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Brevenal Is a Natural Inhibitor of Brevetoxin Action in Sodium Channel Receptor Binding Assays

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Summary

1. Florida red tides produce profound neurotoxicity that is evidenced by massive fish kills, neurotoxic shellfish poisoning, and respiratory distress. Red tides vary in potency, potency that is not totally governed by toxin concentration. The purpose of the study was to understand the variable potency of red tides by evaluating the potential for other natural pharmacological agents which could modulate or otherwise reduce the potency of these lethal environmental events.

2. A synaptosome binding preparation with 3-fold higher specific brevetoxin binding was developed to detect small changes in toxin binding in the presence of potential antagonists. Rodent brain labeled in vitro with tritiated brevetoxin shows high specific binding in the cerebellum as evidenced by autoradiography. Synaptosome binding assays employing cerebellum-derived synaptosomes illustrate 3-fold increased specific binding.

3. A new polyether natural product from Florida's red tide dinoflagellate *Karenia brevis*, has been isolated and characterized. Brevenal, as the nontoxic natural product is known, competes with tritiated brevetoxin for site 5 associated with the voltage-sensitive sodium channel (VSSC). Brevenal displacement of specific brevetoxin binding is purely competitive in nature.

4. Brevenal, obtained from either laboratory cultures or field collections during a red tide, protects fish from the neurotoxic effects of brevetoxin exposure.

5. Brevenal may serve as a model compound for the development of therapeutics to prevent or reverse intoxication in red tide exposures.

Keywords

brevetoxin; brevenal; receptor binding; molecular therapeutics

Introduction

Florida red tides occur in the Gulf of Mexico and result from blooms of the marine dinoflagellate *Karenia brevis* (Daugbjerg *et al.*, 2000; Steidinger and Ingle, 1972). *K. brevis* produces highly potent polyether toxins known as brevetoxins that activate voltage-sensitive sodium channels. Nine natural toxins have been identified and their structures are based on

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two different polyether backbones, brevetoxin-a (eg. PbTx-1) and brevetoxin-b (eg. PbTx-2) (Fig. 1) (Baden, 1983; Lin *et al.*, 1981; Shimizu *et al.*, 1986). By binding to Site 5 associated with the α-subunit of voltage-gated sodium channels, brevetoxins produce four distinct effects: a shift of the activation potential to more negative values; a prolongation of mean open time; inhibition of inactivation; and, visualization of multiple subconductance states (Baden and Adams, 2000).

By virtue of these actions on normal sodium channel function, the brevetoxins cause massive fish kills, produce marine mammal poisoning and are responsible for human health problems (Baden and Mende, 1982; Bossart *et al.*, 1998; Bourdelais *et al.*, 2002; O'Halloran, 2002; Steidinger and Baden, 1984). Aerosolized brevetoxins from natural blooms of *K. brevis* cause respiratory distress resulting from airway constriction (Watanabe *et al.*, 1988), an effect observed at pM concentrations (Abraham *et al.*, 2002). Consumption of contaminated shellfish results in neurotoxic shellfish poisoning, the symptoms of which include excessive salivation, lacrimation, urination, diarrhea, and temperature reversal characteristic of muscarinic stimulants (Steidinger and Baden, 1984).

The severity of human health effects varies annually and temporally in coastal regions. Explanations for the variable toxicity include red tide density, meteorological, biochemical, and dinoflagellate strain toxicity differences (Baden and Tomas, 1988; Pierce, 1986; Roszell *et al.*, 1988). No explanation has been offered to account for the low potency in blooms with high toxin concentrations, or for the high potency demonstrated in blooms with lower toxin concentrations. Recently, we isolated a nontoxic short-chain trans-syn polyether molecule that competes with brevetoxin for its active site on voltage-sensitive sodium channel. Called brevenal, we postulate that nonlethal human health consequences of brevetoxin exposure are most likely modulated in severity by the relative proportions of the known brevetoxins and brevenal in any particular exposure event. We demonstrate, using laboratory cultures, that the relative ratio of brevenal to brevetoxin varies depending upon culture condition. Further, we postulate that compounds such as brevenal may serve as molecular therapeutics for brevetoxin-exposed individuals.

To characterize the binding of brevenal, we refined a synaptosomal binding technique to increase specific binding and reduce nonspecific binding. We localized brevetoxin binding in rodent brain using standard autoradiography using tritium-labeled brevetoxin, selected the cerebellum region of the brain for synaptosome preparation, and demonstrated success of brevenal in competing for brevetoxin binding sites in receptor binding assays at site 5 of the voltage-sensitive sodium channel. We illustrated brevenal's protective effect in vivo during fish bioassay, indicating that brevenal may play a part in the overall observed potency of Florida red tides.

