Brevetoxins Cause Acute Excitotoxicity in Primary Cultures of Rat Cerebellar Granule Neurons

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ABSTRACT

Brevetoxins (designated PbTx-1 to -10) are potent lipid-soluble polyether compounds that are known to bind to and modulate voltage-gated sodium channel activity. To investigate whether brevetoxins produce direct central nervous system neurotoxic effects, cultured rat cerebellar granule neurons were exposed to brevetoxins in Locke’s buffer for 2 h at 22°C. Neuronal injury was quantified by assaying lactate dehydrogenase activity in the exposure buffer and in conditioned growth media collected at 22 h after brevetoxin exposure. Brevetoxins produced acute neuronal injury and death in neurons with a rank order potency of PbTx-1 (EC_{50} = 9.31 ± 0.45 nM) > PbTx-3 (EC_{50} = 53.9 ± 2.8 nM) > PbTx-2 (EC_{50} = 80.5 ± 5.9 nM) > PbTx-6 (EC_{50} = 1417 ± 32 nM), which is similar to their previously determined rank order potency for brevetoxin-induced ictyotoxicity and binding to [3H]PbTx-3-labeled sodium channels on synaptosomes. The neurotoxic response could be prevented by coapplication of the sodium channel antagonist tetrodotoxin or by the competitive or noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonists D-AP5 and MK-801, ketamine, dextromethorphan, and dextrorphan, respectively. NMDA receptor antagonists afforded neuroprotection with rank order potencies comparable to those measured previously for protection against glutamate-induced excitotoxic responses. Further analysis revealed that brevetoxins induced a concentration-dependent release of l-glutamate and l-aspartate into the exposure buffer. These data indicate that brevetoxin-induced injury in cultured rat cerebellar granule neurons is mediated by NMDA receptors that are activated indirectly as a consequence of PbTx-induced sodium channel activation and attendant excitatory amino acid release.

Brevetoxins are potent lipid-soluble polyether neurotoxins produced by the marine dinoflagellate Ptychodiscus brevis, an organism linked to toxic “red tide” blooms that occur periodically in the Gulf of Mexico and along the west coast of Florida (Baden, 1989). P. brevis blooms are known to cause massive fish and marine mammal kills and have been implicated in human intoxication resulting from the ingestion of contaminated shellfish or inhalation of brevetoxin-contaminated aerosols in sea spray (McFarren et al., 1965; Pierce, 1986). At least 10 different brevetoxin derivatives have been characterized and are designated PbTx-1 to PbTx-10 (Poli et al., 1986; Baden, 1989).

Brevetoxins are known to interact specifically with site 5 of the α subunit of voltage-sensitive sodium channels. This interaction causes a shift in the voltage dependence of channel activation to more negative potentials, inhibits sodium channel inactivation, and thereby produces neuronal depolarization at resting membrane potentials (Catterall and Gainer, 1985; Poli et al., 1986; Sharkey et al., 1987; Edwards et al., 1992; Rein et al., 1994). In axonal or neuromuscular preparations, these effects on the sodium channel manifest as transient repetitive neuronal discharges followed by action potential depression and, eventually, a complete blockade of neuronal excitability (Huang et al., 1984; Wu et al., 1985; Huang and Wu, 1989).

Brevetoxins produce a variety of centrally and peripherally mediated effects in vivo; these include a rapid reduction in respiratory rate, cardiac conduction disturbances, and a reduction in core and peripheral body temperatures (Baden, 1989; Templeton et al., 1989a; Poli et al., 1990). Clinical manifestations of brevetoxins in rats include gasping-like respiratory movements, head-bobbing, depression, ataxia, and, in some exposed animals, the development of a head tilt (Templeton et al., 1989a; Poli et al., 1990). In anesthetized cats, brevetoxins induce a triad of bradycardia, hypotension, and bradypnea that correlates with both reflex and central actions inasmuch as it is abolished by either vagotomy (Borison et al., 1980, 1985) or the administration of atropine (Koley et al., 1995). The signs and symptoms of oral brevetoxin exposure in humans accidentally exposed to contaminated...
Cerebellar Granule Cell Culture. Primary cultures of CGNs were obtained from 8-day-old Sprague-Dawley rats as previously described (Berman and Murray, 1996). Isolated cerebella were stripped of meninges, minced by mild trituration with a Pasteur pipette, and treated with trypsin for 15 min at 37°C. Granule cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor- and DNase-containing isolation buffer, centrifuged, and resuspended in Earle’s salts containing 10% heat-inactivated FBS, 2 mM glutamine, 25 mM KCl, and 100 μg/ml gentamicin. The neurons were plated onto poly-l-lysine (mw = 393,000)-coated 6-well (35-mm) culture dishes (Fisher) at a density of ~2.5 × 10^5 cells/well and incubated at 37°C in a 5% CO2/95% humidity atmosphere. Cytosine arabinoside (10 μM) was added after 18 to 24 h to inhibit replication of non-neuronal cells. Cells were fed after 7 to 8 days in culture (DIC) with 50 μM/l of a 25 mg/ml dextrose solution.

