ortho-Substituted Polychlorinated Biphenyls Alter Microsomal Calcium Transport by Direct Interaction with Ryanodine Receptors of Mammalian Brain*

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A stringent structure-activity relationship among polychlorinated biphenyls (PCBs) possessing two or more ortho-chlorine substitutions is observed for activation of ryanodine receptors in mammalian brain, revealing an arylhydrocarbon receptor-independent mechanism through which non-coplanar PCBs disrupt neuronal Ca\(^2+\) signaling. Of the congeners assayed, non-coplanar PCB 95 exhibits the highest potency (EC\(_{50} = 12-24\ \mu\text{M}\)) toward activating high affinity \[^3H\]ryanodine-binding in rat hippocampus, cerebellum, and cerebral cortex. Coplanar PCB 66 and PCB 126 have no ryanodine receptor activity in all brain regions examined. PCB 95 enhances \[^3H\]ryanodine-binding affinity and capacity by significantly altering modulation by Ca\(^2+\) and Mg\(^2+\), thereby stabilizing a high affinity conformation of the ryanodine receptor. Ca\(^2+\) transport measurements using cortical microsomes reveal that PCB 95 discriminates between inositol 1,4,5-trisphosphate- and ryanodine-sensitive stores. PCB 95 selectively mobilizes Ca\(^2+\) from ryanodine-sensitive stores in a dose-dependent manner (EC\(_{50} = 3.5\ \mu\text{M}\)) and is completely inhibited by ryanodine receptor blockers, whereas coplanar PCBs are inactive. These data demonstrate that ortho-substituted PCBs disrupt Ca\(^2+\) transport in central neurons by direct interaction with ryanodine receptors, showing high selectivity and specificity. Alteration of Ca\(^2+\) signaling mediated by ryanodine receptors in specific regions of the central nervous system may account, at least in part, for the significant impact of these agents toward neurodevelopment and neuroplasticity in mammals.

Along with the genetically related inositol 1,4,5-trisphosphate receptors (IP\(_3\)R),\(^1\) ryanodine receptors (RyR) are Ca\(^{2+}\)-selective ion channels which regulate the release of Ca\(^{2+}\) from endoplasmic reticulum (ER) during cell activation. Mobilization of ER Ca\(^{2+}\) is essential for determining the amplitude, spatial and temporal fluctuation of intracellular Ca\(^{2+}\), thereby providing important signaling information to the cell (1). In consonance with their fundamental role in cellular Ca\(^{2+}\) signaling, skeletal (Ry\(_1\)R) and brain (Ry\(_3\)R) isoforms of RyRs possess a cytoskeletal binding motif which could confer a structural and functional association with L-type voltage-dependent Ca\(^{2+}\) entry channels in skeletal muscle (2–5). Data supporting direct coupling of neuronal L-type voltage-dependent Ca\(^{2+}\) entry and RyRs has been recently provided (6). Most significant is the finding that expression of Ry\(_1\)R cDNA in dysgenic muscle cells, which lack constitutive expression of Ry\(_1\)R, restores not only excitation-contraction coupling but also restores L-type voltage-dependent Ca\(^{2+}\) entry (5). Thus mechanical coupling between RyRs and voltage-gated Ca\(^{2+}\) channels within the surface membrane appears to confer reciprocal regulation, a finding that may have broad significance in neuronal cell functions.

Polychlorinated biphenyls (PCBs), polychlorinated dibenzop-dioxins and polychlorinated dibenzofurans are commonly known as halogenated aromatic hydrocarbons (HAHs), a family of persistent and widely dispersed environmental contami-nants. The unique chemical properties and low cost of producing PCBs have contributed to their extensive industrial use (7, 8). The high lipophilicity and chemical stability of PCBs have further resulted in widespread environmental contamination, and there is significant evidence of PCBs accumulation in biota (9). PCBs are found in extracts of virtually all environmental samples as well as in human tissue and breast milk (10, 11).

Among the HAHs various congeners differentially confer ability to bind to a cytosolic receptor, the arylhydrocarbon receptor (AhR). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) binds to the receptor with the highest affinity and confers the highest potency to induce certain toxic responses, such as wasting syndrome, immunosuppression, and teratogenicity. PCB congeners without ortho-chlorine substitutions are able to confer the coplanar structure similar to TCDD and elicit similar toxicity. PCB congeners with single ortho-chlorine slightly favor the non-coplanarity and behave as weak AhR agonists. PCB congeners with two or more ortho-chlorines highly favor the non-coplanar conformation; thus, this group of congeners do not bind to the AhR and exhibit different toxicity. There is emerging evidence indicating that certain ortho-substituted PCB congeners are responsible for the neurotoxic effects of PCBs, including decreased in catecholamine levels in certain brain regions in mammals (12–14) and reduced dopamine lev-

\[^1\] The abbreviations used are: IP\(_3\)R, inositol 1,4,5-trisphosphate receptor; AhR, arylhydrocarbon receptor; t-IP\(_3\), myo-inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; HAH, halogenated aromatic hydrocarbon; t-IP\(_3\), myo-inositol 1,4,5-trisphosphate; MOPS, 3-(N-morpholino)propanesulfonic acid; PCB, polychlorinated biphenyl; PIPES, piperezaine-N, N′-bis(2-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; RyR, ryanodine receptor; Ry\(_i\)R, skeletal isoform of ryanodine receptor; Ry\(_R\)R, brain isoform of ryanodine receptor; SERCA1, skeletal isoform of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase; SERCA2, cardiac isoform of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase; SR, sarcoplasmic reticulum; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.
els in rat phaeochromocytoma cells (PC12 cells) (15). Perinatal exposure of monkeys and rodents to PCBs resulted in behavioral abnormalities including delayed reflex development, altered activity patterns, learning deficits, and impaired memory (16). Rats perinatally exposed to certain ortho-substituted PCB congeners were impaired in learning a delayed spatial alternation task similar to that employed in the behavioral test of the PCB-exposed monkeys (17). Epidemiological studies reveal that children exposed either perinatally or prenatally to PCBs and other HAHs developed long-lasting cognitive function deficits (18–21). Although the compounds that are responsible for these deficits are unknown, results from animal studies have revealed that ortho-substituted PCBs are probably responsible for the neurotoxicity observed.

