Interactive Effects of Environmentally Relevant Polychlorinated Biphenyls and Dioxins on [3H]Phorbol Ester Binding in Rat Cerebellar Granule Cells

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Polychlorinated biphenyls (PCBs) are persistent contaminants that exist as complex mixtures in the environment. One problem faced by risk assessors is that the possible interactive effects of specific PCB congeners and related chemicals found in environmental and biological samples have not been systematically investigated. Some PCBs perturb Ca²⁺ homeostasis and cause protein kinase C (PKC) translocation in neuronal cell cultures and in brain homogenate preparations at concentrations where no cytotoxicity is observed, and these systems are necessary for the growth and normal functioning of neurons. The changes in second messenger systems appear to be associated with the extent of noncoplanarity of the PCB molecule. We studied the interactive effects of selected PCB congeners, a PCB metabolite, and a dioxin on PKC translocation, as determined by [3H]phorbol ester binding in cerebellar granule cells. The binary combinations included coplanar and noncoplanar PCB congeners or PCB congeners with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)/PCB metabolite. In addition, we tested the interactive effects of several PCB congeners (three or more) found in environmental samples such as human milk and blood, contaminated fish, and brain samples from PCB-treated animals. The results indicated that 1) the coplanar congener, 3,3',4,4',5'-tetrachlorobiphenyl (TeCB) did not alter the in vitro activity of the noncoplanar (2,2',5,5'-TeCB) or coplanar (4,4'-dichlorobiphenyl (DCB) congeners; 2) binary mixtures of active PCB congeners (2,2',4,4'-TeCB and 2,2'-DCB; 2,2'-DCB and 3,5-DCB; 2,2',3,5'-6,6-PCB and 2,2',4,4',5,5-PCB) interact in a dose-additive manner; 3) TCDD did not alter the activity of either coplanar (3,3',4,4'-TeCB) or noncoplanar (2,2',5,5'-TeCB) congeners; 4) the interaction between the parent PCB congener and hydroxy metabolite of PCB is additive; 5) PCB congener mixtures at the ratios found in environmental samples are biologically active; and 6) there was no indication of synergism in any of the combinations studied. These results suggest the biological significance of binary mixtures of PCB congeners fit a dose-additive model, indicating that there is a specific site of action for these PCB congeners which is independent of the aryl hydrocarbon receptor. Environmental mixtures contain mostly noncoplanar PCB congeners, and because they appear to be biologically active, the potential human health risk by this group of chemicals should be considered in the risk assessment of PCBs. Key words: binary combinations, cerebellar granule cells, environmental mixtures, interactive effects, neurotoxicity, [3H]phorbol ester binding, polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins. Environment Health Perspect 106:479–486 (1998). [Online 8 July 1998] http://ehpnet1.niehs.nih.gov/docs/1998/106p479-486/kodavantiabstract.html

Polyhalogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins (Fig. 1) (1–3) are persistent environmental pollutants and bioaccumulate throughout the food chain, thus posing a potential health risk to humans and animals (4,5). Neurotoxicity has been reported following environmental and experimental exposure to PCBs (6–9). These effects include memory deficits and intellectual dysfunction in children born to mothers who ate PCB-contaminated food (10–14). In utero exposure to PCBs in animals causes alterations in locomotor activity (15,16) and affects cognitive function (7,17). The mechanism(s) by which PCBs cause these neurological effects remains to be elucidated.

