Neurochemical effects of PCBs: SAR modeling

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1. Introduction

At present, the risk assessment for neurotoxicity of polychlorinated biphenyls (PCBs) is based on information from PCB mixtures. An USEPA workshop on PCBs held in 1992 indicated that mechanistic studies are needed on a congener-specific basis to better evaluate the risk potential of PCBs. Also, this workshop strongly recommended the structure-activity relationship (SAR) approach linking functional effects with exposure to PCBs. Since PCBs were commercially synthesized as mixtures containing 209 possible congeners, congener-specific data are essential in human health risk evaluation of PCB-contaminated sites.

PCBs gained widespread industrial use because of their physical and chemical properties, and it became evident in mid-1960s that PCBs were widespread environmental contaminants[1]. USEPA banned the usage of PCBs after the two major accidental PCB poisoning incidents ("Yusho" in Japan and "YuCheng" in Taiwan) where more than 1000 people were suffered with chloracne, numbness, weakness in limbs and decreased peripheral nerve conduction velocities[2]. Children exposed *in utero* showed clinically evident developmental delays and cognitive deficits[3]. In laboratory animals exposed during development, PCBs have been reported to alter motor function[4,5] and decrease brain neurotransmitter levels including dopamine[6,7]. Shain et al.[8] have studied the effects of forty three PCB congeners on dopamine levels in PC12 cells and reported that *ortho*-substituted congeners were more potent than *meta*- or *para*-substituted congeners in decreasing dopamine levels. The mechanism by which some PCB congeners affect neurotransmitter function is not known.

One possible mechanism is that some PCB congeners may affect intracellular calcium disposition as well as other second messenger systems. Neurotransmitter uptake and release phenomena are regulated through mono- and di-valent cation gradients across the membrane; Ca^{2+} has a key role in regulating neurotransmitter-mediated responses in the nervous system[9]. The distribution of Ca^{2+} within the cell is complex and involves binding to cell macromolecules and compartmentalization within the subcellular organelles[10]. Normal physiological function of the cell is regulated by changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$), which ranges from 0.1 to 0.3 μM. This low concentration is regulated by energy-requiring transport systems located in plasma membrane, endoplasmic reticulum (ER) and mitochondrion[10]. Also, binding of an agonist to membrane receptors leads to the generation of second messengers such as diacylglycerol (DAG) and inositol trisphosphate

(IP_3) from membrane phospholipid hydrolysis[11]. DAG activates protein kinase C (PKC) which, in turn, catalyzes the phosphorylation of a variety of cellular proteins. On the other hand, IP_3 releases Ca^{2+} from ER and regulates several processes including translocation and activation of PKC[12]. Elevated levels of cytosolic $[Ca^{2+}]_i$ within the cell either through Ca^{2+}-influx or by Ca^{2+}-release from intracellular stores, have been reported to activate several intracellular Ca^{2+}-dependent reactions including spontaneous release of neurotransmitters[13], phosphorylation of proteins[14] and activity of proteases[15]. Besides, perturbations in Ca^{2+}-homeostasis as well as sustained activation of PKC have been reported to be involved in pathogenesis of neuronal injury[16,17].

We have now examined the effects of two PCB congeners on Ca^{2+}-homeostasis, phosphoinositide (PI) hydrolysis as well as PKC translocation in cerebellar granule cells. Cerebellar granule cells have been well characterized and investigated by other laboratories to study chemical-induced changes in Ca^{2+} disposition. The selected PCB congeners represent non-dioxin like (2,2'-DCB; *ortho*-substituted, non-coplanar congener) and dioxin-like (3,3',4,4',5-PeCB; non-*ortho*substituted, coplanar congener) classes of PCBs. Our objective was to characterize the neuroactive potential of these two structurally different PCB congeners, as determined by their ability to alter Ca^{2+}-homeostasis, PI hydrolysis and PKC translocation in rat cerebellum or a cultured cerebellar granule cell model.

2. Methods

Granule cells from rat cerebellum were isolated by the enzymatic disruption of cells as outlined by Gallo et al.[18] with modifications[19]. Cerebellar fractions (synaptosomes, microsomes and mitochondria) were isolated as described earlier[19].