Cytotoxicity Assays. CGNs were used for toxicologic assays at 11 to 13 DIC. All assays were carried out in 0.1% dimethyl sulfoxide. Dimethyl sulfoxide alone had no effect on neurons at concentrations as high as 1%. Growth medium was collected and saved, and the neurons washed twice in 1 ml of Locke’s incubation buffer containing 154 mM NaCl, 5.6 mM KCl, 1.0 mM MgCl2, 2.3 mM CaCl2, 8.6 mM HEPES, 5.6 mM glucose, and 0.1 mM glycine, pH 7.4. The neurons were then exposed to brevetoxin in the presence or absence of antagonist compounds in 1.0 ml of Locke’s buffer for 2 h at 22°C. At the termination of brevetoxin exposure, the incubation medium was collected for later analysis of lactate dehydrogenase (LDH) activity, and the neurons were washed twice in 1 ml of fresh Locke’s followed by replacement with 2.0 ml of the previously collected growth medium that had been filtered and supplemented with 1.25 mg/ml dextrose. The cell cultures were returned to the 37°C incubator. At 24 h after brevetoxin exposure, growth medium was collected and saved for analysis of LDH activity. LDH activity was assayed according to the method of Koh and Choi (1987).

Neuronal injury was assessed morphologically by exposing CGNs for 5 min to the vital dye fluorescein diacetate (5 μg/ml). The neurons were photographed at 400× magnification using an Olympus model IX50 inverted microscope equipped with fluorescence optics. Under fluorescence, somata and neurites of nonintoxicated neurons stain bright green, whereas injured neurons stain weakly due to a reduced ability to accumulate and hydrolyze the dye to the UV-excitable fluorescein molecule.

Measurement of Excitatory Amino Acid Release. Exposure conditions in excitatory amino acid (EAA) release studies were identical with those used in excitotoxicity assays. The exposure buffer was collected at specific time points, derivatized with o-phthalaldehyde (OPD), and assayed for EAA content by HPLC according to the method of Hill et al. (1979) with modifications. The derivatization reaction was initiated by the addition of 80 μl of borate buffer (saturated solution, pH 9.5), 200 μl of 100% methanol, and 40 μl of an OPD solution (50 mg in 4.5 ml of 100% methanol, 0.5 ml of borate buffer, 50 μl of ethanol) to 80-μl aliquots of exposure buffer. Twenty microliters of the derivatized sample was injected by autosampler (Beckman 508 with Gold Nouveau software) onto a reverse-phase column (250 × 4.5 mm i.d.; Supelco LC-18) with guard column (15 × 4.6 mm i.d.), both packed with 5-μm particles. The effluent was monitored fluorometrically (model; FS-970 Kratos) with the following settings for detection: excitation monochromator at 229 nm, a 470-nm emission cutoff filter, a 1.0-μA full-scale range setting with a time constant of 0.5 s, and a sensitivity setting of 5.42 units. The mobile phase was 0.0125 M Na2HPO4 (pH 7.2) and acetonitrile at a flow rate of 1 ml/min in a gradient from 9 to 24% over 15 min followed by an increase to 49% over 20 min and then an immediate reduction to 9% and hold for 6 min. 1-Aspartate and L-glutamate were detected at retention times of 8.2 and 10.6 min, respectively.

Quantification of Results. For each brevetoxin or antagonist concentration used in neurotoxicity assays, total LDH activity in triplicate plates was determined, the results were averaged, and LDH efflux in excess of control sister cultures run in parallel was determined. The LDH efflux value obtained from exposure buffer collected at 2 h was added to that obtained from media at 24 h to derive a measure of the cumulative change in LDH activity occurring over time. Nonlinear regression analysis and graphs were generated.
using GraphPAD Prism software (San Diego, CA). EC50 values for brevetoxin neurotoxicity and glutamate receptor antagonist neuroprotection were determined by nonlinear least-squares fitting of a logistic equation to concentration-response data.

