0026-895X/96/040740-12$3.00/0

MOLECULAR PHARMACOLOGY, 49:740–751 (1996).

Ortho-Substituted Polychlorinated Biphenyls Alter Calcium Regulation by a Ryanodine Receptor-Mediated Mechanism: Structural Specificity toward Skeletal- and Cardiac-Type Microsomal Calcium Release Channels

PATTY W. WONG and ISAAC N. PESSAH

Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California 95616

Received November 16, 1995; Accepted January 4, 1996

SUMMARY

We investigated a novel molecular mechanism by which polychlorinated biphenyls (PCBs) alter microsomal Ca^{2+} transport with sarcoplasmic reticulum (SR) membranes isolated from skeletal and cardiac muscles. Aroclors with an intermediate weight percent of chlorine enhance by >6-fold the binding of 1 nM [^{3}H]ryanodine to its conformationally sensitive site on the SR Ca^{2+}-release channel [i.e., ryanodine receptor (RyR)] with high potency (EC_{50} = 1.4 μM), whereas Aroclors with either high or low chlorine composition show little activity. Structure-activity studies with selected pentachlorobiphenyl congeners reveal a stringent structural requirement for chlorine substitution at the *ortho*-positions, with 2,2′,3,5′,6-pentachlorobiphenyl having the highest potency toward the skeletal and cardiac isoforms of RyR (EC_{50} = 330 nM and 2 μM, respectively). In contrast, 3,3′,4,4′,5-pentachlorobiphenyl does not enhance ryanodine binding, suggesting that noncoplanarity of the biphenyl rings is required for channel activation. However, 2,2′,4,6,6′-pentachlorobiphenyl is significantly less active toward RyR, suggesting that some degree of rotation about the biphenyl bond is required. 2,2′,3,5′,6-Pentachlorobiphenyl induces a dose-dependent release of Ca^{2+} from actively loaded SR vesicles with a maximum rate of 1.2 μmol mg^{-1} min^{-1} (EC_{50} = 1 μM), whereas 3,3′,4,4′,5-pentachlorobiphenyl (≤10 μM) does not alter Ca^{2+} transport. The mechanism of PCB-induced channel activation involves a significant decrease in the inhibitory potency of Ca^{2+} and Mg^{2+} (20-fold and 100-fold, respectively). Neither 2,2′,3,5′,6- nor 3,3′,4,4′,5-pentachlorobiphenyl (≤10 μM) alters the activity of the skeletal isoform of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase or the cardiac isoform of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, and PCB-induced Ca^{2+} release can be fully blocked by either μM ryanodine or ruthenium red. These results are the first to demonstrate a selective ryanodine receptor-mediated mechanism by which *ortho*-substituted PCBs alter microsomal Ca^{2+} transport and may have toxicological relevance.

PCBs are a family of bicyclic chlorinated aromatic hydrocarbons composed of 209 possible congeners. Commercial PCB mixtures can be synthesized at low cost through iron-catalyzed chlorination of biphenyl (1). Between 1929 and 1978, technical PCB mixtures, called Aroclors, were marketed for a wide variety of industrial applications, with an estimated worldwide production of 1.5 million metric tons (2, 3). In the United States, PCB mixtures were classified according to the average degree of chlorination: Aroclors 1221–1268, with the first two digits designating the biphenyl structure and the last two digits indicating the weight percentage of chlorine in the respective mixtures. Although the use of PCBs has been completely banned since 1978, improper disposal and persistence of the compounds in the environment have resulted in global contamination (3–5). The high hydrophobicity of PCBs has further promoted accumulation in biota, including human adipose tissue, serum, and milk (2, 6–10).

Oral administrations of the PCB mixture Aroclor 1254 to rats and primates have been shown to decrease dopamine levels in the mammalian brains *in vivo* (11, 12). The decline

The results were presented at the Annual Meeting of Society of Toxicology, Baltimore, MD [*Toxicologist* 15:189 (1995)].

This work was supported by Pilot Project Grant ES05707 from the Center for Environmental Health Sciences (I.N.P.) and Training Grant ES07059 from the National Institute of Environmental Health Sciences (P.W.W.).

ABBREVIATIONS: Ah, arylhydrocarbon; DMSO, dimethylsulfoxide; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; ER, endoplasmic reticulum; FKBP12, FK506 12-kDa binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PCB, polychlorinated biphenyl; RyR, ryanodine receptor; RyR1, skeletal isoform of ryanodine receptor; RyR2, cardiac isoform of ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; 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.

in brain dopamine seems to be caused by the presence of *ortho*-substituted PCB congeners in the mixture, whose structures favor a nonplanar conformation of the phenyl rings (12). Structure-activity studies with selected PCB congeners have further substantiated that several *ortho*-substituted PCB congeners are more potent than those lacking *ortho* chlorines in reducing the dopamine levels of neurogenic cells in culture (13). Recently, Nishida *et al.* (14) demonstrated that repeated oral doses of Aroclor 1254 to adult rats cause a decrease in motor activity. The molecular mechanisms by which neuroactive PCBs reduce cellular and brain levels of dopamine and alter motor activity are not understood. However, perturbations of intracellular Ca^{2+} homeostasis may play a significant role in the biological activity of *ortho*-substituted PCBs. Kodavanti *et al.* (15) demonstrated that nonplanar 2,2'-dichlorobiphenyl, but not coplanar 3,3',4,4',5-pentachlorobiphenyl, increases cytosolic Ca^{2+} in primary cultures of cerebellar granule cells and inhibits Ca^{2+} transport across isolated mitochondria and synaptosomes. This initial study suggests that, at least in part, general inhibition of Ca^{2+}-dependent ATPases may be responsible for the decreased ability of organelles to sequester Ca^{2+}. In support of a Ca^{2+}-dependent mechanism underlying their biological activities, Kodavanti *et al.* recently showed that di-*ortho*-substituted PCB congeners are most potent toward enhancing protein kinase C translocation in rat cerebellar granule cells (16).

Excitation-contraction coupling in striated muscle represents one of the best understood forms of signal transduction (17). Membrane vesicles from regions of the terminal cisternae of SR can be isolated with high purity from skeletal and cardiac muscles (18, 19). These "junctional" SR vesicles possess the major proteins that are responsible for the sequestration and release of Ca^{2+} during relaxation and contraction cycles, respectively. The SERCA1 and SERCA2 isoforms (Ca^{2+}-dependent ATPases) are responsible for the energy-dependent uptake of Ca^{2+} into the lumen of SR, whereas ryanodine-sensitive Ca^{2+}-release channel isoforms RyR1 and RyR2 are responsible for the rapid mobilization of Ca^{2+} from SR in skeletal and cardiac muscles, respectively (17). In addition, these preparations contain additional accessory proteins that are essential for the integrity of SR Ca^{2+} transport, i.e., calsequestrin (20, 21), triadin (22, 23), immunophilins (FK506 binding proteins) (24–26), and calmodulin (27, 28).

