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Development and Testing of an In Vitro Assay for Screening of Potential Therapeutic Agents Active against Na Channel Neurotoxins

Annual Report

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A rapid screening assay based upon the ability of [^{3}H]BTX-B binding to rat brain synaptoneurosomes to report sensitively on ligand-receptor interactions at any of at least five distinct sodium channel binding domains in a single assay has been refined and reduced to practice. Data are presented to demonstrate the expected response from a series of sodium channel-active agents, including tetrodotoxin (TTX), saxitoxin, α-scorpion toxin, local anesthetics, and pyrethroid insecticides. Further radioligand binding assays and electrophysiological testing of HM-197, a simple hexahydropyrimidine analog of TTX, shows that its relatively potent effect to inhibit the binding of [^{3}H]BTX-B is not mediated through an interaction at sodium channel binding sites for TTX, local anesthetics or batrachotoxin. High performance liquid chromatographic separation of the scorpion venom from Leiurus quinquestriatus and testing of the resulting fractions with the screening assay revealed the presence in the venom of a component, called ∂-toxin, at low concentration,

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SUMMARY

A rapid screening assay based upon the ability of [^{3}H]BTX-B binding to rat brain synaptoneurosomes to report sensitively on ligand-receptor interactions at any of at least five distinct sodium channel binding domains in a single assay has been refined and reduced to practice. Data are presented to demonstrate the expected response from a series of sodium channel-active agents, including tetrodotoxin (TTX), saxitoxin, α-scorpion toxin, local anesthetics, and pyrethroid insecticides. Further radioligand binding assays and electrophysiological testing of HM-197, a simple hexahydropyrimidine analog of TTX, show that its relatively potent effect to inhibit the binding of [^{3}H]BTX-B is not mediated through an interaction at sodium channel binding sites for TTX, local anesthetics or batrachotoxin. High performance liquid chromatographic separation of the scorpion venom from *Leiurus quinquestriatus*. and testing of the resulting fractions with the screening assay revealed the presence in the venom of a component, called ∂-toxin, at low concentration, that potently inhibits the binding of [^{3}H]BTX-B in contrast to the polypeptide scorpion α-toxins that enhance binding. The potential of ∂-toxin as a therapeutic agent is discussed. Application of an empirical theory relating to polypeptide ligand-receptor interactions has focussed attention on a short segment of the sodium channel α-subunit sequence as potentially being involved in the binding of α-scorpion toxin to its sodium channel site. Monoclonal antibodies raised against this synthetic peptide cross react with components in the venom of *L. quinquestriatus*.. The implication of these finding for strategies of therapeutic agent development are discussed.

FOREWORD

Citations of commerial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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STATEMENT OF THE PROBLEM UNDER STUDY

The voltage-sensitive sodium channel of excitable cells has the distinction of being the most intensively studied, and best understood, transmembrane ion channel of this type. Such a high level of understanding has been brought about through many years of work using the techniques of electrophysiology, biochemistry, pharmacology and, most recently, molecular biology. In each phase of the work and regardless of the approach, progress has been aided and even enabled by the availability of numerous naturally-occurring neurotoxins that act specifically on this ion channel, binding at distinct sites or domains and eliciting a characteristic functional response. These neurotoxins represent some of the most potent non-protein toxins known, and they have been eagerly sought after and developed precisely because of their value in studying the sodium channel, following the maxim that potency and specificity are often positively correlated with usefulness as a molecular probe. That Nature has provided such an impressive array of these neurotoxins directed against the sodium channel is teleologically understandable when one considers the central importance of this channel to nerve and muscle function.

Against this background it is perhaps to be expected that the development and characterization of potential antitoxins or therapeutic agents has lagged behind, and such is in fact the case. Even in the face of various motivating forces such as the health hazards represented by the occurrence of toxic red tides in the Pacific Northwest (*Gonyaulax catenella*, saxitoxin) and the Florida coast (*Ptychodiscus brevis*, brevetoxin) there has to date been very little work aimed at the identification and development of therapeutic agents directed against sodium channel neurotoxins. In principle, such an effort is likely to be inherently more difficult than the converse, since one must identify agents that are themselves not toxic and the various assays that have been so useful in the identification of toxic agents are simply not designed to detect "inactive" compounds.

Possible approaches to the development of potential antitoxins and therapeutic strategies in general for protection against sodium channel neurotoxins are several. Structure-activity relationships within a known class of neurotoxins may provide clues to new structures that could serve as antagonists and lead to a program of synthesis. Our growing knowledge of the structure of the sodium channel protein itself may eventually provide information on the binding site topography for a particular neurotoxin, and its

relationship to channel function, permitting a rational synthetic design program for the development of antitoxins. Such knowledge could also form the basis for application of modern immunological techniques such as the production of specifically-acting monoclonal antibodies. In addition, the possibility should not be overlooked that Nature has already provided some useful, but as yet undiscovered, antitoxins.

Each of these approaches would be enhanced by the availability of a rapid screening assay that could detect interactions of test compounds at the sodium channel, whether these compounds arise from naturally-occurring sources, synthetic programs, or monoclonal antibody-producing myelomas. This central issue, the development of a rapid, reliable, and general screening procedure, has been a major focus in our current work. This report summarizes progress in the development of this assay and it reduction to practice in the identification of several potential antitoxins active at the voltage-sensitive sodium channel.

BACKGROUND AND REVIEW OF EARLIER WORK

The voltage-sensitive sodium channel protein of excitable membranes mediates the fast rising phase of the action potential in a wide variety of tissues. Electrophysiological studies have provided a precise phenomenological description of the sodium channel and the sequence of events occurring at that channel during an action potential. In brief, sodium channels in the resting state of an excitable cell exist in a closed, non-conducting conformation. In response to a local depolarization of the membrane the channel protein undergoes time- and voltage-dependent conformational changes, progressing through apparently multiple non-conducting states before reaching an open, conducting state permitting, selectively, the flow of sodium ions from extracellular to intracellular spaces down an electrochemical gradient. This process is termed activation. The open state is maintained only transiently before the channel closes, or inactivates, by adopting yet another conformation that is distinct from the closed, resting state. As the membrane potential recovers to resting values, the channel protein returns to the closed, resting configuration without passing again through an open state. The whole cycle is complete within a few milliseconds.

While these electrophysiological studies have provided a wealth of information, further progress in our understanding of the voltage-sensitive sodium channel has been greatly aided by the application of numerous specifically acting, naturally occurring neurotoxins and other pharmacological agents as molecular probes of sodium channel structure and function. These compounds have been instrumental in such diverse tasks as assessing the density of sodium channels in various membranes, probing structure-function relationships, and ultimately the purification to homogeneity and molecular cloning of the channel protein. Three classes of specifically-acting sodium channel neurotoxins in particular have been utilized extensively in these studies, including the heterocyclic guanidinium compounds tetrodotoxin and saxitoxin, the polypeptide neurotoxins from scorpion and sea anemone, and the so-called "lipid soluble" neurotoxins including batrachotoxin, veratridine, aconitine and grayanotoxin.

Experience with this latter group of sodium channel neurotoxins over the last decade has led to the remarkable observation that essentially every functional characteristic or electrophysiological descriptor of the sodium channel is altered or modified upon

binding of these compounds to a single, common site. Thus, binding of batrachotoxin, the prototypical and most potent member of the class, at once affects the voltage-dependent processes of sodium channel activation and inactivation, changes the selectivity of the channel, and alters the single channel conductance. In considering this broad spectrum of effects, one is led to invoke a model for sodium channel function that incorporates not only extensive conformational rearrangements, but also potential allosteric interactions among different protein domains, several of which may reflect upon a key structural element that includes the binding site for the lipid soluble toxins.

This general theme has been supported by work both from this laboratory and others in which the allosteric interactions of a variety of sodium channel neurotoxins and ligands with batrachotoxin binding site have been documented with direct radioligand binding measurements utilizing the biologically active probe [^{3}H]batrachotoxinin-A benzoate (BTX-B) (10). These results are summarized in Table I and have been recently reviewed (11). It is this unique relationship of the batrachotoxin site to other distinct binding domains on the voltage-sensitive sodium channel that has formed the basis for development of a reliable and rapid screening assay of broad scope and application.

The development of the screening assay has been discussed in some detail in the previous Annual Report and may be recalled with reference to the cartoon in Figure 1. Equilibrium specific binding of [^{3}H]BTX-B to sodium channels in rat cerebral cortex synaptoneurosomes is delicately balanced reflecting the opposing allosteric effects of non-labeled tetrodotoxin, deltamethrin, and α-scorpion toxin added to the incubation mixture at approximately half-maximal concentrations. Addition of test substances, such as X or Y in the cartoon, can lead to interaction at any of five at a minimum, and probably more, distinct binding domains on the channel resulting in either an increase or decrease in [^{3}H]BTX-B binding through an allosteric mechanism or an effect on the non-labeled agents. These sites include those for batrachotoxin itself, local anesthetics, tetrodotoxin/saxitoxin, pyrethroids and α-scorpion toxin. In order to maximize the ease of use we also developed procedures for preparation of synaptoneurosomes in bulk and subsequent frozen storage for periods up to four months. The assay has the advantage of casting a rather broad net for "sodium channel-active agents" in one simple step. Once these agents have been flagged by the assay, however, additional tests must follow to determine more specifically the mechanism of action and whether or not a therapeutic potential exists. As described in the previous Annual Report, we have employed both a second assay utilizing [^{3}H]STX and direct

electrophysiological measurements in a rat phrenic nerve-diaphragm preparation as second line measures complementing the screening assay.