The fluorescent detection of L-aspartate and L-glutamate derivatives was recorded and integrated using Beckman Gold Nouveau software. EAA concentrations in exposure buffer were determined by comparing unknown peak area-under-the-curve values with known external amino acid standards.

Materials. Acetoni trile, ethanethiol, and OPD were purchased from Fisher Scientific (Norcross, GA). D-(-)-2-Amino-5-phosphono-pentanoic acid (D-AP5), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), dextromethorphan, and ketamine were purchased from Research Biochemicals Inc. (Natick, MA). Tetrodotoxin was purchased from Sankyo (Tokyo, Japan). PbTx-1 was purchased from BIOMOL (Plymouth Meeting, PA). PbTx-2, PbTx-3, and PbTx-6 were purchased from Calbiochem (La Jolla, CA). Trypsin, basal medium Eagle’s, gentamycin, heat-inactivated FBS, soybean trypsin inhibitor, and DNase were obtained from Atlanta Biologicals (Norcross, GA). Poly-l-lysine and cytosine arabinoside were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

In preliminary experiments, 12 DIC CGNs were exposed for 2 h to 100 nM PbTx-1 at 22°C and observed for morphological signs of toxicity. Noticeable swelling of neuronal somata was apparent within 5 min, followed by progressive membrane blebbing and thinning of the neuronal processes. By 2 h, moderate-to-extensive disintegration of neuronal processes had occurred, whereas the somata remained swollen yet intact. After washing of the neurons and placing them back into conditioned media at 37°C, neuronal necrosis progressed further with only a few intact cell bodies remaining after 24 h. Nonexposed control neurons were unaffected by these manipulations.

The neurotoxic potency of brevetoxins was measured by assaying LDH activity in the exposure buffer of neurons exposed for 2 h to increasing concentrations of four brevetoxin derivatives. Previous experiments in our laboratory have established a close correlation between the degree of neuronal injury and the level of LDH activity in the exposure buffer of CGNs exposed to neurotoxins (Berman and Murray, 1996, 1997). As shown in Fig. 1, the brevetoxin derivatives produced a concentration-dependent increase in LDH activity with the following rank order potency: PbTx-1 (EC50 = 9.31 ± 0.45 nM) > PbTx-3 (EC50 = 53.9 ± 2.8 nM) > PbTx-2 (EC50 = 80.5 ± 5.9 nM) > PbTx-6 (EC50 = 1417 ± 32 nM). LDH activity in the exposure buffer was reduced to 8.3 ± 3.5% of that produced by PbTx-1 alone when CGNs were exposed to 100 nM PbTx-1 in the presence of 1 μM tetrodotoxin, indicating that the neurotoxic response is dependent on sodium channel activation.

Because CGNs are glutamatergic in nature, we hypothesized that the mechanism of brevetoxin-induced neurotoxicity involves the neuronal release of endogenous EAs with subsequent activation of glutamate receptors. To assess the role of NMDA receptors in brevetoxin-induced cytotoxicity, we examined the influence of competitive and noncompetitive NMDA antagonists on CGNs challenged with 100 nM PbTx-1. Initial observations showed that the acute somal swelling induced by PbTx-1 alone could be prevented by each NMDA receptor antagonist that was tested. Moreover, as shown in Fig. 2, CGNs were completely protected from PbTx-1 toxicity in a concentration-dependent manner by the noncompetitive NMDA receptor antagonists MK-801, dextromethorphan, and ketamine and the competitive antagonist D-AP5, with EC50 values of 64.7 ± 2.0 nM, 4.2 ± 0.5 μM, 9.1 ± 0.7 μM, 9.9 ± 1.1 μM, and 20.8 ± 1.4 μM, respectively. As shown in Fig. 3, the rank order potency of the NMDA receptor antagonists against a 100 nM PbTx-1 challenge correlated closely with their previously reported rank order potency against a neurotoxic L-glutamate challenge (r = 0.99; Berman and Murray, 1996, 1997). These results suggest that CGNs may release EAs in response to brevetoxin exposure, which results in NMDA receptor-mediated cell death.