In addition to the subtle neurotoxic effects induced by PCBs, the 209 possible congeners in the PCB family that constitute various industrial PCB mixtures has greatly obstructed progress in understanding the cellular and subcellular mechanisms of PCBs action. Studies have demonstrated that certain ortho-substituted PCB congeners are responsible for the neurotoxic action of PCBs, suggesting a non-AhR-mediated pathway. Shain and co-workers performed an extensive structure-activity relationship study on more than 50 PCB congeners (15). ortho-Substituted, non-coplanar PCB congeners were found to be the most active structures in decreasing dopamine level in PC12 cells, whereas coplanar PCBs were found to be inert. However, the underlying molecular mechanisms by which ortho-substituted PCBs induced neurotoxicity are unknown. We have provided evidence of the direct interaction between certain ortho-substituted PCB congeners and the ryanodine-sensitive Ca$^{2+}$ channel complex (RyRs) localized on sarcoplasmic and endoplasmic reticulum (SR/ER) (22). The present paper demonstrates for the first time a stringent structure-activity relationship among PCBs possessing two or more chlorine substitutions in the ortho positions for activation of RyRs of the mammalian central nervous system, revealing an AhR-independent mechanism through which PCBs disrupt neuronal Ca$^{2+}$ signaling. The most potent congeners at the receptor yet identified, PCB 95 (2,2’,3,3’,5,5’,6-pentachlorobiphenyl), is found to alter Ca$^{2+}$ transport across neuronal microsomal membrane vesicles by a RyR-mediated pathway without affecting the IP$_3$-R-mediated pathway. These actions of PCB 95 at RyRs may underlie its ability to alter neuronal excitability in rat hippocampal slices in vitro (23), as well as both locomotor activity and spatial learning in an in vivo rat model (24). Taken together, these results demonstrate a RyR-mediated mechanism by which certain ortho-substituted PCBs alter neuronal Ca$^{2+}$ signaling, through which they may alter neurodevelopment and neurobehavioral function in mammals.

**Experimental Procedures**

**Chemicals**

Aroclor 1254 and PCB congeners (>99% pure) were purchased from Ultra Scientific (North Kensington, RI). [3H]Ryanoide (60–90 Ci/mmoll; > 99%) and [3H]inositol 1,4,5-trisphosphate (15–30 Ci/mmoll; > 95%) were purchased from DuPont NEN. Flu-3 (pentamethonium salt, ≥90% pure) was purchased from Molecular Probes (Eugene, OR). All other reagents were of the highest purity commercially available.

**Membrane Preparations**

Membrane fractions from cerebellum, cerebral cortex, and hippocampus of rat were prepared as described previously (25). Briefly, tissue from each brain region was homogenized with 10-fold (w/v) ice-cold homogenization buffer consisting of 320 mm sucrose, 5 mm HEPES, pH 7.4, 100 µM phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml leupeptin.

**Whole Particulate Fractions**—The homogenate from each brain region was centrifuged at 1,000 × g for 1 h. The whole particulate fractions were obtained by suspending the pellets in a buffer consisting of 320 mm sucrose, 5 mm HEPES, pH 7.4, at a protein concentration of 8–12 mg/ml (26). The protein preparations were then rapidly frozen in liquid N$_2$ and stored at −80 °C until needed.

**Microsomal Fractions Enriched in IP$_3$R and RyRs**—The homogenate from each brain region was centrifuged at 1,000 × g for 10 min. The membrane pellets were collected, suspended in homogenization buffer with a glass Dounce homogenizer, and re-centrifuged at 1,000 × g. The two supernatant fractions were combined and centrifuged at 8,000 × g for 10 min. The resulting supernatants were then centrifuged at 110,000 × g for 1 h. Finally, the crude microsomal pellets were suspended and stored as described above.

**Radioligand Binding Assay**

**Binding Capacity of Cerebellum**—To examine the protein dependence of [3H]ryanodine binding in the presence or absence of selected PCB congeners, the binding capacity of the whole particulate fraction isolated from rat cerebellum was determined as a function of protein concentration. Specific binding of 10 nM [3H]ryanodine to cerebellar whole particulate fraction (50–250 µg) was measured in an assay buffer consisting of 140 mM KCl, 15 mM NaCl, 20 mM PIPES, pH 7.4, 100 µM PMSF, 10% sucrose, 10 µM CaCl$_2$ in the presence or absence of either 40 µM PCB 95 or 40 µM PCB 126, in a final volume of 250 µl, as described previously (25). All samples were equilibrated at 37 °C for 3 h with constant shaking. Nonspecific binding was determined by addition of a thousandfold excess of cold ryanodine and averaged 70% of the total binding. Each experiment was performed in duplicate and repeated at least three times with different membrane preparations.