Studies from our laboratory found that the ortho-substituted PCB congener, 2,2'-dichlorobiphenyl (DCB; IUPAC no. 4) altered intracellular Ca²⁺ homeostasis by inhibiting Ca²⁺-buffering systems and caused protein kinase C (PKC) translocation at concentrations where no cytotoxicity was observed (18,19). These second messengers have critical roles in the normal functioning of brain. The non-ortho-substituted PCB congener, 3,3',4,4',5'-pentachlorobiphenyl (PeCB; IUPAC no. 126), however, was not effective in inhibiting Ca²⁺-buffering systems, had little effect on Ca²⁺ homeostasis, did not cause PKC translocation, and was not cytotoxic (18,19). Further studies (20–22) indicated that the biological activity of most PCB congeners was associated with chlorination patterns that favored noncoplanarity, whereas those with chlorination patterns that favored coplanarity were less active. Recent in vivo studies from our laboratory indicated that Ca²⁺ buffering and PKC were altered in cerebellum of rats dosed with a commercial PCB mixture, Aroclor 1254 (23), and concentrations as high as 15 ppm (equivalent to 40–50 µM based on the average molecular weight of 326.4 for Aroclor 1254) could be accumulated in brain (24). A significant perturbation in Ca²⁺ homeostasis and PKC can trigger processes that could alter the functioning and growth of the neurons (25) and ultimately could lead to cytotoxicity (26). Hence, it is possible that PCB-induced alterations in Ca²⁺ homeostasis and PKC may form a biochemical basis for the observed neurological effects.

Human exposure to PCBs occurs mainly via oral ingestion of contaminated dairy products, meat, and fatty fish, all of which contain a mixture of noncoplanar and coplanar congeners (5,27). The degree to which the constituents of these mixtures may interact by synergism, additivity, or antagonism of effects has not been extensively studied (28–33). Most research has focused on commercial mixtures or a few congeners. The present study was undertaken to understand the interactive effects of several PCB congeners and dioxins that have different degrees of potency on PKC translocation in neuronal preparations. In the neuron, PKC activation/translocation could be due to increased intracellular free Ca²⁺, formation of diacylglycerol, and increased levels of free fatty acids such as arachidonic acid and lysophospholipids (34). Sustained activation of PKC has been reported to be involved in

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the pathogenesis of neuronal injury (35-37). In the present study, [3H]phorbol ester binding was measured to indicate PKC translocation in the presence of various combinations of PCB congeners, a hydroxy-PCB metabolite, or a dioxin.

Materials and Methods

Chemicals. Radiolabeled 4-β-[3H]phorbol 12,13-dibutyrate (PDBu; 20 Ci/mmole; purity 99%) was purchased from Dupont New England Nuclear Corporation (Boston, MA). Chemicals used in the assays and in cell culture were obtained from commercial sources. PCB congeners, hydroxy-PCB, and 2,3,7,8-tetrachlorodibenzop-p-dioxin (TCDD) (purity >99%) were purchased either from Ultra Scientific (North Kingstown, RI) or from Accu-Standard (New Haven, CT).

Preparation test solutions. Stock solutions of test compounds were prepared by dissolving them in dimethyl sulfoxide (DMSO). We added a 2-μl aliquot of stock solution (different concentrations) to the buffer to yield the desired final concentrations. DMSO (2 μl/ml) had no significant effect on [3H]PDBu binding in cerebellar granule cell cultures.

Preparation of environmental mixtures. We generated environmental mixtures to contain the congeners that represented the major constituents of representative tissue samples (Table 1). For example, of the congeners found in human milk (38), mixture 1 in the present study contained PCB 138 (2,2′,3,4,4′,5,5′-hexachlorobiphenyl; 35% of the total), PCB 153 (2,2′,4,4′,5,5′-hexachlorobiphenyl; 43% of the total), and PCB 180 (2,2′,3,4,4′,5,5′-heptachlorobiphenyl; 21% of the total); no other constituent accounted for more than 1.5% of the total. Mixture 1 was generated by mixing PCB nos. 138, 153, and 180 at ratios of 35:43:21, respectively, to achieve the desired final concentrations.

Animals. Timed pregnant female (gestational day 16) Long-Evans hooded rats were obtained from Charles River Laboratory (Raleigh, NC) and housed individually in American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved animal facilities. Food and water were provided ad libitum. Temperature was maintained at 21 ± 2°C and relative humidity at 50 ± 10% with a 12-hr light/dark cycle (0700-1900 hr).

