ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 343, No. 2, July 15, pp. 149–156, 1997
Article No. BB970149

Brevetoxin-6 (PbTx-6), a Nonaromatic Marine Neurotoxin, Is a Ligand of the Aryl Hydrocarbon Receptor

B. S. Washburn,*,†,[1] K. S. Rein,† D. G. Baden,† P. J. Walsh,† D. E. Hinton,*
K. Tullis,‡ and M. S. Denison‡

*Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, California 95616; †NIEHS Marine and Freshwater Biomedical Sciences Center, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, Miami, Florida 33149; and ‡Department of Environmental Toxicology, University of California, Davis, California 95616

Received December 10, 1996, and in revised form January 28, 1997

Brevetoxins (PbTx) are a family of marine polyether toxins that exert their toxic action by activating voltage-sensitive sodium channels. Two forms of brevetoxin, PbTx-2 and -3, induce hepatic cytochrome P4501A1, measured as ethoxyresorufin *O*-deethylase (EROD) activity, in redfish and striped bass. P4501A1 induction is transcriptionally regulated through the binding of a ligand, typically a planar aromatic compound, to the aryl hydrocarbon receptor (AhR) and subsequent complex formation with the dioxin response element (DRE), an upstream regulatory region of the CYP1A1 gene. To determine if PbTx, a nonaromatic compound, induced EROD by this mechanism, two sets of experiments were performed. Initially, saturation binding assays with PbTx-2, -3, and -6 were carried out to determine if PbTx-2, -3, or -6 was an AhR ligand. Results showed that PbTx-6 inhibited specific binding of dioxin to the AhR, whereas PbTx-2 and -3 had no effect. Subsequently, gel retardation assays showed that PbTx-6 caused a concentration-dependent increase in AhR–DRE complex formation. The most abundant and neurotoxic forms of brevetoxin, PbTx-2 and -3, did not appear to be involved in this process. However, PbTx-6, the epoxide which is a likely biotransformation product, is at least one of the forms of PbTx involved in EROD induction. © 1997 Academic Press

Key Words: **brevetoxin; marine toxins; natural toxins; Ah receptor; EROD; cytochrome P4501A1.**

Brevetoxins are polyether, marine neurotoxins produced by the dinoflagellate *Ptychodiscus brevis.* During "red tides" or blooms of the organism, numerous fish and marine animals in the Gulf of Mexico, the southeastern seaboard of the United States, and other temperate marine environments succumb to the toxin (1). Human consumption of tainted shellfish is associated with a serious intoxication syndrome, neurotoxic shellfish poisoning, the result of binding of brevetoxin (PbTx) to site 5 of the voltage-sensitive sodium channel (VSSC).[2] As a sodium channel activator, PbTx causes VSSC to remain in an open conformation at normal resting membrane potential (2, 3), slows inactivation of the channel, and causes repetitive firing (4). Over time, excitable cells are no longer able to conduct action potentials, causing a series of symptoms including temperature reversal, hypotension, arrhythmias, and, in animals, cardiac and respiratory arrest, leading to death (1).

Recently, we observed that oral exposure of redfish (*Sciaenops ocellatus*) and striped bass (*Morone saxatilis*) to nonlethal concentrations of two distinct brevetoxins, PbTx-2 and -3 (the aldehyde and alcohol forms, Fig. 1), induced cytochrome P4501A1-associated monooxygenase activity, namely ethoxyresorufin *O*-deethylase (EROD) (5, 6). The mechanism of P4501A1 induction by chemicals has been extensively studied by numerous investigators in a wide variety of species. Mechanistically, induction of P4501A1 is mediated by a soluble intracellular protein, the Ah receptor (AhR), to which inducing chemicals bind with high affinity.

[1] To whom correspondence should be addressed at present address: Department of Biological Sciences, University of Texas, El Paso, El Paso, TX 79968. Fax: (915) 747-5808.

[2] Abbreviations used: VSSC, voltage-sensitive sodium channel; EROD, ethoxyresorufin *O*-deethylase; AhR, Ah receptor; DRE, dioxin responsive element; BNF, β-naphthoflavone; DMSO, dimethyl sulfoxide; TCDD, [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DTT, dithiothreitol; HAP, hydroxyapatite adsorption; ANF, α-naphthoflavone; GRA, gel retardation analysis.

0003-9861/97 $25.00
Copyright © 1997 by Academic Press
All rights of reproduction in any form reserved.

