

# Binding of a *Glycera convoluta* Neurotoxin to Cholinergic Nerve Terminal Plasma Membranes

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**ABSTRACT** The crude extract of venom glands of the polychaete annelid *Glycera convoluta* triggers a large  $\text{Ca}^{2+}$ -dependent acetylcholine release from both frog motor nerve terminals and *Torpedo* electric organ synaptosomes. This extract was partially purified by Concanavalin A affinity chromatography. The biological activity was correlated in both preparations to a 300,000-dalton band, as shown by gel electrophoresis. This confirmed previous determinations obtained with chromatographic methods. This glycoprotein binds to presynaptic but not postsynaptic plasma membranes isolated from *Torpedo* electric organ. Pretreatment of intact synaptosomes by pronase abolished both the binding and the venom-induced acetylcholine release without impairing the high  $\text{K}^+$ -induced acetylcholine release. Pretreatment of nerve terminal membranes by Concanavalin A similarly prevented the binding and the biological response. Binding to *Torpedo* membranes was still observed in the presence of EGTA. An antiserum directed to venom glycoproteins inhibited the neurotoxin so we could directly follow its binding to the presynaptic membrane. *Glycera convoluta* neurotoxin has to bind to a ectocellularly oriented protein of the presynaptic terminal to induce transmitter release.

A neurotoxin from the venom glands of the polychaete annelid *Glycera convoluta* enhances several hundred times the frequency of miniature end-plate potentials (mepps)<sup>1</sup> at frog neuromuscular junctions and at *Torpedo* nerve-electroplaque junctions (15). This effect is  $\text{Ca}^{2+}$ -dependent and is observed even in the presence of high concentration of tetrodotoxin (15, 16). The active substance has been recently shown to be a high molecular weight (300,000 daltons) protein that was clearly separated from the low protease and phospholipase activities of the crude venom (24). This substance has also been found to induce acetylcholine (ACh) release from isolated cholinergic *Torpedo* synaptosomes (9, 12). The release starts after a short delay and lasts until exhaustion of the free (cytoplasmic) ACh pool (9). The *Glycera convoluta* venom (GCV) does not induce depletion of synaptic vesicles or other intraterminal organelles (15). Changes in the distribution of intramembrane particles were found in the freeze-fractured plasma membrane of synaptosomes during GCV-induced ACh release (12). These modifications seem related to the ACh release process itself since they were also observed when ACh release was triggered by KCl, gramicidin, or  $\text{Ca}^{2+}$  ionophore A 23187 (10).

In view of the potential usefulness of GCV in studying the mechanism of ACh release, it was important to know whether the active molecule acted after binding specifically to the presynaptic membrane. We chose to study the binding of GCV to preparations of *Torpedo* synaptosomes. This seemed most convenient because the *Torpedo* synaptosome preparation is a homogeneous population of pure cholinergic nerve terminals that retain the properties of nerve terminals in situ including the ability to release ACh. This release can be continuously quantitated by a chemiluminescence method (8, 9). In addition, it is possible to prepare fractions of presynaptic plasma membranes (20) allowing binding studies and evaluation of biological activity on the same preparation. Direct measurements of the neurotoxin binding could not, however, be done since, up to now, this molecule is not available in pure and active form. We therefore demonstrated the binding by immunological methods. For quantitative studies, the binding was indirectly evaluated by measuring the decrease of the soluble activity after incubation of GCV with membranes. These measurements were conducted in parallel at frog neuromuscular junction and on *Torpedo* synaptosomes.

We report that the neurotoxin binds to an ectocellularly oriented protein of the presynaptic plasma membrane. In addition, the neurotoxin was found to be a glycoprotein. This permitted a 100-fold purification of this molecule in a one-step affinity chromatography.

<sup>1</sup> Abbreviations used in this paper: ACh, acetylcholine; GCV, *Glycera convoluta* venom; mepps, miniature end-plate potentials.

## MATERIALS AND METHODS

### Preparation of Venom Extract

*Glycera convoluta* (Keferstein) were collected on the seashore near the Marine Biological Station of Roscoff (France). The venom gland complexes were immediately dissected and kept frozen at  $-80^{\circ}\text{C}$ . After thawing, they were homogenized at  $0^{\circ}\text{C}$  in 10 mM Na phosphate buffer (pH 7.2–7.4), leading to final concentrations of 10–50 glands per ml (gl/ml). The homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was recentrifuged at 160,000 *g* for 10 min (Beckman airfuge, Beckman Instruments, Inc., Richmond, CA). The latter supernatant constituted the crude GCV.

### Partial Purification of Venom Extract

GCV was fractionated by affinity chromatography on Con A-Sepharose 4B (Pharmacia, Inc., Piscataway, NJ). GCV (200–400 glands in  $\sim 10$  ml) was layered on top of a column containing Con A-Sepharose beads (8 ml settled volume) washed with at least 80 ml of frog Ringer's solution. After washing with 100 ml of the same solution, the biological activity was eluted by 0.25 M  $\alpha$ -methyl-D-mannoside and 0.25 M 1-O-methyl  $\alpha$ -D-glucopyranoside (Sigma Chemical Co., St. Louis, MO) dissolved in the Ringer's solution.

When the biological activity had to be estimated on synaptosomal suspensions, an additional filtration through a G 75 Sephadex column was performed either with GCV or with each of the fractions eluted from the affinity column (9). The biological activity was recovered in the excluded volume of the G 75 Sephadex column. Protein was determined either by amidoschwarz staining (23) or by a technique of protein-dye binding described by Bradford (3).