Alterations in $[Ca^{2+}]_i$ were examined with fluorescent dye, Fluo-3/AM[20] using Interactive Laser Cytometer (ACAS 570; Meridian Instruments, Okemos, MI). $^{45}Ca^{2+}$-uptake by mitochondria and microsomes was measured as outlined by Moore et al.[21]. Synaptosomal Ca^{2+}-ATPase was estimated by measuring inorganic phosphate liberated during hydrolysis of ATP[22]. Phosphoinositide hydrolysis was determined by measuring [^{3}H]-inositol phosphates liberated from the incorporated myo-[^{3}H]-inositol into lipids[23]. [^{3}H]Phorbol ester binding was determined by the method of Vaccarino et al.[24]. Lactate dehydrogenase (LDH) leakage was taken as an index of cytotoxicity. LDH activity was determined by the method of Amador et al.[25].

3. Results and Discussion

Ca^{2+}-homeostasis: We have initially compared the effects of two PCB congeners, with different chlorine substitutions (2,2'-DCB and 3,3',4,4',5-PeCB), on cytotoxicity and Ca^{2+}-homeostasis in cerebellar granule cells (6-8 days *in vitro*). 2,2'-DCB was cytotoxic as indicated by a significant increase in LDH leakage at 200 μM after 2 hr of exposure, and at 100 μM after 4 hr exposure. 3,3',4,4',5-PeCB, on the other hand, did not affect LDH leakage even at 200 μM for up to 4 hr. Both PCB congeners increased cerebellar granule cell $[Ca^{2+}]_i$; 2,2'-DCB was more effective than 3,3',4,4',5-PeCB. The increase in $[Ca^{2+}]_i$ produced by both congeners was not transient, but a steady rise was observed with time. 2,2'-DCB was a potent inhibitor of $^{45}Ca^{2+}$-uptake by mitochondria (IC50 = 6.17 $\pm$ 0.53 μM) and microsomes (IC50 = 7.61 $\pm$ 0.35 μM). 3,3',4,4',5-PeCB inhibited Ca^{2+}-sequestration by mitochondria (68% of control) and microsomes (72% of control), but the effects were much less than those produced by equivalent concentrations of 2,2'-DCB. Synaptosomal Ca^{2+}-ATPase was inhibited by 2,2'-DCB, but not by 3,3',4,4',5-PeCB. These results indicate that at concentrations where cytotoxicity was not observed, 2,2'-DCB increased intracellular $[Ca^{2+}]_i$ and inhibited Ca^{2+}-sequestration by intra-cellular organelles, as well as Ca^{2+}-extrusion process in synaptic plasma membrane. Although

3,3',4,4',5-PeCB increased intracellular $[Ca^{2+}]_i$ to some extent, it was not potent in affecting Ca^{2+}-sequestration or Ca^{2+}-extrusion (Figure 1). Hence, 3,3',4,4',5-PeCB-induced increases in $[Ca^{2+}]_i$ levels appear to have been buffered by intracellular organelles. The results of this study suggest that the position and/or number of chlorine substitutions on the biphenyl ring has significant implications for predicting potential effects of PCB congeners in CNS, and perturbations in Ca^{2+}-homeostasis might play a significant role in the effects of PCBs[19].

Inositol phosphates (IP): Further experiments found that neither 2,2'-DCB nor 3,3',4,4',5-PeCB affected basal IP accumulation in cerebellar granule cells. However, at concentrations up to 50 μM, 2,2'-DCB potentiated carbachol (CB)-stimulated IP accumulation, while decreasing it at 100 μM (Figure 1). 3,3',4,4',5-PeCB, on the other hand, had no effect on CB-stimulated IP accumulation in concentrations up to 100 μM[26]. We examined whether direct or indirect activation of PKC may underlie the inhibition of agonist-stimulated IP accumulation. 2,2'-DCB (100 μM) did not alter PKC activity in cytosolic or membrane fractions of cell homogenates. In intact cells, 50 nM phorbol myristate acetate (positive control) inhibited CB-stimulated IP accumulation by 75%, an effect which was blocked completely by the PKC inhibitor, bisindolylmaleimide (2 μM; BIM). However, 2,2'-DCB inhibition of CB-stimulated IP accumulation (75%) was not affected by BIM, suggesting that activation of PKC is not important in 2,2'-DCB effects on IP accumulation. Further studies indicated that Ca^{2+}-overload may be related to inhibition of CB-stimulated IP accumulation where higher concentrations of ionomycin (calcium ionophore; 3 and 10 μM) decreased CB-stimulated IP accumulation as that of 100 μM 2,2'-DCB.