In the current study, we used model membrane preparations from skeletal and cardiac muscles to examine the mechanism by which PCBs alter Ca^{2+} transport across microsomal membranes. Evidence is provided for a highly selective interaction between *ortho*-substituted PCB congeners and the skeletal and cardiac isoforms of ryanodine receptors, RyR1 and RyR2, respectively, whereas no activity toward SERCA1 and SERCA2 pumps could be demonstrated. *Ortho*-substituted PCBs exhibit a stringent structural requirement toward activating ryanodine-sensitive Ca^{2+}-release channels, with 2,2',3,5',6-pentachlorobiphenyl (PCB 95) possessing the highest potency and efficacy of the congeners tested. Considering the essential role of RyR1 and RyR2 in muscle excitation-contraction coupling and, more importantly, their wide distribution in the mammalian central nervous system, the present study provides a receptor-mediated mechanism by which certain PCB structures could alter Ca^{2+} signaling in excitable cells. Moreover, the assay methods provide a fast and efficient way to obtain the complete spectrum of structure-activity relationships of all 209 PCB congeners based on their abilities to modulate SR/ER Ca^{2+} release and ryanodine receptor channel activity.

Experimental Procedures

Materials. Neat technical grade Aroclor PCB mixtures and neat Ultra-certified PCB congeners with purity of >99% were obtained from Ultra Scientific (North Kingstown, RI). [^{3}H]Ryanodine was obtained from New England Nuclear (Wilmington, DE) (specific activity, 60–80 Ci/mmol; purity, >99%). High purity ryanodine (>99%) was obtained from Calbiochem (San Diego, CA). All other chemicals were of the highest grade available commercially.

Preparations of PCB stock solutions. PCB stock solutions (10 mM) were prepared by dissolving the compounds in appropriate amounts of anhydrous DMSO and storing the solutions in borosilicate glass vials sealed with Teflon caps. Lower-concentration stocks were obtained by serial dilution with DMSO with a Hamilton syringe.

Membrane preparations. Membrane vesicles enriched in RyR1 were prepared from fast-twitch (white) skeletal muscles obtained from back and hind limbs of 3–4-kg male New Zealand White rabbits. Freshly ground muscle was homogenized in a Waring blender with 4 volumes ice-cold homogenization buffer consisting of 5 mM imidazole-HCl, pH 7.4, 0.3 M sucrose, 10 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride. The microsomal fraction was obtained by differential centrifugation, and the junctional SR fraction was purified by discontinuous sucrose gradient according to the method of Saito *et al.* (18). The junctional SR fraction was collected from the 38%/45% sucrose interface, pelleted, and then resuspended in ice-cold homogenization buffer at a protein concentration of 3–5 mg/ml. Protein concentration was determined according to the method of Lowry (29) with bovine serum albumin as a standard. Membranes were aliquoted into vials, quickly frozen in liquid nitrogen, and stored at −80°.

Membrane vesicles enriched in RyR2 were prepared from ventricles of male Sprague-Dawley rats (250–300 g) as according to the method of Feher and Davis (19), with the addition of 5 μg/ml leupeptin and 100 μM phenylmethylsulfonyl fluoride to the homogenization buffer. The final pellets containing SR membrane vesicles were resuspended in buffer consisting of 20 mM Tris, pH 7.0, and 30% sucrose, at a protein concentration of ~4 mg/ml (29). Aliquots were quickly frozen in liquid nitrogen and stored at −80°.

[^{3}H]Ryanodine binding assays. Specific binding of [^{3}H]ryanodine to skeletal and cardiac membrane vesicles was determined according to the methods of Pessah *et al.* (30). The ability of selected Aroclor mixtures and PCB congeners to dose-dependently enhance the binding of 1 nM [^{3}H]ryanodine to high affinity sites on skeletal SR (6.3 μg protein) or cardiac SR (44 μg protein) was assayed in a buffer consisting of 20 mM HEPES, pH 7.1, 250 mM KCl, 15 mM NaCl, and 50 μM $CaCl_2$ (skeletal) or 10 μM $CaCl_2$ (cardiac). Aroclor mixture or PCB congener (10 nM–10 μM) was added to the reaction mixture and incubated for 3 hr at 37°.

Saturation curves for high affinity binding of 0.5–35 nM [^{3}H]ryanodine to RyR1 (12.5 μg protein) were measured at 37° with a 3.5-hr incubation in 20 mM HEPES, pH 7.1, 140 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, and 10% sucrose in the presence of 0, 328 nM, or 10 μM 2,2',3,5',6-pentachlorobiphenyl. The modulatory effects of Ca^{2+} and Mg^{2+} on the binding of [^{3}H]ryanodine (1 nM) to RyR1 (12.5 μg) were evaluated in the presence or absence of 10 μM Aroclor 1254 or 2,2',3,5',6-pentachlorobiphenyl by titrating free Ca^{2+} (1 nM–100 μM) or free Mg^{2+} (5 μM–2 M). Free ion concentrations in the assays were adjusted by adding EGTA based on the SPECS computer software and published stability constants (31).

Each binding reaction was initiated by the addition of microsomal

preparation to [^{3}H]ryanodine assay buffer and followed by the addition of PCB with a Hamilton syringe (250 μl final volume). Assays were terminated by rapid filtration, with a Brandel (Gaithersburg, MD) cell harvester, through Whatman GF/B glass fiber filters. Filters were rinsed twice with 1.3 ml of ice-cold harvest buffer (20 mM Tris·HCl, pH 7.1, 250 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$) and soaked overnight in 5 ml of scintillation cocktail. Radioactivity on the filters were measured with a liquid scintillation counter. Nonspecific binding of [^{3}H]ryanodine was determined by the addition of 1000-fold excess of cold ryanodine. Each experiment was performed in duplicate and repeated at least three times with different membrane preparations.

Data analysis of binding assays. Specific binding of [^{3}H]ryanodine to RyR was determined by subtracting nonspecific binding from total binding. PCB enhanced only the specific component of binding; thus, data were analyzed with sigmoidal curve fitting of specific [^{3}H]ryanodine binding (pmol/mg of protein) against log concentrations of PCBs with the use of ENZFITTER (Elsevier BioSoft, London, UK) computer software. EC_{50} values and Hill coefficients for each Aroclor mixture or PCB congener were obtained from linear regression analysis of log-logit transformations [$\log(B/(B_{max} - B)$] versus log concentrations of PCBs) with the use of CA-Cricket Graph III (Computer Associates, Islandia, NY) computer software and nonlinear regression analysis with ENZFITTER, respectively. EC_{50} values for Ca^{2+} activation of the binding of [^{3}H]ryanodine and IC_{50} values for inhibition by mM Ca^{2+} or Mg^{2+} in the presence or absence of Aroclor 1254 or 2,2',3,5',6-pentachlorobiphenyl were analyzed with log-logit analysis as described above. Binding constants (K_D and B_{max}) of ryanodine to its high affinity site on RyR1 or RyR2 were calculated by linear regression analysis of Scatchard plots.