Cerebellar granule cell culture. We isolated granule cells from rat (6- to 8-day-old pups) cerebella by the enzymatic disruption of cells as outlined by Gallo et al. (39) with modifications (18). The final cell suspension contained 1.0 × 10^6 cells/ml, and 1.5 ml cell suspension was added into each well of 12-well culture plates, precoated with poly-L-lysine. These culture plates were maintained in an incubator at 37°C under 5% CO₂/95% filtered room air. Cytosine arabinoside (5 μM) was added after 24–48 hr of plating to prevent the growth of non-neuronal cells. We routinely examined the cultures by immunocytochemistry for neuron-specific enolase (neurons) and glial fibrillary acidic protein (glial). The estimated purity of neurons always exceeded 95%.

[3H]Phorbol ester binding in cerebellar granule cells. Cerebellar granule cells grown on 12-well culture plates (Costar no. 3512, optically clear, virgin polystyrene and radiation sterilized, Cambridge, Massachusetts) were tested at 7 days in culture for [3H]phorbol ester binding according to the method outlined by Vaccarino et al. (40). Briefly, the monolayers were washed with Locke’s buffer (NaCl, 154 mM; KCl, 5.6 mM; NaHCO₃, 3.6 mM; CaCl₂, 2.3 mM; D-glucose, 5.6 mM; HEPES, 5 mM; pH 7.4) containing 0.1% fatty acid-free bovine serum albumin.

![Figure 1. Chemical core structures of polychlorinated biphenyls and dioxins. The letters (o, l), and (p) indicate ortho, meta, and para positions, respectively. The numbers indicate chlorine substitutions.](image1)

![Figure 2. Interactive effects of ortho-substituted active [2,2′,5,5′-tetrachlorobiphenyl (TeCB)] and non-ortho-substituted inactive (3,3′,4,4′-TeCB) congeners on [3H]phorbol ester binding in cerebellar granule cells (see Materials and Methods). [3H]PDBu, [3H]phorbol 12,13-dibutyrate. The plus and minus signs indicate the presence or absence, respectively, of the PCB congener. The binding was represented as percentage of control (538 ± 24 pmol/mg protein/15 min; n = 8). Values are means ± standard errors of four preparations, assayed in triplicate.](image2)

| Table 1. The ratios of selected PCB congeners in each of the environmental mixtures* |
|-----------------------------------------|----------------|---------|---------|---------|---------|---------|---------|---------|---------|
| Mixture no./Reference                  | 28             | 52      | 74      | 82      | 85      | 95      | 99      | 101     | 105     |
| Mixture 1, human milk (38)             |                |         |         |         |         |         |         |         |         |
| Mixture 2, human milk (27)             | 8              |         |         |         |         |         |         |         |         |
| Mixture 3, Wisconsin fish (61)         | 8              |         |         |         |         |         |         |         |         |
| Mixture 4, human blood (62)            | 4              |         |         |         |         |         |         |         |         |
| Mixture 5, rat brain (58)              |                |         |         |         |         |         |         |         |         |
| Abbreviations: IUPAC, International Union of Pure and Applied Chemistry; PCB, polychlorinated biphenyl. |
| *The selected PCB congeners represent the major constituents of tissue samples and were mixed at these ratios (see Materials and Methods for a more detailed explanation). |
After washing, the cells were incubated in Locke's buffer containing 2 nM \(^{3}H\)PDBu (0.04 nCi/ml) for 15 min at room temperature with the test chemicals (10–100 nM). Concentrations ≥ 50 nM, for most of the PCB congeners, were not completely soluble in the assay buffer. An equal amount of DMSO was added to controls. After incubation, the medium was aspirated, and cells were washed three times with Locke's buffer and suspended with 1 ml of 0.1 M NaOH. An aliquot of this sample (0.7 ml) was added to 9 ml Ultima Gold (Packard, Meriden, CT), and the radioactivity was determined using scintillation spectroscopy. A small aliquot was used for protein determination (41). We determined nonspecific binding in the presence of 1.6 nM phorbol myristate acetate, which was always < 20% and subtracted from all the values. The unit of \(^{3}H\)PDBu binding was femtomoles per milligram protein per 15 min.