Methods

Isolation of the Antagonist From Dinoflagellate Cultures

Brevenal was extracted from *K. brevis* cultures (Wilson clone) with chloroform. The chloroform layer was collected and dried and then partitioned between petroleum ether and aqueous methanol to remove pigments and cellular lipid debris. The aqueous methanol layer (90%) was dried under vacuum and the components further separated using a silica gel column (isocratic mobile phase $CHCl_3$: MeOH: acetic acid; 100:10:1 v/v). The antagonist coelutes with toxin. A second low-pressure column employing a C-18 matrix was used to further separate any remaining pigments from the toxin and antagonist using isocratic acetonitrile: water mobile phase (80:20 v/v). The antagonist was separated from the toxin using a preparative silica gel TLC plate (70:30 v/v petroleum ether:acetone). Final purification of the antagonist was achieved by revered phase HPLC (Phenomenex™ phenyl-hexyl column: isocratic elution

with MeOH/H2O; 90:10; with detection using UV absorption at 215 nm). For experiments correlating brevetoxin/brevenal concentrations with cell counts, 24 mL aliquots of *K. brevis* culture were taken semiweekly for 5 weeks from five separate 10 L dinoflagellate cultures. Four mL were used to determine cell counts by Coulter Counter. The remaining 20 mL aliquots were extracted and purified as described above, and quanitified by HPLC. Comparisons were analyzed for statistical significance by one-way ANOVA using GraphPad Prism software.

Isolation of the Antagonist From the Environmental Samples

Samples of seawater (100 mL) were collected during red tide blooms off the west coast of Florida, USA. The antagonist and toxins were extracted from the seawater using 3 × ethyl acetate (100 mL). The organic layer was filtered using 0.2-μm filter and dried. The toxins and antagonist were separated by reverse-phase C18 HPLC and monitored using UV detection at 215 nm. The antagonist was separated using a Phenomenex reversed-phase C18 phenyl-hexyl column and concentration measured using UV detection at 215 nm.

Structure Identification of the Antagonist

NMR spectral data were recorded in a variety of solvents including C_6D_6, d_6-acetone, $CDCl_3$, and d_4-MeOH (Isotech) using a Bruker 400- and 500-MHz spectrometer. HRMS data were obtained from University of California Riverside Mass Spectrometry facilities. FTIR spectra were obtained on 0.5 mg of powdered brevenal in a KBr pellet using a Matteson Cygnus 100 FTIR with WIN98 software upgrade.

Fish Bioassay

Male mosquito fish (*Gambusia affinis*) were placed individually in 50-ml beakers containing 20-ml water. The test compounds (PbTx-3 and the brevenal) were dissolved in ethanol (0.1 mg/mL) and delivered to the fish in a total of 0.2 mL ethanol. The control fish received 200 μL ethanol. The fish receiving toxin alone received the following doses 0.25 μg/mL water and 0.5 μg/mL water. The fish receiving the antagonist alone, received 0.25 μg/mL Water, 0.5 μg/mL water, 1 μg/mL water or 2 μg/mL water again in 0.200 mL ethanol. The fish receiving both brevenal and toxin received the brevenal 3 min before the toxin. After addition of the different compounds the fish were monitored for 24 h or until the time of death (Stuart and Baden, 1988).

Autoradiography of Exposed Mouse Brains Sections

Single hemispheres of mouse brain (Swiss-White) were frozen in 2-methylbutane at −30°C and sectioned with a cryostat. Serial sagittal sections 20-μ thick were mounted on gelatin-coated slides. Sections were transferred to Tris-Mn buffer ±1.0–2.5 nM tritium-labeled brevetoxin (16.5 Ci/mmole PbTx-3) for 30 min at 25°C, followed by a 15-min ice-cold Tris-Mn buffer rinse (Purkerson and Potter, 1998). Sections were dried and apposed to LKB Ultrafilm (Kodak) for 3 weeks at room temperature, and the film was developed. Developed films were evaluated for brevetoxin localization by light microscopy and documented using photomicroscopy.

Synaptosome Binding Assay

Competitive synaptosome binding assays were performed as previously described (Poli *et al.*, 1986) using both whole brain minus cerebellum or isolated cerebellum as source material. Nonlinear regression curves were generated by Graph pad Prizm from binding data (Graphpad Software, San Diego, CA).

Results

The molecular formula of brevenal was established as $C_{39}H_{60}O_8$ by HRMS (MW 656.4043; calc. value 656.4288) and supported by the observation of 39 resonances in the ^{1}H-decoupled ^{13}C NMR spectrum. The 39 carbons were further characterized by ^{13}C-DEPT NMR analysis as 5 quaternary, 16 methines (including one aldehyde, five olefinic), 12 methylenes (including one vinylic group), and 6 methyls (two vinylic, one secondary, three tertiary). In general, both the ^{13}C and ^{1}H NMR spectra were reminiscent of a ladder frame polyether structure, though the molecular formula indicated a smaller structure similar to hemibrevetoxin B (Shimizu *et al.*, 1990). Indeed, brevenal was found to possess a conjugated dienyl side chain (UV max: 227 nm) identical to one found in hemibrevetoxin, as well as a conjugated dienal side chain containing two trans double bonds (UV max: 290 nm; ^{13}C 191.43 ppm; ^{1}H 10.1 ppm) that has no precedent in brevetoxin chemistry. The 2D COSY and TOCSY NMR data was used to establish a novel 7,7,6,7,6 polyether ring system in brevenal, thus accounting for all the double bond equivalents calculated in the molecular formula. Finally, the 2D NOESY data supported the proposed planar structure and confirmed the usual transfusion of polyether rings together with the relative stereochemistry of the molecule. A structure consistent with all of the spectroscopic information is shown in Fig. 1.