To directly examine the relationship between brevetoxin-induced EAA release and neuronal injury, CGNs were exposed to different concentrations of brevetoxins for 20 min, and the exposure buffer was assayed for L-glutamate and L-aspartate content. The resultant concentration-response profiles for EAA release were compared with those for the efflux of LDH. As shown in Fig. 4, brevetoxins produced a
concentration-dependent increase in the release of EAAs into the exposure buffer. Moreover, the amount of EAAs released in response to brevetoxin correlated closely with the degree of neuronal injury that was produced. The brevetoxin EC<sub>50</sub> values for LDH, l-glutamate, and l-aspartate release, respectively, were PbTx-1, 8.65 ± 0.79, 7.57 ± 0.07, and 7.91 ± 0.24 nM; PbTx-2, 37.7 ± 1.8, 59.6 ± 9.1, and 60.7 ± 5.3 nM; and PbTx-3, 30.9 ± 1.4, 45.3 ± 9.8, and 50.2 ± 15.1 nM, respectively.

The temporal relationship between EAA release and neuronal injury was examined by assaying l-aspartate, l-glutamate, and LDH activity in exposure buffer collected from culture plates at specific time points during a 30-min 100 nM PbTx-1 challenge. As shown in Fig. 5, l-glutamate was released earliest, appearing initially at the 1-min time point, and its concentration increased biphasically over the 30-min period, with the most rapid phase being complete after the first 5 min. Similarly, l-aspartate release appeared biphasic but was delayed relative to glutamate release and reached a final concentration of approximately 50% of that of l-glutamate. Neuronal injury, as indicated by the presence of LDH in the exposure buffer, began subsequent to the appearance of EAAs and was detectable 5 min after the start of PbTx-1 exposure. The LDH activity in the media increased monophasically thereafter during the 30-min experiment.

Neuronal injury at the 5-min time point was confirmed morphologically by assessing the ability of CGNs to accumulate the vital dye fluorescein diacetate and to hydrolyze it to fluorescein, which fluoresces green under ultraviolet light. As shown in Fig. 6, the somata and neurites of nonexposed control neurons (Fig. 6A) stained intensely and maintained structural integrity, whereas CGNs exposed to PbTx-1 stained less intensely; had swollen, poorly defined somata; and demonstrated early signs of neurite membrane blebbing (Fig. 6B). Neurons exposed for 5 min to 100 nM PbTx-1 in the presence of the noncompetitive NMDA receptor antagonist MK-801 (1 μM) were protected against excitotoxic injury and appeared to be indistinguishable from nonexposed control neurons (Fig. 6C).

**Discussion**

In the present report, we show that brevetoxins produce a concentration-dependent neurotoxic response in cultured rat CGNs. The neurotoxic rank order potency of the four brevetoxin derivatives tested here matches the previously pub-

![Fig. 3.](image1.png)

Correlation between the ability of NMDA receptor antagonists to protect 11 to 13 DIC CGNs against a 300 μM l-glutamate or 10<sup>-7</sup> M PbTx-1 challenge (r = 0.99, p = .0008). The significance of the correlation was evaluated by two-tailed t test of the probability that r = 0. Glutamate data were obtained from published reports using identical experimental conditions (Berman and Murray, 1996, 1997).

![Fig. 4.](image2.png)

Concentration-response profiles for EAAs (dotted lines) and LDH efflux (solid lines) from 12 DIC rat CGNs exposed for 20 min to brevetoxins. Data are pooled from two experiments performed in triplicate. Each data point represents the mean ± S.E.M. from triplicate plates. Specific LDH activity is that which occurs in excess of parallel run controls. EC<sub>50</sub> values for LDH, l-glutamate, and l-aspartate, respectively, were PbTx-1, 8.65 ± 0.79, 7.57 ± 0.07, and 7.91 ± 0.24 nM; PbTx-2, 37.7 ± 1.8, 59.6 ± 9.1, and 60.7 ± 5.3 nM; and PbTx-3, 30.9 ± 1.4, 45.3 ± 9.8, and 50.2 ± 15.1 nM.