Ca^{2+} transport measurements. Net uptake or release of Ca^{2+} from skeletal and cardiac microsomal vesicles was measured with the metallochromic dye antipyrylazo III with the use of a diode array spectrophotometer (model 8542, Hewlett Packard, Palo Alto, CA). The Ca^{2+} transport buffer consisted of 18.5 mM K-MOPS, pH 7.0, 92.5 mM KCl, 7.5 mM Na-pyrophosphate, 250 μM antipyrylazo III, 1 mM Mg-ATP, 20 μg/ml creatine phosphokinase, 5 mM phosphocreatine, and 50 μg skeletal or 100 μg cardiac SR protein to give a final volume of 1.2 ml (32). Transport assays were performed in temperature-controlled cuvettes at 37° with constant stirring. Vesicles were loaded to near-capacity by serial additions of 24 (skeletal) or 12 (cardiac) nmol $CaCl_2$. SR Ca^{2+} transport function was measured by recording changes in extravesicular free Ca^{2+} determined by subtracting the antipyrylazo III absorbance at 790 nm from the absorbance at 710 nm. Once the loading phase was completed and the dye signal returned to base-line, the ability of PCB to mobilize the accumulated Ca^{2+} from the vesicles was examined in the presence or absence of Ca^{2+} channel blockers. At the end of each experiment, absorbance signals were calibrated by the addition of 1 μg of Ca^{2+} ionophore A23187, followed by 12 or 24 nmol of $CaCl_2$ from a National Bureau of Standards stock solution.

Measurement of Ca^{2+} uptake rate by SERCA2 pump was performed with cardiac SR membrane vesicles. First, 500 μM ryanodine was added to the vesicle suspension to fully block ryanodine-sensitive Ca^{2+}-release channels (33). Once the antipyrylazo III signal returned to base-line, PCB or DMSO (control) was added to the cuvette. After allowing the reaction mixtures to equilibrate for 1 min, a bolus of 24 nmol Ca^{2+} was added to each cuvette, and rate of Ca^{2+} uptake by the cardiac SR vesicles was measured as the decline in antipyrylazo III absorbance over time. Initial rates of Ca^{2+} uptake by cardiac membrane vesicles, in the presence or absence of 10 μM PCBs, were calculated by fitting a single exponential decay function to the initial 100 sec of the uptake data. Rate data were analyzed with paired test (two-tailed, $\alpha = 0.05$) with the use of Excel 4.0 (Microsoft, Redmond, WA) computer software.

ATPase assays. Rates of ATP hydrolysis were determined with a coupled enzyme assay measuring the oxidation of NADH as a linear decrease in absorbance at 340 nm (34). Briefly, 50 μg of skeletal membrane vesicles was added to the temperature-controlled cuvettes (37°) containing assay buffer consisting of 5 mM HEPES, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 60 μM EGTA,, 100 μM $CaCl_2$, 0.3 mM sucrose, 2 mM phospho(enol)pyruvate, 0.8 mM NADH, 24 units/ml LDH, 16.8 units/ml pyruvate kinase, and 1.5 μg/ml A23187 (final volume of 1.2 ml). The spectrophotometer was zeroed, and reactions were started by the addition of 1 mM Na_2ATP. Total ATPase activities, in the presence or absence of PCBs, were recorded for ~30 sec. Ca^{2+}-independent ATPase activities was then measured, followed by the addition of 4 mM K_2EGTA to the reaction mixtures. Ca^{2+}-dependent rates were calculated as the difference between total and Ca^{2+}-independent rates. ATPase activity data were analyzed with Student's t test (two-tailed, $\alpha = 0.05$) with Excel 4.0.

Results

Aroclors enhance [^{3}H]ryanodine binding to RyR1. Aroclor mixtures possessing an increasing percentage of chlorine by weight were examined for their abilities to alter the binding of [^{3}H]ryanodine to high affinity sites on the RyR1/Ca^{2+}-release channel complexes. Fig. 1 shows that commercial Aroclor containing a high (Aroclor 1268) or low (Aroclors 1221 and 1232) weight percentage of chlorine in the mixtures, $\leq$10 μM (based on the average molecular weight of the compounds in the respective mixture), have little influence on the binding of [^{3}H]ryanodine to its conformationally sensitive site on the skeletal Ca^{2+}-release channel complex. In marked contrast, Aroclors with an intermediate weight percentage of chlorine in the mixtures (Aroclors 1248, 1254, and

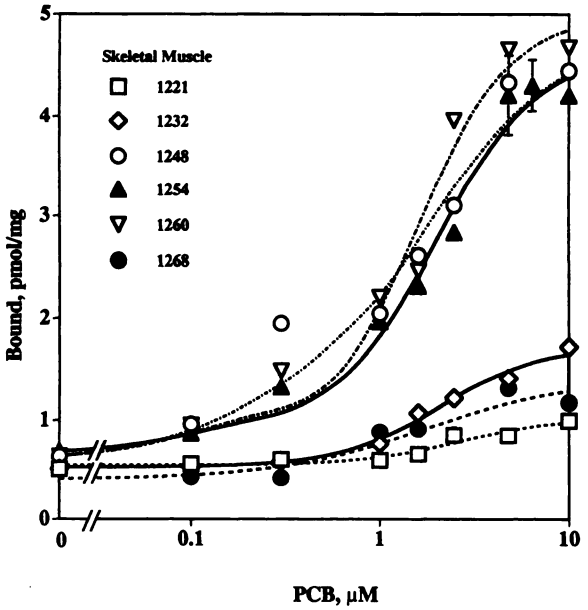

Fig. 1. Aroclors with intermediate weight percentages of chlorine enhance [^{3}H]ryanodine binding to RyR1. Equilibrium binding of [^{3}H]ryanodine to RyR1 from skeletal muscle SR was performed in buffer consisting of 20 mM HEPES, pH 7.1, 250 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, 1 nM [^{3}H]ryanodine, and 6.3 μg skeletal SR protein, as described in Experimental Procedures. Aroclors 1248 (one experiment), 1254 (four experiments), and 1260 (one experiment) show dose-dependent enhancement of [^{3}H]ryanodine binding to RyR1, whereas Aroclors 1221 (one experiment), 1232 (one experiment), and 1268 (one experiment) show no activity toward the receptor. Experiments were performed in duplicate, and data for Aroclor 1254 are the mean ± standard error of four experiments. None of the active Aroclors altered nonspecific [^{3}H]ryanodine binding. Hill coefficients and EC_{50} values of the active Aroclors are summarized in Table 1.

