ACTIVATION OF CALCIUM-DEPENDENT AND -INDEPENDENT PHOSPHOLIPASE A_2 BY NON-COPLANAR POLYCHLORINATED BIPHENYLS IN RAT CEREBELLAR GRANULE NEURONS

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Introduction

Polychlorinated biphenyls (PCBs) are persistent environmental pollutants, widely distributed, and bioaccumulate in animal and human tissues (1). PCBs have been reported to cause neurobehavioral and cognitive deficits in children exposed to PCBs during development and also in animals (2). Recent reports indicate developmental effects of PCBs on long-term potentiation (LTP), a form of synaptic plasticity (3). The toxic equivalency factor approach for PCBs used in risk assessment is based upon TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) equivalency determined by their affinity for the aryl hydrocarbon (AhR) receptor and related endpoints. However, *ortho*-substituted PCBs, which have a low/no affinity for the AhR, unlike the more TCDD-like coplanar PCBs, have been shown to accumulate preferentially in brain, alter signal transduction pathways necessary for neuronal growth, and have been hypothesized to be potentially neurotoxic (2, 4, 5). Reduction of dopamine levels in PC12 cells and brain regions of non-human primates, perturbations of Ca^{2+}-homeostasis, alterations in protein kinase C (PKC) translocation, and disruption of agonist-stimulated inositol phosphate (IP_3) production in rat cerebellar neurons are all effects that have been attributed to non-coplanar PCBs in the nervous system (4, 5).

We have now extended studies to investigate the effects of PCBs on phospholipases . The extracellular release of [^{3}H]arachdionic acid (AA) from the rat cerebellar granule neurons was used to measure the activation of phospholipases. AA can be released by phospholipase C (PLC) from the membrane lipids by the production of IP_3 and diacylglycerol, a substrate for di- and mono-acylgycerol lipases producing AA. Phospholipase D (PLD) can also release AA by the production of phosphatidic acid which can be converted by phosphotidate phosphohydrolase to diacylglycerol which can be converted to AA. However, the major pathway for the AA release is through the activation of phospholipase A_2 (PLA_2). Changes in PLA_2 activity have been implicated in several neurological diseases (6) and AA has also been identified as a retrograde messenger involved in LTP (7). The objective of this study is to: (a) extend structure-activity relationship (SAR) of PCB congeners and their metabolites on the activation of phospholipases; (b) characterize the possible mechanism by which PCBs stimulate [^{3}H]AA release.

Materials and Methods

Chemicals. Radiolabeled [5, 6, 8, 9, 11, 12, 14, 15-^{3}H(N)]arachidonic acid (210 Ci/mmol; >97% pure) was purchased from Dupont New England Nuclear Corporation (Boston, MA). Chemicals used in the assay and cell culture were obtained from commercial sources. The PCBs were obtained from AccuStandard, New Haven, CT, and ULTRAscientific, North Kingstown, RI, and dissolved in dimethylsulfoxide (DMSO). DMSO ($\leq$ 0.4% v/v) had no effect on [^{3}H]AA release.

Cerebellar granule cell culture. Timed pregnant female (16 days gestation) Long Evans rats were obtained from Charles River Laboratory (Portage, OR) and housed individually in AAALAC approved animal facilities. Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6-8 day old pups as outlined by Gallo *et al.* (8) with modifications (9). Cultures were grown in DMEM with 10% FBS and 30 mM KCl in 12-well plates (Corning Costar), with a plating density 1.5 x 10^6 cells/ml. Cytosine arabinoside was added 48 hours after plating to prevent the proliferation of non-neuronal cells. Cultures were assayed at 7 days *in vitro*.

