A Functional Domain on the α-Latrotoxin Molecule, Distinct from the Binding Site, Involved in Catecholamine Secretion from PC12 Cells: Identification with Monoclonal Antibodies†

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ABSTRACT: Seven monoclonal antibodies (mAbs) have been produced against α-latrotoxin (α-Latx), the toxin component of black widow spider venom that stimulates release of neurotransmitters from PC12 cells. These mAbs were characterized by (i) an enzyme-linked immunosorbent assay and by (ii) neutralization analysis of the secretagogue properties of the toxin. The production of a panel of mAbs, possibly directed against different epitopes of α-Latx, provides a useful set of reagents to dissect the molecular regions of the toxin having different functions and to describe steps of its mode of action in responsive cells. Attention was focused on one of these mAbs (4C4.1), which (1) inhibits in a dose-dependent fashion both toxin-stimulated and crude venom stimulated dopamine release from PC12 cells, (2) prevents toxin-induced $^{45}Ca^{2+}$ accumulation in PC12, (3) alters toxin-dependent phosphoinositide breakdown, and (4) prevents toxin-induced channel formation in artificial lipid bilayers. Since, within certain experimental conditions, mAb 4C4.1 is able to recognize the toxin bound to cells, we conclude that its effects were not a consequence of a direct interference with binding. On the basis of kinetic analysis of mAb interference on toxin action, expressed as accumulation of inositol phosphates and transmitter secretion, we suggest that the described effects result primarily from the blockade of an event immediately successive to binding and central for the full expression of toxin action. The availability of mAb 4C4.1 now makes possible the molecular characterization of the toxin moiety responsible for such an event.

The polypeptide neurotoxin α-latrotoxin (α-Latx)[1] (Grasso, 1976; Frontali et al., 1976), isolated from the venom of the italian black widow spider (*Latrodectus mactans tredecimguttatus*), has become in recent years a specific and invaluable natural probe for the study of neurotransmission and presynaptic function. The secretagogue action of this neurotoxin has been quantitatively described (i) at the neuromuscular junction of the mouse and frog (Frontali et al., 1976; Misler & Hurlbut, 1979), where it triggers a massive increase in the frequency of the miniature endplate potentials, followed by vescicles depletion, (ii) in rodent brain synaptosomes (Grasso, 1976; Grasso & Senni, 1979; Nicholls et al., 1982), and (iii) in the cloned neurosecretory cell line (PC12), where it causes a sustained release of neurotransmitters (Grasso et al., 1980). The working hypothesis that, after the primary interaction with the cell, α-Latx acts by forming toxin-specific, voltage-dependent cation channels through which calcium can flow into the cells and trigger neurotransmitter secretion (Grasso et al., 1980) received experimental support from the findings that α-Latx-induced channels in artificial lipid bilayers have properties reminiscent of cellular voltage-dependent ionic channels (Robello et al., 1984). Alternatively, a recent hypothesis based on PIs breakdown observed after toxin treatment of PC12 suggests the involvement of a transmembrane signalling event (Vicentini & Meldolesi, 1984).

The experimental strategy we have adopted to better understand the process of toxin interaction with PC12 leading to the toxin-mediated calcium influx and DA release has been to produce monoclonal antibodies against different epitopes of the toxin molecule (Cattaneo & Grasso, 1984), to be used later as molecular probes for the dissection and identification of the moieties of the α-Latx molecule having different functions.

We describe hereafter a monoclonal antibody reacting with α-Latx polypeptide that totally inhibits its secretagogue action in PC12 cells in terms of calcium influx, transmitter release, and PIs hydrolysis. The antibody also prevents channel formation in artificial lipid bilayers where α-Latx receptors are not present. Thus, the site recognized by the mAb seems to be involved in the events leading to channel formation rather than in the first interaction with cells. Some of the work relative to mAbs production and characterization has been presented in preliminary form (Cattaneo & Grasso, 1984).

Experimental Procedures

Toxin Purification. α-Latx was purified from the venom of female spiders, *Latrodectus mactans tredecimguttatus*, according to published procedures (Grasso, 1976) and to published criteria of homogeneity. α-Latx represents a class of closely related acidic isotoxins of M_r 125 000 (Frontali et al., 1976) possessing toxicity to mouse in the range of 0.1–0.02 μg/g of body weight (LD_{50}) and affecting secretion of endogenous transmitters from synaptosomes or PC12 cells in culture with an ED_{50} ranging from 10^{-10} to 10^{-9} M (Grasso

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[1] Abbreviations: mAb, monoclonal antibody; α-Latx, α-latrotoxin; ELISA, enzyme-linked immunosorbent assay; DA, dopamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, poly(ethylene glycol); HS, horse serum; FCS, fetal calf serum; HR, HEPES-buffered Ringer saline; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HAT, hypoxanthine–aminopterin–thymidine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PIs, phosphoinositides; InPs, inositol phosphates; BLM, black lipid membranes; OPD, 1,2-benzenediamine.

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et al., 1982). ED_{50} is defined as the concentration of toxin causing 50% of its maximum measurable effect.

Generation of Hybridoma Lines. Balb/C female mice were immunized intraperitoneally with 25 μg of a partially purified fraction of α-Latx inactivated by emulsifying with complete Freund's adjuvant, followed after 4 weeks by injection of the same amount of immunogen in incomplete Freund's adjuvant, and eventually challenged with pure soluble α-Latx 6 weeks later. Three days after the last injection, spleen cells were fused with P3-AgX63 myeloma cells (Galfrè & Milstein, 1981) in the presence of PEG 1500 and distributed into 96 wells (10^6 cells/well) of plastic plates containing HAT medium and 10% FCS in DMEM. Following incubation in 5% CO_2 at 37 °C, media with growing colonies were assayed for antibody by ELISA with a purified preparation of toxin as an antigen. Colonies from positive wells were transferred to appropriate wells containing normal culture medium. Cell lines were then cloned by limiting dilution techniques, and ascites was produced by injecting about 2×10^6 cells into Balb/C mice, primed (10 days) with 0.5 mL of Pristane.