1260) enhance the high affinity binding of [^{3}H]ryanodine to the RyR1/Ca^{2+}-release channel complex in a potent and dose-dependent manner. The EC_{50} values for activating radioligand binding by Aroclors 1248, 1254, and 1260 are essentially the same, ranging between 1.3 and 1.4 μM. The respective Hill coefficient for each active PCB mixture ranges between 1.7 and 1.8 (Fig. 1). Under the assay conditions used, a saturating concentration (10 μM) of Aroclor 1248, 1254, or 1260 enhances [^{3}H]ryanodine (1 nM) binding to RyR1 by ~6.7-fold compared with the controls, increasing occupancy from 0.68 ± 0.02 to 4.6 ± 0.1 pmol/mg. Table 1 summarizes the activity of Aroclor mixtures toward RyR1.

***Ortho*-substituted PCB congeners enhance [^{3}H]ryanodine binding to RyR1 and RyR2.** The structure-activity relationships for activation of [^{3}H]ryanodine binding to skeletal RyR1 and cardiac RyR2 were examined for a selected group of pentachlorobiphenyls (Fig. 2, A and B). Under the present assay conditions, coplanar 3,3',4,4',5-pentachlorobiphenyl, which lacks *ortho*-substitution, ≤10 μM, does not alter the binding of [^{3}H]ryanodine to either isoform of the receptor. The mono-*ortho*-substituted, coplanar 2,3,3',4,4'-pentachlorobiphenyl has weak activity toward RyR1. The presence of two *ortho*-chloro substituents favors a nonplanar conformation of the phenyl rings and imparts ryanodine receptor activity. The 2,2',3,3',4- and 2,2',4,6,6'-pentachlorobiphenyl congeners enhance occupancy of [^{3}H]ryanodine to RyR1 in a dose-dependent manner, with EC_{50} values of 1.2 and 0.6 μM, respectively. At saturating concentrations, 10 μM 2,2',3,3',4- and 2,2',4,6,6'-pentachlorobiphenyl enhance ryanodine binding to RyR1 by 4.2-fold and 4.8-fold over the controls, with specific occupancy reaching 2.9 and 3.3 pmol/mg, respectively. The tri-*ortho*-substituted, nonplanar 2,2',3,5',6-pentachlorobiphenyl congener exhibits remarkable potency and efficacy toward RyR1 and RyR2, with EC_{50} values of 0.33 and 2.0 μM, respectively, and Hill coefficients significantly greater than unity (Fig. 2, A and B). At saturating concentration, 10 μM 2,2',3,5',6-pentachlorobiphenyl enhances [^{3}H]ryanodine occupancy of RyR1 and RyR2 by ~11-fold and ~2-fold over the respective controls. Table 1 summarizes the activity of selected pentachlorobiphenyls toward the skeletal- and cardiac-type receptors.

***Ortho*-substituted 2,2',3,5',6-pentachlorobiphenyl induces Ca^{2+} release from skeletal muscle SR via a ryanodine receptor-mediated mechanism.** The addition of 1–10 μM 3,3',4,4',5-pentachlorobiphenyl to skeletal SR vesicles actively loaded to near-capacity with Ca^{2+} has no influence on net Ca^{2+} transport across the membranes, nor does it alter the sensitivity or calibration of the antipyrylazo III Ca^{2+} indicator, as shown by the responses to ionophore A23187 (Fig. 3A). In contrast, the addition of 2,2',3,5',6-pentachlorobiphenyl causes a rapid release of accumulated Ca^{2+}. The initial rate of release is dose dependent in the range of 200 nM to 3 μM and is saturable between 1.5 and 3 μM (Fig. 3B). The concentration of 2,2',3,5',6-pentachlorobiphenyl that induces a half-maximal rate of Ca^{2+} release is 1.2 μM, and the Hill number for the dose-response relationship is 5.5 (Fig. 3B, *inset*). The amount of Ca^{2+} released approximates 64% of the total Ca^{2+} accumulated in the vesicles during the loading phase, regardless of the concentration of 2,2',3,5',6-pentachlorobiphenyl. Final calibrations with Ca^{2+} ionophore A23187 reveal that SR vesicles in each cuvette are loaded equally with Ca^{2+} and that the responses of antipyrylazo III to Ca^{2+} are unaffected by the PCB congener.

Mechanism of *ortho*-substituted PCB-induced Ca^{2+} release from SR. The ability of 2,2',3,5',6-pentachlorobiphenyl to mobilize Ca^{2+} from actively loaded SR vesicles could be the result of (i) activation of a ryanodine-sensitive efflux pathway, and/or (ii) inhibition of the SERCA pump. To discriminate between these two possibilities, experiments with skeletal SR vesicles were performed in the presence or absence of high, channel-blocking, concentrations of ryano-

TABLE 1

Structure-activity relationships for Aroclor mixtures and selected pentachlorobiphenyls for activation of the binding of 1 nM [^{3}H]ryanodine to RyR1/Ca^{2+}-release channel complex of rabbit skeletal SR and RyR2/Ca^{2+}-release channel complex of rat cardiac SR

	Maximal occupancy[b]	EC_{50}	Hill coefficient
	pmol/mg	*μM*	
RyR1 of skeletal SR[a]			
PCB			
Control (11)	0.7 ± <0.1		
Aroclor			
1248 (one)	4.7	1.3	1.8
1254 (four)	4.5 ± 0.1	1.4 ± 0.1	1.7 ± 0.3
1260 (one)	4.7	1.3	1.8
PCB congener			
3,3',4,4',5 (10)	Inactive	Inactive	Inactive
2,3,3',4,4' (one)	1.5	0.3	1.9
2,2',3,3',4 (three)	2.9 ± 0.3	1.2 ± 0.1	1.6 ± 0.3
2,2',3,5',6 (three)	7.2 ± 0.2	0.33 ± 0.02	1.5 ± 0.1
2,2',4,6,6' (three)	3.3 ± 0.1	0.57 ± 0.06	0.94 ± 0.09
RyR2 of cardiac SR[a]			
PCB			
Control (two)	1.1 ± <0.1		
PCB congener			
3,3',4,4',5 (one)	Inactive	Inactive	Inactive
2,2',3,5',6 (one)	1.8	2.1	4.0

The data represent the mean ± standard error of the number of replicate experiments indicated in parentheses.