### Estimation of Biological Activity

**Frog Neuromuscular Junction:** Mepps were recorded intracellularly using conventional electrophysiological methods from single fibers of the cutaneous pectoris muscle which was bathed in a Ringer's solution containing 110 mM NaCl, 2.1 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 4 mM Hepes buffer adjusted to pH 7.2. The Ringer's solution flowed continuously through a 2-ml Perspex chamber at 5.5 ml/min. Crude GCV or fractions thereof were dissolved in 15 or 20 ml of Ringer's solution. They were perfused at the same rate, the first milliliters being eliminated and the last 11 ml being recirculated. After 8 min, the venom solution was washed out with Ringer's solution. Mepp frequency was continuously monitored by a system that detected the rising phase of the mepps. The fiber potential was fed into an active band pass filter with a center frequency of 500 Hz. The filtered output signal was then fed to a Schmitt trigger connected to a microcomputer that permitted on-line storage of each inter-mepp interval on a diskette. Further processing was off-line.

**Torpedo Synaptosomes:** *Torpedo* synaptosomes were isolated from *Torpedo* electric organ as previously described (11, 19). Aliquots of the synaptosomal band (usually 30  $\mu\text{l}$  deriving from 15 mg of electric organ) were resuspended in 250  $\mu\text{l}$  of an alkaline *Torpedo* physiological medium (280 mM NaCl, 3 mM KCl, 3.4 mM  $\text{CaCl}_2$ , 1.8 mM  $\text{MgCl}_2$ , 400 mM sucrose, 5.5 mM glucose, 50 mM Tris buffer adjusted to pH 8.6) to which acetylcholinesterase (EC 3.1.1.7), choline oxidase (EC 1.1.3.17), peroxidase (EC 1.11.1.7), and luminol were added. ACh release was then continuously measured using the chemiluminescence method reported by Israël and Lesbats (8, 9).

### Preparation of Antisera

The fractions eluted from the Con A column and corresponding to the peak of the biological activity were concentrated by dialysis under negative pressure (p Micro-Prodicon, Bio-Molecular Dynamics, Beaverton, OR; 15,000-mol-wt cut-off). The concentrated fractions were emulsified in Freund's complete adjuvant and injected intradermally in rabbit's foot sole at a dose of  $\sim 200$   $\mu\text{g}$  protein/rabbit. Three intradermal booster injections, each consisting of 200  $\mu\text{g}$  of protein in Freund's incomplete adjuvant, were made at 1-mo intervals. Rabbits were bled 10 d after the last injection.

### Adsorption of Venom Fractions on Presynaptic Plasma Membranes

Presynaptic plasma membranes were prepared by subfractionating *Torpedo* synaptosomes, as described (20). Several membrane fractions (M3, M4, M5) were obtained. The fractions M3 and M4 contained the presynaptic plasma membrane; M5 was mainly composed of plasma membranes of the postsynaptic cells. In some experiments, a crude fraction of presynaptic plasma membranes was obtained directly from *Torpedo* electric organ homogenate, as described (18). The membranes were kept frozen at  $-80^{\circ}\text{C}$ . After thawing, they were

washed in frog Ringer's solution and pelleted (160,000 *g* for 10 min) in a Beckman airfuge (Beckman Instruments, Inc.). They were resuspended in Ringer's solution and incubated for 1 to 2 h at room temperature in the presence of aliquots of the venom fractions. After airfuge centrifugation (160,000 *g* for 10 min), the biological activity recovered in the supernatant was immediately assayed. The membrane pellets were kept frozen at  $-80^{\circ}\text{C}$  for further electrophoretic studies.

### Gel Electrophoresis

Gel electrophoresis was performed in a slab gel apparatus as described by Laemmli (14). 40- $\mu\text{l}$  aliquots of the fractions eluted from the Con A column were half-diluted with a dissociation buffer (final concentration 2.5% SDS, 5%  $\beta$ -mercaptoethanol, 10% glycerol, 5 mM Tris, pH 6.8) and incubated in boiling water for 3–5 min. Running gels were either 5–15% gradient acrylamide gels or 5 and 7.5% acrylamide gels. A 3% acrylamide stacking gel was used. Electrophoresis was carried out for 5 h at a constant current of 40 mA. Gels were fixed overnight in 50% methanol and silver stained according to Wray et al. (26). For protein blotting, protein bands were transferred after electrophoresis from the gels to nitrocellulose filters (HAWP 304 FO, Millipore Co., Bedford, MA) according to Bowen et al. (2). Diffusion was allowed to occur at  $4^{\circ}\text{C}$  for 40 h in a buffer solution (in millimolars: NaCl, 100; EDTA, 2; Tris, 20; pH 7.6). The filters were incubated with rabbit antisera directed to venom fractions (see above). The Ig binding was visualized using peroxidase-labeled antibodies to rabbit IgG (Institut Pasteur, Paris) as previously described (18).

## RESULTS

### Quantitation of GCV Biological Activity

We have systematically evaluated the biological activity of GCV by assessing the GCV-induced ACh release at the frog neuromuscular junction and from *Torpedo* synaptosomes. At the frog neuromuscular junction, GCV increased the mepp frequency (14). As shown in Fig. 1, the effect is dose-dependent and easily reversed by washing. It is therefore possible to use this preparation to assay the activity of venom fractions using the same fiber throughout the experiment. In the range 10–160 venom glands/liter, the increase in mepp frequency induced by GCV is directly proportional to the concentration (Fig. 1, *inset*). In the experiments reported below, the mepp frequency was simultaneously recorded from two different muscle fibers and the venom doses were adjusted to bring the final concentration to 50–80 glands/liter.

