Neurochemical Effects of Non-coplanar PCBs: *In vitro* and *In vivo* correlations on Calcium Homeostasis and Protein Kinase C

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Introduction

It is known that some PCBs and other halogenated hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) produce their biological effects through a receptor-mediated response by binding to the cytosolic aryl hydrocarbon (Ah) receptor followed by induction of a number of genes (1,2). It has been proposed that substitutions on the biphenyl ring (lateral substitutions at the *meta*- and *para*- positions) that promote coplanarity are associated with TCDD-like toxic effects of certain PCB congeners (3). PCB congeners that are non-*ortho*-substituted appear to have TCDD-like activity. Ah receptor involvement may be true with reproductive, immunologic, teratogenic, and carcinogenic effects of PCBs (1, 4). However, recent studies indicate that neurotoxic effects of PCBs are not mediated through the Ah receptor (5, 6).

There is now both epidemiological and experimental evidence that developmental exposure to polychlorinated biphenyls (PCBs) causes cognitive deficits, however, the underlying cellular or molecular mechanism(s) is not known (7-9). We have hypothesized that altered signal transduction/second messenger homeostasis by PCBs may be associated with these effects. The reasons are: 1.The most significant neurotoxic effects of PCBs seen in humans are learning and memory deficits. 2. Laboratory studies indicate that PCBs inhibit LTP (a form of synaptic plasticity) and impair learning/memory. 3. LTP is often described as a physiological model for neuronal development, learning and memory. 4. Second messengers such as calcium and inositol phosphates, and PKC have been shown to modulate LTP and play key roles in neuronal development. We have conducted both *in vitro* and *in vivo* studies to understand the cellular mechanism(s) of PCB-induced neurotoxicity. *In vitro* experiments were conducted using cerebellar granule cell cultures as well as brain homogenate preparations while *in vivo* studies were conducted by the repeated exposure of adult rats to a PCB mixture, Aroclor 1254.

Materials and Methods

In Vitro studies: Long Evans Hooded rats (Adult male; 250-300 g) and pregnant dams (GD17) were obtained from Charles River (Portage.OR). Granule cells from rat cerebellum were isolated from 6-8 day old pups following enzymatic disruption method (10, 11). Cerebellar fractions (synaptosomes, microsomes and mitochondria) were obtained from adult cerebella by sucrose-gradient centrifugation method (11, 12). Alterations in intracellular free Ca^{2+} were examined with fluorescent dyes (Fluo-3AM and Fura-2AM) (13). $^{45}Ca^{2+}$-uptake by mitochondria and microsomes was measured as outlined by Moore et al (14). [^{3}H]-Arachidonic acid release was determined by the method of Lazarewicz et al (15). PKC activity was determined by the method

of Chen et al. (16). Nitric oxide synthase activity was determined by Brecht and Snyder (17). *In vitro* studies were conducted with prototypic *ortho* (2,2'-dichlorobiphenyl; DCB) and non-*ortho* (3,3',4,4',5-pentachlorobiphenyl; PeCB) PCBs.

In Vivo studies: Long Evans Hooded rats (Adult male; 200-250 g) were dosed orally by gavage with Aroclor 1254 (Lot # 6024, AccuStandard, New Haven, CT) in corn oil (2 ml/kg). The selected dosages were 0, 10 or 30 mg/kg/day. The rats were dosed once a day, 5 time a week for 4 weeks. At 24 hrs after the last dosage, rats were sacrificed, brains removed, and dissected into frontal cortex, cerebellum, and striatum for neurochemical and PCB analyses. Ca^{2+}-buffering (14) and PKC activity (16) were determined as described before. Congener-specific analysis of PCBs was performed using high-resolution gas chromatography with electron capture detection (18).

Results and Discussion

In Vitro Studies:

Cytotoxicity: 2,2'-DCB was cytotoxic as indicated by a significant LDH leakage at >100 μM. 3,3',4,4',5-PeCB, on the other hand, was not cytotoxic even at 200 μM (Table 1).

Ca^{2+} homeostasis: Both PCBs increased cerebellar granule cell $[Ca^{2+}]_i$; 2,2'-DCB was more effective than 3,3',4,4',5-PeCB. The increase in $[Ca^{2+}]_i$ was slow, and a steady rise was observed with time. 2,2'-DCB was a potent inhibitor of $^{45}Ca^{2+}$-uptake by mitochondria and microsomes. 3,3',4,4',5-PeCB inhibited Ca^{2+}-sequestration, but the effects were much less than those produced by equivalent concentrations of 2,2'-DCB. Synaptosomal Ca^{2+}-ATPase, involved in Ca^{2+}-extrusion process, was inhibited by 2,2'-DCB, but not by 3,3',4,4',5-PeCB (Table 1).

Inositol phosphates (IP): The disruption of Ca^{2+}-homeostasis may have a significant effect on signal transduction pathways (IP second messengers) regulated or modulated by Ca^{2+}. 2,2'-DCB, but not 3,3',4,4',5-PeCB affected basal IP accumulation in cerebellar granule cells. Concentrations of 2,2'-DCB up to 50 µM increased carbachol (CB)-stimulated IP accumulation. At concentration of 100 µM 2,2'-DCB, CB-stimulated IP accumulation was decreased. 3,3',4,4',5-PeCB, on the other hand, had no effect on CB-stimulated IP accumulation in concentrations up to 100 μM. Further studies indicated that any modulation of CB-stimulated IP accumulation is due to Ca^{2+}-overload, but not due to activation of PKC activity (Table 1).