[a] Binding assays were performed in a buffer consisting of 20 mM HEPES, pH 7.1, 250 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, 1 nM [^{3}H]ryanodine, and, in RyR1, 6.3 μg of skeletal SR protein or, in RyR2, 44 μg of cardiac SR protein, as described in Experimental Procedures.

[b] Maximal occupancy at saturating PCB concentrations and nonsaturating [^{3}H]ryanodine (1 nM).

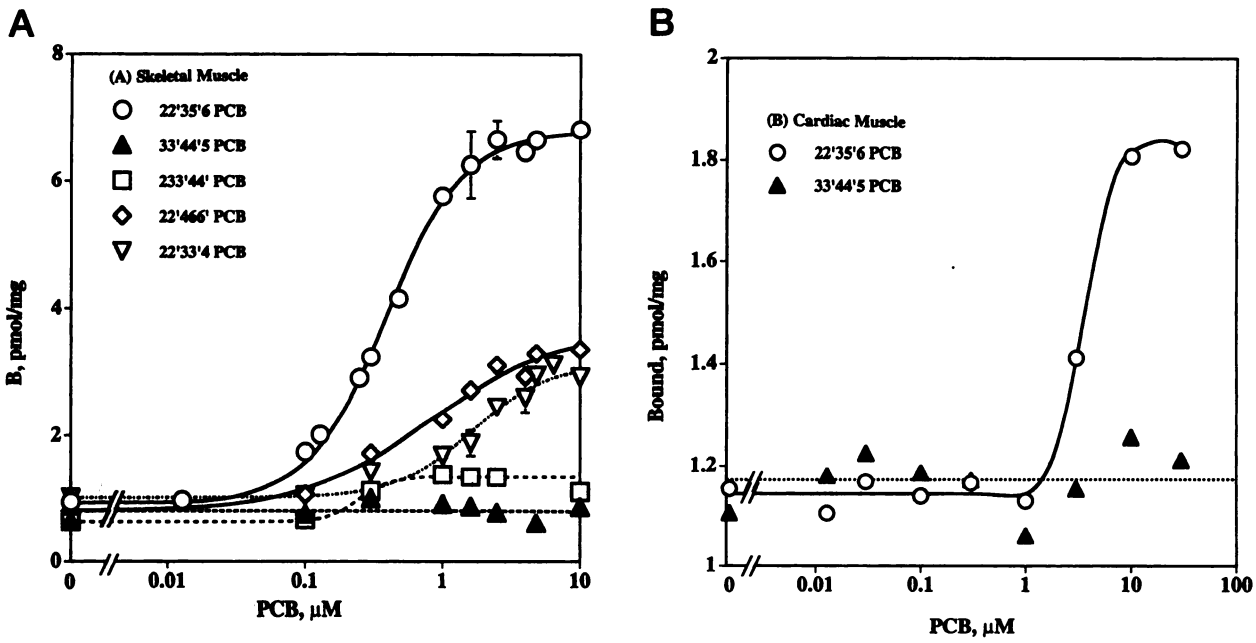

Fig. 2. *Ortho*-substituted PCBs enhance [^{3}H]ryanodine occupancy of both skeletal RyR1 and cardiac RyR2. Equilibrium binding of [^{3}H]ryanodine to (a) skeletal SR was performed as described in legend to Fig. 1 and to (b) cardiac SR was performed in buffer consisting of 20 mM HEPES, pH 7.1, 250 mM KCl, 15 mM NaCl, 10 μM $CaCl_2$, 1 nM [^{3}H]ryanodine, and 44 μg of cardiac SR protein. None of the active PCB congeners altered nonspecific [^{3}H]ryanodine binding. Experiments were performed in duplicate, and data are mean ± standard error of the replicated experiments. Hill coefficients and EC_{50} values are summarized in Table 1.

dine. The addition of 500 μM ryanodine to the actively loaded skeletal SR vesicles suspension induces rapid activation of the SR channels and net Ca^{2+} release, followed by inhibition of the channels and reaccumulation of Ca^{2+} (Fig. 4, *trace a*). The biphasic response to high μM ryanodine is consistent with a time-dependent sequential mechanism (35, 36). The addition of 1 μM 2,2',3,5',6-pentachlorobiphenyl to the transport assay, once ryanodine fully blocks RyR1 and the Ca^{2+} is reaccumulated into the SR lumen, fails to induce net Ca^{2+} efflux (Fig. 4, *trace a*). Furthermore, once saturating (1 μM) 2,2',3,5',6-pentachlorobiphenyl induces Ca^{2+} release from control SR vesicles, the addition of 500 μM ryanodine at the plateau results in complete reaccumulation of extravesicular Ca^{2+} (Fig. 4, *trace b*). The rate of Ca^{2+} reaccumulation after PCB and ryanodine exposure is essentially the same as that observed with ryanodine alone (Fig. 4, compare *traces a and b*), suggesting a RyR1-specific action of the PCB. Similar results were obtained in Ca^{2+} transport studies with cardiac membrane vesicles enriched in RyR2 and SERCA2 (Fig. 5). Like skeletal SR, the addition of 10 μM 2,2',3,5',6-pentachlorobiphenyl releases ~55% of the total Ca^{2+} accumulated within the cardiac SR vesicles (Fig. 5, *trace a*) and the release is completely inhibited by the prior blockade of RyR2 with 500 μM ryanodine (Fig. 5, *trace b*). In contrast, coplanar 3,3',4,4',5-pentachlorobiphenyl does not alter Ca^{2+} transport in isolated cardiac SR regardless of whether the membranes are first exposed to 500 μM ryanodine (Fig. 5, *traces c and d*). These results demonstrate that 2,2',3,5',6-pentachlorobiphenyl can directly and rapidly activate RyR1 and RyR2 Ca^{2+}-release channels without altering the activity of SERCA1 or SERCA2 pumps. The stringent structural requirement observed with *ortho*-chloro substituents for PCB-induced release of SR Ca^{2+} and activation of [^{3}H]ryanodine-binding sites strongly suggests an important role for a ryanodine receptor-mediated mechanism for these bioactive PCBs.