[^{3}H]Arachidonic Acid Release. The release of [^{3}H]AA by the CGNs into the media was determined according to Lazarewicz *et al.* (10) and Tithof *et al.* (11) with modifications. CGNs were labeled for 16-20 hours with 1 µCi [^{3}H]AA per well. The cells were then washed once with modified Locke's buffer and twice with modified Locke's + 0.2% BSA. Preincubation was for 10 min in modified Locke's + 0.2% BSA with or without the addition of pharmacological agents. CGNs were incubated for 60 min with EGTA/AM in DMEM + 0.2% BSA at 37^{o}C in an incubator prior to the preincubation for those treatment groups. The cells were exposed for 20 min to PCBs (1-50 µM) in the presence or absence of pharmacological agents in modified Locke's + 0.2% BSA (1 ml) with or without extracellular calcium (no added $CaCl_2$ and with 0.3 mM EGTA). The media was immediately removed after exposure for counting in scintillation counter, and 1 ml of 0.5 N NaOH was added to lyse the cells to measure total [^{3}H]AA incorporation. The data was calculated as [^{3}H]AA release as a % of total [^{3}H]AA incorporation.

Statistics. All the data (mean ± SEM of 3-5 preparations) were analyzed by a two-way analysis of variance followed by Fisher's LSD test. The significance was set at $p < 0.05$.

Results and Discussion

PCB effects [^{3}H]AA release and Structure-Activity Relationships:

Glutamate, a positive control, stimulated [^{3}H]AA release in a concentration-dependent manner, in agreement with published reports (10). The commercial PCB mixture, Aroclor 1254 also increased [^{3}H]AA release at concentrations as low as 10 µM and the increase was nearly two fold greater than glutamate at 100 µM. *Ortho*-substituted PCB, 2,2'-dichlorobiphenyl (DCB) caused significant release of [^{3}H]AA at 3 µM, while the non*ortho*-PCB 4,4'-DCB had no effect even at 50 µM (Table 1) The release of [^{3}H]AA by 2,2'-DCB is as early as 2 min of exposure, and increased with time. Other non-coplanar PCBs such as 2,2',5,5'-tetrachlorobiphenyl (TeCB), 2,2',3,5',6-pentachlorobiphenyl (PeCB), 2,2',4,4',5,5'-hexachlorobiphenyl (HCB) also stimulated [^{3}H]AA release. The coplanar PCBs such as 3,3',4,4'-TeCB and 3,3',4,4',5,5'-HCB had no effect, however, 3,3',4,4',5-PeCB had marginal effect at 50 µM (Table 1).

2,2',4,4',5,5'-HCB and its potential hydroxy metabolite, 2,2',4,5,5'-pentachloro-4'-biphenylol significantly stimulated [^{3}H]AA release at 30 and 50 µM in a similar fashion suggesting that the metabolite appears to be as effective as the parent compound. On the other hand, 3,3',4,4',5,5'-HCB did not significantly stimulate [^{3}H]AA release, whereas 3,3',5,5'-tetrachloro-4,4'-biphenyldiol caused a significant increase at 30 and 50 µM (Table 1). The low solubility of the parent compound may partially factor into the difference between these two compounds.

These findings are in agreement with SAR demonstrated for Ca^{2+}- buffering and PKC

translocation (4). The hydroxy-PCBs are as active as the parent compound with respect to *ortho*-PCB; whereas non-*ortho* PCB is inactive and its hydroxy derivative was active, suggesting the role of hydroxy-PCBs in the biological effects of PCBs.

Table 1

PCB-stimulation of [^{3}H]Arachidonic acid (AA) release in cerebellar granule neurons and structure-activity relationships (SAR).

PCBs	IUPAC #	[^{3}H]AA release at 50 µM (%control)	Significant effect
Aroclor 1254		388 ± 32	10 µM
2,2'	4	337 ± 27	3 µM
2,2',5,5'	52	548 ± 6	3 µM
2,2',3,5',6	95	423 ± 36	10 µM
4,4'	15	136 ± 8	---
3,3',4,4'	77	126 ± 11	---
3,3',4,4',5	126	157 ± 15	50 µM
2,2',4,4',5,5'	153	303 ± 24	30 µM
2,2',4,5,5'-pentachloro-4'-biphenylol		230 ± 10	30 µM
3,3',4,4',5,5'	169	103 ± 12	---
3,3',5,5'-tetrachloro-4,4'-biphenyldiol		149 ± 6	30 µM

Characterization of 2,2'-DCB-induced [^{3}H]AA release:

Current results indicate that [^{3}H]AA release by non-coplanar PCBs could be mainly due to the activation of PLA_2, but not PLC/PLD. U-73122, an inhibitor of PLC, at 10 µM did not inhibit [^{3}H]AA release. Neomycin, a non-specific PLC inhibitor slightly decreased (30%) [^{3}H]AA release by 2,2'-DCB (Table 2). However, neomycin also blocks voltage-gated calcium channels, inositol phospholipid turnover, and phosphatidylcholine specific PLD. Quinacrine, which inhibits PLA_2 by perturbing the enzyme substrate interface (11), at 50 µM significantly decreased (>50%) the 2,2'-DCB stimulated [^{3}H]AA release. It appears from these results that PLC/PLD is not a primary pathway for the [^{3}H]AA release stimulated by 2,2'-DCB, however, PLA_2 evidently plays a significant role in 2,2'-DCB-induced phospholipid hydrolysis in cerebellar granule neurons.

Removal of Ca^{2+} from extracellular medium ([Ca^{2+}]e) only partially attenuated (14% decrease) the 2,2'-DCB stimulated [^{3}H]AA release (Table 2). Similarly TMB-8 (8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxy-benzoate), a blocker of IP_3 induced intracellular Ca^{2+} mobilization, only partially reduced (34% decrease) the effect of 2,2'-DCB. Also, xestospongin C (10 µM), a blocker of intracellular Ca^{2+} ([Ca^{2+}]i) release from endoplasmic reticulum by the IP_3 receptor (12), partially reduced (14% decrease) 2,2'-DCB-induced [^{3}H]AA release, while dantrolene (50 µM; ryanodine receptor blocker) had no effect (Table 2). These results suggest that 2,2'-DCB-induced [^{3}H]AA release seems to be only partially related to changes in intracellular Ca^{2+} homeostasis.

As various types of PLA_2 exist in the brain, we have attempted to characterize the effects of 2,2'-

DCB. 4-Bromophenacylbromide (4-BPB), an inhibitor of secretory PLA_2 ($sPLA_{2;}$,13), at 1 or 30 μM did not significantly attenuate the 2,2'-DCB stimulated release of [^{3}H]AA suggesting that $sPLA_2$ is not primarily involved in this effect. Methyl arachidonyl fluorophosphonate (MAFP) at 5 μM significantly attenuated (>60% decrease) the 2,2'-DCB (50 μM)-stimulated [^{3}H]AA release. Since MAFP inhibits cytosolic Ca^{2+}-dependent ($cPLA_2$) and -independent PLA_2 ($iPLA_2$) (14), 2,2'-DCB stimulated release of [^{3}H]AA might occur through a $cPLA_2$ / $iPLA_2$ pathway.

Table 2

Characterization of 2,2'-dichlorobiphenyl (DCB)-stimulation of [^{3}H]Arachidonic acid (AA) release in cerebellar granule neurons

Experimental manipulation/ Pharmacological treatment	2,2'-DCB-stimulation of [^{3}H]AA release	Significance
U-73122	no effect	PLC is not involved
Neomycin	marginal effect	PLC/PLD not majorly involved
Quinacrine	Greatly reduced	PLA_2 involved
Removal of [Ca^{2+}]e	only partially reduced	[Ca^{2+}]e partially necessary
TMB-8	only partially reduced	[Ca^{2+}]i partially necessary
Xestospongin/Dantrolene	only partially reduced	[Ca^{2+}]i partially necessary
4-Bromophenacylbromide	no effect	s PLA_2 not involved
MAFP	Greatly reduced	$cPLA_2$ and/or $iPLA_2$ involved

These results suggest that *ortho*-PCBs activate phospholipases, especially PLA_2 at low micro-molar concentrations and as early as 2 min of exposure. These results confirm our previous SAR data on Ca^{2+}-buffering and PKC translocation. [^{3}H]AA release caused by *ortho*-PCBs might be due to activation of cytosolic PLA_2 that are Ca^{2+}-dependent as well as Ca^{2+} -independent. Since predominant $cPLA_2$ activity present in brain (>80%) is Ca^{2+}-independent (15), stimulation of this PLA_2 by non-coplanar PCBs might have a significant role in the neurotoxic effects of PCBs.

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