Purification of mAbs. The crude IgG fractions from culture supernatants were obtained by ammonium sulfate precipitation at 45% saturation. After dissolution and dialysis, the mAbs were affinity purified by absorption onto protein A–Sepharose. The mAbs were then eluted by citrate buffers, 0.1 M, pH 6.0, 4.5, and 3.0. The purity of mAbs was monitored by 10% polyacrylamide gel electrophoresis, in the presence of SDS. The apparent titers of mAbs were determined by adding a serial dilution of mAb to a known α-Latx concentration and determining the resultant dilution able to neutralize 50% of the toxin maximum measurable effect, in terms of DA release from PC12.

Preparation of Radioactively Labeled mAbs. The purified mAbs were labeled with carrier-free ^{125}I by the chloramine T method (Greenwood et al., 1963). Free iodine was separated from labeled antibodies by passage over a Sephadex G-50 column. ^{125}I-labeled mAb 4C4.1 was shown to have an affinity to toxin bound to solid phase of 1.1×10^{-10} M, as measured by displacement of bound radioactivity by increasing amounts of unlabeled antibody. Concentrations of ^{125}I mAb greater than 5×10^{-10} M, were used in binding studies to toxin-treated PC12 cells.

Determination of Antibody Binding. Antibody binding was measured by ELISA. Microtiter plate wells were first treated with 15–60 μg mL^{-1} antigen (50 μL/well) for 120 min at room temperature. The antigen-containing solution was removed, and the wells were washed several times with washing buffer and then exposed to hybridoma supernatants or ascites fluid for 16 h at 4 °C (100 μL). The assay was developed according to the procedure described in the New England Nuclear monoclonal antibody screening system using horseradish peroxidase conjugated sheep Fab antimouse Ig.

Cell Preparation and Dopamine Release Studies. The PC12 cells (Greene & Tischler, 1976) were maintained in DMEM supplemented with 10% HS and 5% FCS. Monolayers [$(5–10) \times 10^6$ cells/plate] were incubated with HR (Greene & Rein, 1977) containing ascorbic acid, pargyline, and 1×10^{-7} M [^{3}H]DA for 1 h at 37 °C. At the end of the uptake period, the medium was sucked off, and the cells were washed 3 times with HR and resuspended after dissociation in a volume such to give a cell suspension of 1×10^6 cells mL^{-1}; 0.5-mL aliquots were then transferred to 1-mL Eppendorf tubes containing from 10 to 50 μL of α-Latx solution in HR, a mixture of α-LaTx solution and hybridoma supernatant, or simply HR. The cell suspension was incubated with these agents for 20 min at 37 °C, the volume was brought to 1 mL by addition of 0.5 mL of HR, and the tubes were centrifuged 1 min in an Eppendorf microcentrifuge. An 0.8-mL aliquot of the supernatant was counted in 5 volumes of Pico Fluor 30 (Packard). The remaining medium was removed by suction, the cells were resuspended in HR and spun again, and the pellet was eventually dissolved in 0.2 mL of 1% SDS and counted in 5 volumes of Pico Fluor 30. The radioactivity released is expressed as the percentage of total radioactivity (radioactivity present in the pellet plus that calculated to dwell in the whole supernatant) amounting to an average of 40 000–50 000 dpm in the various samples.

^{45}Ca Uptake Studies. In ^{45}Ca uptake studies, PC12 cells grown as a monolayer on collagen-coated Petri dishes (35 mm, Falcon) at a density of 10^6 cells per dish were washed with a HR calcium-free medium containing 1 mM EGTA. Cells were then incubated for 10 min at 25 °C with the medium and 5 nM α-Latx. The medium and toxin were removed by careful suction and cultures challenged with 1 mL of HR medium containing 1.8 mM ^{45}Ca (3×10^5 cpm mL^{-1}) for 10 min. The dishes were repeatedly washed with HR–EGTA, and the cells were solubilized in 1% SDS and counted in 5 volumes of Pico Fluor 30.

Generation of [^{3}H]InPs. Myoinositol-free DMEM used for PC12 cell cultures was supplemented with [2-^{3}H]myoinositol, (Amersham) to label overnight the cells previously plated on collagen-treated 35-mm dishes (1 μCi $dish^{-1}$, 2×10^6 cells $dish^{-1}$). The amount of FCS and HS in the modified DMEM was reduced to 0.1 and 0.5% (v/v), respectively. Under these conditions, cells apparently lose their tendency to aggregate but remain perfectly responsive to toxin action. The labeled cells were washed 3 times and then incubated in HR containing 10 mM LiCl. The incubation with toxin, mAb, or both was then started and continued for a maximum of 15 min. By addition of trichloroacetic acid to a final concentration of 2.5%, the incubation was stopped; the cells were resuspended and centrifuged. Analysis of [^{3}H]InPs was carried out by a bulk elution procedure essentially based on the column chromatographic techniques described by Berridge et al. (1983). The supernatant of deproteinized cells was freed of trichloroacetic acid by means of several washings with diethyl ether, buffered, and applied to a column of AG 1X8 (formate, Bio-Rad) that was eluted first with water and then with 1 M ammonium formate/0.1 M formic acid. The radioactivity in this eluate was determined as ^{3}H content of total inositol mono, bis-, and trisphosphate by scintillation counting in the gel phase with Pico Fluor 30.