The assay was successfully tested by noting that, as predicted, compounds or toxins known to affect the sodium channel were indeed flagged in the screening assay. One compound in particular has proven to be of further interest. HM-197, or 5-benzoyloxy-2-aminohexahydropyrimidine, was included among the first test compounds to be evaluated because of its reported tetrodotoxin-like effects at millimolar concentrations (12). However, we had previously found that HM-197 was able to inhibit the binding of [^{3}H]BTX-B much more effectively with a K_d of 35 μM. Since other work from our laboratory (6) demonstrated an inhibitory effect of TTX/STX binding on the binding of [^{3}H]BTX-B, we considered the possibility that HM-197 was interacting with relatively high affinity in the micromolar range at the TTX/SXT site, but that it had little efficacy as a channel blocker. If this were the case, then HM-197 might have some utility as a therapeutic agent against TTX or STX intoxication. Further testing, however, revealed no effect on the binding of [^{3}H]STX, thus ruling out the possibility that HM-197 was acting at the TTX/STX site of the sodium channel. Electrophysiological measurements on the other hand supported the screening assay results in that HM-197 was able to partially antagonize the effects of the depolarizing agent veratridine on phrenic nerve compound action potentials at concentrations where HM-197 alone had no effect. These experiments have been described in the previous Annual Report, leading to the conclusion that additional experiments would be required to elucidate the mechanism of action of this interesting and potentially useful compound.

These studies set the stage for the work to be described in this current report. The screening assay had been developed to the point where it could begin to take its place as a convenient and useful tool in a general strategy to search for and develop potential sodium channel antitoxins. During the last year, additional refinements have been made to the screening assay procedure, and we have focussed our attention on the mechanism of action of HM-197 and on potential antitoxins or therapeutic approaches for α-scorpion toxin poisoning.

RATIONALE

As mentioned previously, several different strategies may be identified that hold some promise for the identification or development of sodium channel antitoxins. Study of structure-activity relationships among families of neurotoxins or study of the receptor sites for these toxins can yield directions for the rational synthetic design of potential antitoxins. Antigenic neurotoxins can provide entries into immunotherapy through the production of specifically-acting monoclonal antibodies. Serendipity might even play a role, as in the case of HM-197. Particularly with respect to the polypeptide sodium channel neurotoxins from scorpion venom, we have felt that a profitable line of investigation might also be found in a closer examination of the components in the venoms as well as their structures *vis a vis* that of the sodium channel. This last point will be addressed further below.

The decision to pursue investigations of HM-197 as a potential therapeutic agent stems from the observation noted in the previous Annual Report that the compound could indeed provide some protection against the depolarizing action of veratridine in the rat phrenic nerve-diaphragm preparation. These effects occur at concentrations that place HM-197 in the same potency range as a number of clinically useful local anesthetics and anticonvulsants acting at the sodium channel. Coupled to the fact that HM-197 is a relatively simple molecule amenable to chemical modifications, these observations provide a strong motive to understand, and perhaps eventually to exploit, its mode of action.

Returning to the subject of scorpion neurotoxins, more than 100 such polypeptides have been purified from various scorpion species to date. The venom of a single species is a complex mixture of as many as 20-30 different components, among which may be several isoforms of α- and/or β-neurotoxins. The isoforms of each group are characterized by extensive sequence homologies, even between different species of scorpion, and similar modes of action, whereas the slight differences in amino acid sequence may be reflected in different potencies or target species specificities. For example, a particular α-toxin may be more potent in insects than in mammals. (See ref. 13 for a recent review). While progress in this area of research has been remarkable, one might adopt a different perspective and note that the majority of scorpion venom components have been ignored since they are not toxic. We have been interested in the possibility that an isoform or isoforms might exist among these unstudied components that, because of critical sequence differences, could still bind the sodium channel yet lack efficacy as a toxin; that is, components that might be

useful as antitoxins. Such components, if present, would be expected to be found at relatively low concentrations in toxic venoms where the antitoxin effects are overridden by the neurotoxins present at higher concentrations. In order to investigate this possibility we have therefore adopted the strategy of fractionating the venom by HPLC and testing the resulting fractions by the screening assay which is well-suited to the purpose.

As more and more information concerning the amino acid sequence, structure and possible functional domains of the voltage-sensitive sodium channel have become available (14, 15), new opportunities have arisen to investigate the particular relationship of channel ligands to their receptor sites. The identity of the receptor site for the polypeptide α-toxins is currently unknown, but we have begun to approach this problem in collaboration with Dr. Donard Dwyer of the Neuropsychiatry Research Program. In the context of some other studies, Dr. Dwyer has developed a novel theory relating to the interaction of polypeptide ligands with their protein receptors. By comparing the amino acid sequences of ligand and receptor site pairs in a series of examples for which such information is available, such as the interleukin II receptor system and the α-bungarotoxin/acetylcholine receptor system, Dr. Dwyer has made the empirical observation that for a given pair, receptor and ligand share discrete regions of significant sequence homology (Dwyer et al., unpublished observations; in preparation). In extending this analysis to α-scorpion toxin interaction with the sodium channel, Dr. Dwyer found one region, and one region only, of the sodium channel sequence that shares significant homology with the amino-terminal portion of α-scorpion toxins such as toxin V from *Leiurus quinquestriatus*. Interestingly, this sodium channel sequence, amino acids 1509 - 1536 as defined by Numa and colleagues (14), is in that region of the channel postulated to be involved in the process of inactivation, i.e. that process which is affected by the binding of α-scorpion toxins (13) and is one of the most highly conserved regions of the protein across isoforms from rat brain and eel electroplax sodium channels (16). This sequence and its relationship to the amino-terminal portion of *L. quinquestriatus* toxin V are shown in Figure 2.

This 27-amino acid channel peptide has been synthesized and, in collaboration with Dr. Dwyer, we have initiated a series of experiments to both evaluate the hypothesis regarding sequence homologies between scorpion toxin ligand and receptor sites on the sodium channel and to investigate the potential within this relationship for the development of therapeutic strategies or antitoxins. As an initial step, Dr. Dwyer has prepared monoclonal antibodies against the synthetic sodium channel peptide. This peptide proved

to be extremely antigenic, since more than 100 positive wells resulted from a single fusion. Approximately 20 of these have now been cloned for experimental use.

EXPERIMENTAL METHODS

A. Buffers. HEPES buffer was composed of 130 mM choline chloride, 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfornic acid), 5.5 mM glucose, 0.8 mM $MgSO_4$, and 5.4 mM KCl, adjusted to pH 7.4 with Trizma base. WASH buffer was composed of 163 mM choline chloride, 5mM HEPES, 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$ adjusted to pH 7.4 with Trizma base. TES buffer consisted of 20 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 112 mM NaCl, 2.5 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 15 mM $NaHCO_3$, 0.4 mM KH_2PO_4, and 10 mM glucose adjusted to pH 7.4 with NaOH. Modified TYRODE'S solution contained 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 15 mM $NaHCO_3$, and 11 mM glucose, pH 7.4. Isotonic SUCROSE solution consisted of 0.32 M sucrose, 10 mM NaH_2PO_4, pH 7.4.

B. Synaptoneurosomes. Synaptoneurosomes were prepared by a modification of the procedure described by Creveling et al. (2). For a detailed explanation, see the Appendix (A rapid screening procedure for the detection of compounds active at the voltage-sensitive sodium channel: A manual). Briefly, freshly dissected cerebral cortical tissue from male Sprague-Dawley rats was homogenized in 2 volumes (wt/vol) of ice cold HEPES buffer using 10 strokes of a loose-fitting glass-glass homogenizer. The homogenate was diluted with 2 additional volumes of cold buffer and centrifuged at 1000xg for 15 min at 5°C. The supernatant was discarded and the pellet was resuspended in HEPES buffer by repetitive pipetting with a 9 inch Pasteur pipette or by gentle homogenization in a loose-fitting glass-glass homogenizer. For preparation of "filtered synaptoneurosomes", the pellet from the 1000xg centrifugation was resuspended in 20 volumes of HEPES buffer (original wt/vol) by homogenization using 3 strokes of a loose-fitting glass-glass homogenizer and filtered first through three layers of HC-3 160 mesh nylon (Tetko, New York) followed by Whatman No. 4 paper. The filtrate was collected in ice cold tubes and subsequently filtered through a Millipore LCWP 047 filter having a 10 µm cut-off. This filtrate was then centrifuged at 1000xg for 30 min at 5°C, and the pellet resuspended in 10 mM phosphate-buffered isotonic sucrose, pH 7.4, for freezing and subsequent storage. Protein concentration was generally adjusted to approximately 6 mg/ml. Protein determinations were performed using the procedure of Peterson (19) with bovine serum albumin as a standard. Alternatively, protein concentratration was estimated by dilution of 75 µl of the tissue suspension in 1.5 ml distilled water and recording the absorbance at 280 nm (uncorrected for light scattering). The absorbance reading was converted to protein concentration with reference to a standard curve calibrated against protein concentrations determined with the Peterson procedure.