Arachidonic acid (AA) release: Aroclor 1254 and 2,2'-DCB increased [^{3}H]-AA release in cerebellar granule cells while 4,4-DCB did not. The release caused by PCBs was linear with time of exposure and a significant release was seen as early as 2 min. PLA_2 inhibitor completely blocked the release. Removal of extracellular Ca^{2+} or inhibition of intracellular Ca^{2+} release only partially blocked the [^{3}H]-AA release.

PKC translocation: [^{3}H]-Phorbol ester ([^{3}H]-PDBu) binding was used as an indirect measurement of PKC translocation. 2,2'-DCB increased [^{3}H]-PDBu binding in a concentration-dependent manner in cerebellar granule cells. 3,3',4,4',5-PeCB had no effect on in concentrations up to 100 μM. The effect of 2,2'-DCB was time-dependent, and also dependent on the presence of external Ca^{2+} in the medium. Several pharmacological agents did not prevent, but sphingosine prevented 2,2'-DCB-induced increases in [^{3}H]-PDBu binding (Table 1).

Nitric oxide synthase (NOS): Both cytosolic (nNOS) and membrane (eNOS) forms of NOS were inhibited by 2,2'-DCB, but not by 4,4'-DCB.

These *in vitro* studies clearly demonstrate that second messenger systems, involved in neuronal function and development, are sensitive targets for the *ortho*-substituted PCBs.

In Vivo Studies: Following Aroclor 1254 treatment, body weight gain in the high-dose group was significantly lower than the control and low-dose groups. Ca^{2+} buffering by microsomes was significantly lower in all three brain regions from the 30-mg/kg group. In the same dose group,

TABLE 1

In Vitro Effects of Prototypic *Ortho*-substituted Non-coplanar (2,2'-DCB) and Non-*ortho*-substituted Coplanar (3,3',4,4',5-PeCB) Congeners on Signal Transduction Mechanisms in Neuronal Cultures and Brain Homogenate Preparations.

		Significant effect	
		Ortho-PCB	Non-*ortho* -PCB
Cytotoxicity:	LDH leakage	100-200 μM	Not toxic at 200 μM
Ca^{2+}-homeostasis:	$[Ca^{2+}]$ levels-fluorescent probe	3 μM	25-50 μM
	Ca^{2+}-buffering by mito and micro	5 μM	50-75 μM
	Ca^{2+}-extrusion by Ca^{2+}-ATPase	10 μM	NOE
Inositol phosphates:	Basal PI metabolism	100 μM	NOE
	Carb stimulated PI metabolism	↑ at 30-50μM; ↓ at 100 μM	NOE
Arachidonic acid Release:	Basal Release	10 μM	NOE
	Characterizing DCB-increased Arachidonic acid release	Seen as early as 2 min after exposure Blocked by PLA_2 inhibitor External Ca^{2+} partially necessary $[Ca^{2+}]_i$ release partially necessary	
PKC translocation:	$[^3H]$Phorbol ester binding		
	Without preincubation	30 μM	NOE
	With preincubation	30 μM	NOE
	Characterizing DCB-increased $[^3H]$PDBu binding	External Ca^{2+} necessary Additive with glutamate No effect with verapamil No effect with Tetradotoxin No effect with MK-801, CPP or CNQX Sphingosine blocked PCB effect	
Nitric Oxide Synthase:	Basal activity	10 μM	NOE

NOE = No effect up to 100 μM

mitochondrial Ca^{2+} buffering was affected in cerebellum but not in cortex or striatum. Similarly, total cerebellar PKC activity was decreased significantly while the % of PKC activity associated with the membrane was significantly elevated at 10 and 30 mg/kg. PKC activity was not altered either in cortex or the striatum (Table 2). These results indicate that *in vivo* exposure to a PCB mixture can produce changes in second messenger systems that are similar to those observed after *in vitro* exposure of neuronal cell cultures and brain homogenate preparations.

TABLE 2

In Vivo Effects of PCBs on Signal Transduction Mechanisms in Different Brain Regions.

Parameter	Cerebellum	Frontal Cortex	Striatum
Ca^{2+} buffering:			
Microsomes	Inhibited	Inhibited	Inhibited
Mitochondria	Inhibited	No effect	No effect
Total PKC	Inhibited	No effect	No effect
Membrane PKC (% total)	Increased	No effect	No effect
Total PCBs	13 ppm	15 ppm	0.64 ppm

Total PCBs accumulated in some brain regions were equivalent to 40-50 μM (13-15 ppm) and most of the PCBs accumulated in brain are *ortho*-substituted, non-coplanar congeners (Table 2). At these concentrations, intracellular second messengers were significantly affected in neuronal cultures and brain homogenate preparations. Current research is focusing on the PCB-induced alterations in intracellular second messengers following developmental exposure.

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