Immunofluorescence. Rat cerebellar neurones were plated and cultured as described (Levi et al., 1984). Cells for immunofluorescence were fixed in 2% paraformaldehyde for 1 min at room temperature. After a short incubation with nanomolar concentrations of α-Latx, a 1:80 dilution of ascites fluid from mAb 4C4.1 was then added to the cells for 30 min at room temperature. After extensive washing in PBS, a rabbit anti-mouse immunoglobulin serum was added (30 min), followed by addition of fluorescein-conjugated goat anti-rabbit IgG antibodies.

Results

Properties of mAbs. The screening procedure employed to select hybridomas directed against α-Latx involved (i) ELISA using pure toxin coupled to solid phase and (ii) neutralization of toxin-induced release of previously accumulated [^{3}H]DA from PC12 cells. The first series of assays on the uncloned hybridomas revealed that only a proportion of the supernatants yielding a positive reaction in the ELISA were able to neu-

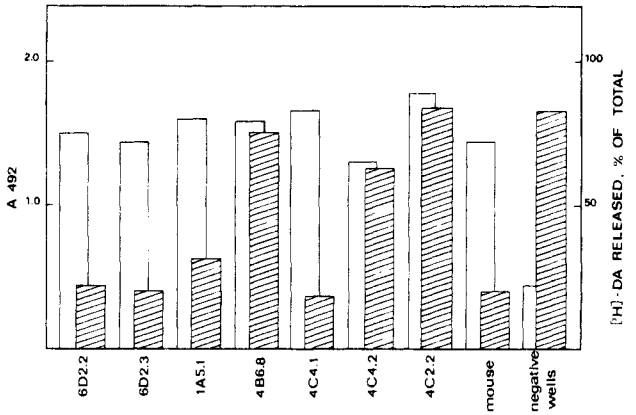

FIGURE 1: Comparison of ELISA response of seven hybridoma supernatants with their ability to neutralize antigen-induced release of [^{3}H]DA from PC12. Results are plotted as A_{492} OPD color developed by HRP-conjugated Fab fragments in the presence of H_2O_2 (open bars) and as percentage of total radioactivity of [^{3}H]DA released by a limiting α-Latx concentration from PC12, in 15 min (dashed bars). ELISA were performed by coating individual wells of polyvinyl microtiter plates with 50 μL of antigen solution equivalent to 13 μg of α-Latx mL^{-1}, by overnight adsorption at 4 °C. For release experiments, 100 μL of the corresponding hybridoma supernatants was incubated for 30 min at room temperature with 25 μL of α-Latx and then added to [^{3}H]DA-loaded PC12. A comparison is made with the results obtained after incubation of α-Latx both with the supernatant of ELISA negative wells and with 10 μL of α-Latx hyperimmune serum of the mouse donor of splenocytes.

tralize the toxin-induced stimulation of transmitter release from PC12 cells. As shown in Figure 1, seven stable hybridomas were isolated and expanded following cloning. Of these, four (6D2.2, 6D2.3, 1A5.1, 4C4.1) were consistently positive both in the ELISA and in the DA-release neutralization assay, while 4B6.8, 4C4.2, and 4C2.2 were ELISA-positive but unable to affect toxin activity. The observation that only four mAbs after binding to toxin did alter its function suggests that the sites to which they bind are of great importance to toxin action. One of these four antibodies (designated mAb 4C4.1) was selected for further characterization of its interaction with α-Latx, since preliminary experiments showed that its inhibitory action was not achieved solely by hindering the binding of the toxin to PC12 cells. The monoclonal nature of this antibody as well as its IgG1 isotype was proven by immunodiffusion and by SDS–polyacrylamide gel electrophoresis of the metabolically labeled antibody. mAb 4C4.1 was easily grown as ascites after being cloned twice by limiting dilution. The affinity of mAb 4C4.1 for α-Latx bound to solid phase was estimated to be 10^{-10} M (see Experimental Procedures).

Effect of mAb on Toxin-Induced [^{3}H]DA Release. The dose–response curve for the inhibition of the α-Latx-stimulated [^{3}H]DA release by mAb 4C4.1 was studied. Increasing amounts of two hybridoma supernatants (4C4.1 and 4B6.8) were added separately to a limited amount of toxin, which releases about 50–60% of the accumulated radiolabeled transmitter. The amount of [^{3}H]DA released from preloaded PC12 cells was referred to a control obtained by adding to the toxin the same amount of a noncompetent monoclonal antibody (mAb 4F1, anti-nerve growth factor; A. Cattaneo, unpublished results) and expressed as percentage of toxin effect in the absence of hybridoma supernatants. A total of 10 μL of mAb 4C4.1 supernatant was able to neutralize more than 50% of the activity of 10^{-9} M toxin, and with 100 μL the release of [^{3}H]DA did not differ from that of toxin-untreated control cultures. Increasing amounts of mAb 4B6.8, up to 200 μL, failed to inhibit the toxin action on transmitter release, while binding strongly to the pure toxin (Figure 1).

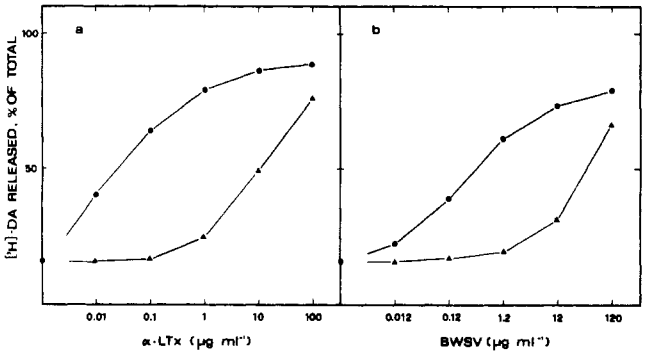

FIGURE 2: Inhibitory effect of mAb 4C4.1 on the dose–response curve for toxin- and venom-induced DA release from PC12 cells. (a) Closed circles represent the releaseing activity induced by untreated toxin and open circles the activity after incubation with a fixed amount of mAb (about 10 nM, based on a 1 μg mL^{-1} hybridoma supernatant estimate). (b) The procedure is identical with (a) except that an amount of black widow spider venom (BWSV) corresponding to the toxin fraction was used. Data depict a typical experiment, which was replicated 3 times.