The polyether antagonist brevenal comprises varying proportions of the total polyether biomass extracted from cells grown in cultures and varies in amount from lag phase through stationary phase (Fig. 2). Our data indicates that brevenal concentrations are low in cultures that are in logarithmic growth phase and increase in cultures that are stationary due to high cell populations or lack of nutrients. In field samples from red tide blooms, the brevenal concentration appears to be low during the early phases of the bloom (lag and logarithmic phases), and is higher during latter phases of the blooms studied. Additionally, the concentration of brevenal was found to be low in instances where human respiratory effects are moderately high. Brevenal concentrations appeared to be higher in situations where human respiratory irritation was low. The concentration of brevenal near the end of bloom events exceeds 32% of the total polyether material in some instances. We have not had the opportunity to measure the relative concentrations of brevenal in bloom events when human respiratory effects were severe. We postulate that brevenal concentrations would show the same inverse correlation with human health effects.

The brevetoxins bind with high affinity to site 5 of the voltage-sensitive sodium channel on neuronal membranes (Catterall and Risk, 1981; Poli *et al.*, 1986). Incubation of tritiated brevetoxin with mouse brain slices results in a pattern of binding as shown in photomicrograph (Fig. 3). The micrograph clearly shows dense binding in the various convolutions of the cerebellum, consistent with the high density of voltage-gated sodium channels in that region. For specific receptor binding experiments, cerebellum was excised and used to prepare synaptosomes. Binding maxima for tritiated brevetoxin in cerebellum often exceeded 3-fold on a per milligram basis the B_{max} observed using whole brain.

Brevenal competitively displaces tritiated brevetoxin in a synaptosome receptor-binding assay (Fig. 4), an assay that evaluates specific sodium channel receptor site binding for natural brevetoxins (Poli *et al.*, 1986). It is evident from Fig. 4(a) that the concentrations of brevenal required to compete for sites occupied by brevetoxin were over two orders of magnitude greater than for brevetoxin against itself. The calculated inhibition constant, K_i, was 685 nM, consistent with its binding affinity. Regressions shown in Figure 4(b) illustrate the pure competitive nature of brevenal competiton.

Binding of natural brevetoxins to voltage-sensitive sodium channels (VSSC) results in a cadre of four distinct effects: a shifting of the activation potential to more negative values; prolonged

channel open times; inhibition of inactivation; and induction of subconductance states. Individual brevetoxins exhibit differential sodium channel binding affinities and potencies (Rein *et al.*, 1994a). Differences in affinity of the natural toxins have been attributed to variations in lipophilicity, topographic features relative to the binding site (Rein *et al.*, 1994a,b), and differential toxicities based on affinity governed by specific molecular or structural differences of each toxin (Purkerson-Parker *et al.*, 2000; Rein *et al.*, 1994a,b). Regardless of affinity, all toxins affect the VSSC resulting in repetitive discharge of neurons and persistent depolarization. Brevenal is the first natural nontoxic polyether ligand described that displaces natural toxin from binding to receptor site 5. Brevenal does not appear to have any effect on sodium channels when applied alone in single sodium channel patch-clamp experiments (personal communication, David J. Adams, University of Queensland, Australia).

In icthytoxicity studies using *Gambusia affinis* (the standard bioassay fish for brevetoxin analyses), brevenal appears to be nontoxic at μMolar concentrations. This is remarkable, considering the half-maximal lethality of brevetoxin PbTx-3 (or any other polyether molecule isolated prior to this description) is in the nMolar range. Simultaneous exposure of *G. affinis* to both brevenal and PbTx-2 or PbTx-3 at equimolar concentrations results in fish living significantly longer than with fish receiving the toxins alone (17.00 ± 2.8 min ($P < 0.01$) for brevenal pretreated; 9.36 ± 0.72 min PbTx-3 alone; 2.7-fold longer, $n = 3$).

Discussion

Brevenal is not the first short polyether natural product isolated from *K. brevis*. Hemibrevetoxin, purified from laboratory cultures by Shimizu *et al.* (1990), precedes it. However, the potential antagonistic properties of that molecule have not been reported. The antagonistic property of gambieric acid, a small polyether compound isolated from the ciguatoxic dinoflagellate *Gambierdiscus toxicus*, on tritiated breve-toxin binding has been reported. A decrease in brevetoxin binding was observed, but site 5-specific competitive receptor blockage was not demonstrated (Inoue *et al.*, 2003). We postulate that brevenal, and other brevetoxin antagonists that may exist, strongly influence the composite potency of *K. brevis* blooms and resulting marine toxic events. Further, the presence or absence of brevenal may provide one explanation for the variable potency of red tides. By competing for the specific binding site on nerve membranes and rendering the site unavailable for brevetoxin binding, brevenal acts as a molecular antagonist or even a therapeutic compound. Our previous work indicated that the H-K ring system of brevetoxin was required for binding to site 5, that the B-G ring ring system essentially acted as a spacer region, and together these two features served to deliver an active A-ring lactone to its site of activity (Rein *et al.*, 1994a,b). Our published work has illustrated, for the most part, that when natural brevetoxins are derivatized in such a manner as to reduce toxicity, there is a concomitant reduction in specific binding (Rein *et al.*, 1994a,b). Recently, we derivatized brevetoxins on the side chain, introducing naphthoyl functions that imparted an antagonistic character to the derivatives. These derivatives competed in receptor binding assays and bound to site 5 associated with the VSSC without apparent activity when administered alone.