**Structure-Activity Relationship of PCBs toward RyRs in Cerebellum**—Dose-response relationship for PCB-activated [3H]ryanodine binding to cerebellar microsomes was determined for selected PCB congeners and Aroclor 1254. Specific binding of 2 nM [3H]ryanodine was measured in the presence or absence of 400 nM to 400 µM PCB with 180 µg of crude cerebellar microsomal protein, in an assay buffer consisting of 140 mM KCl, 15 mM NaCl, 20 mM PIPES, pH 7.4, 100 µM PMSF, 10 µM CaCl$_2$, in a final volume of 250 µl (25). Nonspecific binding was determined by addition of a thousandfold excess of cold ryanodine and averaged 35% of the total binding. The potency (EC$_{50}$ and Hill coefficient of each PCB toward RyRs was determined by linear regression analysis of logit-log transformation of the data obtained between 10 and 90% of maximal binding.

**Saturation Binding of [3H]Ryanodine in Cerebellum**—The mechanism by which PCB 95 enhanced occupancy of [3H]ryanodine to cerebellum was measured by saturation binding experiments (22). Specific binding of 0.5–20 nM [3H]ryanodine was determined in the presence of 50 µM PIPES in an assay buffer (Low Salt Buffer) consisting of 180 µg of cerebellar whole particulate protein, 140 mM KCl, 15 mM NaCl, 20 mM PIPES, pH 7.4, 10% sucrose, 100 µM PMSF, 10 µM CaCl$_2$, 1 mM MgCl$_2$, in a final volume of 250 µl. Control experiments were performed in the absence of PCB by measuring specific binding of 0.5–70 nM [3H]ryanodine in an assay buffer (High Salt Buffer) consisting of 180 µg of cerebellar whole particulate protein, 200 mM KCl, 15 mM NaCl, 20 mM PIPES, pH 7.4, 10% sucrose, 100 µM PMSF, 10 µM CaCl$_2$, 1 mM MgCl$_2$, in a final volume of 250 µl. Binding constants (K$_D$ and B$_max$) of high affinity [3H]ryanodine-binding sites were obtained from linear regression analysis of the Scatchard plots using ENZFITTER (Elsevier Biosoft, London, United Kingdom) computer software.

**Calcium and Magnesium Modulation of [3H]Ryanodine Binding**—The ability of PCB 95 to alter Ca$^{2+}$ modulation of RyRs was performed by measuring specific binding of 2 nM [3H]ryanodine to 200 µg of cerebellar microsomal protein in the presence of 50 µM PCB 95 in Low Salt Buffer and 12 nM to 200 mM free Ca$^{2+}$, in a final volume of 250 µl. Free Ca$^{2+}$ concentrations below 200 µM were adjusted by adding calculated amount of EGTA based on the SPECS computer software and published stability constants (27). Control experiments were performed in High Salt Buffer containing 200 µM to 200 µM CaCl$_2$.

The ability of PCB 95 to alter Mg$^{2+}$ inhibition of RyRs was determined in the presence of 2 nM [3H]ryanodine, 200 µM of cerebellar microsomes, and 2 mM to 1 mM Mg$^{2+}$ in Low Salt Buffer containing 100 µM CaCl$_2$, in a final volume of 250 µl. Mg$^{2+}$ concentrations of 200 µM to 1000 µM were then rapidly frozen in liquid N$_2$ and stored at −80 °C until needed.
RESULTS

ortho-Substituted PCBs Activate Ryanodine Receptors in Vitro—Aroclor 1254 and selected PCB congeners were examined for their ability to modulate the high affinity binding of [3H]ryanodine to microsomes isolated from rat cerebellum, cerebral cortex, and hippocampus in the presence of physiologically relevant concentrations of intracellular K⁺ and Na⁺. Fig. 1 demonstrated that binding of [3H]ryanodine (10 nM) increased linearly as a function of cerebellar protein concentration and that non-coplanar PCB 95 significantly enhanced occupancy to ≥50 µg of protein/assay. In contrast, coplanar PCB 126 did not increase occupancy above control values at any concentrations of protein examined (Fig. 1). Similar enhancement in the occupancy of nanomolar [3H]ryanodine was observed with cortical and hippocampal membrane preparations (data not shown). The influence of ortho-chloro substitutions on the ability of PCB congeners to activate the high affinity binding of [3H]ryanodine was examined with complete dose-response relationships for selected PCB congeners. Coplanar PCB 66 and PCB 126 had no activity toward RyRs in microsomes isolated from rat cerebellum, cerebral cortex, or hippocampus at concentrations up to their solubility limits (200 µM) (Fig. 2, Table I). In marked contrast, non-coplanar PCB 95 enhanced binding of [3H]ryanodine, in a dose-dependent manner, to microsomes isolated from all the brain regions examined. PCB 95 gave EC₅₀ values (in µM) of 17, 12, and 25 with microsomes isolated from rat cerebellum, hippocampus, and cerebral cortex, respectively (Fig. 2, Table I).

An extended structure-activity relationship was performed with cerebellar microsomes. Compared with PCB 95, PCB 4 (2,2'-dichlorobiphenyl) and PCB 52 (2,2',5,5'-tetrachlorobiphenyl) were 2- and 3-fold less potent, respectively. The position of the chlorine substituents about the biphenyl ring structure appeared to be as important as the degree of chlorination for RyR activity, since 2,2',4,5'- and 2,2',3,4,6-pentachlorobiphenyl (PCB 103 and PCB 88, respectively) were 3- and 5-fold less potent than PCB 95. The stringent structural requirement for receptor activity was further demonstrated by the finding that 2,2',4,4',5,5'-hexachlorobiphenyl, 2,2',4,6,6'-pentachlorobiphenyl, and 2,3',4,5-tetrachlorobiphenyl (PCB 153, PCB 104, and...
**PCB 70**, respectively) were approximately 10-fold less potent than PCB 95, whereas 2,3',4,4'-tetrachlorobiphenyl (PCB 66) was inactive in all of the brain regions tested (Table I). Interestingly, the PCB mixture Aroclor 1254 showed appreciable activity toward RyR (EC \textsubscript{50} = 35 \mu M in cerebellum). The values of EC \textsubscript{50}, maximal occupancy, and Hill coefficient for selected PCB congeners and Aroclor 1254 are summarized in Table I.

