 mM Tris HCl, pH 8.4, 10 mg ml^{-1} gelatin. The pooled sample of [^{125}I] tetanus toxin eluting as the first radioactive peak off the column had a specific radioactivity of 3 $\mu Ci\ \mu g^{-1}$. The purity of [^{125}I] tetanus toxin was measured by analyzing the distribution of radioactivity on SDS-PAGE and developing the gels by autoradiography.

RESULTS AND DISCUSSION

PC12 cells have been extensively used as a model system of neurons for studying the molecular mechanism of transmitter secretion (9). In addition when PC12 cells preloaded with [^{3}H] DA or [^{3}H] NE were exposed in a normal medium to nanomolar concentrations of α-LaTx, the presynaptic neurotoxin known to stimulate transmitter secretion at nerve endings, they profoundly released the mediator in the external medium in a calcium dependent manner (4). On the basis of these observations initially we have considered the possibility of studying the effect of a presynaptic toxin like tetanus toxin, but inhibitory of the secretory event on the secretion of transmitters induced by α-LaTx. Preliminary studies indicated that the secretion of [^{3}H] DA induced by 8 nM α-LaTx in NGF-treated and -untreated PC12 cells is not affected substantially by a pretreatment with tetanus toxin. PC12 which had fully differentiated as a consequence of prolonged NGF treatment (4-7 days) showed, when pretreated with μM concentrations of tetanus toxin, a clear and reproducible reduction in the amount of [^{3}H] DA or [^{3}H] NE

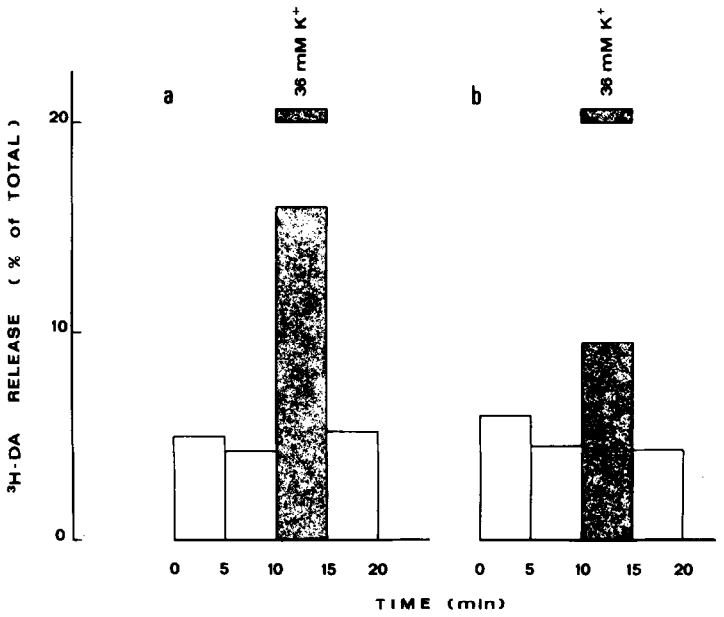

Fig. 1. Influence of tetanus toxin on [^{3}H] DA release in response to K^+ depolarization.
At the end of the uptake period control culture (a) or tetanus toxin treated cultures (b) were washed and brought to 25°C, incubated for 5-min periods with 1-ml aliquots of assay medium. After two 5-min intervals, a medium containing 36 mM K^+ was added (filled bars) to the cultures. Incubation condition are described in the text. Tetanus toxin-treated PC12 showed to take up [^{3}H]DA as effectively as control cells. A statistically significant reduction ($P > 0.01$) in [^{3}H] DA release in response to K^+ was produced by tetanus toxin pretreatment.