When a fixed amount of mAb 4C4.1 is incubated with increasing concentrations of toxin (Figure 2a) or crude venom (Figure 2b), the neutralization effect appears to be specific for the toxic moiety present in the preparation, considering that the presence of other venom components does not affect the dose–response curve. The neutralization appears also to be quantitative, since the toxin constitutes about 10% of the soluble proteins of the gland extract and the concentration of venom giving 50% of maximum measurable effect is in fact shifted to a protein concentration 10-fold higher than that of toxin (compare the ED_{50} in Figure 2).

Effect of mAb on Toxin-Induced ^{45}Ca Uptake. Previous studies have demonstrated that toxin-induced release of DA in PC12 is linked to a massive increase of ^{45}Ca influx. The effect of mAb 4C4.1 on the toxin-induced ^{45}Ca influx was studied as a function of the concentration of toxin preincubated with different amounts of mAb 4C4.1 ascites fluid. A typical experiment is described below. Figure 3 shows that mAb 4C4.1 inhibits also ^{45}Ca influx in PC12 cells. A 1/1000 dilution of the ascites fluid is able to reduce almost to 50% the ^{45}Ca uptake induced by nanomolar concentrations of toxin, lower dilutions being fully inhibitory.

Effect of mAb on Calcium Dependence of Toxin Activity. The Ca^{2+} requirement for activity of α-LaTx on PC12 grown in monolayer was considered an experimental condition that allowed the dissection of cumulative effects of toxin from its binding to cells. In agreement with previous observations (Grasso et al., 1980) on the effect of nanomolar concentrations of toxin given in a nominally Ca^{2+}-free medium, containing 1 mM EGTA, we confirmed that the radioactivity released by toxin-treated PC12 cells was not dissimilar from that of untreated controls (0.6–0.9% min^{-1}). After washing, which allows the removal of unbound toxin, the substitution of the Ca^{2+}-free medium with a Ca^{2+} containing medium releases an amount of transmitter quantitatively comparable with that obtained by an equal amount of toxin added to a Ca^{2+}-containing medium. This indicates that during the incubation in HR–EGTA the saturation of receptor sites occurs. Toxin-induced release of [^{3}H]DA is completely blocked if toxin is challenged with mAb before addition to cultures. If mAbs are added in HR after toxin bound to cells in HR–EGTA, the subsequent Ca^{2+}-triggered effect is partially reduced (Table I). These experiments show that the mAb is able to interact with the toxin and to impair its action after the toxin itself is bound to the cells.

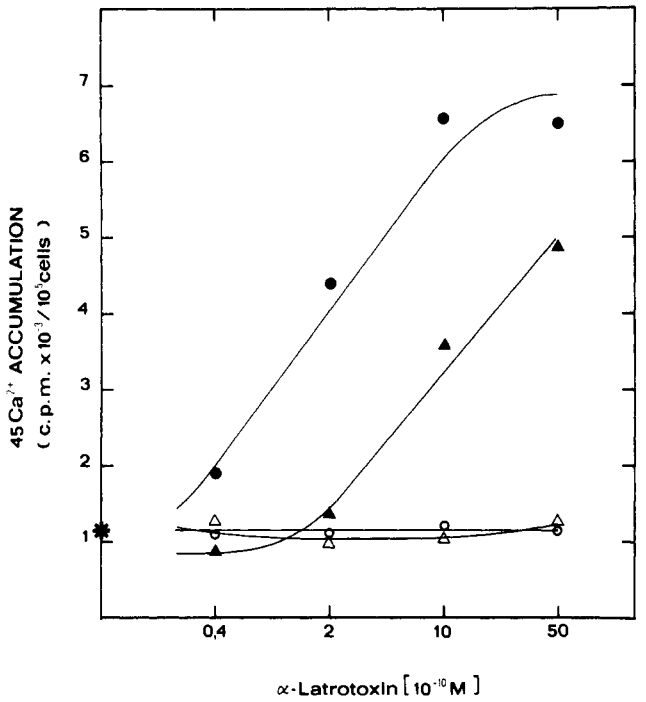

FIGURE 3: Effect of mAbs on toxin-induced ^{45}Ca uptake. PC12 cells grown as monolayer on collagen-coated Petri dishes (35 mm) at a density of 10^6 cells $dish^{-1}$ were washed with EGTA–HR and incubated for 10 min at 25 °C with the same medium containing 5 nM α-Latx (●) or toxin plus 1:10 (▲), 1:100 (○), or 1:1000 (▲) dilutions of ascites fluid. The free toxin was then removed by suction of the medium, and the cells were challenged with 1 mL of HR medium containing 1.8 mM ^{45}Ca (3×10^5 cpm mL^{-1}) for 10 min. The dishes were quickly and repetitively washed and the cells solubilized in 1% SDS and counted in 5 volumes of Pico Fluor 30. (*) Background accumulation.