**PCB 95 Induces Ca\textsuperscript{2+} Release from Cortical Microsomes**—Net Ca\textsuperscript{2+} transport across rat cortical microsomal membrane vesicles was measured with the fluorescent dye fluo-3 under conditions of active Ca\textsuperscript{2+} loading. In Fig. 3A, a typical trace of the Ca\textsuperscript{2+} loading phase is shown, and was invariant for each of the experiments shown below. Ca\textsuperscript{2+} loading was initiated by addition of ATP followed by serial addition of two 2.4-nmol additions of Ca\textsuperscript{2+}. No change in fluorescence intensity, (i.e. net Ca\textsuperscript{2+} transport across the membrane vesicles) was observed before the addition of ATP, suggesting that Ca\textsuperscript{2+} uptake by the membrane vesicles was ATP-dependent (data not shown). Additions of the Ca\textsuperscript{2+} ionophores 4-bromo-A-23187 after completion of the loading phase demonstrated that the accumulated Ca\textsuperscript{2+} could be rapidly released from the vesicles. Addition of Ca\textsuperscript{2+} (2 \times 2.4 nmol) at the end of each experiment verified the linearity and the calibration of the dye signal. In all the measurements reported in the present study, none of the drugs at the concentrations used significantly interfered with either the sensitivity or calibration of the fluo-3 dye (data not shown). Under these assay conditions, F\textsubscript{min} and F\textsubscript{max} were 0.5 ± 0.1 and 8.0 ± 0.3, respectively (mean of three determinations). In Fig. 3B, addition of planar PCB 66 or PCB 126 to cortical microsomes actively loaded with Ca\textsuperscript{2+} had no effect on the net Ca\textsuperscript{2+} flux across the vesicles. In marked contrast, addition of PCB 95 induced a rapid release of Ca\textsuperscript{2+} from the vesicles. The initial rate of Ca\textsuperscript{2+} release from the vesicles induced by PCB 95 (1–10 \mu M) was dose-dependent (Fig. 3C) with an apparent EC \textsubscript{50} of 3.5 \mu M (Fig. 3C, inset).

**RyR Blockers Inhibit PCB 95-induced Ca\textsuperscript{2+} Release from Brain Microsomes**—The mechanism by which PCB 95 induced Ca\textsuperscript{2+} release from brain microsomes was further studied using ruthenium red and ryanodine, known RyR blockers. Fig. 4 shows the response of actively loaded cortical microsomes to 5 \mu M PCB 95 (trace A). Addition of ruthenium red (1 \mu M) after loading the vesicles with Ca\textsuperscript{2+} largely eliminated the response to PCB 95 (~94% inhibition of the initial Ca\textsuperscript{2+} release rate) (Fig. 4, trace B). Prior addition of 500 \mu M ryanodine resulted in a typical biphasic response of the receptor, whereas subsequent addition of PCB 95 failed to mobilize Ca\textsuperscript{2+} from the vesicles (~93% inhibition of the initial Ca\textsuperscript{2+} release rate) (Fig. 4, trace C). These results suggested that PCB 95 induced Ca\textsuperscript{2+} release was inhibited by ryanodine-sensitive Ca\textsuperscript{2+} channel blockers.

**PCB 95 Selectively Targets Ryandine-sensitive Ca\textsuperscript{2+} Stores**—To discriminate which of the Ca\textsuperscript{2+} stores in the microsomal preparation are sensitive to PCB 95, additional pharmacological studies were performed with IP\textsubscript{3} and ryanodine. Fig. 5 illustrates the ability of IP\textsubscript{3} to stereoselectively activate IP\textsubscript{3}Rs in the microsomal preparation, since L-IP\textsubscript{3} is inactive (trace a). Addition of 500 \mu M ryanodine to the preparation caused a biphasic response similar to that seen with junctional SR vesicles isolated from striated muscle (31): initially activating and subsequently inactivating RyRs, resulting in net uptake of the Ca\textsuperscript{2+} into the cortical vesicles (Fig. 5, trace b). Addition of IP\textsubscript{3} after treating with ryanodine demonstrated that the IP\textsubscript{3}Rs maintained their sensitivity to agonist, as the rate and amount of IP\textsubscript{3}-induced Ca\textsuperscript{2+} release was similar to that seen with IP\textsubscript{3} alone (Fig. 5, compare traces a and b). Addition of ryanodine subsequent to IP\textsubscript{3}-induced Ca\textsuperscript{2+} release mobilized stored Ca\textsuperscript{2+} with magnitude and kinetics quantitatively similar to ryanodine-induced Ca\textsuperscript{2+} release in the absence of IP\textsubscript{3} (Fig. 5, compare traces a and b). Taken together, the results suggested that the IP\textsubscript{3}- and ryanodine-sensitive efflux pathways in the cortical microsomal preparation were on distinct vesicles.