The brevenal molecule does not fit the “binding” motif we previously hypothesized for binding at site 5 (a prerequisite for blocking brevetoxin binding in our opinion). There is no corollary structure in brevenal corresponding to the H-K ring system of brevetoxin. For this reason, we believe that the character of binding regions of molecules that interact at Site 5 may require revisit. The brevenal molecule and its possible synthetic derivatives may be an important tool for this further work.

We have demonstrated the efficacy of brevenal in reducing brevetoxins' effects in two different biological systems, one in vivo and one in vitro. We presently have no hypothesis as to the

function of brevenal in the dinoflagellate, nor any clue as to its synthesis. We believe that brevenal may be exploited as a template for the development of therapeutic drugs to reduce or prevent brevetoxin effects in humans and aquatic animals. We further believe that polyether molecules such as the brevenals—or other polyether materials which are yet to be discovered, will play an important role in the further elucidation of voltage-sensitive sodium channel structure and function.

Acknowledgements

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References

Abraham, WM. Animal models of asthma. In: Busse, WW.; Holgate, ST., editors. Asthma & Rhinitis. Blackwell Scientific; Boston: 1995. p. 1205-1227.

Abraham WM, Ahmed A, Sabater JR, Lauredo IT, Botvinnikova Y, Bjercke RJ, Hu X, Revelle BM, Kogan TP, Dupré B, Scott IL, Dixon RAF, Yeh ETH, Beck PJ. Selectin blockade prevents antigen-induced late bronchial responses and airway hyperresponsiveness in allergic sheep. Am J Respir Crit Care Med 1999;159:1205–1214. [PubMed: 10194167]

Abraham WM, Bourdelais AJ, Baden DG. Polyether brevetoxin (PbTx) aerosols cause bronchoconstriction in sheep. Am J Respir Crit Care Med 2002;165(8):A20.

Abraham WM, Sielczak MW, Ahmed A, Cortes A, Lauredo IT, Kim J, Pepinsky B, Benjamin CD, Leone DR, Lobb RR, Weller PF. α_4 integrins mediate antigen-induced late bronchial responses and prolonged airway hyperresponsiveness in sheep. J Clin Invest 1994;93:776–787. [PubMed: 8113411]

Baden DG. Marine food-borne dinoflagellate toxins. Intl Rev Cytol 1983;82:99–150.

Baden, DG.; Adams, DJ. Brevetoxins: Chemistry, mechanism of action, and methods of detection. In: Botana, LM., editor. Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection. Marcel Dekker; New York: 2000. p. 505-532.

Baden DG, Mende TJ. Toxicity of two toxins isolated from *Ptychodiscus brevis*. Toxicon 1982;20:457–461. [PubMed: 6896247]

Baden DG, Tomas CR. Variations in major toxin composition for six clones of *Ptychodiscus brevis*. Toxicon 1988;26(10):961–963. [PubMed: 3201483]

Bossart GD, Baden DG, Ewing RY, Roberts B, Wright SD. Brevetoxicosis in manatees (*Trichechus manatus latirostris*) from the 1996 epizootic: gross, histologic, and immunohistochemical features. Toxicol Pathol 1998;26:276–282. [PubMed: 9547868]

Bourdelais AJ, Tomas CR, Naar J, Kubanek J, Baden DG. New fish-killing alga in coastal Delaware produces neurotoxins. Environ Health Perspect 2002;110(5):465–470. [PubMed: 12003749]

Catterall WA, Risk MA. Toxins T46 from *Ptychodiscus brevis* (formerly *Gymnodinium breve*) enhances activation of voltage-sensitive sodium channels by veratridine. Mol Pharmacol 1981;19:345–348. [PubMed: 6262621]

Daugbjerg N, Hansen G, Larsen J, Moestrup O. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. Phycologia 2000;39:302–317.

Inoue M, Hirama M, Satake M, Sugiyama K, Yasumoto T. Inhibition of brevetoxin binding to the voltage-gated sodium channel by gambierol and gambieric acid-A. Toxicon 2003;41:469–474. [PubMed: 12657316]

Lin YY, Risk MA, Ray SM, VanEngen D, Clardy J, Golik J, James JC, Nakanishi K. Isolation and structure of brevetoxin B from the “red tide” dinoflagellate *Ptychodiscus brevis*. JACS 1981;103:6773–6775.

O'Halloran. Brevetoxin and brevetoxin-producing species in Monterey Bay, California. Xth International Conference on Harmful Algae; St Petersburg, FL. October 21–25; 2002. p. 218Abstract

Pierce RH. Red tide (*Ptychodiscus brevis*) toxin aerosols: A review. Toxicon 1986;24:955–966. [PubMed: 3824403]

Poli MA, Mende TJ, Baden DG. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. Mol Pharmacol 1986;30:129–135. [PubMed: 2426567]

Purkerson SL, Potter LT. Use of antimuscarinic toxins to facilitate the study of striatal m4 muscarinic receptors. J Pharmacol 1998;284:707–713.