**Statistics.** We analyzed the data by a two-way analysis of variance (ANOVA) using the raw data (fmol/mg protein/15 min) and also by using the percentage of control. In both cases, the statistical information is similar. We chose to represent the data in percentage of control form for the sake of clarity. The factors used in two-way ANOVA were the PCB concentrations of each of two congeners. Comparisons between control and dosed groups were made using Dunnett's test (42). The accepted level of significance was p < 0.05.

**Results**

PKC translocation was determined by measuring \(^{3}H\)PDBu binding in rat cerebellar granule cells. The interactive effects of PCBs and dioxins in binary combinations were studied on \(^{3}H\)PDBu binding in cerebellar granule cell cultures and are presented in Figures 2–10. The selected combinations include a mixture of coplanar PCBs, noncoplanar PCBs, TCDD, and a hydroxy metabolite of PCB.

**Interactive effects between coplanar and noncoplanar PCBs on \(^{3}H\)PDBu binding.**

Figure 2 shows the combined effects of an active noncoplanar \(2,2',5,5'\)-tetrachlorobiphenyl (TeCB) and an inactive coplanar \(3,3',4,4'\)-TeCB) PCB congener. The ANOVA indicated no significant interaction between two congeners. \(3,3',4,4'\)-TeCB (10–100 nM) had no significant effect on \(^{3}H\)PDBu binding, nor did it affect the response of 30 nM \(2,2',5,5'\)-TeCB. \(2,2',5,5'\)-TeCB significantly increased \(^{3}H\)PDBu binding from 10–100 nM, an effect that was not modified by the presence of 50 nM \(3,3',4,4'\)-TeCB. A similar result was obtained with the combination of an active noncoplanar \(2,2',4,6\)-TeCB

![Interactive effects of ortho-substituted active [2,2',4,6-tetrachlorobiphenyl (TeCB)] and ortho-substituted inactive (2,2',6,6'-TeCB) congeners on [H]phorbol ester binding in cerebellar granule cells (see Materials and Methods). [H]PDBu, [H]phorbol 12,13-dibutyrate. The plus and minus signs indicate the presence or absence, respectively, of the PCB congener. The binding was represented as percentage of control (325 ± 19 fmol/mg protein/15 min; n = 9). Values are means ± standard errors of four to five preparations, assayed in triplicate.](image1)

![Interactive effects of [2,2'-dichlorobiphenyl (DCB)] and non-ortho-substituted active (3,5-DCB) congeners on [H]phorbol ester binding in cerebellar granule cells (see Materials and Methods). [H]PDBu, [H]phorbol 12,13-dibutyrate. The plus and minus signs indicate the presence or absence, respectively, of the PCB congener. The binding was represented as percentage of control (369 ± 27 fmol/mg protein/15 min; n = 9). Values are means ± standard errors of four to five preparations, assayed in triplicate.](image2)

![Interactive effects of two ortho-substituted active congeners [2,2',4,4'-tetrachlorobiphenyl (TeCB) and 2,2'-dichlorobiphenyl (DCB)] on [H]phorbol ester binding in cerebellar granule cells (see Materials and Methods). [H]PDBu, [H]phorbol 12,13-dibutyrate. The plus and minus signs indicate the presence or absence, respectively, of the PCB congener. The binding was represented as percentage of control (374 ± 16 fmol/mg protein/15 min; n = 12). Values are means ± standard errors of four preparations, assayed in triplicate.](image3)
and an inactive noncoplanar (2,2',6,6'-TeCB) congener (Fig. 3). 2,2',6,6'-TeCB(10–100 μM) had no effect on [3H]PDBu binding, and there was no interaction with 30 μM 2,2',4,4'-TeCB. Figure 3 also shows that 2,2',4,4'-TeCB produced a concentration-dependent increase in [3H]PDBu binding and that this response was not affected by 50 μM 2,2',6,6'-TeCB.