To further determine whether the actions of PCB 95 were selective toward the ryanodine-sensitive store, PCB 95 was added prior or subsequent to addition of IP\textsubscript{3}. With either experimental protocol, PCB 95-induced Ca\textsuperscript{2+} release was quantitatively similar (Fig. 6, compare traces a and b). Addition of 1 \mu M ruthenium red after depleting the IP\textsubscript{3}-sensitive stores completely blocked PCB 95-induced Ca\textsuperscript{2+} release (Fig. 6, trace c). Similarly, prior treatment of the Ca\textsuperscript{2+}-loaded vesicles with 1 \mu M ruthenium red completely negated the response to PCB 95.

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**Table I**

| Ballschmiter no. | Chlorine positions | EC\textsubscript{50} \mu M | Hill coefficient | Maximal occupancy filol/mg protein |
|------------------|--------------------|-----------------------------|------------------|----------------------------------|
| A. Cerebellum    |                    |                             |                  |                                  |
| Control (n = 11) |                    |                             |                  |                                  |
| Aroclor 1254 (n = 4) | Mixture | 35.5 ± 4.5 | 0.9 ± 0.2 | 2.4 ± 1.3 |
| 4 (n = 3) | 2,2 | 34.3 ± 4.9 | 1.1 ± 0.1 | 39 ± 3.0 |
| 52 (n = 2) | 2,2,5,5' | 52.1 ± 5.6 | 1.0 ± 0.1 | 30 ± 3.8 |
| 66 (n = 2) | 2,3,4,4' | Inactive | Inactive | Inactive |
| 70 (n = 2) | 2,3,4,5 | 166 ± 40 | 0.8 ± 0.2 | 34 ± 7.4 |
| 88 (n = 2) | 2,9,3,6 | 89.3 ± 7.9 | 0.9 ± 0.1 | 20 ± 2.0 |
| 95 (n = 5) | 2,2,3,5,6 | 17.1 ± 4.0 | 1.0 ± 0.2 | 37 ± 1.0 |
| 103 (n = 3) | 2,2,4,5,6 | 50.8 ± 4.6 | 1.0 ± 0.1 | 33 ± 2.0 |
| 104 (n = 2) | 2,2,4,6,6 | 157 ± 24 | 1.0 ± 0.1 | 33 ± 3.6 |
| 126 (n = 5) | 3,3,4,4,5 | Inactive | Inactive | Inactive |
| 153 (n = 2) | 2,2,4,4,5,5 | 178 ± 35 | 1.2 ± 0.3 | 36 ± 11 |
| B. Hippocampus  |                    |                             |                  |                                  |
| 66 (n = 2) | 2,3,4,4' | Inactive | Inactive | Inactive |
| 95 (n = 2) | 2,2,3,5,6 | 12.1 ± 1.9 | 1.5 ± 0.3 | 15 ± 1.9 |
| 126 (n = 2) | 3,3,4,4,5 | Inactive | Inactive | Inactive |
| C. Cerebral cortex |                    |                             |                  |                                  |
| 66 (n = 2) | 2,3,4,4' | Inactive | Inactive | Inactive |
| 95 (n = 2) | 2,2,3,5,6 | 24.8 ± 4.4 | 1.9 ± 0.4 | 18 ± 0.5 |
| 126 (n = 2) | 3,3,4,4,5 | Inactive | Inactive | Inactive |
without altering the response to D-IP$_3$ (Fig. 6, trace d). Addition of 60 m[M] of heparin to Ca$^{2+}$-loaded vesicles resulted in an instantaneous jump in the fluo-3 response due to contamination of the drug with 34 pmol of Ca$^{2+}$. However, subsequent addition of D-IP$_3$ failed to induce the release of Ca$^{2+}$ even though the preparation remained completely responsive to PCB 95 (Fig. 6, compare traces a, b, and c). These results further demonstrated the presence of distinct IP$_3$- and ryanodine-sensitive vesicles in the microsomal preparation and that PCB 95 selectively mobilized Ca$^{2+}$ by directly interacting with vesicles possessing RyRs. PCB 95 (5 m[M]) did not appear to alter the Ca$^{2+}$ transport properties of the fraction of IP$_3$R-containing vesicles. In consonance with this observation, PCB 95 (±50 m[M]) did not significantly alter the binding of [3H]ryanodine to cerebellar microsomes, although higher concentrations did produce a statistically significant enhancement of occupancy that was not seen with coplanar PCB 126 (Fig. 7).

PCB 95 Stabilizes a High Affinity Conformation of RyRs—The mechanism by which PCB 95 enhanced the high affinity binding of [3H]ryanodine to cerebellar microsomes was further elucidated by performing saturation binding measurements and examining changes in modulation by Ca$^{2+}$ and Mg$^{2+}$. In the presence of physiologically relevant concentrations of intracellular Na$^+$ (15 m[M]) and K$^+$ (140 m[M]), negligible specific binding of [3H]ryanodine (0.5–25 n[M]) was detected (data not shown), whereas inclusion of PCB 95 (50 m[M]) to the same assay medium produced a significant enhancement in the number of high affinity binding sites for [3H]ryanodine exhibiting a $B_{max}$ of 68 ± 5 fmol/mg and a $K_D$ of 3.7 ± 0.6 n[M] (Fig. 10). By contrast, control measurement in the presence of high salt (200 m[M] KCl) revealed that [3H]ryanodine bound to RyRs on cerebellar microsomes with $B_{max}$ of 52 ± 1 fmol/mg and $K_D$ of 24 ± 3 n[M] (Fig. 8).

In the presence of 50 m[M] PCB 95 and physiological concentrations of Na$^+$ and K$^+$, the EC$_{50}$ for activation of [3H]ryanodine-binding sites by Ca$^{2+}$ was 61 n[M] (Fig. 9a, Table II). Interestingly, no consistent inhibition of [3H]ryanodine binding was observed at Ca$^{2+}$ as high as 200 m[M]. In contrast, control measurements performed in the presence of 200 m[M] KCl exhibited an IC$_{90}$ for Ca$^{2+}$-activated binding of 1.6 m[M] and a Hill coefficient of 3, with near-complete inhibition at 10 m[M] Ca$^{2+}$.