Table I: Effect of mAb 4C4.1 on DA Release Induced by a Ca^{2+}-Containing Medium from PC12 Previously Challenged with α-Latx in HR–EGTA[a]

	[^{3}H]DA release (% of total)		
	5 nM α-Latx in HR–EGTA (0–10 min)	HR (10–20 min)	HR + mAb (10–20 min)
control cells (n = 6)	6.4 ± 0.3	52.5 ± 5	
mAb-treated cells (n = 8)	6.5 ± 0.3		43.2 ± 4

[a] Cells grown as monolayer on collagen-coated Petri dishes at a density of about 10^6 cells $dish^{-1}$, accumulated [^{3}H]DA in HR according to Grasso et al. (1980). At the end of the uptake period, cultures were brought at 25 °C, washed with Ca-free HR containing 1 mM EGTA, and then treated in the same medium with 1 mL of 5 nM α-Latx. After 10-min incubation, the medium with toxin was collected from the dishes by careful suction directly into scintillation vials and replaced with 1 mL of HR with or without mAbs. At the end of the incubation with HR (10 min), the media were removed and counted and the cells solubilized in 1% SDS and counted. Values are means ± SEM.

Time Dependence of mAb Action. Thus, mAb 4C4.1 reacts with an antigenic determinant of α-Latx molecule having a central function in toxin mode of action and that is possibly distinct from the binding site. As an attempt to understand the functional role of this specific structure, the effect of the antibody on toxin-induced transmitter release was studied as a function of incubation time of the toxin with the antibody and monitored by the secretive response of the cells. The neutralization of toxin action occurs rapidly since addition of the mAbs to the toxin 15 s before its addition to the cells almost completely prevents toxin effect. The virtually simultaneous addition of toxin, mAb, and cells reduces toxin action to 50% of maximum measurable effect, indicating its

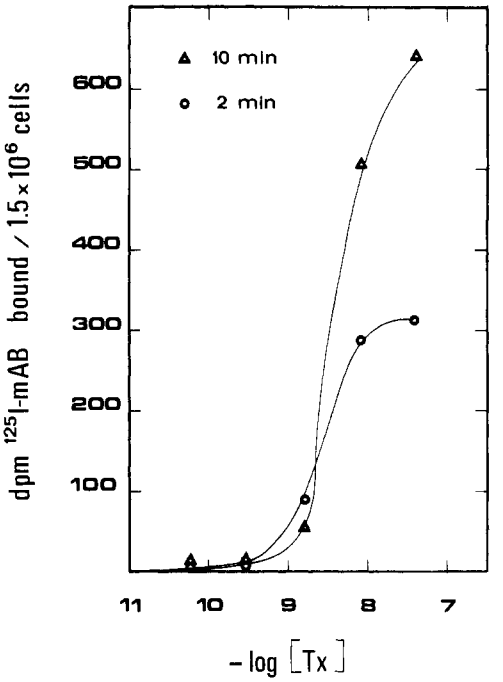

FIGURE 4: Specific binding of ^{125}I-labeled mAb to PC12 cells treated with α-Latx for 2 and 10 min. The 0.5-mL aliquots of a PC12 cell suspension (3×10^6 cells mL^{-1}) incubated for 2 (○) or 10 (▲) min with the indicated concentration of α-Latx were pelleted by a rapid centrifugation in Eppendorf microfuge. The resuspended cells were incubated for 20 min with a fixed amount of ^{125}I-labeled mAb (20 000 dpm) and then centrifuged through oil according to Meldolesi et al. (1983). The radioactivity specifically bound to the cells was determined after cutting the tip of the centrifuge tube containing the pelleted cells and subtracting the amount of radioactivity present in toxin-untreated cells. Equivalent controls were obtained by incubating toxin-treated cells with ^{125}I-labeled mAb in presence of an excess of unlabeled mAb (ascite fluid).

equal partition between cells and mAb, in keeping with the comparable affinity of α-Latx for its membrane receptors and for mAb 4C4.1. By contrast, addition of the antibody a short time (30 s) after the first interaction of the toxin with the cells is ineffective in neutralizing the toxin action. These data still leave unsolved the question as to whether the neutralizing action of the antibody simply results from an inhibition of the binding of the toxin to the cells or from preventing its action successive to binding. A series of different experiments that allow us to make some inferences on the antigenic determinant of the toxin molecule recognized by the antibody are described in the next paragraph.

mAb 4C4.1 Recognizes Toxin Bound to Cells. Affinity-purified mAb 4C4.1 labeled with ^{125}I was allowed to bind to the toxin molecule incubated for different lengths of time with PC12 cells. Was the antibody directed to the site of the toxin involved in binding to the cell receptor, it would be difficult to detect cell-bound toxin molecules. Specifically bound ^{125}I-labeled mAb was measured in toxin-treated PC12 cells pelleted through oil according to Meldolesi et al. (1983) in order to remove α-Latx from solution. From the data shown in Figure 4 it can be calculated that about 800 bound toxin molecules per cell are recognized by ^{125}I-labeled mAb. This figure is significantly lower than the about 6400 binding sites on PC12 cell measured in suspension by radioactively labeled α-Latx (Grasso et al., 1982; Meldolesi et al., 1983). One explanation for this discrepancy is that ^{125}I-labeled mAb most likely reacts with that proportion of toxin molecules bound to cells having the relevant epitope still prone to be recognized by the antibody and this population may not comprise all cell-bound toxin molecules.