Purkerson-Parker SL, Fieber LA, Rein KS, Baden DG. Brevetoxin derivatives that inhibit toxin activity. Chem Biol 2000;7:385–393. [PubMed: 10873835]

Rein KS, Baden DG, Gawley RE. Conformational analysis of the sodium channel modulator, brevetoxin A, comparison with brevetoxin B conformations, and a hypothesis about the common pharmacophore of the ‘site’ 5 toxins. J Org Chem 1994a;59:2101–2106.

Rein KS, Lynn B, Gawley RE, Baden DG. Brevetoxin B: Chemical modifications, synaptosome binding, toxicity, and an unexpected conformational effect. J Org Chem 1994b;59:2107–2113.

Roszell, LE.; Schulman, LS.; Baden, DG. Toxin profiles are dependent on growth stages in cultured *Ptychodiscus brevis*. In: Graneli, E.; Sundstrom, B.; Elder, L.; Anderson, DM., editors. Toxic Marine Phytoplankton. Elsevier; New York: 1988. p. 403-406.

Shimizu, YS.; Gupta, S.; Prasad, AVK. Biosynthesis of dinoflagellate toxins. In: Graneli, E.; Sundstrom, B.; Edler, L.; Anderson, DM., editors. Toxic Marine Phytoplankton. Elsevier; New York: 1990. p. 62-76.

Shimizu YS, Chou HN, Bando H, VanDuyne G, Clardy J. Structure of brevetoxin A (GB-1), the most potent toxin in the Florida red tide organism *Gymnodinium breve*. JACS 1986;108:514–515.

Steidinger, KA.; Baden, DG. Toxic marine dinoflagellates. In: Spector, DL., editor. Dinoflagellates. Academic Press; New York: 1984. p. 201-299.

Steidinger KA, Ingle RM. Observations on the 1971 summer red tide in Tampa Bay, Florida. Environ Lett 1972;3:271–277. [PubMed: 4627989]

Stuart A, Baden DG. Florida redtide brevetoxins and binding in fish brain synaptosomes. Aquatic Toxicol 1988;13:271–280.

Watanabe T, Lockey RF, Krzanowski JJ. Airway smooth muscle contraction induced by *Ptychodiscus brevis* (red tide) toxin as related to a trigger mechanism of bronchial asthma. Immunol Allergy Pract 1988;185:25–32.