C. Measurement of tritiated toxin binding. Measurement of specific [3H]BTX-B binding was performed as reported previously (29). Briefly, standard binding reactions were initiated by addition of 150 µl of synaptoneurosome suspension in HEPES buffer containing approximately 1 mg protein to a solution in HEPES buffer of [3H]BTX-B and various concentrations of unlabeled effectors as indicated. The concentration of labeled toxin was generally 8-9 nM, and the total assay volume was 320 µl. The effectors were added from concentrated stock solutions (*L. quinquestriatus* scorpion venom, 2 mg/ml

water; TTX, 100 μM in water; veratridine, 10 mM in MeOH;, pyrethroids, 5 mM in MeOH; all others as indicated). For the standard screening assay, the concentrations of TTX, deltamethrin, and scorpion venom in the assay were 25 nM, 1 μM, and 2.5 μg/ml, respectively. Incubations were carried out for 45 min at room temperature and were then terminated by addition of 3 ml cold WASH buffer. The tissue was immediately collected on Whatman GF/C glass fiber filters using a Brandel 30-place filtration manifold, and washed 3 more times with 3 ml cold WASH buffer. Radioactivity associated with the tissue was determined by liquid scintillation spectroscopy of the filters suspended in 10 ml scintillation cocktail (3a70B, RPI). Nonspecific binding was determined from parallel assays containing 250 μM veratridine and has been subtracted from the data. For further details of the standard screening assay, see the Appendix.

For the determination of [^{3}H]BTX-B dissociation rates, synaptoneurosomes were pre-equilibrated with 9 nM labeled toxin and 12.5 μg/ml *L. quinquestriatus* scorpion venom with and without test agent in parallel. Following the pre-equilibration period, at time = t_0, excess veratridine was added (300 μM) to all tubes. At various time points over the next two hours, triplicate samples from assays both with and without test agent were filtered and the specific binding determined by liquid scintillation spectroscopy as above.

Data points for all binding experiments were determined in triplicate and are presented as the mean ± S.D. of these determinations. Unless otherwise indicated, figures show the results of 1 experiment that is representative of 2 or more separate determinations.

D. Scorpion venom fractionation. *L. quinquestriatus* scorpion venom was dissolved in 0.1 M ammonium acetate, pH 6.9/15% isopropanol at a concentration of 20 or 40 mg/ml by stirring at 5°C for one hour. The mixture was then centrifuged at 100,000 x g at 5°C for one hour to pellet the insoluble mucopolysaccharides. The clear supernatant was harvested and passed through a Rainin LX-N66045 pre-filter prior to storage at -20°C or fractionation by HPLC. Reverse phase gradient elution HPLC over a C18 column (generally using 0.1 M ammonium acetate, pH 6.9 with a conductance of 12.9 mSiemens and isopropanol as the two solvents) was performed on a Rainin instrument consisting of two HP-Rabbit pumps, pressure monitor, mixing chamber, Rheodyne injection manifold with either a 20 μL (analytical scale) or 100 μL (preparative scale) sample loop. The system was interfaced to an Apple computer for control of flow rates and gradient production. Column effluent was monitored with an ISCO V^4 variable absorbance monitor set at 280 nm and led to a Gilson model 201 programmable fraction collector. Fractions were concentrated for further use by rotary evaporation in a 40°C water bath to strip the isopropanol followed by lyophilization to remove water and the ammonium acetate. The proteinacious residues were generally redissolved in a minimal volume of 0.1 M ammonium acetate, pH 6.9, for further use or storage at -20°C.

E. Electrophoresis and Western blotting. Scorpion toxin fractions were subjected to SDS gel electrophoresis according to the Laemmli procedure (19) using a Hoeffer Mini-Gel apparatus and a 15% gel. Native gel electrophoresis was carried out as described by Catterall (20). SDS gels were blotted onto nitrocellulose membranes using the Poly-Blot apparatus from American Bionectics, Inc., according to the manufacturer's instructions.

Blotted proteins were probed with antibodies using the procedures of Birk and Kcepsell (21) to minimize nonspecific antibody binding and visualized with an HRP-coupled second antibody developed with diaminobenzidine as a substrate (22).

E. Electrophysiological measurements. The diaphragm and phrenic nerve were dissected from 150 g rats under pentobarbital anesthesia and a 5-10 mm diameter strip of muscle with nerve attached was suspended in an apparatus permitting measurement of the nerve compound action potential. The apparatus is a modification of that described earlier by Pagala (23). The muscle was fixed at the bottom of a glass chamber and connected to a force transducer at the top to measure muscle tension. Two pairs of platinum electrodes contacted the phrenic nerve for stimulation and recording of the nerve compound action potential. The muscle could be stimulated directly *via* two plate electrodes and the muscle compound action potential recorder by another platinum electrode situated just below the top tendon. The entire arrangement was immersed in TYRODE'S solution and bubbled continually with 95% O_2 and 5% CO_2. The apparatus was surrounded by a thermostatted circulating water jacket and the temperature controlled at 30°C. In order to collect the data presented here, the solution level in the apparatus was lowered below the level of nerve insertion in the muscle, and the distal nerve electrodes used to deliver supramaximal stimulating square wave pulses of 0.1 msec duration at a frequency of 100 Hz for 1 sec unless otherwise indicated. The resulting train of nerve compound action potential recorded at the proximal pair of electrodes was displayed on a storage oscilloscope and photographed with Polaroid film to produce a permanent record. Following these brief recording periods, the fluid level in the bath was restored to full height. Control experiments have demonstrated that, under these conditions, reproducible responses of the preparation may be obtained over a period of 5-6 hours.

RESULTS

Rapid screening assay. Testing or validation of the rapid screening assay as presented in the previous Annual Report has been extended by the inclusion of additional test compounds and determination of of complete dose response curves and calculation of $K_{0.5}$ values for each. These final results are tabulated in Table II, and representative dose response curves for nesacaine, THP and benzimidazole are shown in Figure 3. THP and THP-OH are tetrahydropyrimidine derivatives supplied by Dr. B. J. Gabrielsen, Fort Detrick, that were reported to have effects at the neuromuscular junction (B. J. Gabrielsen, personal communication). These compounds were of interest to the present studies because of the reduced pyrimidine nucleus reminiscent of that in HM-197, which is a hexahydropyrimidine. The list of compounds includes those known to act at the sodium channel with predictable effects on the binding of [^{3}H]BTX-B as well as others whose effects, if any, were unknown. As indicated in the table, the response to each of the sodium channel-active agents was as predicted, showing that under standard screening assay conditions this measurement reports faithfully on ligand-channel interactions at at least five sites, i.e. BTX, TTX/STX, local anesthetic, pyrethroid, and α-scorpion toxin binding domains.

In working with the standard screening assay over a period of time, we noticed some variability between duplicate experiments, not in the overall result but in the absolute amount of specific [^{3}H]BTX-B binding, even using the same tissue preparation. This prompted a re-evaluation of the binding of [^{3}H]BTX-B as a function of thawing conditions for the frozen synaptoneurosomes and time post-thawing before start of the assay. Two different tissue preparations were thawed at both room temperature and 5°C, centrifuged to pellet the tissue away from the sucrose buffer used for frozen storage, resuspended in HEPES buffer, and incubated on ice for varying periods of time before being aliqotted to assay tubes for measurement of [^{3}H]BTX-B binding under standard screening assay conditions with no other additions. The results of these experiments are shown in Figure 4. The method of thawing the tissue seems to have little effect on the overall level of binding, but in both cases there is an obvious decrease in that binding with time as the tissue sits on ice in HEPES buffer prior to the start of the assay. There is an initial rapid decrease that is essentially completed in the first 15 min followed by a much slower, yet steady decrease over the next two hours. Although we have not investigated the reason for this directly, we suspect that it is related to the equilibration of the tissue with the HEPES

buffer and establishment of a stable membrane potential. Since in the 45 min period following the initial rapid decrease, i.e. the time required for the assay itself, there is only about a 10% further loss in specific binding, we have elected to standardize the procedure by routinely thawing the tissue at room temperature and incubating the preparation on ice, following resuspension in HEPES buffer, for 15 min prior to the start of an assay.

Mechanism of action of HM-197. In view of the result previously reported that HM-197 effects on the binding of [^{3}H]BTX-B could not be correlated with binding at the TTX/STX site as originally suspected, we have begun to examine other possibilities to account for its mechanism of action. The possibility that HM-197 inhibits the binding of BTX-B by direct competition for the BTX-B binding site was evaluated by measuring the dissociation rate for BTX-B binding in the presence and absence of HM-197. A directly competitive mode of inhibition would be obviated if the dissociation rate for BTX-B were found to be altered in the presence of HM-197, since by definition HM-197 could not bind to any site that was occupied by BTX-B and therefore could not exert an influence on the off binding kinetics. As illustrated in Figure 5, the half-time for BTX-B dissociation, or the time at which ln $B(t)/B(0) = -0.69$, is reduced from 110 min in the absence of HM-197 to 70 min in the presence of 50 μM HM-197. This result demonstrates that HM-197 does not compete for the BTX-B binding site, but exerts its inhibitory effect through an allosteric mechanism upon binding to a different site.