Table II: Recognition of α-Latx Bound to Target Structures by mAb 4C4.1

assay	target structure	α-Latx concn (nM)	α-Latx treatment (min)	mAb treatment (min)	response
ELISA (A_{492})[a]	PC12 cells		0		100
		5	10		64
immunofluorescence[b]	granule cells of rat cerebellum (8th day in culture)		none	20	negative
		1	2	20	strongly positive
		1	15	20	positive
conductance measurements[c]	artificial lipid bilayers	0.5	30	30	increase in conductance
		0.5	preincubation with mAb	30	base-line conductance

[a] A limiting dilution of mAb 4C4.1 was incubated with a fixed amount of PC12 cell suspension obtained from monolayers previously treated or untreated with 5 nM α-Latx. After the cells were pelleted 30/μL of the supernatant was serially diluted and assayed for residual antibodies in ELISA. Data are reported as percent of net specific A_{492} (A_{492} produced by mAb 4C4.1 minus A_{492} produced by a noncompetent hybridoma supernatant directed against nerve growth factor). [b] See Experimental Procedures. [c] The 0.5 nM α-Latx generally increases the bilayer conductance up to 2 orders of magnitude over base line within 30 min.

A different type of experiment was performed on rat cerebellar neuron primary cultures (Levi et al., 1984), with a fluorescent second-stage antibody to visualize the bound toxin (see Experimental Procedures). These cultures are highly enriched in granule neurons, and experiments to be reported elsewhere have shown that α-Latx induces a massive secretion of [^{3}H]-D-aspartate from these neurons. The significantly positive response obtained both on the cell body and on neurites (Table II), besides confirming that mAb 4C4.1 is able to recognize the cell-bound toxin following its interaction with the surface receptors, allows us to evaluate the distribution of the binding sites for the α-Latx on primary neuronal cell cultures (work in progress). Thus, the antibody binds to an epitope that is left accessible soon after the binding of the toxin to the receptor. This inference was confirmed by further experiments whereby PC12 cells incubated with α-LaTx were used to subtract antigen binding capacity from a given dilution of mAb 4C4.1. After centrifugation of cells, the absorbed solutions were then titrated for the presence of anti-toxin antibodies serially diluted by an ELISA assay. The results clearly showed that toxin-treated PC12 cells were very effective in subtracting the mAb 4C4.1 in comparison with the amount of mAb subtracted by untreated cells (Table II).

Effect of mAb on Toxin-Dependent Increase of Conductance in BLM. α-Latx has been shown to induce voltage-sensitive calcium-selective channels in BLM (Finkelstein et al., 1976; Robello et al., 1984). It was of interest to assess the influence, if any, of the mAb 4C4.1 on the insertion of α-Latx in the lipid bilayer and on the resulting ionic channel formation. The experiment was performed essentially as described by Robello et al. (1984). Toxin concentrations ($\sim 10^{-10}$ M) increasing the bilayer conductance up to 2 orders of magnitude over base line after 30 min were preincubated with serial dilutions of the antibody for 30 min at RT. The antibody treatment completely abolishes the increase in conductance that usually accompanies toxin action on BLM, the amounts of mAb necessary being comparable with those effective in reducing toxin action on PC12 (Table II). Thus, mAb 4C4.1 inhibits also an effect of α-Latx that does not require interaction with the membrane receptor. If mAbs were added after interaction of α-Latx with BLM, both on the cis and on the trans sides, they did not affect the macroscopic properties of the formed ion-permeant channels. This experiment suggests that the epitope recognized by mAb 4C4.1 does not protrude from the membrane and is probably of hydrophobic nature. Moreover, mAb 4C4.1 appears not to interfere with channel function per se but rather with the process of insertion into the membrane and consequent channel formation.

Effect of mAb on Toxin-Dependent InPs Production. In agreement with a recent paper (Vicentini & Meldolesi, 1984), treatment of [^{3}H]inositol-labeled PC12 with nanomolar concentrations of α-Latx (4nM) induced in a short time a large accumulation of InPs, which was 3.2-fold that produced by untreated controls. A short-term preincubation (30 s) of the toxin with mAb before addition to the cells, a condition that totally abolishes toxin-dependent DA release, reduces InPs to 1.8-fold that of controls. Addition of mAbs to cells treated with toxin for 30 s does not modify the amount of InPs produced by toxin. An intermediate value (2.1-fold) was obtained at time zero when mAb and toxin were added simultaneously to [^{3}H]inositol-labeled cells. Thus, breakdown of PIs in response to "receptor" stimulation by α-Latx was affected by mAb at a rate that appears to be different from that occurring in the DA release experiments (see above), indicative of the existence of a further step after binding to receptor necessary for stimulation of transmitter release. Of particular interest is the finding that a condition can be found whereby the inhibitory effect of mAb on the toxin-induced PI turnover can be dissected from DA release: namely, by incubation of the toxin with the mAb for a short time before the addition to the cells, the DA release is completely inhibited, while the PI turnover is significantly higher than that of controls. As to the issue raised by such experiments, whether toxin-induced PI turnover is per se sufficient or not for its subsequent action (DA release), only future experiments will provide the answer.

Discussion

High-affinity monoclonal antibodies specific for α-Latx provide a useful set of reagents for the study of toxin–effector cell interaction and consequently can be used in considering the secretagogue action of the toxin for the understanding of the secretory mechanism of the nerve cell in general.

On the basis of the preexisting experimental evidence, the mechanism of action of α-Latx on its target structures (for example PC12, rat brain synaptosomes, nerve terminal of the neuromuscular junction) was schematically suggested (Finkelstein et al., 1976; Robello et al., 1984) to occur as follows: (i) toxin–acceptor interaction, (ii) toxin insertion into the cell membrane, and (iii) expression of channel functions by the inserted toxin. According to a recent alternative view (Vicentini & Meldolesi, 1984), toxin acceptor interaction triggers in PC12 a transmembrane signal coupled with the PIs hydrolyzing system, which per se would be sufficient to induce toxin-dependent increase of calcium accumulation and DA release. Protease-sensitive and high-affinity binding sites have been described in α-Latx responsive cell preparations (Tzeng & Siekevitz, 1979; Meldolesi, 1982).