released upon high (36 mM) K^+ stimulation (Fig. 1). High K^+ medium was replaceable as a releaser agent by veratridine (10). The effect of either agent was depressed to a comparable degree by tetanus toxin pretreatment as shown in Fig. 2 reporting response data for three different concentrations of the agents. The efficacy of tetanus toxin was strongly temperature dependent being maximal at 37°C, and within certain limits (2 hr) was depending on the length of treatment. Experiments correlating pretreatment time with the inhibition of release (0.5, 1, 2, 3 hr) showed that incubation for 3 hr did not substantially improve the effect after 2 hr and this was the incubation time chosen for the majority of experiments reported. The consideration that a culture dish of NGF-treated PC12 (4-6 days), reflects in various degree a collection of cells in slow transition from the round shape,

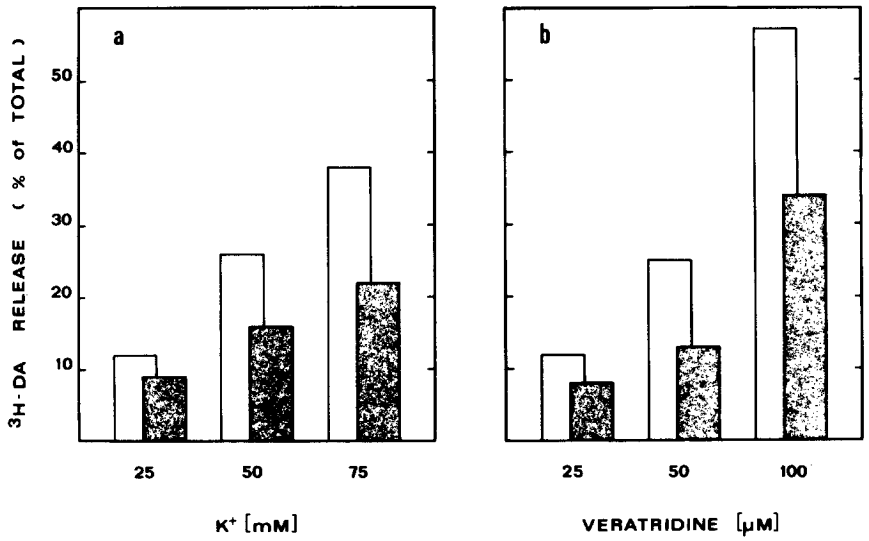

Fig. 2. Dose response relationship between three different K^+ a) and veratridine b) concentrations in control (open bars) and tetanus toxin treated cells (filled bars). Incubation condition as in Fig. 1. Each bar represents the mean (n = 4) of [^{3}H]DA released during the 5 min incubation with the indicated agent, corresponding with the filled area of Fig. 1. The S.D. for each group was less than 10% of the respective mean.

undifferentiated cell to the fully differentiated neurone-like one, sensitive to tetanus toxin, resulted very important in order to augment the inhibitory effect of tetanus toxin. NGF-treated cells were then synchronized by a treatment with FUDR, 48 hrs before the release experiment. While non dividing cells survive the treatment with an inhibitor of DNA synthesis, the dividing ones (not NGF committed) will degenerate and perish (11). Following FUDR the release of[^{3}H]NE induced by 50 mM K^+ after tetanus toxin poisoning is reduced to 35% that of toxin-untreated controls. There are reasons to believe that the K^+-dependent secretory mechanism of NGF-treated cells is not different from that of the untreated ones since in both conditions catecholamines release can be equally stimulated by depolarizing agents (2) or by agonists of the acetylcholine receptor (3). It was concluded that the differential response to tetanus toxin was probably consequence of the fact that only NGF-treated cells express a membrane's constituent which acts as a receptor for tetanus toxin. To test this prediction we used tetanus toxin jodinated by Bolton and