**Fig. 3.** PCB 95 selectively and specifically induces Ca$^{2+}$ release from cortical microsome. Net Ca$^{2+}$ flux across microsomal membrane vesicles isolated from rat cerebral cortex was measured with fluorescence dye fluo-3 as described under “Experimental Procedures.” A, typical trace of the Ca$^{2+}$-loading phase and calibration of fluo-3. B, trace a, 5 m[M] non-coplanar PCB 95 induced Ca$^{2+}$ release from the vesicles, whereas 5 m[M] coplanar PCB 126 (trace b) and PCB 66 (trace c) did not cause any net change in extravascular Ca$^{2+}$. C, PCB 95 induced Ca$^{2+}$ release from microsomal vesicles in a dose-dependent manner. In the control, 6 μl of Me$_2$SO was added. Inset, the mean initial rate of Ca$^{2+}$ release ± S.E. was plotted against the PCB 95 concentration. EC$_{50}$ value and Hill coefficient were 3.5 μM and 2.7, respectively. The experiments shown were repeated three times with identical results.

**Fig. 4.** PCB 95 induces Ca$^{2+}$ release from cortical microsomes by RyR-mediated pathway. Vesicles were loaded with Ca$^{2+}$ as described in Fig. 3. Trace A, addition of 5 μM PCB 95 caused a rapid release of Ca$^{2+}$ from loaded vesicles. Trace B, with prior addition of 1 μM ruthenium red, 5 μM PCB 95 failed to mobilize Ca$^{2+}$ from loaded vesicles. Trace C, prior addition of 500 μM ryanodine caused an initial release of Ca$^{2+}$ followed by a slow re-uptake. Subsequent addition of 5 μM PCB 95 failed to alter the Ca$^{2+}$ flux across of the membrane vesicles. The time break in trace C represents an interval of 650 s during the re-accumulation of Ca$^{2+}$ into the vesicles. The experiment shown was repeated three times with identical results.
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**FIG. 5.** IP$_3$- and ryanodine-sensitive Ca$^{2+}$ vesicles are distinct in cortical microsomal preparations. Ca$^{2+}$ loading of brain microsomes was performed by adding a single 2.4-nmol bolus of Ca$^{2+}$ in the initial loading phase. Trace a, addition of 5 $\mu$M t-IP$_3$ did not cause any change of Ca$^{2+}$ transport across the vesicles. Subsequent addition of t-IP$_3$ induced an instantaneous robust release of Ca$^{2+}$ from the loaded vesicles. Further addition of 500 $\mu$M ryanodine after depletion of IP$_3$-sensitive stores caused release of Ca$^{2+}$ from ryanodine-sensitive stores (inset a). Trace b, addition of 500 $\mu$M of ryanodine caused an initial release of Ca$^{2+}$ followed by a subsequent re-uptake of Ca$^{2+}$ (inset b). Subsequent addition of 5 $\mu$M t-IP$_3$ induced another robust release of Ca$^{2+}$ from IP$_3$-sensitive stores. Insets a and b are rescaled from the respective regions indicated by the boxes. The experiments shown were repeated three times with identical results.

Furthermore, in the presence of 50 $\mu$M PCB 95, the IC$_{50}$ for Mg$^{2+}$ was 19 mM and the Hill coefficient was 1.3, and only $\sim$50% of the binding sites could be inhibited even with 1 mM Mg$^{2+}$ (Fig. 9B, Table II). Control measurement in the presence of 200 mM KCl revealed an IC$_{50}$ of 1 mM and Hill coefficient of 3.7 for Mg$^{2+}$, with $>$90% inhibition at 10 mM Mg$^{2+}$.

**DISCUSSION**

ortho-Substituted PCBs Alter Ca$^{2+}$ Regulation in Rat Brain by a Novel Mechanism Independent of the AhR—The present study demonstrates that certain ortho-substituted PCB congeners alter Ca$^{2+}$ regulation of central neurons by a RyR-mediated mechanism. In neuronal tissues, two types of ligand-gated Ca$^{2+}$ release channels have been found to be localized to the ER (32, 33): 1) IP$_3$R channels and 2) Ca$^{2+}$-induced Ca$^{2+}$ release channels (i.e., RyR). Because of structural homologies shared by IP$_3$Rs and RyRs, it is necessary to determine if ortho-substituted PCBs discriminate between these microsomal Ca$^{2+}$ release channels. In neurons, the ryanodine-sensitive stores have thus far been found mainly localized in cell soma, whereas the IP$_3$-sensitive stores appear to be equally distributed in neuronal soma and processes (34, 35). Using autoradiography, Snyder and co-workers (33) have demonstrated the differential localization of IP$_3$Rs and RyRs within the central nervous system. Direct Ca$^{2+}$ transport measurements are conducted in the present study using microsomal membrane isolated form rat cerebral cortex. The results demonstrate that IP$_3$Rs and RyRs do not share the same population of vesicles. Despite the presence of a large fraction of IP$_3$-sensitive vesicles in the microsomal preparation used in the present study, the potent actions of PCB 95 are found to be completely selective toward the ryanodine-sensitive microsomes. In addition, PCB 95-induced Ca$^{2+}$ mobilization from brain microsomes is completely blocked by $\mu$M ryanodine or ruthenium red, but not heparin. The inability of PCB 95 ($\geq$50 $\mu$M) to significantly alter the binding of $[^{3}H]$IP$_3$ to cerebellar microsomes offers further support for the hypothesis that PCB 95 selectively targets RyRs. However, at the present time we cannot discount the possibility that other PCB structures possess IP$_3$R activity. In light of the current findings, PCB congeners exhibiting potent activity toward RyR proteins in the mammalian brain would be expected to alter Ca$^{2+}$ signaling and Ca$^{2+}$-dependent processes in affected neurons. Although it is currently unclear what the exact role of ryanodine-sensitive Ca$^{2+}$ channels is in the adult mammalian brain, the distinct heterogeneity in the distribution of neuronal RyRs suggests distinct Ca$^{2+}$-associated brain functions for each isoform. Given the potency and specificity of PCB congeners, they may represent a new class of molecular probes to define the function of RyRs in the brain. Because of the wide environmental distribution of PCBs, and our emerging understanding on the role of RyRs in neurodevelopment and neuroplasticity, this newly identified mechanism by which PCBs alter Ca$^{2+}$ signaling in mammalian brain may underlie the neurotoxicity that has been attributed to non-coplanar PCBs (14).