On *Torpedo* synaptosomes, the venom triggers ACh release which can be continuously monitored by a chemiluminescence method. In the absence of precursor recycling, the transmitter release is maintained until complete exhaustion of the free ACh compartment (9). To eliminate interferences of GCV with the chemiluminescent reaction, it was necessary to pass GCV through a G 75 Sephadex column. The time course of ACh release triggered by different GCV doses is shown in Fig. 2. After a short delay, the rate of ACh release is directly proportional to the venom concentration whereas the total amount of ACh released is dose-independent (Fig. 2, *inset*). In the experiments reported below, the venom doses were adjusted to give a final concentration of  $\sim 0.5$  glands/ml.

### Partial Purification on Con A-Sepharose Column

GCV was fractionated by affinity chromatography on Con A-Sepharose 4B. The biological activity was recovered in the fraction eluted in one step by a mixture of 0.25 M  $\alpha$ -methyl-D-mannoside and 0.25 M 1-O-methyl  $\alpha$ -D-glucopyranoside in Ringer's solution (Fig. 3). The elution profiles were very reproducible throughout the experiments. The biological ac-

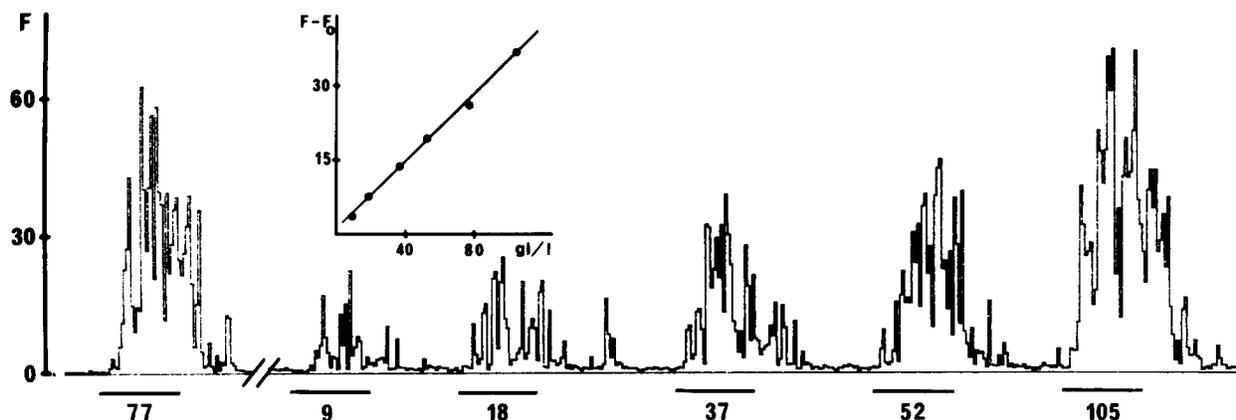


FIGURE 1 Dose-dependence of GCV effect and assay of venom fractions at the frog neuromuscular junction. Mepps are intracellularly recorded from the same muscle fiber and their mean frequency is evaluated during successive 15-s intervals. At the beginning of the experiment, a test dose of GCV is applied for 8 min (bar), then washed out. The fractions to be tested are then applied under the same conditions during the interval denoted by  $\text{---}$ . At the end of the experiment (150 min later in this case), increasing doses of GCV are perfused for 8 min (bars) and washed out. For each dose, the final GCV concentration is indicated in venom gland/liter. The mean mepp frequency  $F$  is evaluated during periods of 9 min starting 2 min after venom introduction to take into account the initial and washing delays. The resting mepp frequency  $F_0$  is subtracted and  $F - F_0$  is plotted against the GCV concentration (*inset*). The activity of the venom fractions is determined from the regression line.

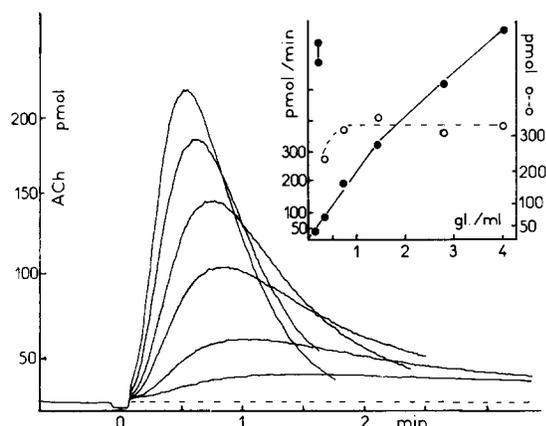


FIGURE 2 Dose-dependence of GCV effect on synaptosomal ACh release. Time-course of ACh release triggered by increasing doses of GCV. The slope of the rising phase ( $\bullet$  in *inset*) increases with the venom concentration. The total amount of ACh released ( $\circ$  in *inset*), estimated from the surface of the light response, is independent of the venom dose and corresponds to the free ACh pool (9). For low venom doses, the long duration of the response leads to underestimation of the total amount released. 20  $\mu$ l of synaptosomes deriving from 10 mg of electric organ were used for each determination in 0.28 ml of reaction medium. The light responses were calibrated by comparison with known ACh standards injected in each tube.

tivity estimated using *Torpedo* synaptosomes paralleled that measured at frog neuromuscular junctions. The mean total recovery of biological activity was good (72% for synaptosomes and 85% for frog neuromuscular junction). Since only 2% of the GCV proteins were recovered in the eluted fractions, such an affinity column permits an important increase in the specific activity of the neurotoxin. The highest purification was obtained in fraction 5 ( $\times 165$  when determined with synaptosomes and  $\times 245$  when determined at frog neuromuscular junctions). In all experiments, the recovery of biological activity was higher when estimated at the frog neuromuscular junction.