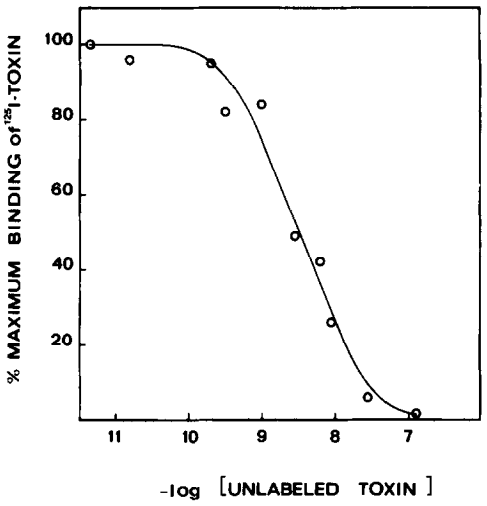

Fig. 3. Displacement of [^{125}I] tetanus toxin binding to NGF-treated PC12 by unlabeled tetanus toxin.
[^{125}I] tetanus toxin (20.000 cpm) was incubated with NGF-treated PC12. Incubations were performed in triplicate in 16 mm wells of tissue culture cluster originally plated with 3 x 10^5 cells $well^{-1}$ for 2 hr. The cells were then washed twice with incubation medium resuspended, centrifuged in a Eppendorf microfuge and counted in a LKB gamma counter after suction and wash of the supernatant. Specifically bound [^{125}I] tetanus toxin is defined as the excess binding over control wells containing the same amount of [^{125}I] tetanus toxin, but an increasing excess of unlabeled toxin. Each point is the mean of 2 triplicate determinations.

Hunter procedure in order to show the presence of specific binding sites in these cells. The [^{125}I] labeled tetanus toxin used was homogenous on SDS-PAGE and autoradiographs of the gels showed that the labeling procedure did not give rise to bands attributable to degradation products. The evaluation of tetanus toxin affinity for PC12 cells was determined by displacing [^{125}I] tetanus toxin from NGF-treated and -untreated PC12 cells with unlabeled toxin (Fig. 3). The concentration of unlabeled toxin that displaced 50% of [^{125}I] tetanus toxin binding was $4x10^{-8}$ M for NGF-treated PC12 cells, while even 10^{-7} M tetanus toxin had a negligible displacing effect on the radioactivity bound to NGF-untreated cells. This observation was confirmed by indirect immunofluorescence experiments whereby only NGF-treated PC12 cells showed to give a positive reaction (data not shown). These results allow to conclude that, i) the plasma membrane of PC12 cells

before and after treatment with NGF can be distinguished on the basis of its affinity to tetanus toxin, ii) the expression of tetanus toxin-specific recognition sites, belonging to a group of delayed responses promoted by NGF (12), makes the cells susceptible to toxin action. The nature and composition of these sites (gangliosides), has however not been investigated. Conceivably, as is known to occur in several neuronal preparation in culture (see inter alia, 13,14,15), after interaction, the toxin is internalized. Thus, the next questions arising concern the molecular action of the toxin, and the cell mechanism by which the depolarization-coupled release of transmitters is depressed. To test wether tetanus toxin treatment might affect depolarization-dependent calcium influx, we studied ^{45}Ca accumulation by NGF-treated PC12 upon the action of 56 mM K^+. While K^+-dependent DA release is reduced (Fig. 1 and Fig.2), the ^{45}Ca accumulated by tetanus toxin-poisoned cells is not significantly different from that of the untreated control cells (6898±854 n=8 and 7266±1002 n=8 respectively; values given as cpm/dish/15 min, are means ±SEM). These findings rule out as a plausible mechanisms of action, a direct effect of the toxin on calcium fluxes but leave open the possibility of an effect at a step in transmitter release after calcium entry into the cells.

In summary we have shown that following prolonged NGF treatment, PC12 express cell surface receptors for tetanus toxin, become vulnerable to toxin action and depolarization-evoked release of catecholamines is depressed. The toxin has however little or no effect on the associated influx of extracellular calcium. The action of α-Latx, which evokes catecholamines release from PC12 probably by raising the level of calcium in the cytosol (4) is not affected.

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