Using an electrophysiological approach, we also examined the possibility that HM-197 is acting at a sodium channel site for local anesthetics since local anesthetics similarly reduce the dissociation rate constant for BTX-B binding resulting in a decreased binding affinity. Earlier experiments described in the last Annual Report showed that HM-197, at concentrations where the binding of BTX-B was profoundly inhibited, had essentially no effects on the compound action potential of rat phrenic nerve. If HM-197 were acting at a local anesthetic binding site, one might expect the effects of local anesthetic blockade of sodium channels as reflected in compound action potential height to be antagonized by inclusion of HM-197 in the solution bathing the nerve. The experiment shown in Figure 6 demonstrates that this was not the case. Control responses of a rat phrenic nerve mounted in the chamber described in Experimental Methods are shown in panel A. The nerve was stimulated supramaximally at 100 Hz (0.5 msec pulse width) for 1 second and the resulting 100 action potentials superposed on a storage oscilloscope. The effects of nesacaine (2-chloroprocaine hydrochloride) at a concentration of 150 μM following 30 min pre-incubation are presented in panel B. In preliminary experiments, this concentration of

nesacaine was found to produce approximately half-maximal effects on the compound action potential height. Panel C shows that pre-equilibration of the nerve with 100 μM HM-197 prior to addition of nesacaine did not appreciably alter the response to the local anesthetic.

Fractionation of *L. quinquestriatus* scorpion venom. The objective of these experiments has been primarily to ascertain if the venom contains potential antitoxins that might bind to the α-scorpion toxin site yet have little efficacy in terms of effects on the process of sodium channel inactivation. Secondarily, we have also focussed on the purification of toxin V, the most potent α-toxin in the venom, as a source of material for subsequent chemical modification and other studies. Initial analytical scale runs suggested that the venom could be substantially fractionated using a C18 reversed phase column and a gradient elution system comprised of the two solvents: A solvent = 0.1 M ammonium acetate, pH 6.9, conductance 12.9 mSiemens; B solvent = isopropyl alcohol. After testing a number of different conditions, we have adopted the following standard procedure at the semi-preparative level. Two to four mg of the venom dissolved in 100 μL of solvent A made 15% in isopropanol is injected onto a 1cm x 25 cm C18 separating column (Rainin Dynamax) preceded by a guard column of the same material. Elution is carried out at a flow rate of 1.5 ml/min with the following gradient: 85% A / 15% B for 10 min followed by an increase to 30% B over the next 20 min which is then held constant for 7.5 min before beginning a final increase to 70% B over the next 7 min. This solvent composition is then maintained for the duration of the run. A typical chromatogram run under these conditions is illustrated in Figure 7. Although individual peaks were originally tested for activity in the standard screening assay, leading to the identification, among others, of toxin V in fraction 3, we subsequently adopted the collection scheme indicated in the figure to keep the number of fractions collected from multiple runs at a manageable level. Toxin V has been substantially purified by rechromatography of fraction 3 under the conditions noted in Figure 7.

Initial tests did indeed reveal components that were inhibitory to the binding of BTX-B under standard screening assay conditions, both in fraction 1 and fraction 6. Subsequent work has failed to confirm this activity in fraction 1, and it is possible that the activity is labile to our conditions of fraction work-up and storage. Inhibitory activity first found in fraction 6 however has been more robust and stable, allowing some progress to be made in its isolation and characterization. Rechromatography of fraction 6 as indicated in Figure 7 resulted in collection of a single sharp peak which retained inhibitory activity.

When subjected to electrophoresis in a 15% SDS gel an amount of this material corresponding to that contained in 2 mg of whole venom produced a very faint Coomassie Blue-stained band which ran slightly ahead of the major components in fractions 3 and 4 that are primarily 6000-7000 Dalton isoforms. Native gel electrophoresis run to the cathode did not reveal a visible band. It is clear that this component is present at extremely low concentrations in the whole venom. This has hampered work with the material, which we have called ∂-toxin, but it also suggests very high potency. Assay of activity from three separate preparations of fraction 6 under standard screening assay conditions produced the result that ∂-toxin recovered from the equivalent of 100 μg of whole venom inhibits the binding of [^{3}H]BTX-B by 42 ± 4% (mean ± S.D. of three assays run in triplicate). An estimation based on the absorbance at 280 nm for this fraction relative to other components in the venom suggests that ∂-toxin comprises not more, and probably less, than 0.1% of the total protein constituents. The inexactness of such an estimation notwithstanding, it appears that on a weight basis ∂-toxin is as potent an inhibitor of BTX-B binding as α-toxin V is an enhancer.

Synthetic sodium channel peptide and monoclonal antibodies. Monoclonal antibodies raised against a synthetic sodium channel peptide corresponding to amino acids 1509 - 1536 in the sequence of eel electroplax sodium channel were provided by Dr. Donard Dwyer as supernatant solutions from myeloma cultures grown in RPMI medium. These antibodies were given the designation "PB". In preliminary tests, several of these antibodies, including PB6,7, and 29, were found to give a positive test against *L. quinquestriatus* venom in an ELISA assay (D. Dwyer, personal communication). When tested in this manner against fractions from HPLC chromatography of the venom, PB29 reacted positively with fractions 1,3 and 4. Based upon these results, we were interested to see if these antibodies would also give a positive test in the standard screening assay as might be anticipated if the antibodies were recognizing an α-toxin. Antibodies were added directly to the assay tubes as solutions in RPMI medium (20 μL/tube). The results for a series of PB antibodies assayed in this way are shown in Figure 8. There is no robust effect for any of these antibodies, although there is an indication of slight inhibition of BTX-B binding by PB6,7,and 9. Other antibodies, including PB3,8,11,15,18,24, and 28, have also been screened with similar results.

To further assess the relationship between the PB antibodies and scorpion venom components, fractions 1,3, and 4 from our standard HPLC separation were subjected to electrophoresis on a 15% SDS gel and then transferred to nitrocellulose paper by

electroblotting. The blotted proteins were probed with PB29 (diluted two-fold from the myeloma supernatant) and stained with an HRP-conjugated second antibody by reaction with diaminobenzidine. The results are presented in Figure 9 and demonstrate that in agreement with the ELISA data PB29 recognizes some, but not all, of the proteins in each of the fractions. At this point we have no data to suggest that these proteins are α-toxins or closely related isoforms, but this must be considered a distinct possibility. The failure to see any robust effects in the screening assay may be related to the concentration of antibodies in the incubation mixture. In making the additions to the assay tubes the concentration of antibodies was diluted 17-fold over that present in the supernatants. If that concentration were 10 μg/ml as is typical for such cultures, then the concentration in the assay may have been inadequate. Further experiments using more concentrated preparations of antibodies are required to resolve this point.

DISCUSSION

Based upon what is now a significant amount of accumulated experience and data, the conclusion can be drawn that the rapid screening assay does indeed provide a reliable and useful tool that should be of value to a variety of research strategies aimed at the development of sodium channel antitoxins or therapeutic agents. In our laboratory the assay has been reduced to practice as a routine procedure, and has been used to a distinct advantage in the screening of multiple samples resulting both from the fractionation of scorpion venom and the production of monoclonal antibodies. As demonstrated by the data in Table II, the assay reports sensitively on ligand-receptor events at any of at least five distinct binding domains on the channel protein, reflecting the unique relationship of the batrachotoxin binding site to other regions of the sodium channel. The casting of such a broad net is limiting in one sense, since information is not recovered concerning either the site or mechanism of action of active agents. Witness, for example, the story of HM-197. On the other hand, this approach can be comforting given the extensive allosteric nature of sodium channel pharmacology (see e.g. ref. 11), since too narrow a focus might miss a potentially useful observation. Again, the case of HM-197 provides a good illustration. Based on the early electrophysiological testing of this compound and the fact that it was synthesized as a TTX analog (12), it would have been logical to assess its potential as a therapeutic agent solely by testing its interaction with the binding of labeled TTX or STX. As our results have shown, this approach would have led to the conclusion that HM-197 was of little interest. Based upon testing of HM-197 with the screening assay, however, such a conclusion would seem to be premature. We suggest that the screening assay can be an extremely useful tool when used with an appropriate appreciation of its advantages and limitations.

Before leaving the subject of HM-197, we note that the results presented in this report concerning possible sites of action do not provide an answer, but only add to the list of sites where the compound is <u>not</u> acting. We know that HM-197 inhibits the binding of BTX-B at low micromolar concentrations where it has no obvious effects on nerve compound action potentials, but this action does not appear to be mediated by an interaction at either the TTX/STX, local anesthetic, or BTX sites. We are continuing to check other possibilities, including a possible interaction with the brevetoxin site. This work is being carried out in collaboration with Dr. Michael Adler of the Neurotoxicology Branch, USAMRICD, Aberdeen Proving Grounds.

In the original contract application we have discussed the possibility that scorpion venoms, in addition to containing several α- and/or ß-sodium channel toxins, could also contain isoforms of these polypeptides that could serve as antitoxins. Since the venoms themselves are quite toxic, it seemed apparent that such components, if indeed they were present, would be found at low concentrations relative to the toxins. By fractionation using high performance liquid chromatography and testing of individual fractions with the standard screening assay we have identified at least one, and perhaps two, candidates for potential antitoxins in the venom of *L. quinquestriatus*. This work is still in the initial stages and the only criterium for such status applied thus far has been the ability to inhibit the binding of BTX-B under standard screening assay conditions. Based on the results presented here, the so-called "∂-toxin" found in fraction 6 of the HPLC for *L. quinquestriatus*. venom exhibits a potency in this aspect that appears to be on the same order of magnitude as the enhancing activity of α-toxin V. The challenge remains to collect enough of the ∂-toxin, found in very low concentrations in the venom, to characterize it biochemically and functionally. Our guiding hypothesis is that the ∂-toxin is a homolog of the α-toxins and may inhibit the binding of BTX-B by competition with the α-toxin for a common site on the sodium channel, thus blocking the positively cooperative allosterism responsible for high affinity BTX-B binding. If this is the case, and if ∂-toxin alone does not adversely affect sodium channel function, then sequence information for the ∂-toxin will be critical to the development of structure-activity relationships for this model of scorpion polypeptide antitoxins.