PKC translocation: Subsequent studies were concentrated on the events that could be altered by changes in $[Ca^{2+}]_i$ and IPs. PKC translocation was selected in this respect. PKC is a family of ubiquitous phospholipid-dependent serine/threonine kinases, which play pivotal roles in cellular signal transduction[11]. PKC activation/translocation in the neuron could be due to several factors including a rise in intracellular $[Ca^{2+}]_i$, formation of DAG, and increased levels of free fatty acids such as arachidonic acid and lysophospholipids[27]. PKC has been reported to play a key role in a variety of physiological/toxicological phenomena[17]. [^{3}H]-Phorbol ester ([^{3}H]-PDBu) binding was used to determine PKC translocation.

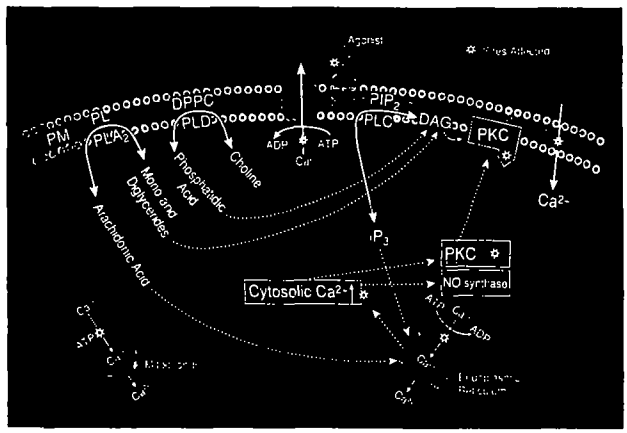
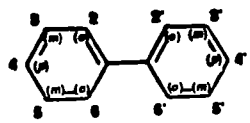


Figure 1. Schematic showing the sites affected by non-dioxin like congener.

2,2'-DCB increased [^{3}H]-PDBu binding in a concentration-dependent manner and a twofold increase was observed at 100 μM in cerebellar granule cells. 3,3',4,4',5-PeCB had no effect on [^{3}H]-PDBu binding in concentrations up to 100 μM. The effect of 2,2'-DCB on [^{3}H]-PDBu binding was time-dependent, and also dependent on the presence of external Ca^{2+} in the medium. To test the hypothesis that 2,2'-DCB increases [^{3}H]-PDBu binding by acting on receptor-activated calcium channels, the effects of 2,2'-DCB were compared with those of L-glutamate. The effects of glutamate (20 μM) and 2,2'-DCB (50 μM) were additive. MK-801, a non-competitive NMDA antagonist, blocked the effects of glutamate, but had no effect on 2,2'-DCB-induced increase in [^{3}H]-PDBu binding. Other pharmacological pretreatments such as incubations with CPP (competitive NMDA antagonist), CNQX (AMPA antagonist), verapamil (Ca^{2+}-channel antagonist) and tetradotoxin (Na^+ channel antagonist) also had no effect on 2,2'-DCB-stimulated [^{3}H]-PDBu binding. These studies indicate that 2,2'-DCB, a putative neuroactive PCB congener causes translocation of PKC in cerebellar granule cells[28] (Figure 1).

These results indicate that non-dioxin-like congener increased Ca^{2+} accumulation and inhibited Ca^{2+} buffering, resulting in increased $[Ca^{2+}]_i$. The increased $[Ca^{2+}]_i$ could cause PKC translocation and cytotoxicity (Figure 1). With dioxin-like congener, there is increased Ca^{2+} accumulation, but little effect was observed on Ca^{2+} buffering resulting in marginal changes in $[Ca^{2+}]_i$ and no change in PKC translocation and the absence of cytotoxicity.

Structure-Activity Relationships (SAR): For SAR study, PCB congeners were selected based on their presence in the environment (soil and water samples), food and in humans. For SAR, We selected two events that were differentially affected by the congeners in the mechanistic studies, including: PKC translocation and Ca^{2+}-buffering.



Polychlorinated Biphenyls

SAR of 3 PCB mixtures, 24 PCB congeners, and 1 dibenzofuran was established on these two parameters. All the PCB mixtures studied increased [^{3}H]-PDBu binding significantly and in a concentration-dependent manner. However, Aroclor 1016 and Aroclor 1254 were more potent when compared to Aroclor 1260. Of the 24 congeners studied, di-*ortho* congeners like 2,2',5,5'-tetrachlorobiphenyl (-TeCB), 2,2',4,6,6'-PeCB, 2,2',4,6'-TeCB, and 2,2'-DCB were the most potent while non-*ortho* congeners like 3,3',4,4'-TeCB and 3,3',4,4',5-PeCB were not effective.