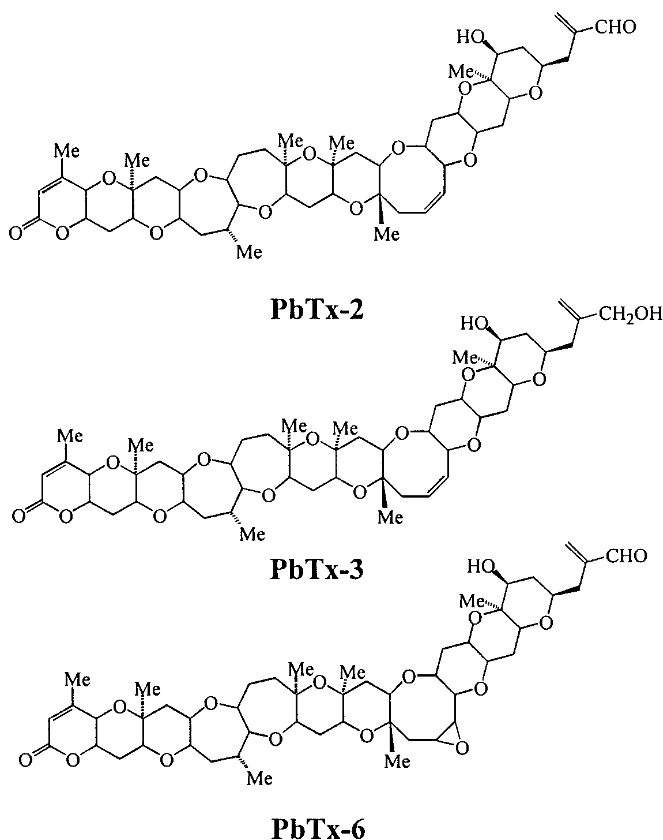

FIG. 1. Brevetoxin molecules. PbTx-2 and -3 are the aldehyde and alcohol forms, respectively. PbTx-6 contains an aldehyde as well as an epoxide on ring H.

Following ligand binding, the ligand–AhR complex is transformed into its DNA binding form and accumulates within the nucleus. After dimerization with a second protein (ARNT, Ah receptor nuclear translocator), the AhR complex binds to DNA. The interaction of the ligand–AhR–ARNT complex with its specific DNA recognition site, the dioxin response element (DRE), results in transcriptional activation of an adjacent promoter and gene (CYP1A1 in this case) (7, 8).

To date, no endogenous ligand for the receptor has been found; however, numerous high-affinity AhR ligands and classes of ligands have been identified. The highest affinity ligands described are halogenated aromatic hydrocarbons (HAHs), including polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls. In addition, a variety of nonhalogenated polycyclic, aromatic hydrocarbons, i.e., benzo(*a*)pyrene and 3-methylcholanthrene, nitrogen heterocycles such as indole 3-carbazole (9), UV and metabolic products of tryptophan (10), constituents of wood pulp (11), and other chemicals have been shown to be potent AhR ligands (12). Although the AhR appears to bind a wide diversity of chemical structures, all of these inducers exhibit one common physical feature: they contain at least one aromatic carbon ring. Interestingly, the specific inducing brevetoxins, PbTx-2 and -3, are polyether ladder toxins (Fig. 1) which are not aromatic. Further, computer modeling studies have shown these compounds to be nonplanar (2, 3). If PbTx-2 and/or -3 induce P4501A1 activity via the AhR-dependent mechanism, then this would represent a novel and potentially important observation. Here, we describe studies of the mechanism by which PbTx induces P4501A1 activity and demonstrate an interaction between PbTx and the AhR.

MATERIALS AND METHODS

Chemicals. Solutions of three forms of brevetoxin, PbTx-2, -3, and -6 (Fig. 1), and β-naphthoflavone (BNF) were prepared in DMSO. Because PbTx is a potent neurotoxin, extra precautions were taken when handling lyophilized toxin. These included the use of a respirator, gloves, and protective clothing. PbTx-2, an aldehyde; PbTx-3, an alcohol; and PbTx-6, an epoxide were diluted to various concentrations in DMSO appropriate for each assay. All three are naturally occurring forms of brevetoxin produced by *P. brevis.* Culture and purification of the PbTx-2 and -3 have been described (6). Since PbTx-6 is in low abundancy naturally, it was chemically synthesized from PbTx-2 using dimethyldioxorane to oxidize the double bond on the eight-membered ring (ring H) (3). BNF was purchased from Sigma Chemical (St. Louis, MO).

[^{3}H]2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD; 37 Ci/mmol) and unlabeled TCDBF were obtained from Dr. S. Safe (Texas A & M University). [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from New England Nuclear. Molecular biological reagents were from Bethesda Research Laboratories and Boehringer-Mannheim. TCDD and TCDBF are extremely toxic substances and were handled with great care.

Animals and preparation of cytosol. Male Hartley guinea pigs [250–300 g; Michigan Department of Public Health (Lansing, MI)] were exposed to a 12:12 light:dark cycle daily and were allowed free access to food and water. Hepatic cytosol was prepared in HEDG buffer [25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol] as previously described (13) and was stored at −80°C until use. Protein concentrations were determined by the method of Bradford (14), using bovine serum albumin as the standard.

AhR ligand binding assay. Specific binding of ligands to AhR was measured using hydroxyapatite adsorption (HAP) (15) and sucrose density gradient assays (10). For HAP assays, diluted guinea pig cytosol, containing the AhR (8 mg protein/ml HEDG buffer), was incubated at 25°C on a rotating shaker for 2 h with [^{3}H]TCDD and a 100-fold molar excess of PbTx-2, -3, or -6, BNF, or DMSO. To separate ligand-bound [^{3}H]TCDD from free [^{3}H]TCDD, 0.2 ml of the mixture was added to 0.5 ml Bio-Gel HTP (Bio-Rad, Hercules, CA) in a clean tube, vortexed, and incubated at 4°C for 30 min with mixing every 10 min. The protein-bound HAP was then washed three times with 2 ml ice-cold buffer with 0.5% Tween 80. Following the last wash, 1.0 ml of scintillation cocktail was added to the tube and vortexed, and the sample was transferred to a scintillation vial. The sample tube itself was subsequently washed with 95% ethanol and the contents were added to the HAP sample along with 3.0 ml of scintillation cocktail. The radioactivity was quantitated by liquid scintillation counting (Tricarb 2000, Packard Instruments).