The possible actions of PCBs on the enzymatic activity of skeletal muscle SERCA1 were further investigated by measuring changes in Ca^{2+}-dependent ATPase activity with the use of a coupled enzyme assay that monitors the rate of NADH utilization (Table 2). Neither 5 μM 2,2',3,5',6- nor 3,3',4,4',5-pentachlorobiphenyl significantly altered SERCA1 activity. SERCA2 pump activity was examined by measuring the rate of active Ca^{2+} accumulation into cardiac SR membrane vesicles in the presence or absence of PCB. The RyR2/Ca^{2+}-release channel complex of cardiac SR was first blocked with 500 μM ryanodine. After 5 min, DMSO (control) or 10 μM 2,2',3,5',6- or 3,3',4,4',5-pentachlorobiphenyl was added to separate vesicle suspensions. To initiate active Ca^{2+} uptake, a bolus of 24 nmol $CaCl_2$ was added to each cuvette, and the initial rate of Ca^{2+} accumulation was measured. No significant differences were found between the initial rates of Ca^{2+} uptake by cardiac SR vesicles treated with DMSO and those exposed to either of the pentachlorobiphenyl congeners when RyR2 was fully blocked (Table 2). SERCA2 pump activity was also examined in the absence of Ca^{2+} channel blocker. In the presence of 10 μM 2,2',3,5',6-pentachlorobiphenyl, the net rate of Ca^{2+} uptake on addition of 24 nmol $CaCl_2$ was significantly slower than that of the control, as indicated from the shallower slope of the uptake curve (Fig. 6). However, the rate of Ca^{2+} uptake in the PCB-treated sample was increased on subsequent addition of 20 μM ruthenium red, suggesting that 2,2',3,5',6-pentachlorobiphenyl slows the initial rate of Ca^{2+} accumulation, principally by activating the RyR2/Ca^{2+}-release channel complex.

***Ortho*-substituted 2,2',3,5',6-pentachlorobiphenyl enhances [^{3}H]ryanodine binding to RyR via a novel mechanism.** The mechanism by which 2,2',3,5',6-pentachlo-

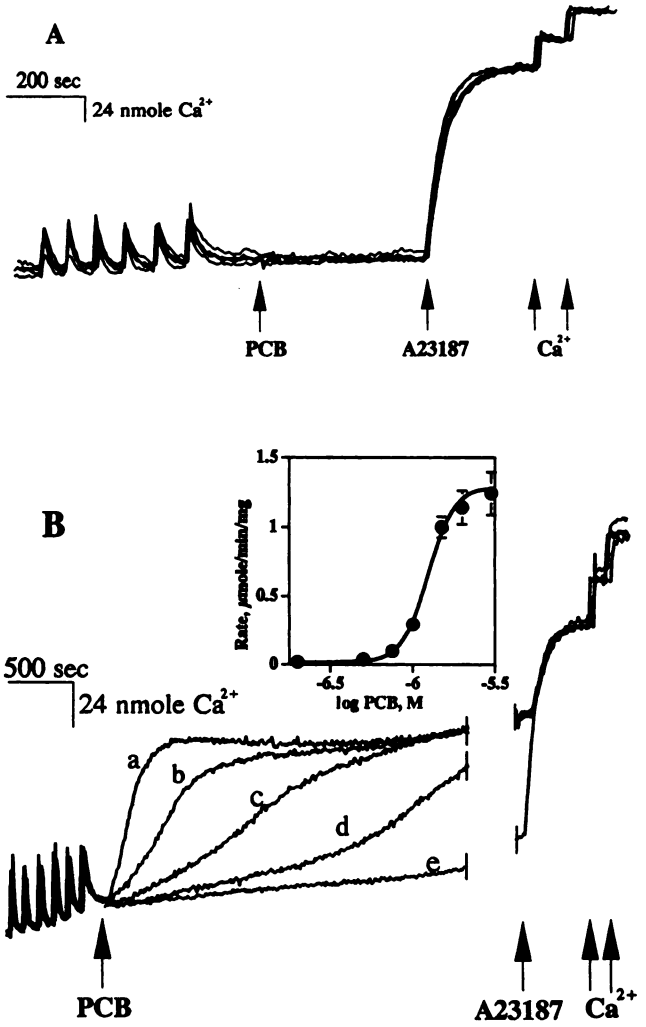

Fig. 3. *Ortho*-substituted 2,2′3,5′,6-pentachlorobiphenyl induces Ca^{2+} release from skeletal SR vesicles in a dose-dependent manner. Ca^{2+} transport across skeletal muscle SR vesicles was measured in buffer consisting of 18.5 mM K-MOPS, pH 7.0, 92.5 mM KCl, 7.5 mM Na-pyrophosphate, 250 μM antipyrylazo III, 1 mM Mg-ATP, 20 μg/ml creatine phosphokinase, 5 mM phosphocreatine, and 50 μg of skeletal SR protein, as described in Experimental Procedures. Total Ca^{2+} accumulated during the loading phase was 144 nmol. A, DMSO (control) and 1, 2, 5, and 10 μM 3,3′,4,4′,5-pentachlorobiphenyl do not induce any Ca^{2+} release from loaded membrane vesicles. B, 2,2′,3,5′,6-Pentachlorobiphenyl (0.2–3 μM) induces Ca^{2+} release from loaded membrane vesicles in a dose-dependent manner. *Trace a*, 1 μM; *trace b*, 750 nM; *trace c*, 500 nM; *trace d*, 200 nM; and *trace e*, DMSO. The experiments shown in A and B are representative of three replicate traces. B, *inset*, mean ± standard error Ca^{2+}-release rates of three replicated experiments for 2,2′,3,5′,6-pentachlorobiphenyl (EC_{50} = 1.2 ± <0.1 μM).

robiphenyl enhances the high affinity binding of [^{3}H]ryanodine to RyR1 was further elucidated by examining changes in saturation binding constants and altered responses to Ca^{2+} and Mg^{2+}, physiologically important modulators of SR Ca^{2+} release. Results of equilibrium binding experiments with [^{3}H]ryanodine are shown as binding isotherms and Scatchard plots in Fig. 7, and the binding constants are summarized in Table 3. Scatchard analysis reveals that 328 nM 2,2′,3,5′,6-pentachlorobiphenyl increases the maximal binding capacity (B_{max}) by 1.4-fold and enhances the apparent affinity ($1/K_D$) for the radioligand by 1.6-fold, whereas congener (10 μm) increases B_{max} by 2-fold and reduces K_D by 3.6-fold compared