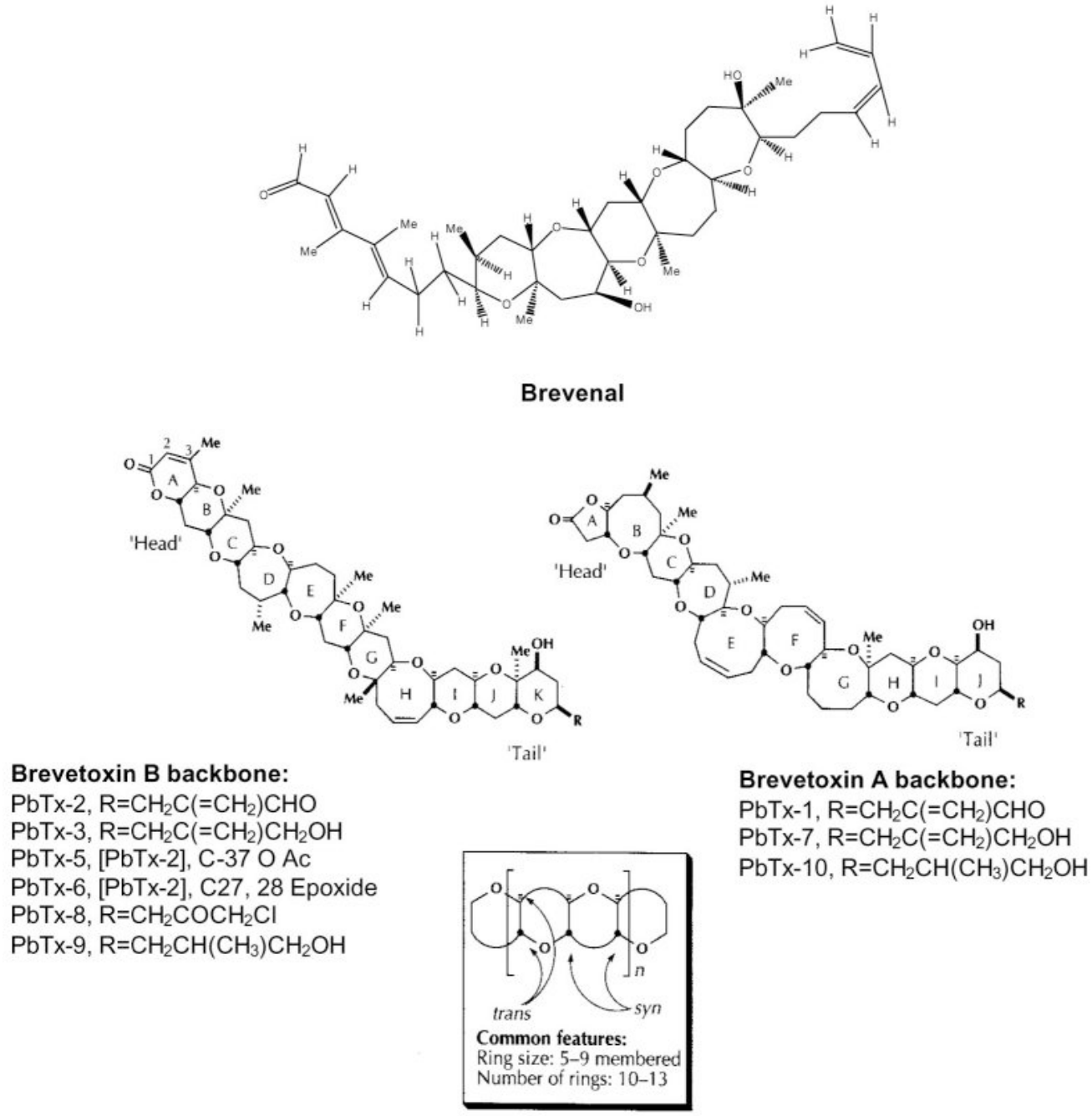

Fig. 1.

Polyethers isolated from Karenia brevis. The nine known brevetoxins are illustrated, all of which are sodium channel depolarizing agents that act on site 5 associated with the voltage-gated sodium channel. Also illustrated is the structure of brevenal, the first natural antagonist of brevetoxin.

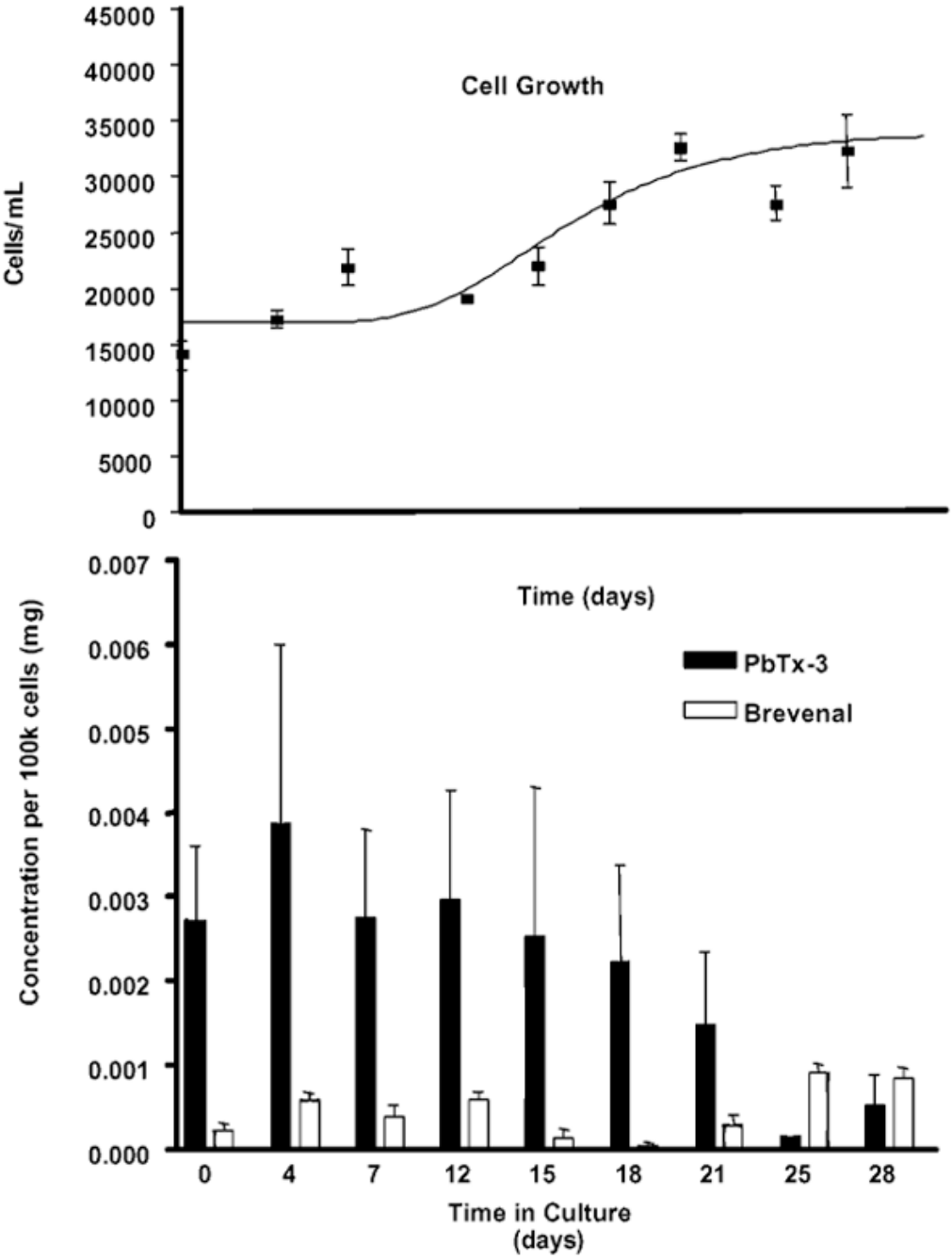

Fig. 2.