Understanding the relationship between a ligand and its specific binding site offers significant promise for the development of therapeutic pharmacologies directed at the blockade of neurotoxin binding and its untoward physiological consequences. For the sodium channel protein, this approach has thus far gone unrealized since the binding sites for sodium channel neurotoxins are not yet known at the level of the protein sequence. We have therefore been intrigued by the empirical theory developed by Dr. Donard Dwyer as discussed under Rationale. In collaboration with Dr. Dwyer several predictions of this theory regarding the interaction of polypeptide scorpion toxins with the sodium channel protein have been tested with positive results. Thus monoclonal antibodies against a synthetic sodium channel peptide identified by sequence homologies to scorpion α-toxins have been found to cross react with several components of *L. quinquestriatus* scorpion venom. Two of these components are found in HPLC fractions 3 and 4 which we have shown to contain α-toxin activity. On the surface, this cross-reactivity may not seem

surprising given the sequence homologies. However, in view of the fact that the antibodies were raised against a sodium channel peptide sequence, not against the scorpion toxins, and that this sequence has been associated with the sodium channel inactivation process through modeling studies, we consider these results to be quite striking. They are at a minimum consistent with the theory which holds that there is an evolutionary relationship between peptide ligands and receptors that is reflected in residual, yet significant sequence homology. In view of the implication of this theory for the development of therapeutic treatments, we feel that further experimentation is warranted. One very interesting possibility is that the synthetic sodium channel peptide may provide a soluble scorpion toxin "pseudoreceptor" that could essentially scavenge the toxin and prevent its interaction with the sodium channel. In preliminary experiments, a Sephadex affinity column containing the covalently bound sodium channel peptide was found to retain as yet uncharacterized polypeptide components from the venom of *L. quinquestriatus*.. Our plans call for characterization of these components as well as direct testing of the synthetic sodium channel peptide by radioligand binding assays.

LITERATURE CITED

1. Catterall, W.A., Morrow, C.S., Daly, J.W., and Brown, G.B. (1981). *J. Biol. Chem.* **256**, 8922-8927.

2. Creveling, C.R., McNeal, E.T., Daly, J.W., and Brown, G.B. (1983). *Mol. Pharmacol.* **23**, 350-358 .

3. Postma, S.W., and Catterall, W.A. (1984). *Molec. Pharmacol.* **25**, 219-227.

4. Willow, M., and Catterall, W.A. (1982). *Molec. Pharmacol.* **22**, 627-635.

5. Brown, G.B., and Olsen, R.W. (1984). *Soc. Neurosci. Abstr.* **10**, 865.

6. Brown, G.B. (1986). *J. Neurosci.* **6**, 2064-2070.

7. Gonoi, T., Ashida, K., Feller, D., Schmidt, J., Fujiwara, M., and Catterall, W.A. (1986). *Molec. Pharmacol.* **29**, 347-354.

8. Catterall, W.A., and Risk, M. (1981). *Molec. Pharmacol.* **19**, 345-348.

9. Sharkey, R.G., Jover, E., Couraud, F., Baden, D.G., and Catterall, W.A. (1987). *Molec. Pharmacol.* **31**, 273-278.

10. Brown, G.B., and Daly, J.W. (1981). *Cell. Molec. Neurobiol.* **1**, 361-371.

11. Brown, G.B. (1988). *Internat. Rev. Neurobiol.* **29**, 77-116.

12. Spiegelstein, M.Y. and Kao, C.Y. (1971). *J. Pharmacol. Exptl. Therap.* **177**, 34-39.

13. Watt, D.D. and Simard, J.M. (1984). *J. Toxicol.-Toxin Rev.* **3**, 181-221.

14. Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H., and Numa, S. (1986). *Nature* **320**, 188-192.

15. Guy, H.R. and Seetharamulu, P. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 508-512.

16. Numa, S, and Noda, M. (1986). In Tetrodotoxin, Saxitoxin and the Molecular Biology of the Sodium Channel, C.Y. Kao and S.R. Levinson, eds., Annals of the New York Academy of Sciences, New York, New York, pp. 338-355.

17. Kopeyan, C., Martinez, G., and Rochat, H. (1985). *FEBS* **181**, 211-217.

18. Peterson, G.L.(1977). *Anal. Biochem.* **83**: 346-356.

19. Laemmli, U.K. (1970). *Nature* **227**, 680-685.

20. Catterall, W.A. (1976). *J. Biol. Chem.* **251**, 5528-5536.

21. Birk, H.-W. and Koepsell, H. (1987). *Analyt. Biochem.* **164**, 12-22.

22. Towbin, H. and Gordon, J. (1984). *J. Immunol. Meth.* **72**, 313-340.

23. Pagala, M.K. (1983). *J. Electrophysiol. Technol.* **10**: 111-118.

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Compound	Effect	Reference
α–Scorpion toxin, sea anemone toxin	Increase affinity	(1)
Local anesthetics	Decrease affinity	(2,3)
Diphenylhydantoin and carbamazepine (anticonvulsants)	Decrease affinity	(4)
α-Cyano pyrethroid insecticides	Increase affinity	(5)
TTX and STX	Decrease affinity	(6)
Ciguatoxin	Increase affinity (?)	(7)
Ptychodiscus brevis toxin	Increase affinity	(8,9)

Table I. Allosteric Modifiers of BTX-B Binding

Compound	$K_{0.5}$	Predicted Effect	Observed Effect
BTX-B	300 nM	Inhibit	Inhibit
TTX	20 nM	Inhibit	Inhibit
ScTX	2 µg/ml	Enhance	Enhance
Deltamethrin	1 µM	Enhance	Enhance
CGA-98496	1 µM	Inhibit	Inhibit
CGA-98479	3 µM	Inhibit	Inhibit
HM-197	35 µM	Inhibit	Inhibit
Benzimidazole	>600 µM	?	None
2-ß-aminoethyl-benzimidazole	>600 µM	?	None
Nesacaine	15 µM	Inhibit	Inhibit
THP	>600 µM	?	None
THP-OH	>600 µM	?	None

Table II. Effects of ligands on [^{3}H]BTX-B binding under standard screening assay conditions.

The compounds listed above were included at varying concentrations in the standard screening assay and the effects on the specific binding of [^{3}H]BTX-B at each concentration was recorded as a percentage of control binding without added test agent. The resulting dose response curves were used to determine the $K_{0.5}$ values, i.e. that concentration of test agent that produced a half-maximal effect. ScTX refers to the venom of *L. quinquestriatus*. CGA-98496 and CGA-98479 are non-toxic stereisomers of the pyrethroid insecticide cypermethrin. TPH and TPH-OH are tetrahydropyrinidine derivatives supplied by Dr. B. J. Gabrielsen, Fort Detrick.

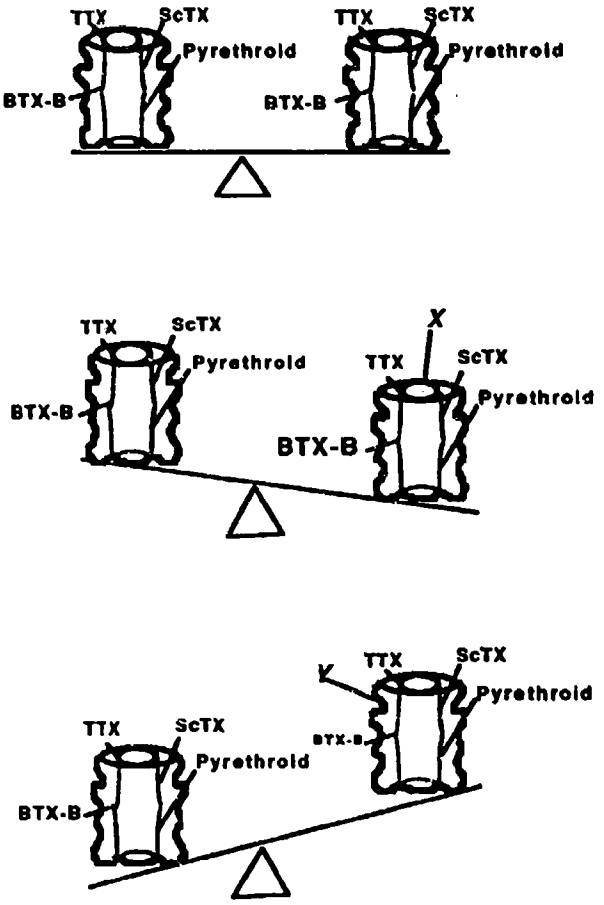

Figure 1. Rapid screening assay for compounds acting at the sodium channel.

