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Monoclonal antibodies against the presynaptic calcium channel antagonist ω-conotoxin GVI A from cone snail poison

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Monoclonal antibodies have been prepared against ω-conotoxin GVI A, a peptide isolated from marine snails of the genus *Conus* (*Conus geographus* and *Conus magus*). This toxin is a blocker of select presynaptic Ca^{2+} channels in the central nervous system. Antigenic ω-conotoxin GVI A was synthesized as a covalent conjugate with bovine serum albumin and injected s.c. An ELISA assay combined with a competitive inhibition assay was used to select and characterize monoclonal antibodies able to recognize and bind the free toxin. Several of the antibodies were found to block ω-conotoxin GVI A inhibition of ^{45}Ca transport into rat brain synaptosomes and to block ω-conotoxin GVI A binding to membranes from the same preparation. The antibodies recognize native, synthetic toxin, and are useful for analysis of toxin in biological fluids.

Conotoxin; Calcium; Monoclonal; Channel; Presynaptic; Transport

1. INTRODUCTION

ω-Conotoxin GVIA (ω-ctx GVIA) is a bicyclic 27 amino acid peptide isolated from the marine snail, *Conus geographus* and *Conus magus* [1,2], which inactivates select presynaptic calcium channels in the central nervous system [3-6]. The bicyclic structure is defined by disulfide bonds between 6 cysteines (see fig.1). Other structurally related polypeptide toxins which are less well characterized also occur in these snails, including α-conotoxin, a nicotinic antagonist and μ-conotoxin, a Na^+ channel antagonist [1,2]. ω-ctx GVIA does not seem to have an effect on the dihydropyridine-sensitive Ca^{2+} channels at the postsynaptic level [7-9], and indeed appears to bind to brain calcium channels at separate sites from the binding sites of verapamil or dihydropyridine analogues [7,8]. However, there is presently no method to measure ω-ctx GVIA in tissue extracts or biological fluids, other than biological activity, and there exist no specific antagonists to the ω-ctx GVIA. This problem has thus severely limited the pharmacologic exploration of the molecule. Indeed, in recent studies on the activity of ω-ctx GVIA on chromaffin cells [10] and islets of Langerhans [11], it was necessary to verify ω-ctx GVIA potency by measuring behavioral effects in rats subjected to intracerebroventricular administration of the toxin [10].

To provide a useful tool for the study of the physiology of these Ca^{2+} channels in the nervous system, we have now prepared monoclonal antibodies against the ω-ctx GVIA molecule. In this report we describe the procedures required to synthesize an immunogenic form of ω-ctx GVIA [12,13], and the method for successful production of monoclonal antibodies to the free toxin. These antibodies do not exhibit cross-reactivity with other toxic peptides with related chemical structures. In at least one instance synthetic ω-ctx GVIA has been shown to be equally toxic to authentic native toxin [14].

2. MATERIALS AND METHODS

2.1. *Preparation of immunogen*

Synthetic ω-ctx GVIA (Peninsula Lab. Inc., Belmont, CA) (0.5 mg) solubilized in 100 μl of dimethylformamide (Sigma Chemical Co., St. Louis, MO) was added with stirring to a solution containing 1.0 mg of bovine serum albumin (BSA) (Sigma Chemical

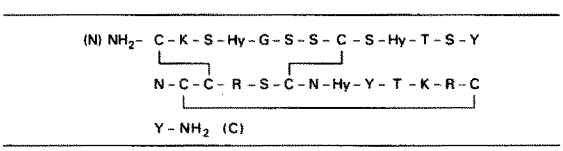

Fig.1. Structure of ω-ctx GVIA. The letters are the usual one letter codes for the 20 amino acids. Hy is hydroxyproline. Heavy lines between C (cysteine) residues represent disulfide bonds. Data are from the literature [1].

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Abbreviations: ω-ctx GVIA, ω-conotoxin GVIA; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin

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Co., St. Louis, MO) and 2.5 mg of EDC (1-ethyl-3,3'-dimethyl-amino-propyl-carbodiimide, Sigma Chemical Co., St. Louis, MO) in 3 ml of distilled water. The reaction was stirred at room temperature for 18 h and pH maintained at 5.5. The conjugated toxin was dialyzed through a Spectrapor dialysis membrane with a molecular weight cutoff of 3500 against distilled water for 72 h and then lyophilized [15].