As an attempt to describe some part of these schemes, we have selected out of a panel of mAbs directed against α-Latx, previously developed (Cattaneo & Grasso, 1984), an antibody

that was able to interact with the toxin with high affinity and to neutralize its action in terms of PIs hydrolysis, calcium influx, and dopamine release. We have first provided evidence that mAb 4C4.1 recognizes an epitope of the toxin molecule that is still exposed after the toxin is bound to its acceptors and, second, shown that this epitope is important for the action of the toxin. The fact that we have not been able to titrate with ^{125}I-labeled mAb 4C4.1 all the presumed toxin molecules bound to the cells (~6400) may be explained by supposing that the channel formed by α-Latx is in fact an oligomer. If mAb is added to toxin first, then all monomers would be tied up with antibody and the oligomer could not be formed. But if the channel is first formed, only one or a part of them could bind mAb, just for steric reasons. In other words, one might speculate that mAb 4C4.1 is titrating the oligomers (channels) formed.

The finding that mAb 4C4.1 completely prevents channel formation in the reconstituted system of BLM, a process that is not mediated by binding to protein acceptor sites, suggests that the site of the toxin recognized by the mAb has probably a function related with insertion into the membrane. Furthermore, the observation that the electrophysiological properties of the membrane endowed with α-Latx channels are not modified by subsequent addition of mAbs on either side of the cell seems to exclude that the protruding portion of the toxin channels (Robello et al. 1984) is the site recognized by mAb 4C4.1 and support the view of a hydrophobic nature of such epitope.

The question arising now concerns the role, if any, of the epitope recognized by mAb 4C4.1 in the mechanism of action of α-Latx. The bulk of evidence presented allows us to conclude that this epitope is not involved in the binding of the toxin to membrane acceptors but is directly involved in the expression by the toxin itself of its secretagogue action. The given demonstration of the existence of such an epitope provides evidence in favor of the view that α-Latx has to exert a further action, following binding to the cell membrane, in order to cause neurotransmitter secretion. Accordingly, toxin-induced phosphoinositide hydrolysis would not be sufficient per se, without conceiving a further action of the toxin, to cause neurotransmitter secretion. Previous experimental evidence suggests that this step may be represented by toxin insertion into the cell membrane. The results of two experiments presented above are relevant to this issue. (i) The first is that the demonstration that the action of the toxin can be inhibited by mAb 4C4.1 after toxin is bound to cells (in conditions where no toxin molecules are available for interaction with the antibody in the soluble phase; Table I). The partial inhibition resulting from this experiment can be explained by a limited accessibility of the relevant toxin epitope, as supported by the calculation of the toxin molecules bound to membrane and prone to be recognized by ^{125}I-labeled mAb 4C4.1 (see Figure 4). (ii) An experimental condition was determined whereby toxin-induced PIs hydrolysis was clearly displayed, while neurotransmitter release was completely inhibited. It has been previously reported (Vicentini & Meldolesi, 1984), that the extent of α-Latx-induced PI turnover and DA secretion are tightly correlated and that the first event is casually related with the second. The apparent dissection of the process of PI hydrolysis from DA release obtained by mAb 4C4.1 experiments makes it difficult to reconcile the latter view with the results here obtained. It is more plausible to conceive the InPs generation as the result of a toxin-induced membrane perturbation while DA release is ruled by the toxin itself through a direct or indirect involvement of the epitope recognized by mAb 4C4.1. This experiment, moreover, provides evidence that the inhibition of toxin-induced DA release in these conditions is not due to inhibition of binding to the cells. We cannot exclude that the inhibition of toxin action observed after long preincubation of toxin and mAb 4C4.1 may be due to inhibition of binding by steric hindrance; nevertheless, different lines of experimental evidence (Table II and Figure 4) demonstrate that incubation of the α-Latx with target cells prior to addition of mAb 4C4.1 allows the binding of the antibody to the toxin-treated cells. Among these experiments, the immunofluorescence performed on granule cells prospects the possibility of studying the distribution of α-Latx on target cells, a study that hereby has been possible only on the neuromuscular junction of the frog (Valtorta et al., 1984), due to the presence in polyclonal antisera of complex mixtures of epitope specificities. Moreover, the availability of mAb 4C4.1 makes it now possible to investigate the nature and the function of the toxin epitope recognized, as well as to characterize, along with detailed electrophysiological studies, the membrane binding sites by suitable double-immunoaffinity chromatography.

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Registry No. α-Latx, 65988-34-3; dopamine, 51-61-6; calcium, 7440-70-2.

REFERENCES

Berridge, M. J., Dawson, R. M. C., Downes, P. C., Heslop, J. P., & Irvine, R. F. (1983) Biochem. *J. 212*, 473–482.

Cattaneo, A., & Grasso, A. (1984) in *Proceedings of the 6th European Symposium on Animal, Plant and Microbial Toxins* (Meier, J., Stocker, K., & Freyvogel, T. A., Eds.) p 82, Basle.

Finkelstein, A., Rubin, L. L., & Tzeng, M. C. (1976) *Science (Washington, D.C.) 193*, 1009–1011.

Frontali, N., Ceccarelli, B., Gorio, A., Mauro, A., Siekevitz, P., Tzeng, M. C., & Hurlbut, W. P. (1976) *J. Cell Biol. 68*, 462–479.

Galfrè, G., & Milstein, C. (1981) *Methods Enzymol. 73*, 3–46.

Grasso, A. (1976) *Biochim. Biophys. Acta 439*, 406–412.

Grasso, A., & Senni, M. I. (1979) *Eur. J. Biochem. 102*, 337–344.

Grasso, A., Alemà, S., Rufini, S., & Senni, M. I. (1980) *Nature (London) 283*, 774–776.