The cartoon represents the binding of various ligands to the voltage-sensitive sodium channel under conditions of the screening assay. Synaptoneurosomes are incubated with a tracer amount of [^{3}H]BTX-B (9 nM) and approximately 1/2 maximal concentrations of TTX (25 nm), *L. quinquestriatus* scorpion venom (ScTX, 2.5 µg/ml), and deltamethrin (1 µM) in the presence and absence of test agents (X and Y). Depending on the site of action, the test agent may lead to either an increase or decrease in specific binding (right) compared to controls in the absence of the agent (left). In the absence of test agents, equilibrium binding of 9 nM BTX-B to synaptoneurosomes at a concentration of 3 mg protein/ml yields approx. 4000 dpm of total binding, of which 50% is specific.

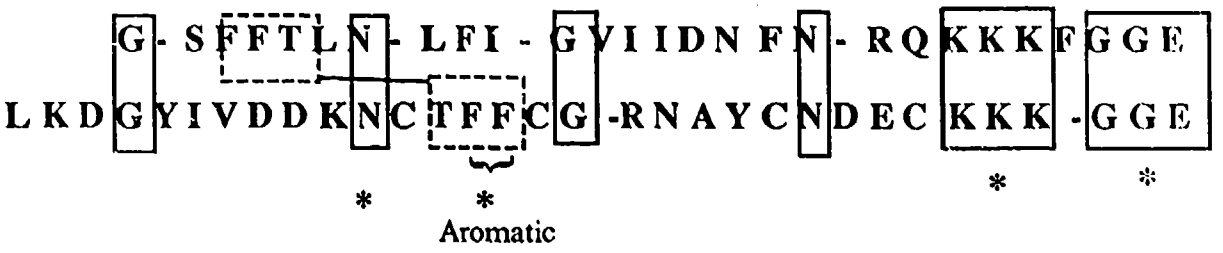

Figure 2. Sodium channel - α-Scorpion Toxin Sequence Homology

The amino acid sequence of residues 1509-1536 for rat brain sodium channels I and II as defined by Numa and Noda (16) (upper line) is compared to the first 32 amino terminal residues of toxin V from L. quinquestriatus (17) (lower line) using standard one-letter notation for the amino acids. Deletions inserted to maximize the alignment homology are indicated by dashes (-). Amino acids shared in normal order sequence are surrounded by solid line boxes, whereas the dashed box indicates inverse sequence homology reflected around the common asparagine. In this representation, 13 out of 27 or 48% of sodium channel residues are shared with the scorpion toxin sequence. The asterisks mark those amino acids in toxin V that are invariant among α-toxins from various scorpion species (17).

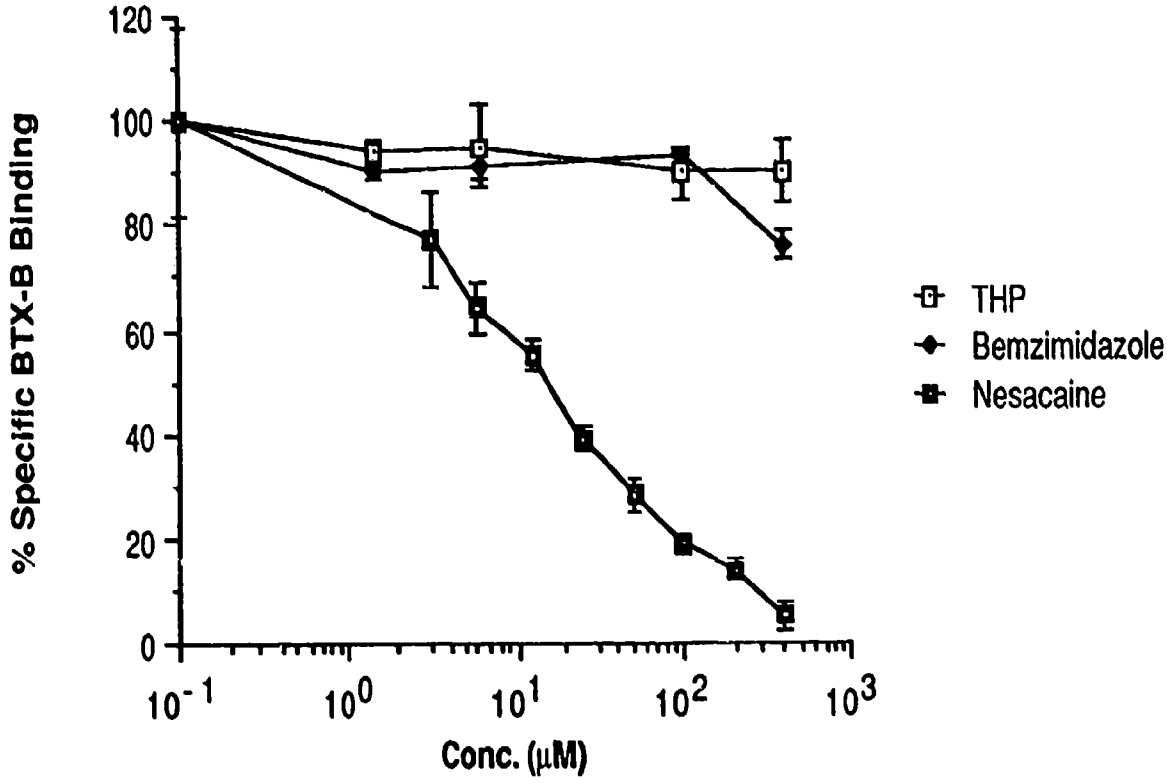

Figure 3. Effects of nesacaine, benzimidazole, and THP on BTX-B binding under standard screening assay conditions.

Specific binding of [^{3}H]BTX-B under standard screening assay conditions was measured as a function of varying concentrations of the local anesthetic nesacaine and the two heterocyclic compounds benzimidazole and THP. The data are expressed as a percentage of the control binding of [^{3}H]BTX-B in the absence of these compounds. Each data point is the mean ± S.D. of triplicate determinations. Points without error bars indicate errors that were less than the size of the symbol.

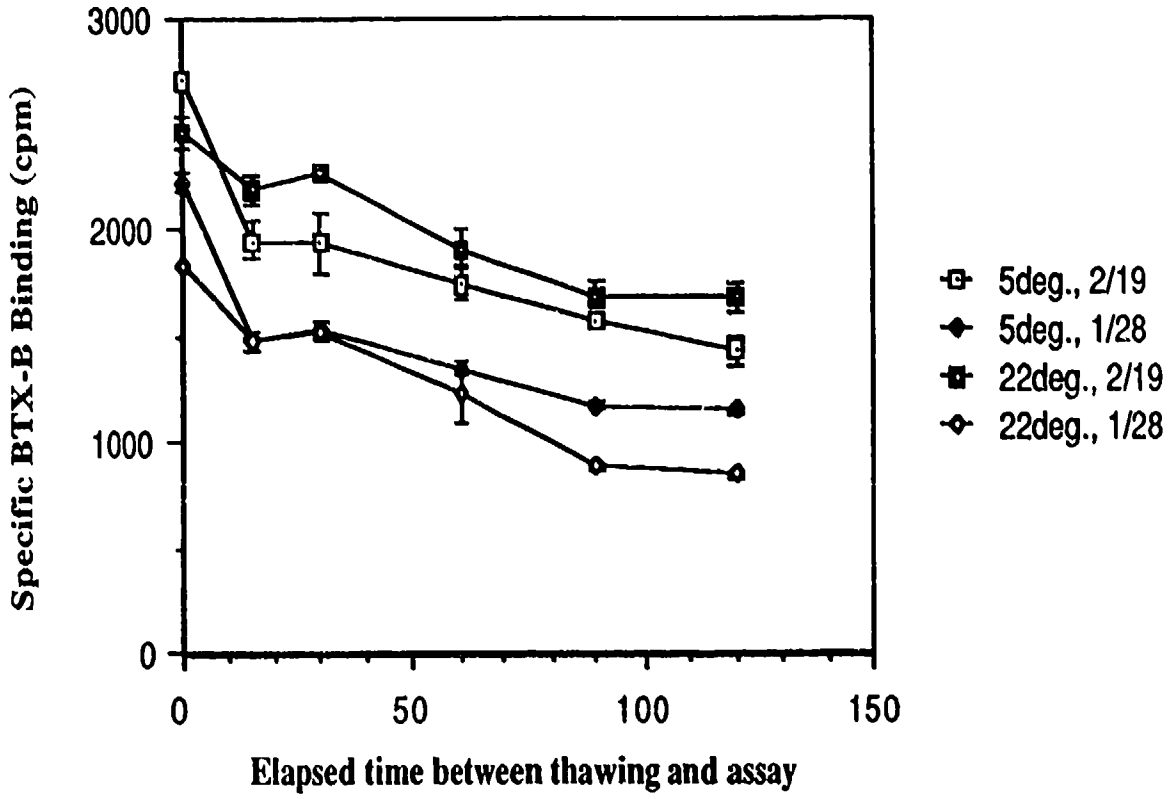

Figure 4. Effects on specific [^{3}H]BTX-B binding of thawing temperature and elapsed time between thawing and use in the standard screening assay.

Synaptoneurosomes, prepared and initially frozen on the indicated dates, were thawed at either 5 or 22°C, reconstituted in HEPES buffer and incubated on ice for the indicated times before being utilized to determine specific binding of BTX-B under standard screening assay conditions. The data points are the means ± S.D. of triplicate determinations.