Three isoforms of RyRs have been shown to be expressed in the mammalian central nervous system, where they are thought to be responsible for Ca$^{2+}$-induced Ca$^{2+}$ release (36). In situ hybridization and immunolocalization studies have revealed a cell type-specific pattern of expression in the different regions of the central nervous system (37–40). Several recent studies have provided evidence that RyRs are under strict
developmental control. First, Ry1R and Ry3R expression have been shown to be regulated by cytokines and growth factors (41–43). Second, using polymerase chain reaction analysis, Futatsugi et al. (44) have identified two alternatively spliced regions in mRNA of mouse Ry1R, which are characterized by the presence or absence of amino acid sequences that exist within the region where modulatory sites for phosphorylation and binding of Ca\(^{2+}\), calmodulin, and ATP are postulated to exist. The ratio of Ry1R splice variants changes abruptly in the cerebrum between embryonic days 14 and 18. Third, RyRs have been found to be expressed in neural growth cones, where they are thought to play an important role in buffering and releasing Ca\(^{2+}\) during intracellular Ca\(^{2+}\) oscillations (45). Interestingly, RyRs appear to regulate the amplitude of Ca\(^{2+}\) spiking behavior in the growth cone, suggesting a role in signal amplification. Since the homeostatic mechanisms controlling intracellular Ca\(^{2+}\) dynamics of growth cones are likely to be important determinants of growth cone migration during development, it is worthwhile to speculate how prenatal exposure to PCB 95 might alter RyR function and its relationship to reduced motor activity and radial arm maze performance (24).

**Fig. 7.** PCB 95 (50 \(\mu M\)) does not affect IP\(_3\) binding to cerebellar microsomes. Specific binding of 0.5 nM \([\text{3H}]\text{IP}_3\) in the presence of 0–100 \(\mu M\) PCB 95 (○) or 100 \(\mu M\) PCB 126 (-schema) was determined as described under “Experimental Procedures.” Note that neither low \(\mu M\) PCB 95 (1–50 \(\mu M\)) nor PCB 126 (100 \(\mu M\)) altered the IP\(_3\) binding significantly as compared with the control value. However, at 25 °C, 25–60 \(\mu M\) PCB 95 significantly enhanced the \([\text{3H}]\text{IP}_3\) binding. The figure shows the mean ± S.E. of two and four determinations at 4 °C and 25 °C, respectively. (*, \(p < 0.05\), two-tailed Student’s \(t\) test, \(\alpha = 0.05\)).

**Fig. 8.** PCB 95 enhances \([\text{3H}]\text{ryanodine binding by stabilizing the high affinity binding conformation of the receptors. Representative saturation curves of the binding of 0.5–70 nM \([\text{3H}]\text{ryanodine to brain microsomes isolated from rat cerebellum in the presence of 50 \(\mu M\) PCB 95 and physiological salt (○) and the respective control in the presence of 200 mM K\(^+\) (●), performed as described under “Experimental Procedures.” Inset, Scatchard plot of the binding data. The mean (± S.E.) \(K_D\) and \(B_{max}\) from four replicated PCB 95 experiments each performed in duplicate were 3.7 ± 0.6 nM and 67.9 ± 4.6 fmol/mg, respectively. \(K_D\) and \(B_{max}\) from four replicated control experiments were 24.5 ± 3.3 nM and 51.6 ± 1.3 fmol/mg.}