Figure 4 shows the combined effects of an active noncoplanar [2,2'-dichlorobiphenyl (DCB)] and a moderately active coplanar (3,5-DCB) congener; the results indicated no interaction between these two congeners. 2,2'-DCB (10–100 μM) produced a concentration-dependent increase in [3H]PDBu binding. At a concentration of 50 μM, 3,5-DCB increased [3H]PDBu binding by 40% and did not interact significantly with 2,2'-DCB. 3,5-DCB also produced a concentration-dependent increase in [3H]PDBu binding; the maximal effect at 100 μM was only 169% of control. There was no significant interaction with the presence of 50 μM 2,2'-DCB, which also produced 72% increase in [3H]PDBu binding in the absence of 3,5-DCB. Figure 5 shows the results from the experiment with two active noncoplanar (2,2',4,4'-TeCB and 2,2',6,6'-TeCB) congeners. 2,2'-DCB produced a concentration-dependent increase in [3H]PDBu binding; the maximal effect at 100 μM was >250% of control. In the presence of 50 μM 2,2',4,4'-TeCB, ANOVA indicated no significant interaction (p = 0.235). 2,2',4,4'-TeCB also increased [3H]PDBu binding in a concentration-dependent manner, and this response was not affected by the presence of 30 μM 2,2',6,6'-TeCB (Fig. 5).

Figure 6 shows the interactions of a very active noncoplanar congener (2,2',3,5',6-PeCB) with that of a slightly active congener (2,2',4,4',5-PeCB). The ANOVA indicated a significant interaction (F,4,50 = 5.01, p=0.0033). A post hoc test showed that 2,2',3,5',6-PeCB increased [3H]PDBu binding in a concentration-dependent manner with maximal effect >250% of control at 100 μM, and this effect was reduced significantly in the presence of 50 μM 2,2',4,4',5-PeCB, 2,2',4,4',5-PeCB alone increased [3H]PDBu binding in a concentration-dependent manner (Fig. 6). There was a significant interaction with the presence of 2,2',3,5',6-PeCB (F,4,50 = 3.73, p=0.0099), which alone produced 94% increase in [3H]PDBu binding in the absence of 2,2',4,4',5-PeCB.

Figure 7 shows the lack of any interactive effects of the inactive noncoplanar (2,2',6,6'-TeCB) congener with an inactive coplanar (3,3',4,4'-TeCB) congener. Figure 7 also shows the lack of any interaction between two inactive coplanar congeners (3,3',4,4'-TeCB and 4,4'-DCB).

**Interactive effects between PCBs and TCDD on [3H]PDBu binding.** The combined effects of 100 nM TCDD with an active noncoplanar (2,2',5,5'-TeCB) and an inactive coplanar (3,3',4,4'-TeCB) congener are shown in Figure 8. TCDD alone at concentrations up to 200 nM had no significant effect on [3H]PDBu binding (data not shown). TCDD at a concentration of 100 nM did not affect the concentration-dependent increases produced by 2,2',5,5'-TeCB or the lack of activity of 3,3',4,4'-TeCB.

**Interactive effects between a parent PCB and a hydroxy PCB metabolite on [3H]PDBu binding.** There is growing concern about the contribution of PCB metabolites to the biological effects of PCBs because hydroxy metabolites have been detected in mammalian tissue and blood samples (49). To study the interaction between parent compound and metabolites, we selected a noncoplanar PCB (2,4,4',5-TeCB) and a noncoplanar hydroxy PCB (2,2',5'-trichloro-4-biphenylo1, 2,4,4',5-TeCB (10–100 μM) produced a concentration-dependent increase in [3H]PDBu binding (Fig. 9), which did not interact significantly with the hydroxy metabolite. 2,2',5'-Trichloro-4-biphenylo1 increased [3H]PDBu binding in a concentration-dependent manner (44), and an approximately 20% increase was observed at 50 μM (Fig. 9).