Sucrose density gradient centrifugation. To further characterize PbTx competitive binding to the AhR, sucrose density gradient centrifugation was carried out as previously described (16). Guinea pig cytosol (20 mg/ml) was incubated with 2 nM [^{3}H]TCDD with 100-fold excess DMSO, TCDBF, or PbTx-6 for 1 h at room temperature. After

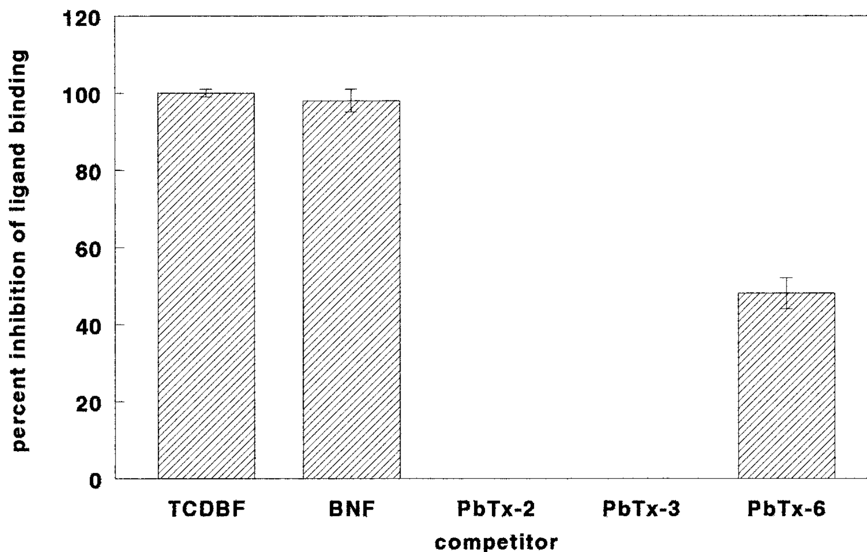

FIG. 2. Inhibition of ligands on [^{3}H]TCDD binding to the AhR. Cytosol containing the Ah receptor was incubated with [^{3}H]TCDD and 100 molar excess of TCDBF, BNF, PbTx-6, PbTx-2, or PbTx-3. Free or loosely bound ligand was separated from bound [^{3}H]TCDD with HAP. Bars represents the means of two replicates performed in triplicate. Bracket indicates range of data.

treatment with dextran-coated charcoal to remove unbound and loosely bound [^{3}H]TCDD, samples were centrifuged in 10–30% sucrose (v/v) gradients and fractionated as previously described (13). The radioactivity present in each fraction was determined by liquid scintillation counting. Inhibition of binding was calculated by dividing the area under the specific binding curve (AUC) for PbTx-6 by AUC for [^{3}H]TCDD and subtracting this value from 1.

Gel retardation analysis. A complementary pair of synthetic oligonucleotides containing the sequence 5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGAAGAGCCA-3′ (corresponding to the AhR binding site of the DRE3 and designated as the DRE oligonucleotide) was synthesized, purified, annealed, and radiolabeled with [γ-^{32}P]ATP as described (10). Guinea pig hepatic cytosol (16 mg protein/ml) was incubated with DMSO and various concentrations of BNF, PbTx-2, PbTx-3, or PbTx-6 for 2 h at 25°C, followed by gel retardation analysis. In the binding assay, cytosol was mixed with poly(dI–dC) and incubated for 15 min at 25°C, followed by the addition of the ^{32}P-labeled DNA oligonucleotide to the mixture and further incubation for 15 min. The optimal incubation mixture contained 80 μg of protein, 225 ng of poly(dI–dC), $\simeq$1 ng (100,000 cpm) ^{32}P-DNA, 25 mM Hepes, 1 mM EDTA, 1 mM DTT, 10% glycerol (v/v), and 80 mM KCl. After the final incubation, the samples were mixed with Ficoll sample buffer [to 10% (v/v)] and loaded onto a 4% nondenaturing polyacrylamide gel which had been preelectrophoresed for 1 h. The samples were subjected to electrophoresis with continual buffer recycling, the gels dried, and protein–DNA complexes visualized by autoradiography. Analysis using 5 nM TCDD was also performed for comparative purposes.

RESULTS

AhR binding assays. To assess the ability of several brevetoxins (PbTx-2, -3 and -6) to bind the AhR, saturation binding assays using [^{3}H]TCDD were carried out using HAP binding assays. BNF, an AhR ligand used as a positive control, and PbTx-6, but not PbTx-2 or PbTx-3, inhibited [^{3}H]TCDD binding to the Ah receptor (Fig. 2). Nonspecific binding averaged 44% (data not shown). BNF inhibited specific TCDD binding by 98% while PbTx-6, the epoxide form of brevetoxin, inhibited specific binding by 48%. In contrast, neither PbTx-2 nor -3 demonstrated any ligand binding ability.