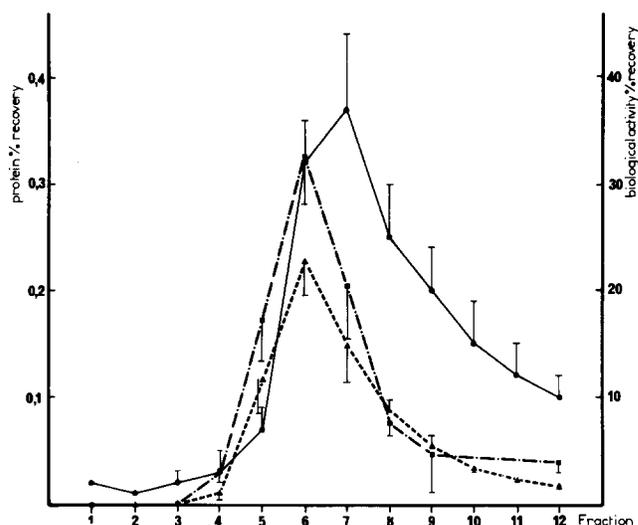


FIGURE 3 Chromatography of GCV on Con A-Sepharose. The protein content ( $\bullet$ ) and biological activity on *Torpedo* synaptosomes ( $\blacktriangle$ ) and frog neuromuscular junctions ( $\blacksquare$ ) are expressed as percents of the same parameters for the sample layered on top of the column. Five experiments were pooled. The mean protein content of the initial sample was  $213 \pm 36 \mu\text{g/gl}$  (mean  $\pm$  SEM).

### Loss of Biological Activity Following Incubation with Presynaptic Plasma Membranes

In seven experiments, where GCV was incubated with presynaptic plasma membranes prepared from either *Torpedo* electric organ or synaptosomes, the biological activity was no longer detectable either on synaptosomes or at frog neuromuscular junctions. To determine whether this effect was specific for the presynaptic membranes, GCV (2.7 glands) was incubated for 2 h at room temperature with different *Torpedo* electric organ membrane fractions (20) in 180  $\mu$ l of frog Ringer's solution. After centrifugation, the biological activity of the supernatant was assayed. The results obtained using different concentrations of each membrane fraction are

shown in Fig. 4. In a previous work (20), it was shown that after fractionation of *Torpedo* synaptosomes, the presynaptic plasma membranes are recovered in fractions M3 and M4 whereas fraction M5 contains essentially plasma membrane fragments of the postsynaptic cell. Fraction M5, even at high concentrations, failed to remove the biological activity. By contrast, fractions M3 and M4 could completely suppress this activity for membrane protein concentration of the order of 50  $\mu\text{g}$  protein per venom gland. It is worth noting that, though incubation was carried out with *Torpedo* membranes, measurements based on either frog mepp frequency or ACh release from *Torpedo* synaptosomes led to identical results for both the relative potency of the membrane fractions and the concentrations inhibiting 50% of the biological activity.

Control experiments were carried out to rule out a possible degradation of the neurotoxin during incubation with membranes. The residual activity was studied as a function of the duration of incubation with crude presynaptic plasma membranes (18). GCV and membrane concentrations were adjusted so as to obtain a large but not total loss of activity. Fig. 5 shows that the loss of activity occurred rapidly and then stabilized: for incubations longer than 2 min, no further decrease was observed.

### Membrane-Venom Interactions in $\text{Ca}^{2+}$ -free Media

Removal of the GCV neurotoxin by presynaptic plasma membranes was not abolished when  $\text{Ca}^{2+}$  concentration was strongly reduced. Presynaptic membranes directly prepared from *Torpedo* electric organ homogenates were incubated for 1 h with GCV (90  $\mu\text{g}$  protein/gland) in the presence of 2.5 mM EGTA (free Ca: 20  $\mu\text{M}$ ). A total inhibition of the biological activity on both frog neuromuscular junction and

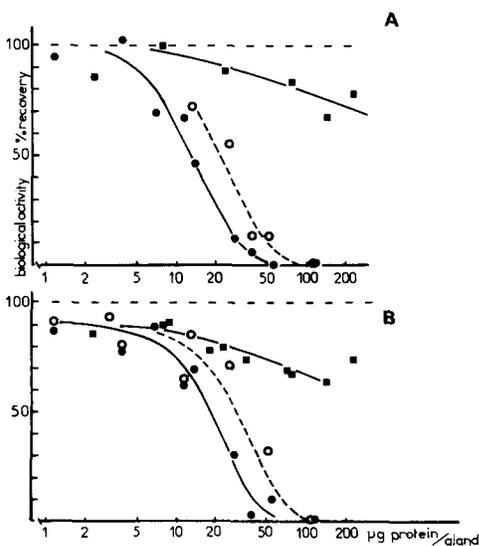


FIGURE 4 Inhibition of venom activity following incubation with *Torpedo* membranes. After filtration through G 75 Sephadex, GCV was incubated for 2 h with fractions containing presynaptic plasma membranes M3 (●) and M4 (○) or membranes of the postsynaptic cell M5 (■). After centrifugation, the biological activity of the supernatant (residual activity, expressed as percent of filtrated GCV activity), was measured at the frog neuromuscular junction (A) and on *Torpedo* synaptosomes (B). It is plotted against the membrane protein concentration expressed in micrograms of membrane protein per venom gland on a logarithmic scale. Results from two experiments.