Correlation of Karenia brevis cell counts with brevetoxin and brevenal concentrations. Cultures were grown in 5–10 L quantities for a period of 35 days. Twenty-four mL aliquots from each culture were taken semiweekly for 5 weeks. Each data point in the top panel represents the average cell count ± SEM for $n = 5$ as determined by Coulter counter on 4-mL subfractions. Twenty mL fractions from each culture were extracted and quantified for brevetoxin and brevenal by HPLC (Bourdelais *et al.*, 2002).

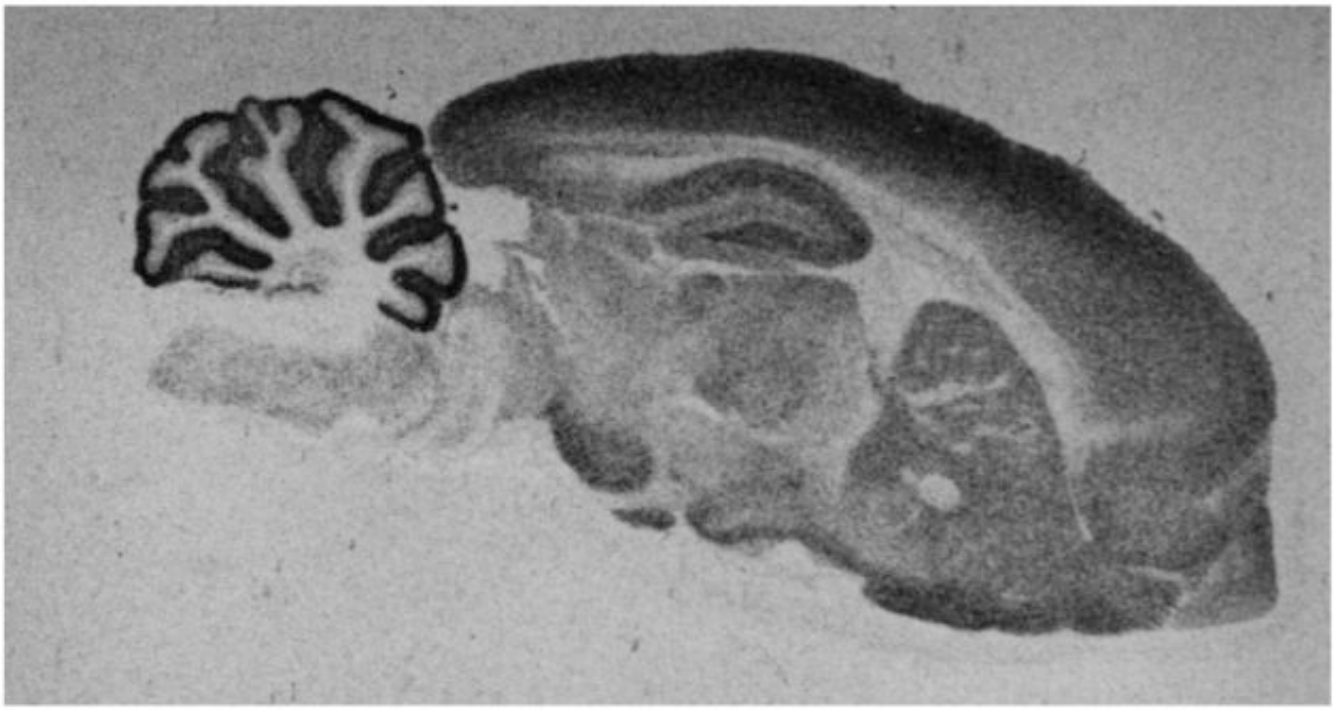

Fig. 3.

Labeling mouse brain with tritium-labeled brevetoxin. Mouse brain hemispheres were prepared and exposed to tritiated brevetoxin PbTx-3 (16.5 Ci/mmole) for 30 min at room temperature. Following development for 3 weeks at room temperature, light microscopy of films exposed to brain sections revealed a high localization in cerebellum. Cerebellum was chosen as the region of brain used subsequently for the preparation of brain synaptosomes.