**Interactive effects of environmental mixtures on [3H]PDBu binding.** Biota in the environment (including the human population) are exposed to mixtures of PCB
congeners, and all environmental samples contain several of these congeners. In the present study, mixtures selected for testing included the ones found in human milk (mixtures 1 and 2), contaminated lake fish (mixture 3), blood of general population (mixture 4), and in brains of pups born to rats exposed to commercial PCB mixtures (mixture 5). Artificial mixtures were generated to reflect the ratio of the major constituent PCB congeners in these environmental mixtures (Table 1).

Figure 10 shows the effects of the selected environmental mixtures on [3H]PDBu binding in rat cerebellar granule cells. All the mixtures increased [3H]PDBu binding in a concentration-dependent manner (Fig. 10). Post hoc analysis indicated that mixtures 1, 4, and 5 were significantly different from control starting at 30 μM, whereas mixtures 2 and 3 were significantly different from control starting at 50 μM (Fig. 10). Of all the selected mixtures, mixture 3 was the most active.

To understand the interactions between PCB congeners in these mixtures, the data were fit into a previously established "logistic second-order response surface model," which is based on dose-addition assumption [for details, see Svendsgaard et al. (45)]. The observed and predicted E50 values (the concentration that increases the control activity by 50%) along with their 95% confidence limits (CL) are presented in Table 2. The observed E50 values for these environmental mixtures are within the 95% CL of the predicted E50 values using the model based on dose-addition assumption, suggesting that PCB congeners in these selected mixtures might exhibit dose additivity. Although the average observed E50 values for mixtures 3 and 4 were lower than the predicted values, 95% CLs of observed and predicted values overlap, and strong synergism does not seem to exist in any of these environmental mixtures.

Discussion

In the environment, humans are exposed to complex mixtures containing many PCB congeners and contaminants such as dioxins and furans. Because of different metabolic fates of the individual congeners and their contaminants, the pattern of composition in biological and environmental samples differ from that of the original commercial mixture (5,46). Human populations are exposed to complex mixtures of PCBs, but most laboratory research has focused on commercial mixtures or individual congeners. The objective of this research was to understand more completely the potential interactions of some of the components in these mixtures.

Until now, studies on the interactive effects of PCBs and related compounds...
have been restricted to nonneuronal systems. For example, there are few reports indicating that the effects of these compounds are additive using end points of toxicokinetics (29,30), liver enzyme induction (47,48), teratogenicity (28), or immunotoxicity (49). An in vivo study by Sargent et al. (50), however, indicated more-than-additive effects of 2,2',5,5'-TeCB and 3,3',4,4'-TeCB on hepatocytes and lymphocytes. On the other hand, the immunosuppressive activity of PCB mixtures studied by Harper et al. (51) was found to be less than additive. These investigators concluded that the TEF (toxic equivalence factor) approach may overestimate the toxicity of these compounds. Jansen et al. (52) reported that 2,2',5,5'-TeCB was estrogenic and 3,3',4,4'-TeCB was antiestrogenic, suggesting these compounds may be antagonistic in combination. Bager et al. (48) reported a greater-than-additive effect of 3,3',4,4'-5-PeCB (PCB 126) and 2,2',4,4',5-5'-HCB (PCB 153) on γ-glutamyltranspeptidase-positive hepatic loci and antagonistic effect of PCB 126 on the induction of pentoxysorin-α-deethylase CYP2B1/B2 activity by PCB 153. The induction of ethoxyxysorin-α-deethylase CYP1A1 activity by these two congeners was approximately additive. Aarts et al. (53) reported that 2,2',5,5'-TeCB and 2,2',4,4',5-5'-HCB antagonize the TCDD- and 3,3',4,4'-TeCB-induced luciferase expression. Walker et al. (54) found that the toxic potency of mixtures of PCBs, furans, and dioxins was additive in causing early life-stage mortality in lake trout. Recently, van Birgelen et al. (32) reported synergistic effects of 2,2',4,4',5,5'-HCB and TCDD on hepatic porphyrin levels in the rat. These reports suggest that there are multiple mechanisms for the effects of PCBs and related compounds. Although there are several reports on the effects of PCB congeners in neural tissue (6,7,15,17,55-57), there are no reports on the potential interactive effects of these congeners in the nervous system.