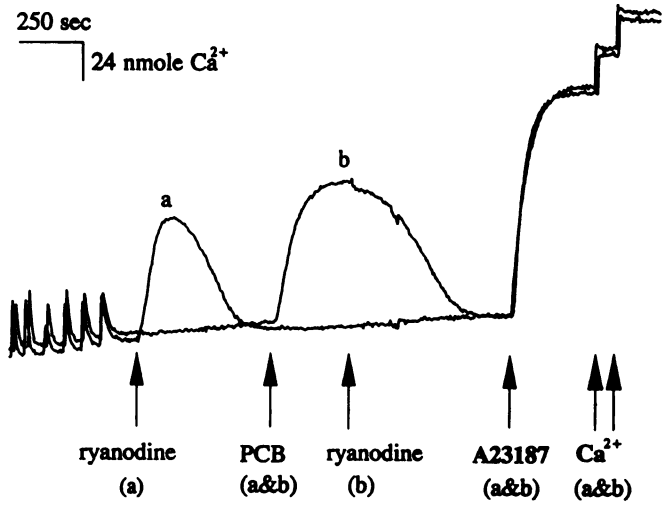

Fig. 4. *Ortho*-substituted PCB induces Ca^{2+} release from skeletal SR via a ryanodine receptor-mediated mechanism. Ca^{2+} transport across skeletal SR vesicles was measured in buffer consisting of 18.5 mM K-MOPS pH 7.0, 92.5 mM KCl, 7.5 mM Na-pyrophosphate, 250 μM antipyrylazo III, 1 mM Mg-ATP, 20 μg/ml creatine phosphokinase, 5 mM phosphocreatine, and 50 μg of skeletal SR protein, as described in Experimental Procedures. Total Ca^{2+} accumulated during the loading phase was 144 nmol. *Trace a*, addition of 500 μM ryanodine rapidly activates Ca^{2+} release followed by sustained inhibition of the channel and reaccumulation of Ca^{2+} by SERCA1 pumps. Subsequent addition of 1 μM 2,2′,3,5′,6-pentachlorobiphenyl cannot mobilize Ca^{2+} from the vesicles. *Trace b*, Ca^{2+} release induced by 1 μM 2,2′,3,5′,6-pentachlorobiphenyl is blocked by subsequent addition of 500 μM ryanodine. Once RyR1 is blocked, Ca^{2+} reaccumulates in SR vesicles at essentially the same rates in *traces a and b*. The experiment shown was repeated four times with identical results.

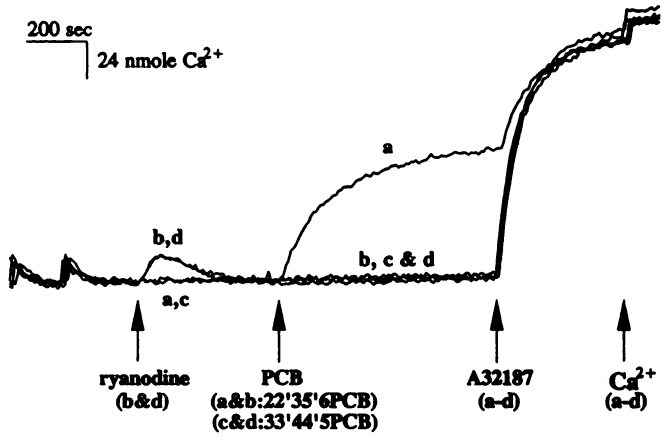

Fig. 5. *Ortho*-substituted PCB induces Ca^{2+} release from RyR2-enriched cardiac SR vesicles. Ca^{2+} transport across cardiac SR vesicles was measured as described in legend to Fig. 4 with 100 μg of cardiac SR protein. Membrane vesicles actively accumulated 72 nmol of Ca^{2+} during the loading phase. *Trace a*, addition of 10 μM 2,2′,3,5′,6-pentachlorobiphenyl induces Ca^{2+} release from actively loaded vesicles. *Trace b*, Ca^{2+} release induced by 10 μM 2,2′,3,5′,6-pentachlorobiphenyl is inhibited by prior addition of 500 μM ryanodine. In the absence (*trace c*) or presence (*trace d*) of 500 μM ryanodine, 10 μM 3,3′,4,4′,5-pentachlorobiphenyl does not induce any Ca^{2+} release from Ca^{2+}-loaded vesicles. The experiment shown was repeated twice with the same results.

with the controls. Therefore, 2,2′,3,5′,6-pentachlorobiphenyl increases [^{3}H]ryanodine occupancy by stabilizing the high affinity state of the receptor.

The interaction between 2,2′,3,5′,6-pentachlorobiphenyl

TABLE 2

Neither 2,2',3,5',6- nor 3,3',4,4',5-pentachlorobiphenyl significantly alters Ca^{2+}-dependent ATPase activity of skeletal or cardiac SR membrane

Condition	Skeletal SERCA1 pump[a]	
	SERCA1 activity	Associated p[b]
	μmol/mg/min	
Control	4.7 ± <0.1	
5 μM 2,2',3,5',6-pentachlorobiphenyl	4.6 ± <0.1	0.544
5 μM 3,3',4,4',5-pentachlorobiphenyl	4.6 ± <0.1	0.788
	Cardiac SERCA2 pump[c]	
	Rate of Ca^{2+} uptake *μmol/mg/min*	Associated p[d]
Control	5.9 ± 0.2	
10 μM 2,2',3,5',6-pentachlorobiphenyl	5.2 ± 0.2	0.340
10 μM 3,3',4,4',5-pentachlorobiphenyl	5.1 ± 0.4	0.155

The data represent the mean ± standard error of three experiments.

[a] ATPase assays in SERCA1 were performed in a buffer consisting of 5 mM HEPES, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 60 μM EGTA, 100 μM $CaCl_2$, 0.3 mM sucrose, 2 mM phospho(enol)pyruvate, 0.8 mM NADH, 24 units/ml LDH, 16.8 units/ml pyruvate kinase, 1.5 μg/mg A23187, and 50 μg skeletal SR protein, as described in Experimental Procedures.

[b] No significant difference from the control mean (Student's t test, two-tailed with $\alpha = 0.05$).

[c] Ca^{2+}-uptake rates were determined in a buffer consisting of 18.5 mM K-MOPS, pH 7.0, 92.5 mM KCl, 7.5 mM Na-pyrophosphate, 250 μM antipyrylazo III, 1 mM Mg-ATP, 20 μg/ml creatine phosphokinase, 5 mM phosphocreatine, and 100 μg cardiac SR protein, as described in Experimental Procedures.

[d] No significant difference from the paired controls (paired t test, two-tailed with $\alpha = 0.05$).