To confirm that the reduction in [^{3}H]TCDD binding to the AhR was actually due to competitive inhibition by PbTx-6, sucrose density centrifugation analysis was carried out (Fig. 3). The single [^{3}H]TCDD specific binding peak observed in fractions 13–20 has previously been determined to represent binding to the AhR (16). The results demonstrated that PbTx-6, at a concentration of 200 nM, can inhibit binding of [^{3}H]TCDD to the AhR by 72% of that observed with TCDBF. Overall, the above data confirm that PbTx-6 is a ligand of the AhR.

Gel retardation assays. Although competitive ligand binding analysis confirms the ability of PbTx-6 to bind the AhR, it does not address whether PbTx-6 exhibits antagonist/agonist activity. To address this question, we carried out gel retardation analysis which examined the ability of a chemical not only to bind the AhR, but to transform it into its DNA binding form. Although this assay does not confirm the ability of the chemical to alter gene expression, the high degree of correlation we have observed between the ability of a chemical to induce AhR transformation/DNA binding and gene expression would strongly support its AhR agonist activity.

To examine the ability of the various PbTxs to induce AhR transformation and DNA binding, we carried out GRA using 1–100 μM PbTx-2 and -3, 1–1000 nM PbTx-6, and, as a positive control, 1–1000 nM BNF. Results

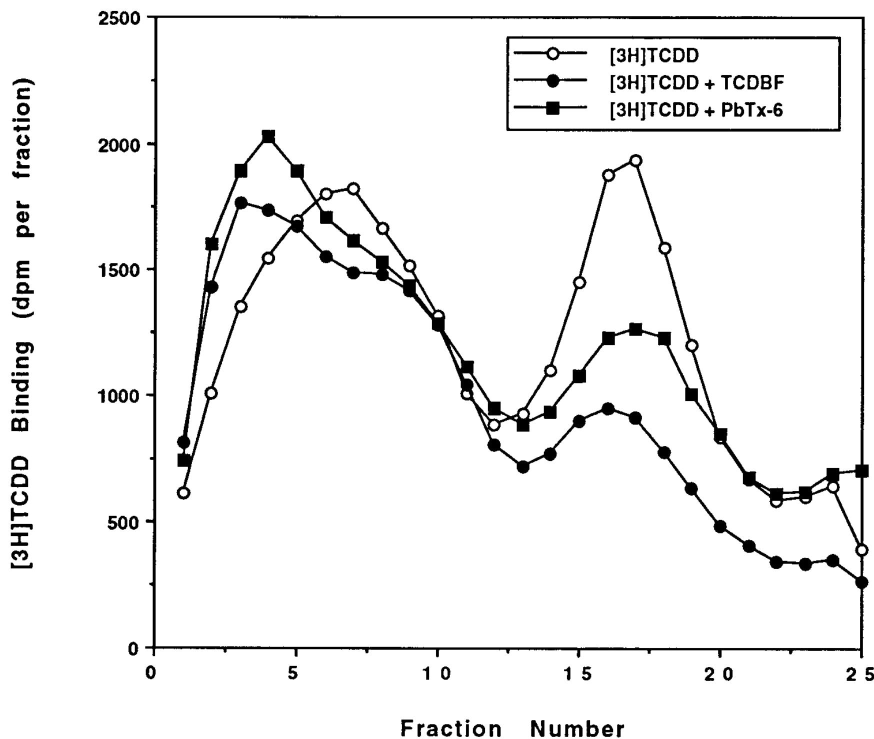

FIG. 3. Effect of PbTx-6 on [^{3}H]TCDD binding using sucrose density gradient assay. Lines represent incubation of guinea pig cytosol containing the AhR with [^{3}H]TCDD and DMSO (vehicle), TCDBF (100-fold excess, nonspecific binding), and PbTx-6 (100-fold excess). Data points represent means of triplicate assays. The first peak is associated with nonspecific binding; the second peak is AhR-specific binding.

of the initial assay using 5 nM TCDD are shown in Fig. 4. The arrow indicates the typical retarded band associated with TCDD–AhR–DRE complex formation. The data in Fig. 5 revealed that nanomolar concentrations of BNF induced AhR–DRE complex formation, whereas only minimal complex formation was seen with micromolar concentrations of PbTx-2 and -3. These results suggest that PbTx-2 and -3 are, as anticipated, weak AhR ligands. In contrast, PbTx-6 induced protein–DNA complex formation in a concentration-dependent manner with a potency similar to that of BNF (Fig. 6).

To confirm that the protein–DNA complex induced by PbTx-6 and which migrates to the same position as that induced by BNF represents the AhR–DRE complex, we examined the DNA binding specificity of the complex. The ability of the two ligands to induce protein–DNA complex formation with both wild-type and mutant DRE containing oligonucleotides was compared. We have previously demonstrated that a mutant DRE containing a single nucleotide substitution within the core DRE sequence prevents complex formation. As shown in Fig. 7, the expected inducible protein–DNA complex was observed when the wild-type DRE was used (lanes 1, 2, and 3), whereas the inducible complex could not be identified when the mutant DRE was used in the assay (lanes 4, 5, and 6). These results confirm that the protein–DNA complex induced by BNF and PbTx-6 represented the binding of transformed AhR complex to the DRE and was not associated with nonspecific DNA binding.