*Torpedo* synaptosomes was then observed as in control incubations carried out in the presence of 1.8 mM  $\text{Ca}^{2+}$ . It has been checked that no loss of activity occurred when GCV was incubated with 2.5 mM EGTA alone.

In view of the fact that GCV-induced ACh release was found to be  $\text{Ca}^{2+}$ -dependent on in situ nerve terminals (15, 16), this problem was reinvestigated on isolated *Torpedo* synaptosomes (Fig. 6). The synaptosomes were prepared in  $\text{Ca}^{2+}$ -free media, and GCV was passed through a G.50 Sephadex column equilibrated with 10 mM phosphate buffer at pH 7.4. Various amounts of  $\text{Ca}^{2+}$  were added in the reaction mixture just before GCV introduction. In these conditions, a clear  $\text{Ca}^{2+}$ -dependence was observed.  $\text{Mg}^{2+}$  was not able to substitute for  $\text{Ca}^{2+}$ .

### Absence of Membrane-Venom Interactions in Synaptosomes Pretreated with Pronase

To determine the kind of interaction between the neurotoxin and the presynaptic plasma membrane, it was of interest to see if an ectocellularly oriented membrane protein was involved. We knew that pronase induces the loss of ~30% of membrane proteins without impairing the depolarization induced ACh release (Morel et al., in preparation). This enzyme was thus used to treat synaptosomes which were first tested for their ability to release ACh upon venom application, then subfractionated so that their plasma membranes might be tested for their ability to remove the biological activity from the crude extract.

Intact synaptosomes were incubated for 2 h at room temperature either with or without 0.25 mg/ml pronase (Boehringer Mannheim Biochemicals, Indianapolis, IN). ACh release

FIGURE 5 Time course of inhibition of GCV activity by presynaptic membranes. The residual activity after variable durations of incubation with crude presynaptic plasma membranes (100  $\mu\text{g}$  protein/gland) was measured on *Torpedo* synaptosomes. Activity is expressed as percent of that of GCV incubated in the absence of membranes.

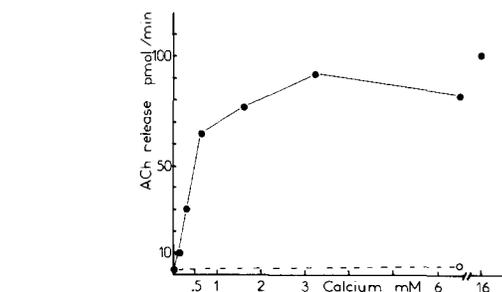


FIGURE 6  $\text{Ca}^{2+}$  dependence of GCV-induced ACh release from *Torpedo* synaptosomes. 30  $\mu\text{l}$  of synaptosomes (corresponding to 15 mg of electric organ) prepared in  $\text{Ca}^{2+}$ -free media were exposed to GCV (0.3 glands/ml) in the presence of 6.5 mM  $\text{Mg}^{2+}$  (○) or of various  $\text{Ca}^{2+}$  concentrations (●). The rate of ACh release was estimated from the slope of the light emission rising phase and calibrated by internal ACh standards.

induced by 100 mM KCl or GCV was then tested (Fig. 7). While the KCl-induced release was only slightly decreased after pronase treatment, the venom-induced release was abolished.

Control and pronase-treated synaptosomes were then diluted and centrifuged (10,000 *g* for 20 min), a procedure that allows elimination of pronase. They were disrupted (osmotic shock associated with freezing and thawing) and their membranes recovered in parallel as described (20). The membranes were incubated for 1 h at room temperature with GCV (55  $\mu$ g protein/gland). As expected, GCV incubated with control membranes was no longer active either at the frog neuromuscular junction or on *Torpedo* synaptosomes. By contrast, the activity was completely recovered after incubation with membranes of pronase-treated synaptosomes (respectively 94 and 96% recovery for the two test preparations). The above results show that pronase destroys a membrane protein interacting with the neurotoxin without affecting the ACh release process itself.

#### Absence of Membrane Venom Interactions in Preparations Treated by Con A

After application of a test dose of GCV and washing with Ringer's solution, a frog muscle was exposed for 50 min to 500  $\mu$ g/ml Con A dissolved in Ringer's solution. After a 15-min wash-out of the unbound Con A, the same test dose of

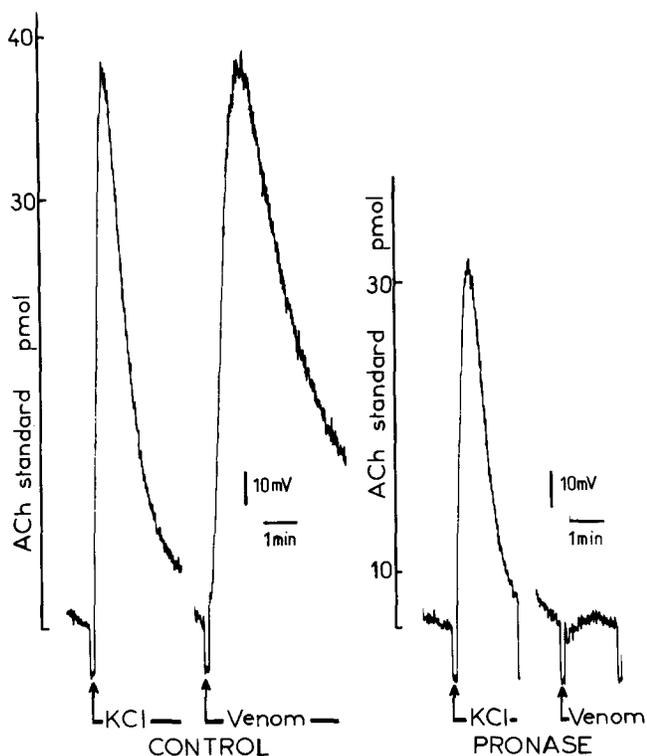


FIGURE 7 ACh release from control and pronase-treated synaptosomes. ACh release from 30  $\mu$ l of synaptosomes (corresponding to 17 mg of electric organ) was triggered by addition of either KCl (100 mM final concentration) or Sephadex filtered GCV (0.2 g/ml final concentration). Synaptosomes had been incubated for 2 h at room temperature without (CONTROL) or with (PRONASE) 0.25 mg/ml pronase. The ACh release was quantified by comparison with ACh standards injected in the same test tube. Note that pronase present in the synaptosomal samples reduces the sensitivity of the chemiluminescent reaction.