![Fig. 5.](image3.png)

Time course of EAAs (solid lines) and LDH efflux (dotted line) from 12 DIC rat CGNs exposed to 100 nM PbTx-1. Data are from a representative experiment. Each data point represents the mean ± S.E.M. from triplicate plates. Specific LDH activity is that which occurs in excess of parallel run controls.
lished rank order potency for brevetoxin ichthyotoxicity and affinity for [3H]PbTx-3-labeled sodium channels in synaptosomes (Rein et al., 1994; Gawley et al., 1995). Prevention of the response by tetrodotoxin confirmed that brevetoxin neurotoxicity is dependent on the activation of voltage-sensitive sodium channels; however, sodium channel activation did not directly cause neurotoxicity in CGNs. These data show clearly that brevetoxin-induced neurotoxicity is mediated entirely by NMDA receptors that are activated secondarily as a consequence of brevetoxin-induced stimulation of EAA release. This interpretation is based on the following findings: first, brevetoxins stimulated the release of L-glutamate and L-aspartate from CGNs in a concentration-dependent manner. The EC₅₀ values measured for EAA release were nearly identical with the EC₅₀ values determined simultaneously for brevetoxin-induced neuronal injury (Fig. 4). Second, competitive and noncompetitive NMDA receptor antagonists completely and concentration-dependently protected CGNs against brevetoxin neurotoxicity. The causal involvement of NMDA receptors in glutamate-induced neuronal necrosis has been well documented (Choi and Rothman, 1990). The competitive and noncompetitive NMDA receptor antagonist compounds used here act either by preventing the interaction of glutamate with its binding site or by binding to and blocking the ion channel, respectively. Both actions prevent the EAA-stimulated influx of Ca²⁺ and Na⁺ into neurons. Third, there was an excellent correlation between the rank order potency of the NMDA receptor antagonists to protect against PbTx-1 neurotoxicity and their neuroprotective rank order potency reported previously against neurotoxic L-glutamate challenge in CGNs under identical conditions (Berman and Murray, 1996, 1997). Finally, the temporal relationship between the appearance of EAAs and LDH in the exposure buffer is consonant with a cause-effect relationship between brevetoxin-stimulated release of EAAs from neurons and induction of neuronal injury.

In a previous study, we investigated the toxicologic mechanisms of another marine neurotoxin, domoic acid, in CGNs using identical experimental conditions (Berman and Murray, 1997). Domoate is a tricarboxylic amino acid produced by various species of marine diatom that can cause severe neurologic dysfunction and necrosis in areas of the brain associated with learning and memory (Tietelbaum et al., 1990). Similar to the present findings on brevetoxin neurotoxicity, domoate was found to produce a largely NMDA receptor-mediated neurotoxic response in CGNs even though its primary target is the AMPA/kainate subtype of glutamate receptor. Further investigation revealed that the domoate-stimulated neuronal degeneration in CGNs is produced secondarily to the AMPA/kainate receptor-mediated release of EAAs and subsequent activation of NMDA receptors. These findings are supported by a variety of in vitro and in vivo reports demonstrating that AMPA/kainate receptor agonist-induced neurotoxicity and brain damage are substantially mediated by NMDA receptors (Favaron et al., 1988; Manev et al., 1989; Lerner-Natoli et al., 1991; Berg et al., 1993). The present study extends these earlier findings and suggests that a variety of neurotoxins that depolarize neurons through different effectors may be capable of releasing excitatory neurotransmitters and producing excitotoxic cell death in the CNS. Brevetoxins and domoic acid are therefore able to act via a common indirect excitotoxic mechanism.

The data presented here suggest that brevetoxins have the potential to cause central neuronal injury in exposed humans and animals. By inference, this notion may be extended to the related polyether neurotoxin, ciguatoxin, which also binds to toxin binding site 5 on sodium channels and produces a toxicologic syndrome similar to that of brevetoxins (McFarren et al., 1965; Lombet et al., 1987; Baden, 1989; Swift and Swift, 1993). Ciguatera fish poisoning is the most frequently reported seafood-related disease in the United States and is a common ichthyosarcotoxin endemic throughout the Caribbean and Indo-Pacific islands (Swift and Swift, 1993). The signs of ciguatoxosis can be more severe than those associated with brevetoxins and are generally more persistent, lasting from weeks to months as opposed to hours. More than 175 manifestations of ciguatera toxicity have been documented, which include chronic paresthesias, arthralgias, and headaches, or, when severe, coma, paralysis, respiratory depression, and even death (Sims, 1987). To our knowledge, however, no other investigations have specifically addressed whether these polyether marine neurotoxins cause permanent injury to CNS neurons. The present demonstration of brevetoxin-induced excitotoxicity in CGNs renders this issue relevant and in need of further investigation.

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References


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