DISCUSSION

Previous research with redfish and striped bass demonstrated increased EROD activity in fish exposed to brevetoxin (5, 6); however, the mechanism by which this occurred was not examined. PbTx-2 and -3 are the most abundant forms of brevetoxin in nature and we initially thought that they were the forms responsible for EROD induction. However, the present series of experiments demonstrates that they play little if any role in the AhR mechanism of P4501A1 induction. In HAP adsorption binding assays, PbTx-2 and -3 failed to cause inhibition of [^{3}H]TCDD binding to the AhR.

Although the receptor binding experiments demonstrated specific binding of PbTx-6 to the AhR, it remained to be demonstrated if this form of brevetoxin acted as a AhR agonist or antagonist. If PbTx-6 induced a conformational change which prevented the PbTx-6–AhR from complexing with the DRE, then it could not be responsible for the induction of EROD observed in

FIG. 4. Gel mobility shift induced by TCDD. Guinea pig cytosol incubated with 5 nM TCDD or DMSO for 2 h at room temperature. After a brief incubation with [^{32}P]DRE, gel electrophoresis was performed and autoradiography followed. Lane 1 contains DMSO, lane 2 contains TCDD. Arrow indicates retarded band induced by TCDD–AhR–DRE complex formation.

previous experiments. When analyzed by gel retardation assays, a concentration of 100 μM PbTx-2 and -3 was necessary to observe a shift in mobility (Fig. 5). This contrasts sharply with BNF for which a shift was observed at 10 nM. The very large concentration of PbTx-2 or -3 needed to induce complex formation suggests it is unlikely to have an effect *in vivo.* LD_{50} for PbTx-2 and -3, the most toxic forms of brevetoxin (1), is approximately 5000-fold less than the concentration which induced a shift, suggesting that it is highly unlikely that these forms of brevetoxin could be responsible for EROD induction noted in the sublethal exposures of redfish and striped bass to brevetoxin in previous experiments (5).

In contrast, PbTx-6 acted as a AhR competitive inhibitor of TCDD, both with HAP adsorption and sucrose gradient density assays. HAP adsorption assays demonstrated that PbTx-6 competitively inhibited [^{3}H]-TCDD specific binding by 47 $\pm$ 0.5%. To confirm these results, sucrose density gradient assays were also performed. Again, we observed inhibition of labeled TCDD specific binding to the AhR; in this case 79% inhibition was noted. The discrepancy in the results of the two assays is likely due to nonspecific binding of [^{3}H]TCDD to the HAP since specific and nonspecific binding cannot be easily differentiated with adsorption assays of this type (17). Although this assay-based discrepancy was noted, both methods of analysis confirm that PbTx-6 acts as an AhR ligand and is able to inhibit [^{3}H]TCDD binding (Fig. 2). The ability of PbTx-6 to compete with TCDD for ligand binding is of the same magnitude seen with other AhR ligands, such as benzo[a]pyrene and 3-methylcholanthrene (100 and 10 nM BaP and 3-MC respectively with 10 nM [^{3}H]TCDD) (18). Poland and Glover reported a positive relationship between relative AhR binding affinity and relative biological potency among dibenzo-*p*-dioxin congeners (19). This analysis, if applied to the three brevetoxins, would suggest that PbTx-6 is the form responsible for CYP1A1 induction.

The results of the gel mobility shift assays confirmed the findings of the receptor binding studies, i.e., PbTx-6 induced a gel shift. The ability of PbTx-6 to cause a concentration-dependent shift suggests that the toxin also causes conformational changes in the AhR that promote DRE complex formation which would be ex-

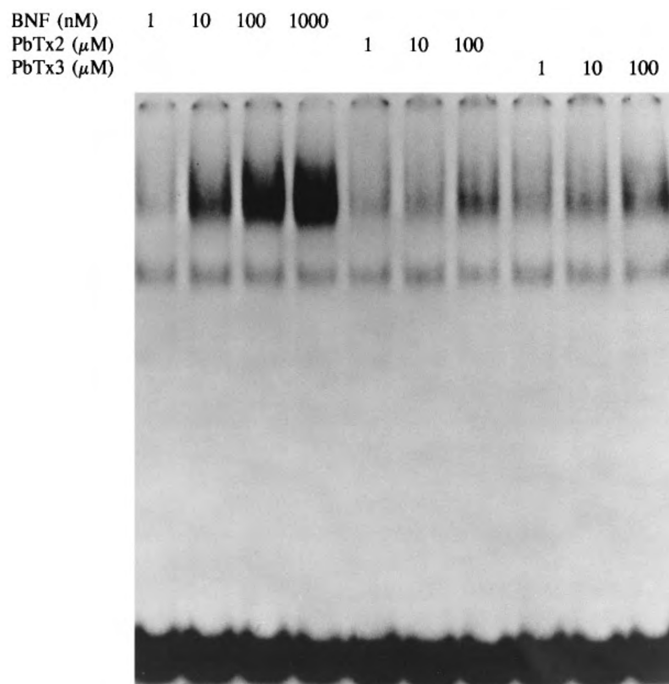

FIG. 5. Gel mobility shift induced by PbTx-2, PbTx-3, and BNF. Various concentrations of two forms of PbTx and BNF, a positive control, were incubated with [^{32}P]DRE to determine the ability of these ligands to induce a gel shift. Note that the concentrations of PbTx are in the micromolar range, while those of BNF are in the nanomolar range. Arrow represents shifted band induced by ligand–AhR–DRE complex formation. Data represent one determination; $N = 4$.