Grasso, A., Pelliccia, M., & Alemà, S. (1982) *Toxicon 20*, 149–156.

Greene, L. A., & Tischler, A. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2424–2428.

Greene, L. A., & Rein, G. (1977) *Brain Res. 129*, 247–263.

Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114.

Levi, G., Aloisi, F., Ciotti, M. T., & Gallo, V. (1984) *Brain Res. 290*, 77–86.

Meldolesi, J. (1982) *J. Neurochem. 38*, 1559–1569.

Meldolesi, J., Madeddu, L., Tora, M., Gatti, G., & Niutta, E. (1983) *Neuroscience 10*, 997–1009.

Misler, S., & Hurlbut, W. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 991-995.
Nicholls, D. G., Rugolo, M., Scott, I. G., & Meldolesi, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7924-7928.
Robello, M., Rolandi, R., Alemã, S., & Grasso, A. (1984) *Proc. R. Soc. London, Ser. B 220*, 477-487.
Tzeng, M. C., & Siekevitz, P. (1979) *J. Neurochem. 33*, 263-274.
Valtorta, F., Madeddu, L., Meldolesi, J., & Ceccarelli, B. (1984) *J. Cell Biol. 99*, 124-132.
Vicentini, L. M., & Meldolesi, J. (1984) *Biochem. Biophys. Res. Commun. 121*, 538-544.

Glutathione Reductase from *Escherichia coli*: Cloning and Sequence Analysis of the Gene and Relationship to Other Flavoprotein Disulfide Oxidoreductases†

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ABSTRACT: A glutathione reductase negative strain of *Escherichia coli* K-12 was isolated as a thermoresistant survivor when a *gor*::Mu*cts*Ap lysogen was subjected to elevated temperature. It was found that in addition to being ampicillin sensitive this mutant was hypersensitive to arsenate, which may be connected with the fact that the *gor* gene maps between 77 and 78 min on the *E. coli* genome, close to the *pit* locus encoding the major arsenate transport system of *E. coli*. A derivative of this mutant was used as the recipient in a screen of the Clarke and Carbon hybrid plasmid bank of *E. coli* DNA. A plasmid, pGR, was isolated that encodes both an arsenate-resistance element and glutathione reductase. Restriction mapping of this plasmid showed that the insert DNA is approximately 10 kilobase pairs in length, and a fragment of the *gor* gene was identified that allowed the *gor* gene to be accurately mapped on pGR by a combination of restriction analysis and Southern blotting. The DNA sequence of the *gor* gene was determined and found to encode a protein of 450 amino acid residues. The glutathione reductase of *E. coli* is very homologous to the human enzyme and is also related (though less closely) to other flavoprotein disulfide oxidoreductases whose sequences are available. These enzymes have retained a common mechanism while evolving different specificities.

Glutathione reductase (EC 1.6.4.2) is a widespread enzyme that catalyzes the reduction of oxidized glutathione by NADPH:

$$GSSG + NADPH + H^+ \rightarrow NADP^+ + 2GSH$$

It is a member of an important class of flavoprotein enzymes, the disulfide oxidoreductases, which appear to share a common catalytic mechanism. Other members of the class are dihydrolipoamide dehydrogenase (EC 1.6.4.3) (Reed, 1974; Packman et al., 1984), thioredoxin reductase (EC 1.6.4.5) (Holmgren, 1980), and mercuric reductase (Fox & Walsh, 1982). Studies of their amino acid sequences reveal homologies between dihydrolipoamide dehydrogenase, glutathione reductase, and mercuric reductase, implying that they have arisen by divergent evolution from a common ancestor (Perham et al., 1978; Williams et al., 1982; Fox & Walsh, 1983). On the other hand, thioredoxin reductase is sufficiently different for it to be likely that this enzyme has arisen by convergent evolution toward a common mechanism (Perham et al., 1978).

The *lpd* gene of *Escherichia coli*, encoding dihydrolipoamide dehydrogenase (Stephens et al., 1983), and the *mer*A gene of transposon Tn 501 from *Pseudomonas aeruginosa*, encoding mercuric reductase (Brown et al., 1983), have been cloned and their nucleotide sequences determined. This in turn has enabled their primary structures to be inferred. The complete amino acid sequence (Krauth-Siegel et al., 1982) and X-ray crystallographic structure at 2-Å resolution (Thieme et al., 1981) of human glutathione reductase are also known, which has shed considerable light on the reaction mechanism (Pai & Schulz, 1983).

Glutathione is not an essential metabolite in *E. coli* (Apontoweil & Berends, 1975; Fuchs & Warner, 1975). Mutants (*gor*) that lack glutathione reductase have been isolated from *E. coli* mutated by bacteriophage Mu insertion and shown to be similarly unimpaired in growth (Davis et al., 1982). This phenotype has placed considerable difficulty in the way of attempts to clone the *gor* gene, but we now describe further experiments with the *gor* mutation that have allowed the isolation of a plasmid carrying the *E. coli gor* gene from the Clarke and Carbon (1976) bank of recombinant plasmids. The determination of the DNA sequence of this gene was undertaken to add another flavoprotein oxidoreductase sequence to those few already known. Apart from advancing our understanding of the mechanism and evolutionary history of this important group of enzymes, this should also permit more stringent tests of mechanism to be applied by means of in vitro mutagenesis.

Materials and Methods

Materials. Complex bacteriological media were from Difco Laboratories Ltd., East Molesey, Surrey. NADPH, 5,5′-dithiobis(2-nitrobenzoic acid), and oxidized glutathione were supplied by Sigma Chemical Co. Ltd., Poole, Dorset. Agarose and low melting point agarose (Seaplaque) were from Miles

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