Our previous structure–activity relationship studies (20,22) used stimulation of [3H]PDBu binding as an indicator of PKC translocation due to altered intracellular Ca2+ homeostasis by PCBs. Both Ca2+ homeostasis and PKC translocation, which are involved in the neurotoxicity of a variety of environmental chemicals (26,37,58), were affected by PCB congeners that have a noncoplanar configuration (18,19,59). Higher concentrations and longer exposures to 2,2'-DCB, a noncoplanar PCB, result in cytotoxicity (18). The present study extends our structure–activity relationship work by examining binary combinations of PCBs, a hydroxy-PCB or TCDD to determine 1) if the active congeners are “dose additive,” indicating that they act through a common site, or “effect additive,” indicating that, although the end point ([3H]PDBu binding) is common, the congeners act through independent sites (60); 2) if the nonactive congeners enter the active site and are ineffective at inducing [3H]PDBu binding or if these congeners simply are unable to enter the active site; and 3) if a dioxin or a hydroxy-PCB alters the response of PCBs. In addition, mixtures were generated to match the ratio of congeners found in environmental and biological samples and studied their effects on [3H]PDBu binding in neuronal cultures.

The inactivity of 2,2',6,6'-TeCB and 3,3',4,4'-TeCB could be due either to their inability to enter the active site or to inability to elicit the response after entering. When these congeners were tested in combination with active congeners, there was no significant interaction. The nonactive coplanar congener (3,3',4,4'-TeCB) also did not alter the activity of either the noncoplanar (2,2',5,5'-TeCB) or active coplanar (3,5-DCB) congeners. The inactive, noncoplanar congener 2,2',6,6'-TeCB also failed to alter the effects of an active PCB congener (2,2',4,6,6'-TeCB). When the dose response of 3,3',4,4'-TeCB was measured with 2,2',6,6'-TeCB, no interaction was seen. The nonactive congener failed to block the effects of the active congeners, suggesting that these nonactive congeners do not bind to the response-inducing site.

A pair of active congeners, 2,2'-DCB with 2,2',4,4'-TeCB (Fig. 5), in which one congener was more active than the other, was tested (maximum stimulations of 163% and 93% for 2,2'-DCB and 2,2',4,4'-TeCB, respectively). If the congeners with different activities acted at different sites to increase [3H]PDBu binding, the results would be additive as in an “effect additive” model. If, on the other hand, the congeners act at the same site, the effects would not be additive, fitting a dose additive model. As shown in Figure 5, the interactions between 2,2'-DCB and 2,2',4,4'-TeCB indicate a less-than-additive effect, but no statistically significant interaction was found. This interaction is not limited due to saturation of effect (end point measured is [3H]PDBu binding) because we have previously reported that [3H]PDBu binding can be increased as high as 3.5-fold (19). Solubility of PCB congeners might play a key role at concentrations greater than 100 μM.

Following these experiments, a second pair of congeners was selected with greater difference in activity between two congeners where an interaction would be more clearly demonstrated. The results of the binary combinations of a very active congener, 2,2',3,5,6-PeCB (maximum stimulation of 150%), and a less active congener, 2,2',4,4',5-PeCB (maximum stimulation of 56%), are shown in Figure 6. This combination resulted in a significant interaction (Fig. 6), where the less active congener, instead of adding to the total [3H]PDBu binding, decreased this effect, indicating that the less active congener interacted at the same site but lowered the total activity, thus resulting in a less-than-additive response. On the other hand, the response of a mixture of 3,5-DCB with and without 2,2'-DCB (which have similar activity) showed simple additive response and no interaction. A similar additive interaction was observed with the parent PCB and the hydroxy metabolite (Fig. 9). Taken together, these data indicate that the increase in [3H]PDBu binding induced by certain PCB congeners and their hydroxy metabolites results from interaction at a specific site and not a generalized nonspecific interaction. In addition, the nonactive congeners did not interfere with the active congeners, indicating that they do not enter the active site.