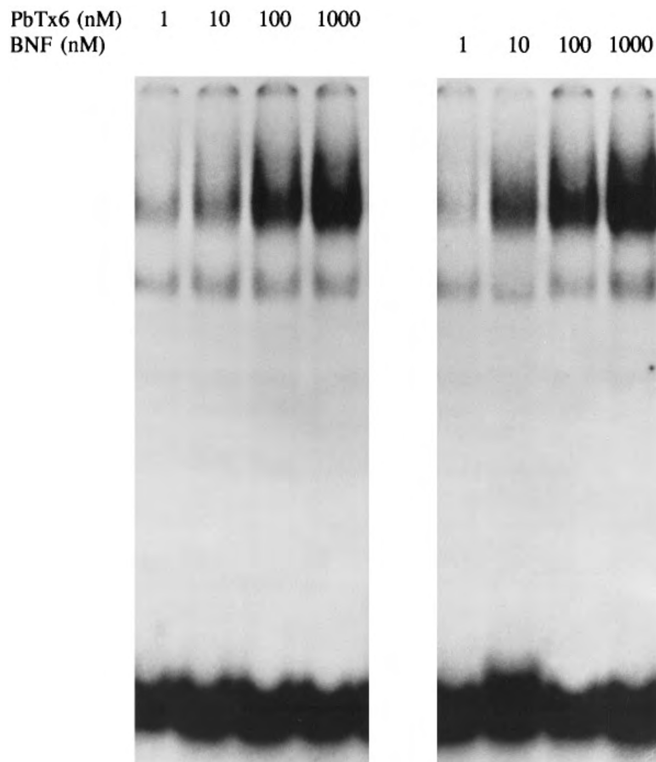

FIG. 6. Gel mobility shift induced by PbTx-6 and BNF. Four concentrations (nM) of PbTx-6 and BNF, a positive control, were incubated as described in the legend to Fig. 5. In both cases, a concentration-dependent shift was observed. $N = 5$.

produced three polar metabolites, suggesting that biotransformation of PbTx-2 occurs in fish (Washburn and Jones, unpublished observations). Although at present we have not positively identified these metabolites, epoxides are often a minor product of Phase I metabolism (21). Therefore, epoxidation of the H ring on PbTx-2 could occur as a consequence of P450 activity. The preliminary data we have in animals and the fact that PbTx-6 is produced in plants suggest that PbTx-6 is probably formed *in vivo* in vertebrates.

Most epoxides are highly labile compounds and are susceptible to nucleophilic attack from the face opposite the oxygen, resulting in opening of the epoxide ring. However, PbTx-6 does not appear to behave in this typical fashion. Our attempts to subject it to nucleophilic ring opening have not been successful (results not shown). This epoxide appears to be quite stable. Inspection of the three-dimensional models of the molecule reveals that the face of the epoxide opposite the oxygen (α) is sterically hindered and inaccessible to nucleophiles. *In vivo,* PbTx-6 might be sufficiently stable to allow it to bind to the AhR, induce CYP1A1, and produce the increased EROD activity that we have observed.

Our results show that PbTx-6 acts as an Ah receptor agonist, not an antagonist. PbTx-6 competes with TCDD for binding to the AhR. This behavior is characteristics of both agonists and antagonists. The fact that PbTx-6 also caused complex formation with the DRE

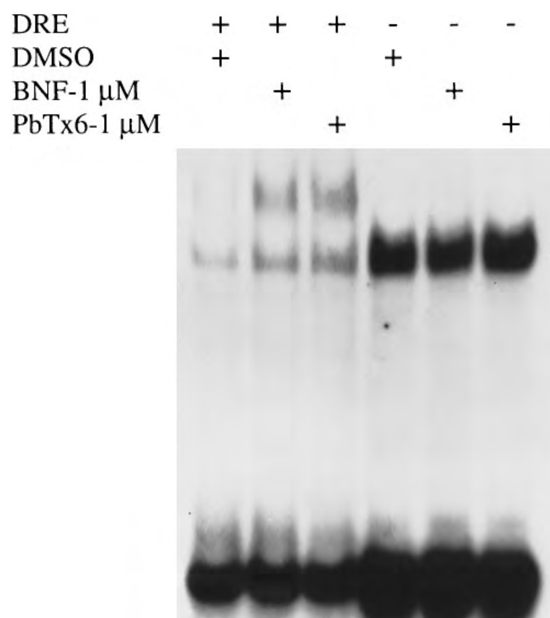

FIG. 7. Formation of ligand–AhR–DRE complex using wild-type and mutant DRE. Lanes 1, 2, and 3, wild-type DRE. Lanes 4, 5, and 6, mutant DRE (single base pair substitution in core DRE sequence). Nonspecific binding in lanes 4, 5, and 6 appears greater due to the higher specific activity of mutant DRE.