GCV was completely inactive. Similarly, *Torpedo* synaptosomes were treated for 90 min at 4°C by 500  $\mu$ g/ml Con A. Unbound Con A was removed by dilution and centrifugation of the synaptosomes which were then tested for their ability to release ACh. Whereas ACh release triggered by 60 mM KCl was not affected, the venom-induced release was reduced to 30–50% of the control. When the dimeric derivative succinyl-Con A was used in place of the tetrameric lectin at 500  $\mu$ g/ml, neither KCl-induced nor venom-induced ACh release was reduced. No attempt was made to quantify the inhibitory effect of Con A since the synaptosomal suspension contains sugars (5 mM glucose and 400 mM sucrose) which interact with the lectins.

Crude presynaptic plasma membranes (250  $\mu$ g of protein in 180  $\mu$ l of Ringer's solution) were incubated at 4°C for 50 min with various concentrations of Con A or succinyl-Con A. The lectins were washed out by three successive centrifugations and resuspensions (Beckman airfuge, Beckman Instruments, Inc. 160,000 *g* for 5 min). The membranes were then incubated with GCV (92  $\mu$ g protein/venom gland) for 1 h at room temperature. After centrifugation, the biological activity of the supernatant was tested on synaptosomes and frog muscle (Fig. 8). Whereas control membranes (incubated without lectin) totally removed the activity, Con A-treated membranes were much less efficient. Membranes treated with 500  $\mu$ g/ml Con A retained only 10% of the activity. The dimeric form of the lectin was about 10 times less potent in preventing the binding of the neurotoxin.

#### Inhibition of the Venom Activity by Antiserum

Rabbits were immunized against the partially purified neurotoxin obtained by Con A affinity chromatography (fractions 5, 6, and 7 of Fig. 3). Because of interferences of rabbit sera with the chemiluminescent reaction, the effects of the antisera were tested only on frog neuromuscular preparations. GCV passed through G 75 Sephadex (11 glands/ml final concentration) was incubated with antiserum at various dilutions in Ringer's solution at 4°C for 3 h. After centrifugation (160,000 *g* for 15 min in Beckman airfuge, Beckman Instruments, Inc.), the biological activity was assayed in the supernatant. The results are shown in Fig. 9. A complete inhibition was obtained for antiserum concentration of the order of 1  $\mu$ l

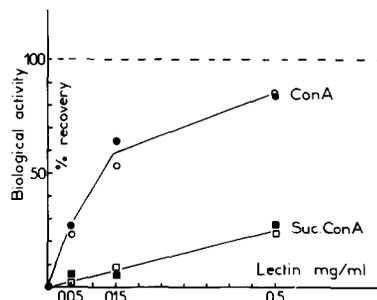


FIGURE 8 GCV biological activity after incubation with presynaptic membranes pretreated by Con A or Suc. Con A. Isolated presynaptic plasma membranes were incubated with various concentrations of Con A (○, ●) or Suc. Con A (□, ■), and unbound lectin was washed out (see Results). They were then incubated with GCV. After centrifugation, the biological activity recovered in the supernatant was measured on *Torpedo* synaptosomes (●, ■) and at the frog neuromuscular junction (○, □), and compared with GCV activity. Percent recovery is plotted against the lectin concentration used for the isolated membranes pretreatment.

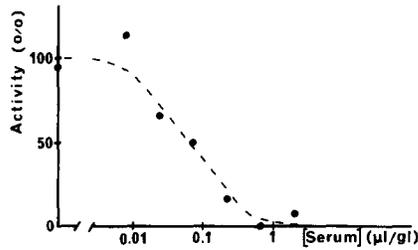


FIGURE 9 Inhibition of venom activity by a rabbit antiserum raised against GCV glycoproteins. After incubation with different antiserum concentrations, the biological activity was measured at the frog neuromuscular junction.

antiserum per venom gland. This demonstrated the immunoreactivity of the neurotoxin. The antisera have been used to characterize the venom glycoproteins.

### Correlation between Protein Patterns and the Biological Activity of GCV Fractions

GCV was partially purified on Con A-Sepharose column. The biological activity of the eluted fractions was measured on *Torpedo* synaptosomes and at frog neuromuscular junctions. An aliquot of the fraction exhibiting the highest specific activity (4 in Fig. 10) was incubated with crude presynaptic plasma membranes for 20 min at room temperature (~150 µg of protein for a biological activity equivalent to that of one venom gland). After centrifugation, no activity was recovered in the supernatant (fraction 4'). Equal aliquots of the different fractions were boiled for 4 min in the presence of 2.5% SDS and 5% β-mercaptoethanol, then submitted to gel electrophoresis. Numerous bands were observed after silver staining. One of them (~300,000 mol wt) seemed to correlate with the biological activity in the fractions. In particular, it was no longer detectable in fraction 4' which had been incubated in the presence of presynaptic plasma membranes. It must be emphasized that, even in the purest fraction, this protein represents only a few percent of the fraction glycoproteins.