**Fig. 9.** PCB 95 alters Ca\(^{2+}\) and Mg\(^{2+}\) modulation of RyRs. Equilibrium binding of 2 nM \([\text{3H}]\text{ryanodine to cerebellar microsomes in the presence of 50 \(\mu M\) PCB 95 (○) or control in the presence of 200 mM K\(^+\) (●) was performed as described under “Experimental Procedures.” A, PCB 95 (50 \(\mu M\)) altered the Ca\(^{2+}\) modulation of \([\text{3H}]\text{ryanodine binding. Maximal specific binding of \([\text{3H}]\text{ryanodine (at 200 mM Ca}\(^{2+}\)) is 44.5 ± 1.2 fmol/mg in the presence of PCB 95, and is 8.7 ± 0.6 fmol/mg for 200 mM K\(^+\) control. The data shown are the mean % of maximal binding ± S.E. of four determinations. B, PCB 95 (50 \(\mu M\)) altered the potency and extent of inhibition of \([\text{3H}]\text{ryanodine binding by Mg}\(^{2+}\). Maximal specific \([\text{3H}]\text{ryanodine binding (in the absence of Mg}\(^{2+}\)), in the presence of PCB 95 and 200 mM KCl control, are 30.7 ± 3.8 and 9.9 ± 1.6, respectively. The data shown are the mean % of maximal binding ± S.E. of four determinations. Hill coefficients and EC\(_{50}\) value for Ca\(^{2+}\), and IC\(_{50}\) values for Ca\(^{2+}\) and Mg\(^{2+}\) are summarized in Table II.**
PCB congeners with two or three chlorine substituents in the ortho-position confer the highest potency and efficacy toward RyRs of the mammalian brain. Of the congeners assayed, PCB 95 (2,2',3,5',6-pentachlorobiphenyl) and PCB 4 (2,2'-dichlorobiphenyl) exhibit the highest potency and efficacy. Therefore, the position of the chlorine substituents on PCBs is more important toward conferring RyR activity than the degree of chlorination. The four ortho-chloro substituents of PCB 104 (2,2',4,6,6'-pentachlorobiphenyl) contribute significant steric constraint which severely limit rotation about the biphenyl bond. The finding that PCB 104 has significantly lower receptor potency than PCB 95 and PCB 4 suggests that although a non-coplanar conformation of the biphenyl structure appears to be critical for receptor activity, a certain degree of rotational flexibility about the biphenyl bond seems to be required to produce maximum activation of RyRs. The high receptor activity exhibited by PCB 4 can reflect an induced fit of this congener with its binding domain on the receptor complex. Other than three ortho-chloro substituents, substitutions at the meta- and para-positions are also important for optimal activity at RyRs. This is exemplified by the stringent structure-activity relationship among pentachlorobiphenyls, which reveals a ranked potency of 2,2',3,5,6- > 2,2',4,5,6- > 2,2',4,6,6-pentachlorobiphenyl.

PCBs Modulate RyRs by a Novel Mechanism—Typically, studies of the high affinity binding of [3H]ryanodine to RyRs of mammalian brain microsomes have been performed in the presence of high salt (1 mM KCl or NaCl) (25, 48–50). [3H]Ryanodine binding to its high affinity sites was shown to be modulated by Ca$^{2+}$, Mg$^{2+}$, caffeine, and adenosine nucleotides (25, 50), in a manner qualitatively similar to those reported for skeletal and cardiac SR preparations. The requirement for high salt may be important to stabilize an open conformation of the receptors which recognizes [3H]ryanodine with high affinity but the underlying mechanism has remained unclear. Certain ortho-substituted PCB congeners (e.g. PCB 95) effectively eliminate the requirement of high salt in the assay medium. Results from saturation binding of [3H]ryanodine to cerebellar microsomes in the presence of a minimal concentration of salt (200 mM K$^+$) to permit measurement of high affinity binding of [3H]ryanodine reveal a 6.5-fold lower affinity and 1.5-fold lower capacity compared with an assay medium containing physiologically relevant K$^+$ and Na$^+$, and PCB 95. Moreover, the value of $K_D$ in the presence of PCB 95 is similar to that previously reported by Zimanyi and Pessah ($K_D = 1–3$ nM) and Padua et al. ($K_D = 2.4$ nM), in the presence of 1 mM KCl (25, 50). Thus PCB 95 appears to significantly stabilize a single high affinity state (Ca$^{2+}$-conducting state) even in the presence of physiologically relevant K$^+$ and Na$^+$.

The mechanism by which PCB 95 favors the high affinity state of brain RyR appears to be related to its ability to dramatically alter the responses of the channel to two important physiological ligands, Ca$^{2+}$ and Mg$^{2+}$. It has been previously demonstrated that micromolar Ca$^{2+}$ is required to activate high affinity binding of [3H]ryanodine to brain microsomes in the presence of 1 mM K$^+$ (50). Under the same conditions, millimolar Ca$^{2+}$ has been shown to fully inhibit the binding of [3H]ryanodine to its high affinity site (50). In the present paper, we report that PCB 95 significantly alters the sensitivity of RyRs to activation and inhibition by Ca$^{2+}$, shifting the $EC_{50}$ value to 61 nM and essentially eliminating inhibition. Zimanyi and Pessah have reported an $IC_{50}$ for Mg$^{2+}$ of 10 mM in high salt (1 mM K$^+$) (25), and Padua and co-workers (50) have reported $IC_{50}$ values for Mg$^{2+}$ of 2 mM and 5 mM assayed in 200 mM K$^+$ and 1 mM K$^+$, respectively. In this respect, PCB 95 alters Mg$^{2+}$ inhibition in two important ways; 1) it shifts the $IC_{50}$ for...
susceptible sites nearly 20-fold compared with control lacking PCB, and 2) it completely eliminates inhibition for approximately 50% of the measurable sites. Therefore, altered modulation by Ca$^{2+}$ and Mg$^{2+}$ appears to underlie the ability of PCB 95 to stabilize a high affinity [3H]ryanodine-binding conformation of RyRs of the brain.

In conclusion, ortho-substituted PCBs are shown for the first time to directly and selectively activate the RyR/Ca$^{2+}$ release channel complex in adult rat brain. Structure-activity relationship studies performed with selected PCBs indicate a stringent structural requirement for activation of RyRs in central nervous system. ortho-Substituted PCB 95, the most potent congener studied, mobilizes microsomal Ca$^{2+}$ selectively and specifically from ryanodine-sensitive microsomes. Disruption of Ca$^{2+}$ homeostasis in the affected regions of the brain by certain ortho-substituted PCBs may contribute significantly in altering neurodevelopment and neuropsychiatric function in mammals.

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