pected to induce P4501A1 gene expression. Taken together with the receptor binding studies, these data strongly suggest that PbTx-6 is responsible for the induction of P4501A1. It is possible, however, that one of the untested forms of PbTx might be involved in the observed response. PbTxs have two structural backbones, PbTx A and B, that contain minor variances in the carbon ring structure. PbTx-2, -3, and -6 are variants of the A backbone. It is possible that other variants of the PbTx A or either of the two forms of PbTx B could account for our findings. However, it should be noted that all forms of PbTx are nonplanar and nonaromatic.

PbTx-6 is roughly fivefold less toxic than PbTx-2 or -3 to mosquito fish (*Gambusia affinis*); LC_{50} is 77 nM for PbTx-6 and 14–15 nM for PbTx-2 and -3. Furthermore, PbTx-2 has a roughly sixfold lower K_d at site 5 of the VSSC than PbTx-6 (2). Although the binding affinity is lower for the epoxide, its K_d is still significant (43 nM), and this molecule is considered quite toxic. At this point, it is not known if PbTx-6 is a human or animal metabolite. An *in vivo* study has identified five to six metabolites of PbTx-3 in toadfish (20). Incubation of [^{14}C]PbTx-2 with BNF-stimulated rainbow trout hepatic microsomes and an NADPH regenerating system

suggests it acts as an agonist, although the effect of PbTx-6 on gene expression remains to be confirmed. However, almost all chemicals we have analyzed that produce an inducible gel shift also induce gene expression (M. Denison, unpublished observations). BNF acted in a similar fashion and is a well-recognized AhR agonist. In contrast, its isomer, α-naphthoflavone (ANF), has antagonist activity (15, 22, 23) and competes with TCDD and BNF for AhR binding but with significantly lower affinity. At relatively low concentrations (<10 μM), ANF competes with TCDD and BNF to inhibit their ability to induce gel shifts and gene expression. At concentrations greater than 10 μM, however, ANF can act as a partial agonist. BNF, on the other hand, binds to the Ah receptor in a competitive manner and induces complex formation in a concentration-dependent fashion, as indicated by the presence of a retarded band that has the same mobility as TCDD–AhR–DRE. Taken together, findings from this and previous work show that PbTx-6 acts similarly to BNF as an AhR agonist.

One of the interesting aspects of these results is the demonstration that a nonplanar, nonaromatic molecule has the ability to bind to the AhR and induce a classical P4501A1 response. All of the molecules that are known ligands of the AhR contain aromatic rings and many exhibit planar conformation. Synthetic toxicants, such as TCDD, polycyclic aromatic hydrocarbons, heterocyclic amines, polychlorinated dibenzofurans, and certain members of the polychlorinated biphenyl family, all contain aromatic rings. Natural products such as the phytochemicals harmane and tropolone, derived from wood pulp, and ultraviolet light photoproducts of the amino acid tryptophan also contain aromatic rings (11, 10). Regardless of the origin of these inducers, all share the common feature of containing at least one aromatic ring and induce P4501A1.

The eight-membered H ring in brevetoxins is not planar nor is it aromatic. PbTx-2 and -3 may adopt one of two conformations, the boat chair or crown, with the boat chair favored by 4.5 kcal/mol (2). Thus, the relative population, according to the Boltzman distribution, is 2000:1 boat chair vs crown. The epoxidation of the H ring double bond essentially fixes the ring in the boat chair conformation (no other conformations were identified within a 10 kcal/mol energetic window). Consequently, the epoxidation of PbTx-2 to form PbTx-6 does not change the overall shape of the brevetoxin backbone. Thus, for brevetoxins, the shape of the molecule is unlikely to be a major factor contributing to binding affinity for the AhR.

Initially, structural features that were thought to be important factors defining AhR ligands were their planarity and aromaticity (24). Additional factors such as lipophilicity, electron affinities, and entropies of the molecule have been shown to characterize AhR ligands (25, 26). For example, PCBs with high electron affinity, but low lipophilicity and entropy, are more toxic than those with the reverse characteristics (26). With regard to the brevetoxins, PbTx-6 is less lipophilic than PbTx-2 or -3. The relative retention time of PbTx-6 on C_{18} reverse-phase HPLC using a 85% methanol mobile phase is about one-half that of the other congeners of brevetoxin. Thus, lower lipophilicity may be an important factor allowing PbTx-6 to act as an AhR ligand while other forms of PbTx apparently do not share this same characteristic.