The results of the present study confirm and extend the hypothesis that the PCB congeners in neuronal system do not act through a dioxinlike mechanism. For instance, TCDD at concentrations up to 200 nM was ineffective in this system (data not shown). In addition, TCDD (100 nM)

### Table 2. The observed and predicted \( E_{50} \) values for the interactive effects of selected environmental mixtures on [3H]phorbol 12,13-dibutyrate binding in rat cerebellar granule cells

| Mixture no. | Observed \( E_{50} \) values (μM)      | Predicted \( E_{50} \) values (μM) |
|------------|--------------------------------------|----------------------------------|
| 1          | Human milk                           | >100                             | 125 (90–203)                      |
| 2          | Human milk                           | >100                             | 184 (130–260)                     |
| 3          | Wisconsin fish                       | 61 (47–82)                       | 82 (70–95)                        |
| 4          | Human blood                          | 88 (70–100)                      | 158 (127–196)                     |
| 5          | Rat brain                            | >100                             | 145 (108–195)                     |

| \( E_{50} \) | Effective concentration that increases the control activity by 50%.
|-------------|------------------------------------------------------------------|
| \( E_{50} \) | Predicted \( E_{50} \) values (mean and 95% confidence limits) were calculated from the regression line fit to the linear portion of the curve.]
| \( E_{50} \) | Indicates that the mixture increased [3H]phorbol ester binding significantly, but the increase was below \( E_{50} \) at 100 μM.
did not interact with an active congener (2,2',5,5'-TeCB) or a nonactive congener (3,3',4,4'-TeCB). These results indicate that PKC translocation in neuronal cultures represented as [3H]PDBu binding is independent of the aryl hydrocarbon receptor mode of action and that the current TEF approach does not include effects reported in the present study. Therefore, application of the TEF approach based on TCDD-like activity for risk assessment of PCBs would be inappropriate and must be used with caution.

Complex mixtures were generated to more nearly mimic human exposure. These mixtures are representative of the ratios of the main constituents of the PCB load, but no attempt was made to match a total dose. Mixtures 1 and 2 were generated to represent the ratios of PCB congeners found in human milk (27,38) and would be a measure of infant exposure. Mixture 3 represents the PCB congener ratio found in Wisconsin fish (61), which represents adult and infant exposure. Mixture 4 is the congener’s mix from human blood representing maternal exposure (62). Mixture 5 represents the ratio of PCB congeners detected in the brains of rats at postnatal day 21 that had been exposed to PCBs from conception to weaning via feeding dams with adulterated chow containing the commercial mixture Acorol 1254 (55). All these mixtures increased [3H]PDBu binding. Mixture 3 was the most active among the mixtures tested, followed by mixtures 4 and 5. To understand the interactions between congeners in these mixtures, the data were fit in a previously established empirical model based on dose additive assumption. The observed $E_{50}$ values are close to the predicted values obtained from the empirical model, suggesting that the interactions between PCB congeners in these mixtures may follow dose additivity.

In summary, the present data suggest that the biological effects of some PCB congeners are dose additive and that the coplanar congeners do not alter the activity of noncoplanar congeners, indicating a common mechanism for all noncoplanar congeners distinct from that of coplanar congeners. Some interactions showed inhibition of a very active congener by a moderately active congener, indicating that the congeners act at the same site. The dose-additive model, which implies a single site of action, seems to be a more appropriate model for predicting the results. There was no indication of synergism in any of the combinations studied. Because samples from human and environmental mixtures of PCB congeners were biologically active, careful attention must be paid for the role of coplanar congeners in PCB exposure assessment process.
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