2.2. *Mice and immunization*

Mice used in this study were Balb/c female (NIH stock). Mice (age 6–10 weeks) were immunized by injecting with 100 μg immunogen resuspended in 0.9% in NaCl, emulsified in complete Freunds adjuvant [16] (ratio 1:1), and injected subcutaneously. Three weeks later the animals received 50 μg of immunogen emulsified in incomplete Freunds adjuvant, and a booster injection with 50 μg immunogen resuspended in a saline solution 4 days prior to fusion.

2.3. *Myeloma cell line*

The myeloma cell line used was P3-NSI-1-Ag4-1, and was routinely maintained in tissue culture flasks in Dulbecco Modified Eagles Minimal Essential Medium (DMEM) containing 10% fetal calf serum (FCS) (Biofluids Inc., Rockville, MD).

2.4. *Cell fusion technique*

The spleens from immunized mice were aseptically removed and single cell suspensions were prepared. Spleen lymphocytes were fused with mouse myeloma cells (ratio 5:1, lymphocytes:myeloma) in the presence of polyethylene glycol (M.W. 1000) (Sigma Chem. Co., St. Louis, MO) according to the method of Kohler and Milstein [17]. Following three washes in FCS-free medium, the cells so produced were then resuspended in DMEM containing HAT (hypoxanthine, 10^{-4} M; aminopterin, 10^{-5} M; and thymidine, 4×10^{-5} M) and 10% FCS.

The cells were then plated out into ten 24 well plates which were incubated at 37°C in an humidified incubator containing 10% CO_2. The fused cells were fed with the same HAT medium 5 days after plating and the colonies were observed to be growing between days 12 and 15 [12]. Wells with a growth of cells were screened for specific immunoglobulin content using an ELISA assay [18–20].

2.5. *Enzyme-linked immunosorbent assay*

To select hybridoma producing specific immunoglobulins (IgGs), supernatants were tested in parallel against the free ω-ctx GVIA, the BSA-conjugated toxin and BSA alone. Assays were carried out in 96 well flat bottom, white polystyrene Dynatech microfluor plates (Dynatech Laboratories Inc., Alexandria, VA). The plates were prepared as follows: a 100 μl aliquot of a solution of phosphate-buffered saline pH 7.4 containing free ω-ctx GVIA, or BSA-conjugated ω-ctx, or BSA at a concentration of 10 μg/ml was added to all wells on the plate. After incubation overnight, the plates were washed three times with PBS containing 0.05% Tween 20. The plates were then blocked in 5% dry fat-free milk in PBS for 2 h at 37°C. 100 μl of the hybridoma supernatants were mixed with 0.01% Tween 20 and added to wells in a volume of 100 μl and incubated at 37°C for 2 h. They were then washed as above with PBS-Tween 20 before addition of 100 μl of β-galactosidase-conjugated sheep anti mouse IgG F(ab)2 fragment (Amersham Lab.) diluted in PBS-Tween 20 (1:250). The plates were then incubated for an additional 2 h at 37°C. 100 μl of a solution in PBS of the fluorogenic substrate 4-methylumbelliferyl-β-galactopyranoside was added to each well. Fluorescence was read in a Dynatech Microfluor reader 30 min after the addition of the substrate. Wells were considered positive when the fluorescent units were more than 1800 fluorescent units greater than the negative control (full scale = 4000).

2.6. *Competitive inhibition assay*

Competitive inhibition assays were carried out in 96 well flat-bottom, white polystyrene Dynatech microfluor plates, as described above [21,22].