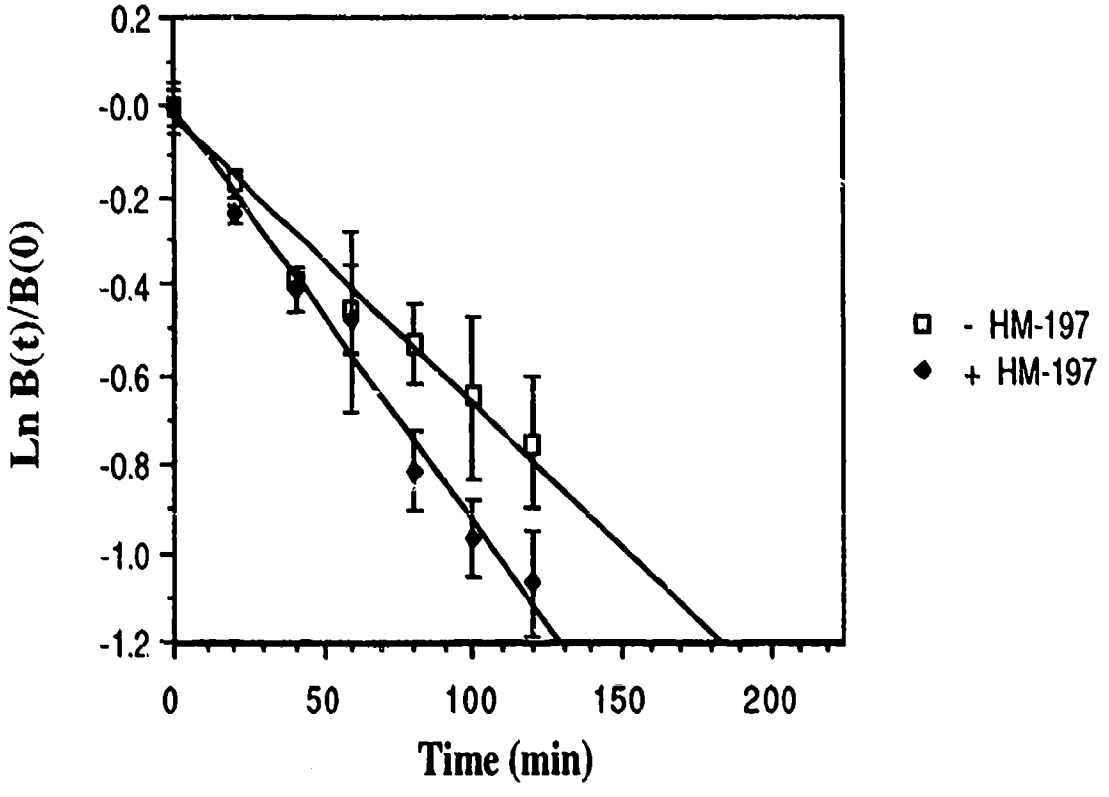

Figure 5. Effect of HM-197 on the dissociation rate for BTX-B binding.

Rat brain synaptoneurosomes were equilibrated with 9 nM [^{3}H]BTX-B and a saturating concentration of *L. quinquestriatus* scorpion venom (12.5 µg/ml) for 1 hr, with or without 50 µM HM-197. Excess veratridine (300 µM) was then added at time 0 and specific binding remaining at the indicated times (t) was measured by filtration assay. The logarithm of the specific binding at each time point, B(t), divided by the specific binding at time 0, B(0), is plotted as a function of time. The data are the means ± S.D. of triplicate determinations, and the straight lines were fit to the data using linear regression analysis.

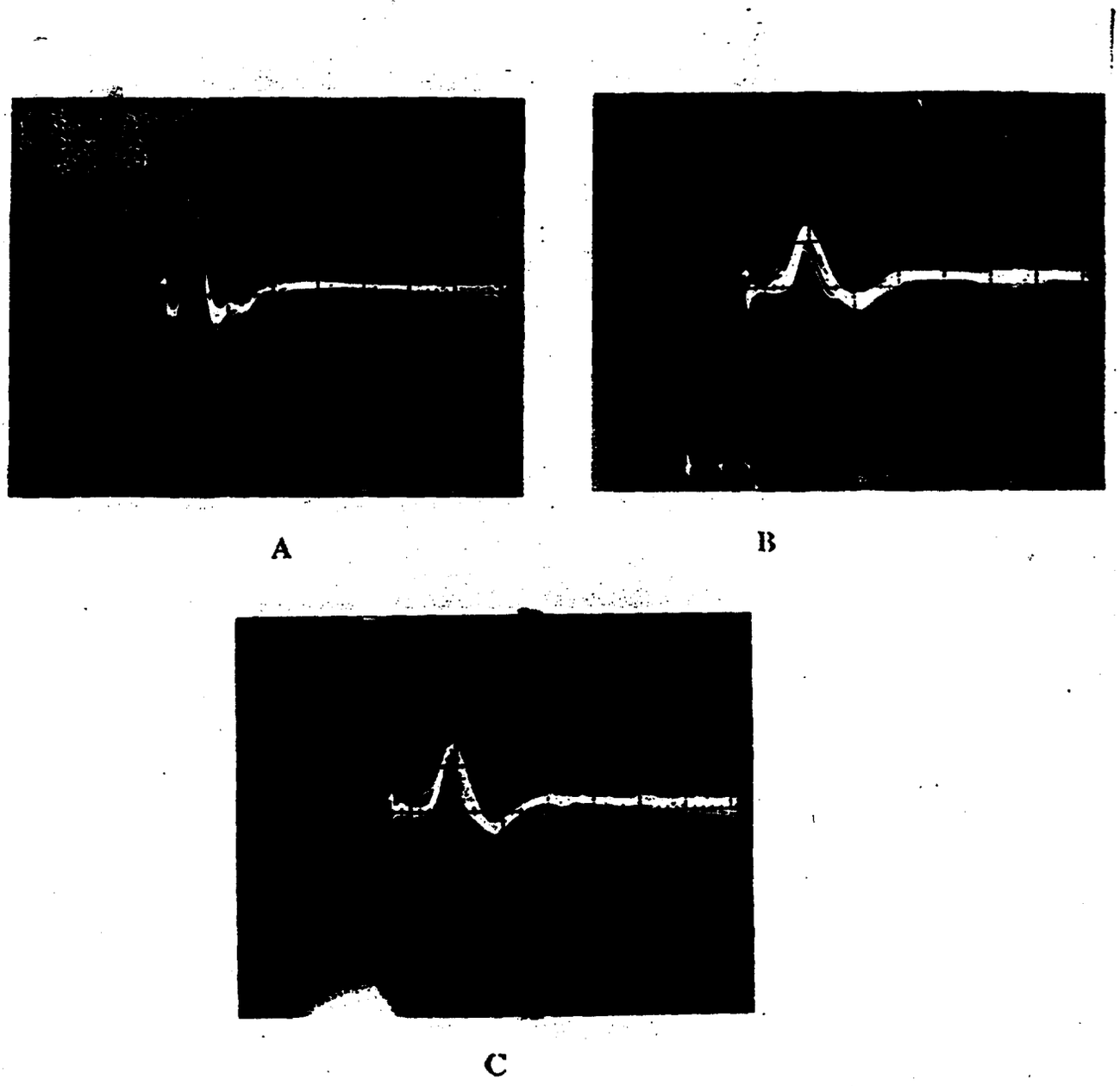

Figure 6. Effect of HM-197 on nesacaine-induced block of rat phrenic nerve compound action potential.

Panel A. Control nerve stimulated supramaximally at 100 Hz. 100 compound action potentials are superposed. Panel B. The nerve was equilibrated with 150 µM nesacaine at 30° C for 30 min prior to stimulation for 1 sec at 100 Hz as in the control. Note the decreased size in the amplitude of the compound action potential and the slight decrementing response indicating a use-dependent effect. Panel C. The nerve was pre-equilibrated with 100 µM HM-197 followed by 150 µM nesacaine. After 30 min the nerve was stimulated as before. Note the lack of effect of pre-equilibration with HM-197.