In conclusion, this study suggests that PbTx-6 is responsible for the induction of EROD activity observed in previous work. The toxin is a ligand of the AhR and induces DRE complex formation. As one of the so-called "orphan receptors," an endogenous ligand has not been identified that binds to the AhR. Although PbTx is not an endogenous ligand, the fact that PbTx-6 binds to the receptor suggests two potentially useful observations. First, it contributes a better understanding of the characteristics of AhR ligands. Second, it is potentially another piece in the puzzle regarding the evolution of the AhR. Phylogenetically, the AhR is first observed in jawed vertebrate fish (27). It has not been identified in animals lower on the evolutionary scale than these fish. In the primordial oceanic environment, one that was full of plant life including primitive plants such as algae, it may have been that the AhR played a role in enabling fish to metabolize natural toxins. It is possible that an ability to detoxify marine algal toxins played a role in survival of fishes. The ability of these toxins to bind to the AhR and begin the process of biotransformation and detoxification could have been important in the success of fish in this environment. Additional research with other forms of brevetoxin and other marine toxins, such as saxitoxin and okadaic acid, will help shed additional light on this question.

ACKNOWLEDGMENTS

Support for this work came from the NIEHS Marine and Freshwater Biomedical Sciences Center, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami (ES05705), NMFS Saltonstall-Kennedy funds (NA 37FD0078), NIEHS (ES07685), and Superfund Basic Research Grant (ES04699).

REFERENCES

1. Baden, D. G. (1989) *FASEB J.* **3,** 1807–1817.
2. Rein, K. S., Lynn, B., Gawley, R. E., and Baden, D. G. (1994) *J. Org. Chem.* **59,** 2107–2113.
3. Rein, K. S., Baden, D. B., and Gawley, R. E. (1994) *J. Org. Chem.* **59,** 2101–2106.
4. Baden, D. G., Fleming, L. A., and Bean, J. A. (1995) *in* Handbook of Clinical Neurology: Intoxification of the Nervous System, Pt. II, (Delwolff, F. A., Ed.), pp. 141–175, Elsevier, Amsterdam.

5. Washburn, B. S., Vines, C. A., Baden, D. G., Hinton, D. E., and Walsh, P. J. (1996) *Aquat. Toxicol.* **35,** 1–10.
6. Washburn, B. S., Baden, D. B., Gassman, N. J., and Walsh, P. J. (1994) *Toxicon* **32,** 799–805.
7. Landers, J. P., and Bunce, N. J. (1991) *Biochem. J.* **276,** 273–287.
8. Hankinson, O. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 307–340.
9. Bjeldanes, L. F., Kim, J.-Y., Grose, K. R., Bartholomew, J. C., and Bradfield, C. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9543–9547.
10. Helferich, W. G., and Denison, M. S. (1991) *Mol. Pharmacol.* **40,** 674–678.
11. Kiparrisis, Y., and Metcalfe, C. D. (1994) *Society of Environmental Toxicology and Chemistry.* Abstracts, 15th Annual Meeting, p. 136.
12. Giesy, J. P., Ludwig, J. P., and Tillitt, D. E. (1994) *in* Dioxins and Health (Schecter, A., Ed.), pp. 249–307, Plenum Press, New York.
13. Denison, M. S., Vella, L. M., and Okey, A. B. (1986) *J. Biol. Chem.* **261,** 10189–10195.
14. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
15. Gasiewicz, T. A., and Neal, R. A. (1982) *Anal. Biochem.* **124,** 1–11.
16. Bank, P. A., Yao, E. F., Phelps, C. L., Harper, P. A., and Denison, M. S. (1992) *Eur. J. Pharmacol. Environ. Toxicol. Pharmacol.* **228,** 85–94.
17. Okey, A. B., Bondy, G. P., Mason, M. E., Kahl, G. F., Eisen, H. J., Guenthner, T. M., and Nebert, D. W. (1979) *J. Biol. Chem.* **254,** 11636–11648.
18. Okey, A. B., and Vella, L. M. (1982) *Eur. J. Biochem.* **127,** 39–47.
19. Poland, A., and Glover, E. (1976) *J. Biol. Chem.* **251,** 4936–4946.
20. Kennedy, C. X., and Walsh, P. J. (1992) *Aquat. Toxicol.* **22,** 2–14.
21. Manahan, S. E. (1991) Toxicological Chemistry, pp. 70–76, Lewis, Chelsea, MI.
22. Gasiewicz, T. A., and Rucci, G. (1991) *Mol. Pharmacol.* **40,** 607–612.
23. Santostefano, M., Merchant, M., Arellano, L., Morrison, V., Denison, M. S., and Safe, S. (1993) *Mol. Pharmacol.* **43,** 200–206.
24. Safe, S., Bandiera, S., Sawyer, T., Bozena, Z., Mason, G., Romkes, M., Denomme, M. A., and Fujita, T. (1985) *Environ. Health Perspect.* **61,** 21–33.
25. Kafafi, S. A., Said, H. K., Mahmoud, M. I., and Afeefy, H. Y. (1992) *Carcinogenesis* **13,** 1599–1605.
26. Kafafi, S. A., Afeefy, H. Y., Ali, A. H., Said, H. K., Abd-Elazem, I. S., and Kafafi, A. G. (1993) *Carcinogenesis* **14,** 2063–2071.
27. Hahn, M. E., Poland, A., Glover, E., and Stegeman, J. J. (1994) *Arch. Biochem. Biophys.* **310,** 218–228.