The plates were prepared as follows: 100 μl of a solution of 0.1 M carbonate/bicarbonate buffer pH 8.6 containing ω-ctx GVIA at a concentration of 10 μg/ml was added to all wells on the plate. The plates were incubated for 2 h at 37°C, the non-adsorbed ω-ctx GVIA removed and the wells washed with PBS/Tween 20 as described above. An initial experiment was performed to determine the concentration of anti-toxin IgG that bound approximately 50% of the available sites on the solid phase-bound ω-ctx GVIA. The monoclonal IgGs were diluted to this concentration (50 μg/ml in PBS/Tween 20) for the competitive inhibition assay. Aliquots of 100 μl of serial log diluted toxin were then mixed with 100 μl of aliquots of monoclonal IgGs and incubated for 2 h at 4°C. Aliquots of 100 μl of these mixtures were added to ω-ctx GVIA-coated microtiter plates and incubated for 2 h at 37°C.

Toxin-coated wells incubated with monoclonal IgGs alone served as the non-inhibited positive controls, while wells coated with BSA only incubated with monoclonal IgGs were controls for nonspecific binding of monoclonal antibody. After incubation the plates were washed with PBS-Tween, as above, and detection of toxin-antibody reaction was determined with fluorogenic substrate 4-methylumbelliferyl-β-galactopyranoside as described above.

2.7. *Subcloning*

The clones with the highest reading over the control were selected for subcloning performed by the standard procedure of limiting dilution [16].

2.8. *Immunoglobulin purification and subclass identification*

Monoclonal immunoglobulin was concentrated from culture medium using microporous membranes on a Minitan Ultrafiltration System (Millipore Co., Bedford, MA) and then purified on a Protein-A-Sepharose column (Pierce Chemical Co., Rockport, IL) [23,24]. Monoclonal immunoglobulins were lyophilized and stored at −20°C.

Immunoglobulin subclass was determined by enzyme immunoassay kit for isotyping mouse monoclonal antibody provided by Zymed (Zymed Lab. Inc., San Francisco, CA).

3. RESULTS

3.1. *Preparation of monoclonal antibodies*

Small toxic molecules such as ω-conotoxin GVIA are notoriously difficult to use as antigens, and in preliminary studies we attempted to use the in vitro technique [18]. This method was unsuccessful, and we resorted to construction of a BSA–ω-ctx GVIA adduct and conventional in vivo methods. We chose BSA because it yields soluble conjugates, does not elicit significant production of antibody, and its reactivity is well known and predictable [12,13]. To form the adduct we used carbodiimide, since ω-ctx GVIA has a free amino terminal, suitable for forming amide linkages with carboxyl residues on BSA. We also enhanced the immunogenicity of the toxin–BSA conjugate by emulsifying in Freunds adjuvant prior to immunization.

The results of the successful fusion that yielded the monoclonal antibodies we describe in this report are summarized in table 1. As summarized, the ensuing successful fusion produced a high number of fused cells (337) compared to number of wells seeded (456). In our initial ELISA screening assay we tested supernatants for antibodies that recognize free ω-ctx GVIA or the ω-ctx GVIA–BSA conjugate. Of the 337 growing hybridomas, 26 were positive, with a specific efficiency of

Table 1

Production of monoclonal antibodies positive for reactivity of ω-ctx GVIA

Number of wells seeded	456
Number of wells with growth	337
Seeded wells with growth (%)	73
Number of positive culture supernatants detected by ELISA	26
Specific efficiency	7.7
Number of positive culture supernatants detected by competitive inhibition assay	3

7.7%. We also tested the specificity of the 26 hybridoma producing antibodies for ω-ctx GVIA binding by examining their reactivity to unconjugated toxin peptides with related structure. α-Conotoxin M1 and α-conotoxin G1 are two toxic peptides also found in the crude venom which are blockers of nicotinic receptors but which are homologous to ω-ctx GVIA. They have only 13 amino acids each, and only two disulfide bridges, rather than the three found in ω-conotoxin GVIA [1]. No reactivity of the 26 hybridoma supernatants was found against either the α-conotoxins G1 or M1 when both peptides were tested in their free state.