Since antisera directed to GCV glycoproteins were able to inhibit the neurotoxin activity (Fig. 9), it was necessary to check that they recognized the 300,000-mol-wt protein. Fractions 4 and 4' were concentrated by dialysis under negative pressure and boiled as described above. Protein bands were separated on a 7.5% acrylamide gel and transferred by diffusion on nitrocellulose filters. After incubation with rabbit antisera directed to GCV glycoproteins (diluted to 1:500), the Ig binding was visualized using peroxidase-labeled antibodies to rabbit IgG (Fig. 11, left). As expected, numerous bands were recognized by the antisera. The 300,000-mol-wt band was labeled in fraction 4 whereas it was not detected in fraction 4'. No other obvious differences were observed between these two fractions, a finding that agrees with previous determination of the neurotoxin molecular weight using other methods (24).

The presynaptic membranes that had been used to adsorb the neurotoxin of fraction 4 and control presynaptic membranes were solubilized in parallel in 5% cholate in 10 mM Tris buffer pH 8. The protein concentration was ~1.5 mg/ml. The glycoproteins were purified on Con A-Sepharose as described for the venom, except for the presence of 1% cholate in all solutions. The eluted proteins were concentrated by dialysis against 10 mM Tris buffer pH 8 under negative pressure. They were boiled for 4 min in 2.5% SDS-5 β-

mercaptoethanol and then submitted to gel electrophoresis in a 5% acrylamide gel. The proteins were transferred, incubated with antiserum directed to venom glycoproteins, and labeled as described above. No binding of the antiserum to control presynaptic membranes was detected (Fig. 11, right). This made it possible to detect the venom glycoproteins bound to the incubated presynaptic membranes. A band (mol wt, 180,000) was heavily labeled. Other bands were much less labeled. Since they are heavily labeled in the venom fractions

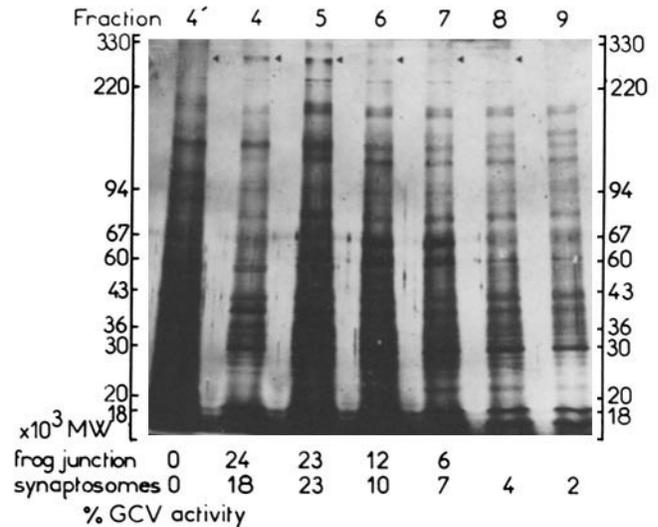


FIGURE 10 Protein patterns of the purified venom fractions. Venom glycoproteins were purified on Con A-Sepharose, boiled in 2.5% SDS 5% β-mercaptoethanol for 5 min and electrophoresed in a 5–15% acrylamide gradient gel (40 mA, 5 h). Proteins were silver stained. The biological activity recovered in each fraction is indicated in percent of GCV activity. Each slot contains a fraction aliquot deriving from six venom glands. Fraction 4' corresponds to fraction 4 after incubation with presynaptic plasma membranes and removal of membrane-bound proteins by centrifugation. Protein standards (Pharmacia) were used for the determination of molecular weights.

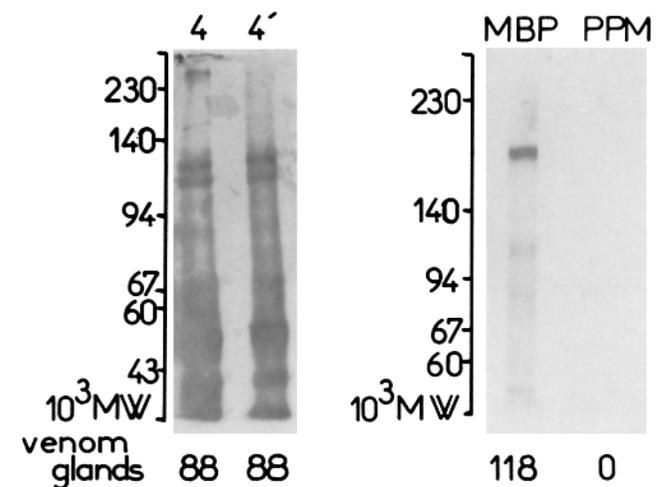


FIGURE 11 Immunological labeling of GCV glycoproteins. Left: labeling of glycoproteins of fraction 4 (active) and 4' (4 inactivated by incubation with presynaptic membranes). Each fraction derived from 88 venom glands. Right: labeling of glycoproteins recovered from presynaptic plasma membranes incubated (MBP) or not (PPM) with fraction 4 prepared from 118 venom glands. See Results for experimental procedure.

and since the membrane pellet was not washed, they most likely represent venom components unspecifically trapped in the membrane pellet.