The potential contaminant of PCB mixtures, 1,2,3,7,8-pentachlorodibenzofuran has no effect on [^{3}H]-PDBu binding. The SAR among these congeners revealed: (i) congeners with *ortho*-chlorine substitution such as 2,2'-DCB (E50 = 43 $\pm$ 3 μM) or *ortho*-lateral (*meta, para*) chlorine substitutions such as 2,2',5,5'-TeCB (E50 = 28 $\pm$ 3 μM) and 2,2',4,6-TeCB (E50 = 41 $\pm$ 6 μM) were most potent; (ii) congeners with only *para*-substitution such as 4,4'-DCB or high lateral content in the absence of *ortho*-substitution such as 3,3',4,4',5,5'-hexachlorobiphenyl were not effective and (iii) increased chlorination was not clearly related to the effectiveness of these congeners, although hexa- and hepta-chlorination is less effective when compared to di- and tetra-chlorination. The relative potency of these congeners was given in Table 1. Low lateral substitution, especially without *para*-substitution, or lateral content in the presence of *ortho*-substitution, may be the most important structural requirement for the *in vitro* activity of these PCB congeners in neuronal preparations[28]. The SAR results on Ca^{2+}-buffering are generally consistent with the [^{3}H]-PDBu binding data.

Table 1

Relative potency of PCB mixtures, PCB congeners and a dibenzofuran in increasing [^{3}H]PDBu binding in rat cerebellar granule cells.

Very active (E_{50} = <50 μM)	Moderately active (E_{50} = 50-100 μM)	Slightly active (E_{50} = >100 μM)	Not active (E_{50} = NEO)
PCB mixtures:			
	Aroclor 1016	Aroclor 1260	
	Aroclor 1254		
PCB congeners:			
2,2'-DCB	3,3'-DCB	2,4,4'-TCB	4,4'-DCB
2,2',4,6-TeCB	3,5-DCB	2,3',4,4',5-PeCB	2,2',6,6'-TeCB
2,2',4,6'-TeCB	2,2',6-TCB	2,2',3,3',4,4'-HCB	3,3',4,4'-TeCB
2,2',5,5'-TeCB	2,2'4,4'-TeCB	2,2',3,3',5,5'-HCB	3,3',4,4',5-PeCB
2,2',4,6,6'-PeCB	3,3',5,5'-TeCB	2,2',4,4',5,5'-HCB	3,3',4,4',5,5'-HCB
	2,3,3',4,4'-PeCB	2,3,3',4,4',5-HCB	2,2',3,4,4',5,5'-HeCB
	2,2',3,3',6,6'-HCB		
Polychlorinated dibenzofuran:			
			1,2,3,7,8-PeCDF

E_{50} value indicates the effective concentration that increases the control activity by 50%. NEO = no effect observed up to 100 µM. (Adapted from Kodavanti et al., Toxicol. Appl. Pharmacol. 130: 140-148, 1995).

The SAR studies indicated that activity of more PCB congeners was associated with chlorination that favored non-coplanarity while those with chlorination that favored coplanarity were less active. To support this observation further, studies with a group of chemicals, in which coplanarity is difficult regardless of degree and pattern of chlorination, were initiated. All the polychlorinated diphenyl ether (PCDE) congeners studied, increased [^{3}H]PDBu binding in a concentration-dependent manner. The order of potency was 2,4,4'-trichlorodiphenyl ether > 4,4'-dichlorodiphenyl ether > diphenyl ether, 3,3',4,4'-tetrachlorodiphenyl ether, 2,2',4,4',5-, and 2,3',4,4',5-pentachlorodiphenyl ethers. The PCDE analogs, nitrofen, *o,p'*-DDT, and *p,p'*-DDT increased [^{3}H]PDBu binding to a similar extent (28-35% stimulation at 100 µM). All PCDE congeners and their analogs inhibited $^{45}Ca^{2+}$-sequestration by microsomes and mitochondria. Of all the chemicals, diphenyl ether is the least active. These results support our hypothesis that coplanarity of certain chlorinated aromatic hydrocarbons seems to weaken their potency *in vitro*.

The development of empirical and molecular models are currently in progress to gain a better understanding about the relative importance of the pattern of chlorine substitutions in the activity of PCBs *in vitro*.

4. References

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