3.2. *Development of a competitive inhibition assay for ω-ctx GVIA*

To use the monoclonal antibodies for quantitative analysis we attempted to develop a competitive inhibition enzyme immunoassay. The principle of the method is to measure the ability of free toxin to inhibit binding of antitoxin antibody to solid phase-bound ω-ctx GVIA, under conditions of a non-saturating concentration of affinity purified antibody. As shown in fig.2, we incubated various dilutions of affinity purified monoclonal IgGs in wells coated with 10 μg/ml of ω-ctx GVIA. IgG concentration above 500 μg/ml saturated the binding sites, but below this concentration the curve was linear to 1 μg/ml of antibody. We therefore used a concentration of 50 μg/ml of monoclonal IgG in the remainder of experiments for development of the competitive inhibition assay. As shown in fig.3, the binding of anti-ω-ctx GVIA antibody to ω-ctx GVIA can be inhibited in a dose-dependent manner by free ω-ctx GVIA. The curve is linear between 1.0×10^{-4} M to 1.0×10^{-8} M, allowing the quantification of toxin in test samples over a 4 log range. The maximum sensitivity of the competitive inhibition assay is defined by the lowest toxin concentration that falls in the range of linear inhibition. By this method, however, only 3 of the original 26 hybridoma supernatants positive by the ELISA were found suitable.

3.3. *Evidence that antibodies detect native structure of ω-conotoxin GVIA*

The successful competitive assay also allowed us to examine structural features of the native ω-ctx GVIA

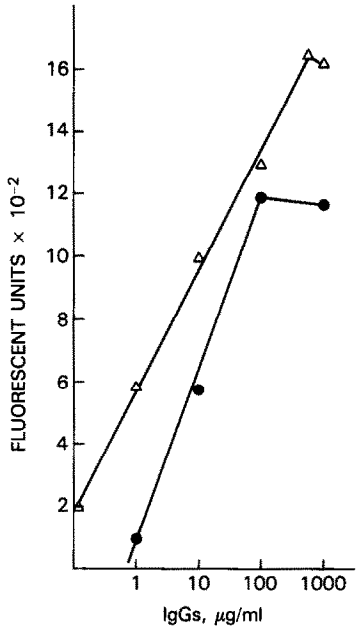

Fig.2. Titration curve for the binding of two different affinity purified monoclonal antibodies anti-ω-ctx GVIA to solid phase-bound unconjugated ω-ctx GVIA. The data are pooled from three experiments with four replicates for each point. SE was less than 10% of the mean in all cases.

detected by various antibodies, and to further test the possible reactivity of the α-conotoxin homologues. ω-ctx GVIA was boiled in PBS for 10 min, and tested for reactivity in the competitive assay. As shown in fig.3, it was inactive even at 10^{-5} M. We also reduced and alkylated the toxin with DTT and iodoacetamide. After reducing excess iodoacetamide with DTT we found the resulting toxin inactive in the competition assay (see fig.3). We concluded that our anti-ω-ctx GVIA antibodies recognize exclusively the native form of the toxin.

3.4. *Detection of toxin in biological samples*

A valuable attribute for specific antibodies is the ability to detect an antigen in biological samples. We therefore added ω-ctx GVIA to human serum, and tested for the toxin directly. As shown in fig.3, recovery in the concentration range of 10^{-5}–10^{-8} M was nearly that of buffer alone. Less was recovered at lower concentrations.

We observed that ω-conotoxin GVIA is quite toxic to the goldfish (*Carassius auratus*). We therefore injected toxic doses of toxin i.p. into goldfish, and froze the individual fish when evidence of intoxication occurred. Such evidence included loss of balance and subsequent death, in a period dependent on the dose of toxin. The fish were homogenized in ice-cold PBS, particulate material spun down at $20000 \times g$ for 30 min, and the supernatant solution assayed for ω-ctx GVIA. Recovery from intoxicated fish (range 1–100 nmol of ω-ctx GVIA) was ≥80%.