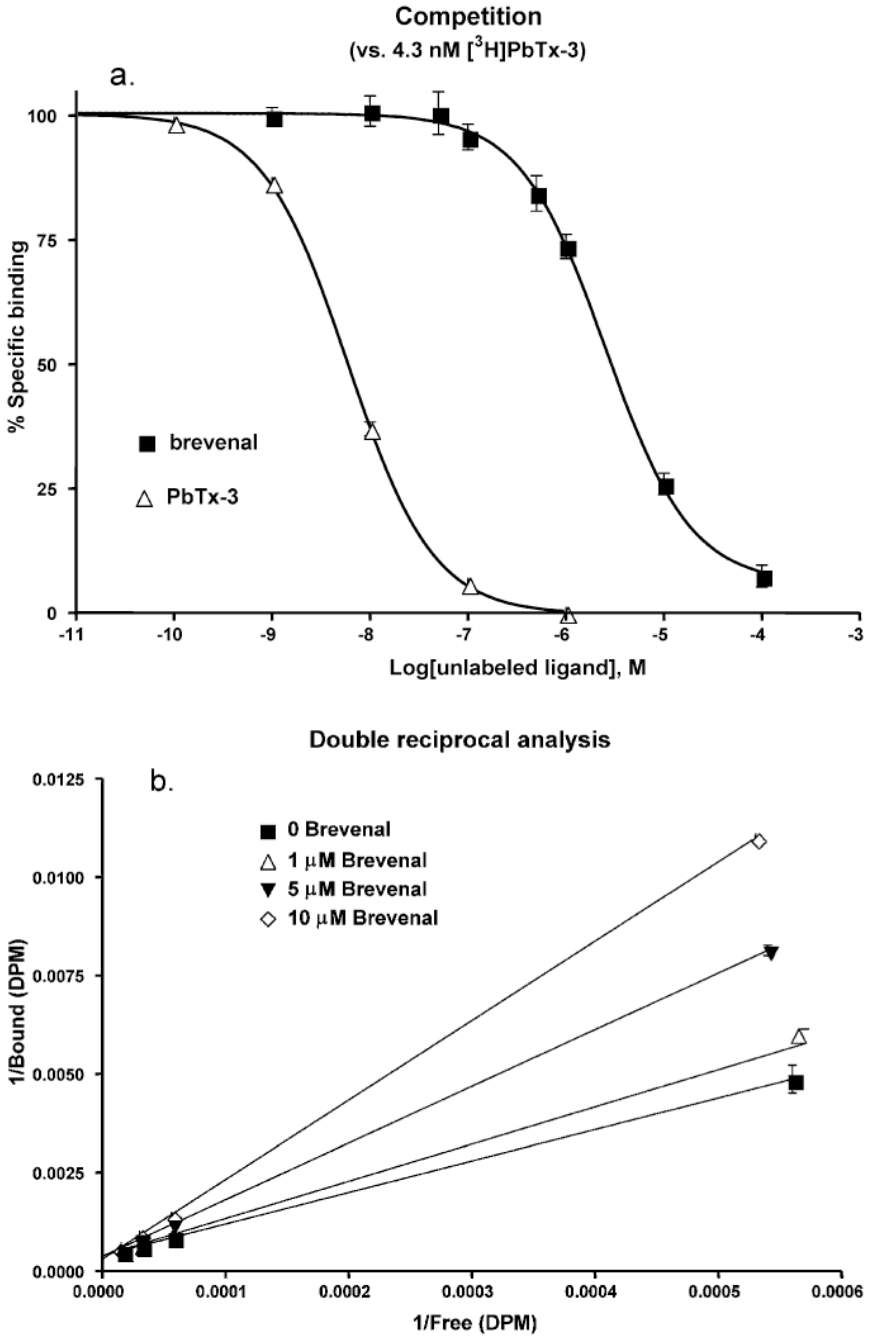

Fig. 4.

Displacement of tritium-labeled PbTx-3 ([^{3}H]PbTx-3) from rat brain synaptosomes. Binding experiments were performed as described previously (Poli *et al.*, 1986). Briefly, synaptosomes were prepared from frozen whole rat brains (male, Sprague–Dawley) by homogenization in 0.32-M sucrose (containing protease inhibitors: 1-mM iodoacetamine, 1 mM 1,10-phenanthroline, 0.1-mM phenylmethylsulfonyl fluoride, 1-μM pepstatin A) followed by three centrifugation steps. The P3 fraction was diluted to 1 mg/mL protein (determined by a modified Lowry technique) with HEPES binding medium containing protease inhibitors. Binding was determined at ice temperature in 1 mL total volume in the presence of 1 mg/mL bovine serum albumin. Serial dilutions of competing ligands were prepared in ethanol and added (10 μL) to

790 μL of binding medium in 1.5-mL Eppendorf centrifuge tubes (triplicates at each concentration of competitor). A solution of [^{3}H]PbTx-3 in binding medium (100 μL) was added to yield a final concentration of approximately 5 nM, near the reported K_D value for rat brain synaptosomes (2.2 nM). After addition of 100 μL of synaptosome suspension, the contents of each tube were vortexed and allowed to equilibrate for >1 hr on ice. Tubes were then centrifuged, supernatant was quickly aspirated, the pellet washed with immediate aspiration, and the bottom of the tube (containing the pellet) removed. The radioactivity associated with the pellet was quantified by liquid scintillation spectroscopy. Nonlinear regression analysis was performed on the resulting competition binding data using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, California, www.graphpad.com). All derived values are the mean of at least three experiments, with triplicate determinations at each concentration of competitor in each experiment. a. A displacement curve for PbTx- is provided for reference. b. Binding experiments were performed as described above at four concentrations of [^{3}H]PbTx-3 (in nM: 0.1, 1.5, 3, and 6). Radioactivity associated with the pellet (bound) and the supernatant (free) was determined. Linear regression analysis of each of the double reciprocal plots yielded r^2 values > 0.98. Convergence of the plots near the ordinate indicates that the interaction between the ligands is competitive. K_i for brevenal is 685 nM.