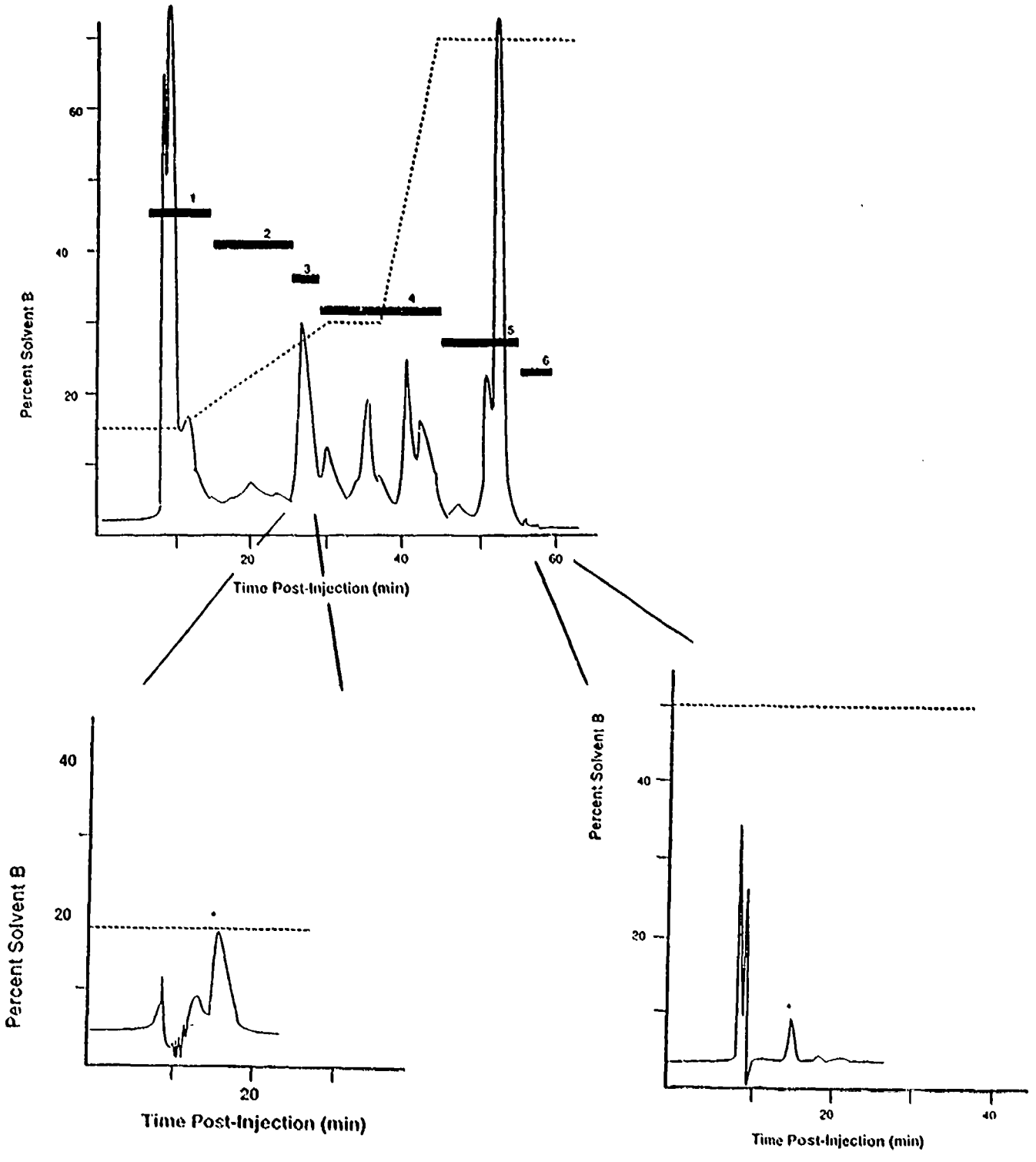

Figure 7. HPLC of *L. quinquestriatus* scorpion venom

Gradient elution high performance liquid chromatography of *L. quinquestriatus* scorpion venom over a C18 Dynamax column preceded by a C18 guard column is diagrammed in the upper panel. Eluting solvents "A" and "B"are described in the text. The percent "B" in the mixture was varied as a function of time as indicated by the dashed line which refers to the ordinate. Injection of samples was at time 0. Flow rate was 1.5 ml/min. The solid bars refer the fractions that were collected. The effluent was monitored by adsorption at 280 nm. Rechromatography of fractions 3 and 6 is indicated below. Notations are as above except that for these HPLC runs, the C18 guard column was replaced with a C8 guard column. The peaks from these rechromatographies containing activity in the standard screening assay (enhancing and inhibiting for fractions 3 and 6, respectively) are indicated by the asterisks.

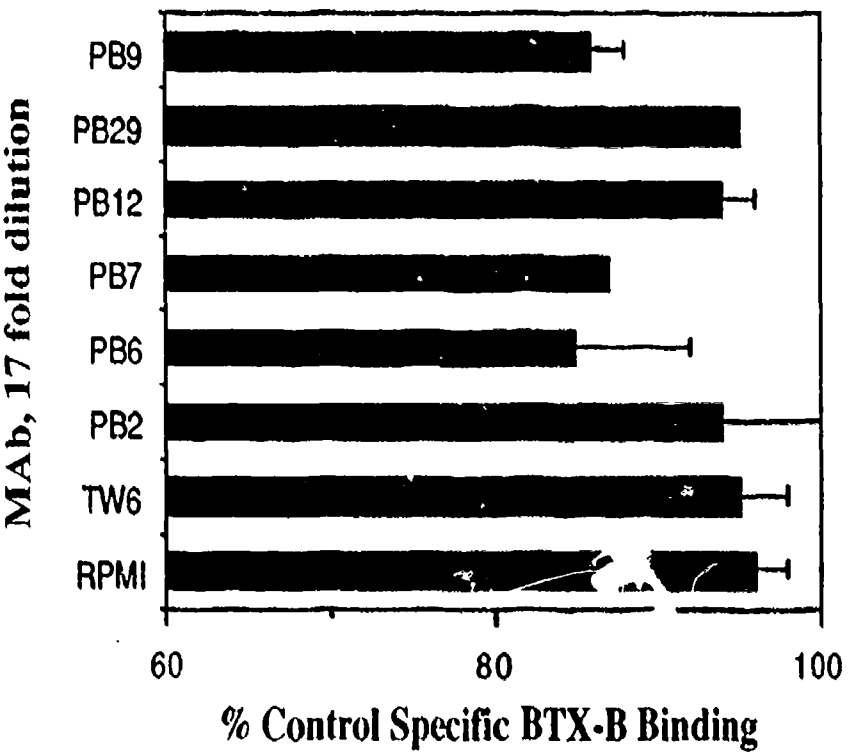

Figure 8. The effects of PB series monoclonal antibodies on BTX-B binding.

Using standard screening assay conditions, the effects of the indicated monoclonal antibodies on specific binding of BTX-B was evaluated. Antibodies were added to the assay tubes as solutions in RPMI cell culture medium. Twenty µL were diluted into a total assay volume of 338 µL, yielding a dilution factor of 17 for the antibody titer. The results are expressed as a percentage of specific BTX-B binding in control tubes containing no added antibodies. TW6 is a monoclonal antibody raised against an irrelevant antigen and has been used as a control unrelated to the PB series. RPMI refers to the addition of 20 µL of RPMI medium containing no antibodies. The data are expressed as the mean ± S.D. of triplicate determinations. The standard error for PB7 and PB29 was 1% and was too small to be visualized upon initial computerized production of the graph.

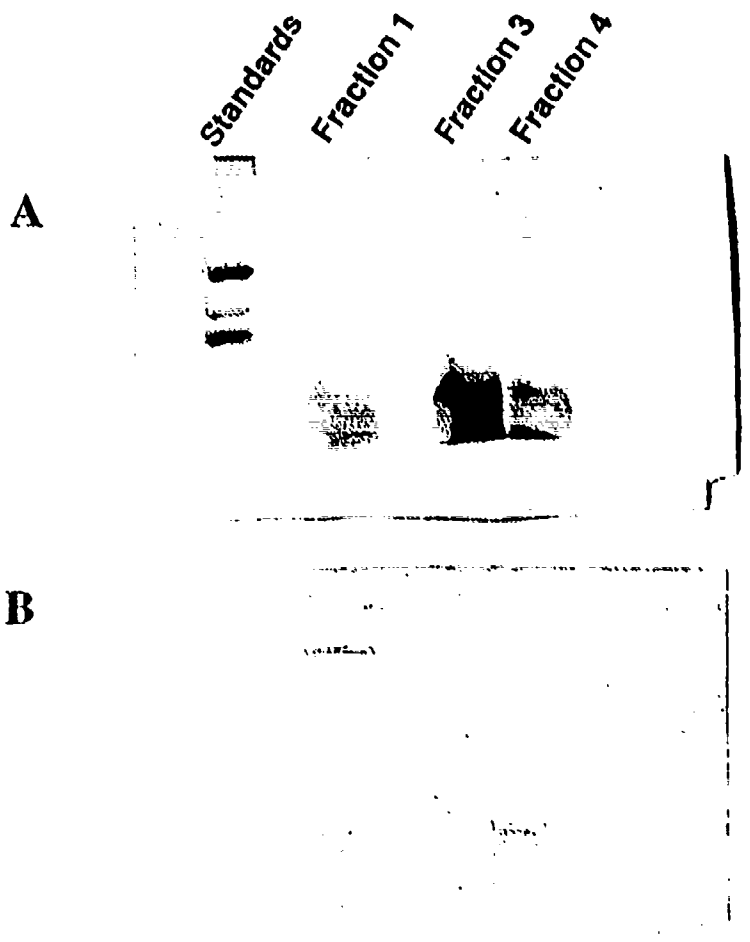

Figure 9. PB29 reaction with *L. quinquestriatus* scorpion venom components.

Panel A shows a 15% SDS gel stained with Coomassie Blue. Fractions 1,3, and 4 refer to those bands recovered from HPLC of whole *L. quinquestriatus* venom. The amounts loaded on the gel correspond to amounts contained in 100μg, 100μg, and 50μg of whole venom for fractions 1,3, and 4, respectively. The fastest migrating band in the standards lane has a molecular weight of 14,200 Daltons. Panel B is the corresponding nitrocellulose paper blot probed with PB29 from a gel run in parallel with that shown in Panel A. (The bands have been slightly retouched for purposes of reproduction.)