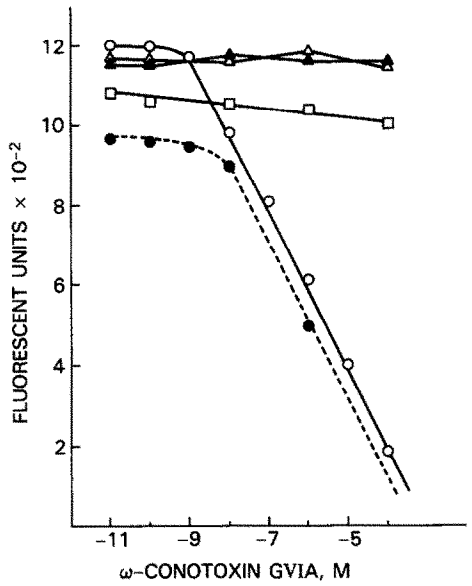

Fig.3. Inhibition of binding of affinity purified monoclonal antibody to solid phase-bound ω-ctx GVIA by various concentrations of free ω-ctx GVIA diluted in PBS (○—○) or in fetal calf serum (●—●). (△—△) and (▲—▲) represent the inhibition of binding of anti-ω-ctx GVIA antibody to ω-ctx GVIA by α-conotoxins G1 and M1. (□—□) represents inhibition of binding of anti-ω-ctx GVIA antibody by boiled and alkylated ω-ctx GVIA. Each point is the mean of eight replicates. The standard error was less than 10% of the mean.

3.5. *Antitoxin activity of antibodies*

ω-ctx GVIA elicits profound behavioral changes when instilled into rat brain, i.c.v. [1], and [^{125}I]ω-ctx GVIA binds specifically to regions of rat brain [25]. We found that preincubation of 10^{-6} M [^{125}I]ω-ctx GVIA with anti-ω-ctx GVIA monoclonal antibody for 30 min at 4°C blocked toxin binding to rat brain synaptosomes (data not shown).

4. DISCUSSION

In the present study we show that monoclonal antibodies can be prepared against ω-ctx GVIA, a specific blocker of presynaptic Ca^{2+} channels in the vertebrate nervous system [3-5]. The antibodies can detect ω-ctx GVIA immobilized on surfaces, and in some cases can be used as the basis for a sensitive competition assay. In the latter case the toxin added exogenously can be detected in biological fluids, such as serum, or in aqueous extracts of biological tissue. Our preliminary studies have also shown that our reagents have the potential to be adaptable to dipstick technology, should detection in environmental and biological samples prove of value.

All of the antibodies found suitable for the competition assay proved specific for native toxin. While we anticipated that reduction/alkylation would be inimicable to the interaction with antibody, it was not obvious that boiling would profoundly denature the otherwise triple-disulfide-bonded ω-ctx GVIA. Clearly, secondary and tertiary structure of the toxin is based on more than the constraints afforded by the disulfide bonds. These data provide us with an unexpected advantage, in that recovered activity in biological specimens represents, by definition, native molecules. The additional meaning is that the antigenic site for the monoclonal antibody must also be the site of binding of the toxin to the presynaptic calcium channel on the target membrane.

ω-ctx GVIA is similar in structure to a number of endogenous peptides. These range from endothelin [26], which is the most powerful hypertensive agent now available, to toxins such as α-conotoxins G1 and M1 [1], scorpion toxin [26], sarafotoxin [27] and apamin [26]. All have sequences with less than 30 amino acids, and have two or three disulfide bonds. We have only tested the most closely related α-conotoxins for cross-reactivity. However, the lack of cross-reactivity encourages us to expect that endogenous compounds yet to be tested or discovered, or indeed other toxins, will not be the basis of false positive reactions. Indeed, our methods may prove useful for developing immunological reagents with specificity for these other molecules.

Finally, the ability of our anti-ω-conotoxin GVIA monoclonal to inhibit binding of ω-ctx GVIA to rat brain synaptosomes emphasizes the likelihood of using our reagents as antitoxins for affected organisms. For example, we have shown that a monoclonal antibody against the trichothecene mycotoxin T-2 is capable of neutralizing T-2 toxin in human B-lymphoblastoid cells and in rats [28]. Another possible use is the production of anti-idiotype antibodies to our monoclonals. Such anti-idiotypes ought to mimic the action of the original toxin, and to provide ready delineation of presynaptic calcium channels in cells and tissues. The production of anti-idiotypes to this toxin will be aided by the fact that in mammals peripheral nerves and organs lack sensitivity to ω-conotoxin GVIA.

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