## DISCUSSION

In the present work, we confirm that the venom biological activity is correlated with the presence of a 300,000-mol-wt protein band. This band was observed after gel electrophoresis of samples boiled for 5 min in the presence of 2.5% SDS and 5%  $\beta$ -mercaptoethanol, suggesting that this protein is made up of a single polypeptide chain. The neurotoxin could be partially purified on a Con A affinity column and is therefore a glycoprotein. The close similarity between results obtained using either frog neuromuscular junctions or *Torpedo* synaptosomes, especially adsorption of the activity on isolated *Torpedo* presynaptic plasma membranes, strongly suggests that the same molecule is responsible for the venom-induced ACh release in both preparations. The neurotoxin represents only a small fraction of the venom gland proteins. From Fig. 10, the neurotoxin represents at most 5% of the proteins of the most enriched fraction (fraction 4) which was enriched  $\sim 200$ -fold when compared with GCV (213  $\mu\text{g}$  protein/gland, see Fig. 3). An upper limit of the neurotoxin content of a venom gland can thus be estimated to  $0.05 \times 213/200 = 0.05 \mu\text{g}$  protein (300,000 mol wt) corresponding to  $5 \cdot 10^{-8} \times 6 \cdot 10^{23}/3 \cdot 10^5 = 10^{11}$  molecules/gland. Since the effect of a solution containing 10 glands/liter is easily detectable (Fig. 1), the neurotoxin is active in the picomolar range.

The large size of the GCV neurotoxin, the short delay, and reversibility of the effect at neuromuscular junctions argued against the penetration of the toxin into the nerve terminal and suggested a direct action on the presynaptic plasma membrane. Binding of the neurotoxin to membranes prepared from *Torpedo* electric organ synaptosomes was directly demonstrated by immunological labeling: the antiserum that was shown not to react with any protein of the presynaptic membrane, but reacted with many GCV glycoproteins, heavily labeled a single band (180,000 mol wt) associated with incubated membranes (Fig. 11). Since the 300,000-mol-wt band corresponding to the neurotoxin is labeled by the antiserum and is the only band of fraction 4 (Figs. 10 and 11) to disappear after incubation with presynaptic membranes, the 180,000-mol-wt band bound to presynaptic membranes necessarily derives from the neurotoxin. The difference in molecular weight could be explained by a modification of the neurotoxin once bound or by its proteolysis during the steps (solubilization, purification on Con A column, and concentration) which precede the gel electrophoresis.

However, such an approach did not allow a quantitative evaluation of the binding that was indirectly obtained by measuring the residual activity of the supernatant of GCV incubated with presynaptic plasma membranes. The loss of biological activity during incubation (Fig. 5) rapidly stabilized at a constant level. This ruled out the possibility of an inactivation of the neurotoxin by a membrane-bound protease since a complete inactivation following an exponential decay would then be observed. The binding appeared to be specific for the presynaptic plasma membrane since incubation with other plasma membrane fractions from the electric organ only slightly affected the GCV activity (Fig. 4). Externally applied pronase suppressed the binding, thus demonstrating that the neurotoxin bound to an ectocellularly oriented protein. Pre-treatment of the membranes by Con A inhibited the binding

of the neurotoxin, succinyl-Con A being 10 times less potent. The difference in potency between the dimeric and tetrameric form of the lectin suggests that blockade of the binding results from a reduced accessibility due to cross-linkage of glycoproteins (5). Such an aggregation is likely to be passive since it was obtained on isolated membranes. Nerve terminals pre-treated by pronase or Con A still released transmitter following depolarization. By contrast, GCV-induced ACh release was abolished. This strongly suggested that the binding of the neurotoxin was necessary for inducing the biological effect.

The stability of the binding with *Torpedo* presynaptic membranes is in apparent contrast to the rapid washing of the effect at frog neuromuscular junctions. However, it must be remembered that the GCV-induced discharge is different at frog neuromuscular junctions and at *Torpedo* nerve-electroplaque junctions (15, 24). At the former, the effect results from the summation of mepp bursts whereas at the latter, the miniature potential discharge is more continuous. It is thus possible that, at frog nerve terminals, the neurotoxin unbinds after having exerted its effect.

The GCV-induced ACh release from nerve terminals in situ has been shown to be  $\text{Ca}^{2+}$ -dependent (15, 16). In the present work, GCV action on isolated *Torpedo* nerve terminals was also found to be  $\text{Ca}^{2+}$ -dependent, a feature that could not be detected in earlier experiments carried out with less reliable and less sensitive methods (12, 21). Since the neurotoxin binding does not require  $\text{Ca}^{2+}$ , GCV very likely acts by triggering a  $\text{Ca}^{2+}$  entry into the nerve terminal. The prerequisite of a binding cannot completely rule out a channel-forming action for GCV. Such an action has been reported to occur in artificial lipid bilayers exposed to gland extract of the related species *Glycera dibranchiata* (13). However, *G. dibranchiata* extracts were not able to trigger ACh release when applied to our preparations (unpublished results). Alternatively, the protein that binds GCV could be involved in the control of  $\text{Ca}^{2+}$  membrane permeability.

Among the neurotoxins that act presynaptically (see reference 6 for a review),  $\alpha$ -latrotoxin purified from black widow spider venom (4) resembles GCV in its ability to trigger quantal transmitter release (7). Its effect occurs also after binding to the presynaptic plasma membrane (17, 25). The binding site is a protein not found in non-neuronal tissues (17, 25). Prior treatment with Con A abolishes both the binding (17, 25) and the toxin-induced release (1, 17, 22). The binding of  $\alpha$ -latrotoxin or GCV neurotoxin to a protein found only in presynaptic plasma membrane raises the question of the function of these proteins. At the present time, no data are available that would permit deciding whether or not they are directly involved in the release process itself.

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