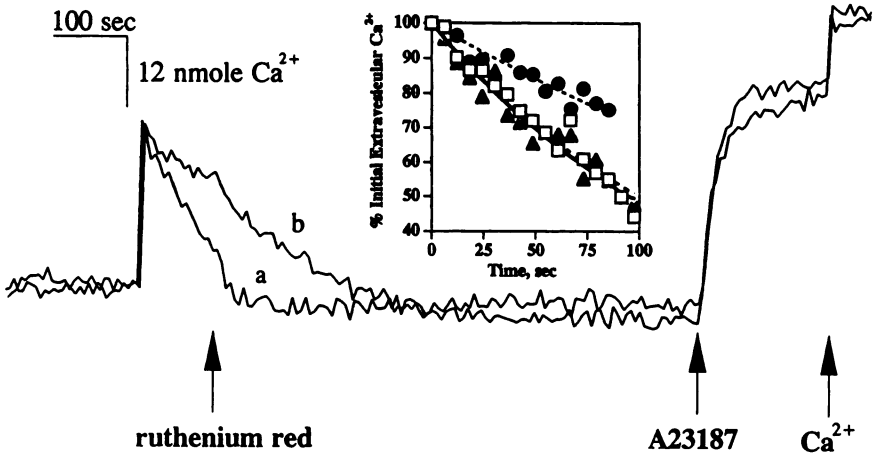

Fig. 6. *Ortho*-substituted 2,2',3,5',6-pentachlorobiphenyl reduces Ca^{2+} uptake rate by activation of RyR2/Ca^{2+}-release channel complex. Ca^{2+} fluxes across the cardiac SR vesicles (100 μg of protein) were measured in the buffer as described in legend to Fig. 4. Membrane vesicles were treated with (*trace a*) an equivalent volume of DMSO (control) or (*trace b*) 10 μM 2,2',3,5',6-pentachlorobiphenyl. Ca^{2+} uptake was initiated by the addition of 24 nmol $CaCl_2$. *Trace b*, rate of Ca^{2+} uptake is significantly reduced in the presence of 2,2',3,5',6-pentachlorobiphenyl but returns to the level of the control after the addition of 20 μM ruthenium red. *Inset*, normalized extravesicular Ca^{2+} concentration versus time in the presence of 500 μM ryanodine (□), of 10 μM 2,2',3,5',6-pentachlorobiphenyl (●), and of 10 μM 2,2',3,5',6-pentachlorobiphenyl and 20 μM ruthenium red (▲). For the experiment shown, the Ca^{2+} uptake rate was 6.7 μmol/mg/min (□), 3.2 μmol/mg/min (●), and 6.4 μmol/mg/min (▲). The data for the first 100 sec after Ca^{2+} or ruthenium red addition were fitted using a single exponential. This experiment was repeated twice with similar results.

and the Ca^{2+}-release channel complex dramatically alters the response of the receptor to Ca^{2+}. When assayed in the presence of physiological concentrations of intracellular monovalent cations, 10 μM 2,2',3,5',6-pentachlorobiphenyl not only enhances the maximal occupancy achieved at optimal free Ca^{2+} by >7-fold (from 0.45 ± 0.02 to 3.3 ± 0.04 pmol/mg) but also shifts both activation and inhibition constants for Ca^{2+} at RyR1 (Fig. 8 and Table 4). The half-activation constant for Ca^{2+} at RyR1 is reduced by 8.9-fold in the presence of 10 μM 2,2',3,5',6-pentachlorobiphenyl (EC_{50} shifts from 1.3 to 0.15 μM; Fig. 8A), whereas the potency for inhibition by Ca^{2+} decreases by 22-fold (IC_{50} shifts from 0.5 to 10 mM; Fig. 8B). Furthermore, at very low (<50 nM) or very high (>10 mM) free Ca^{2+}, 2,2',3,5',6-pentachlorobiphenyl partially negates the Ca^{2+} requirement for binding of nanomolar [^{3}H]ryanodine to high affinity sites on RyR1. Interestingly, the mixture Aroclor 1254 (10 μM) also decreases the potency with which Ca^{2+} inhibits the binding of [^{3}H]ryanodine by 4-fold (Table 4).

The ability of 2,2',3,5',6-pentachlorobiphenyl to alter inhibition of RyR1 by Mg^{2+} is demonstrated in Fig. 9. The PCB congener dramatically shifts the Mg^{2+} inhibition curve to the right by more than 2 log units, by increasing the IC_{50} from 0.3 mM to 28 mM for control and 10 μM 2,2',3,5',6-pentachlorobiphenyl, respectively.

Discussion

Commercial PCB mixtures such as Aroclor 1254 exhibit an array of hepatotoxic (37, 38), immunotoxic (39), and carcino-

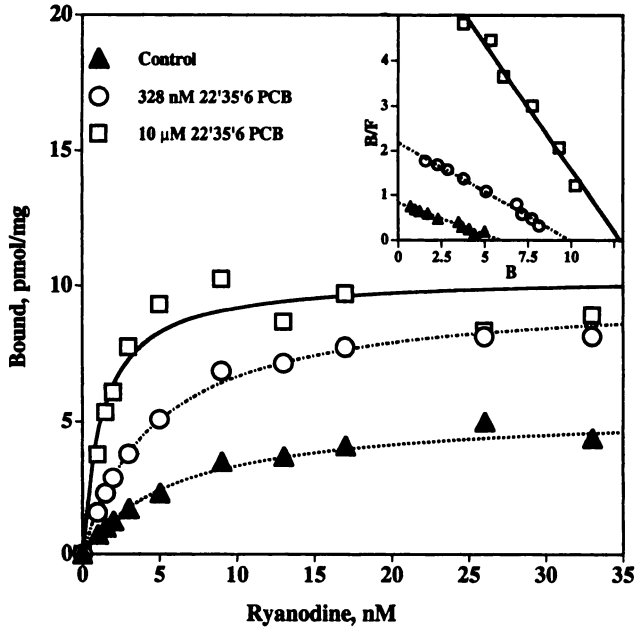

Fig. 7. *Ortho*-substituted 2,2',3,5',6-pentachlorobiphenyl increases maximal [^{3}H]ryanodine binding capacity (B_{max}) and affinity ($1/K_D$) by stabilizing the high affinity state of RyR1. Equilibrium binding of 0.5–32 nM ryanodine to 12.5 μg of skeletal SR was performed in buffer consisting of 20 mM HEPES, pH 7.1, 140 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, and 10% sucrose, as described in Experimental Procedures. *Inset*, Scatchard plots of the binding isotherms. *Ortho*-substituted 2,2',3,5',6-pentachlorobiphenyl increases B_{max} and apparent binding affinity in a dose-dependent manner. Binding constants for four experiments are summarized in Table 3.

TABLE 3

2,2',3,5',6-Pentachlorobiphenyl increases binding affinity and maximal binding capacity of [^{3}H]ryanodine to RyR1

Condition[a]	K_D	B_{max}
	nM	*pmol/mg*
Control	6.3 ± 0.3	5.8 ± 0.3
328 nM 2,2',3,5',6-pentachlorobiphenyl	4.0 ± 0.1	8.2 ± 0.3
10 μM 2,2',3,5',6-pentachlorobiphenyl	1.8 ± <0.1	11.7 ± 0.3

The data represent the mean ± standard error of four experiments.

[a] Binding assays were conducted in 20 mM HEPES, pH 7.1, 140 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, 10% sucrose, 0.5–32 nM [^{3}H]ryanodine, and 12.5 μg of skeletal SR protein, as described in Experimental Procedures.

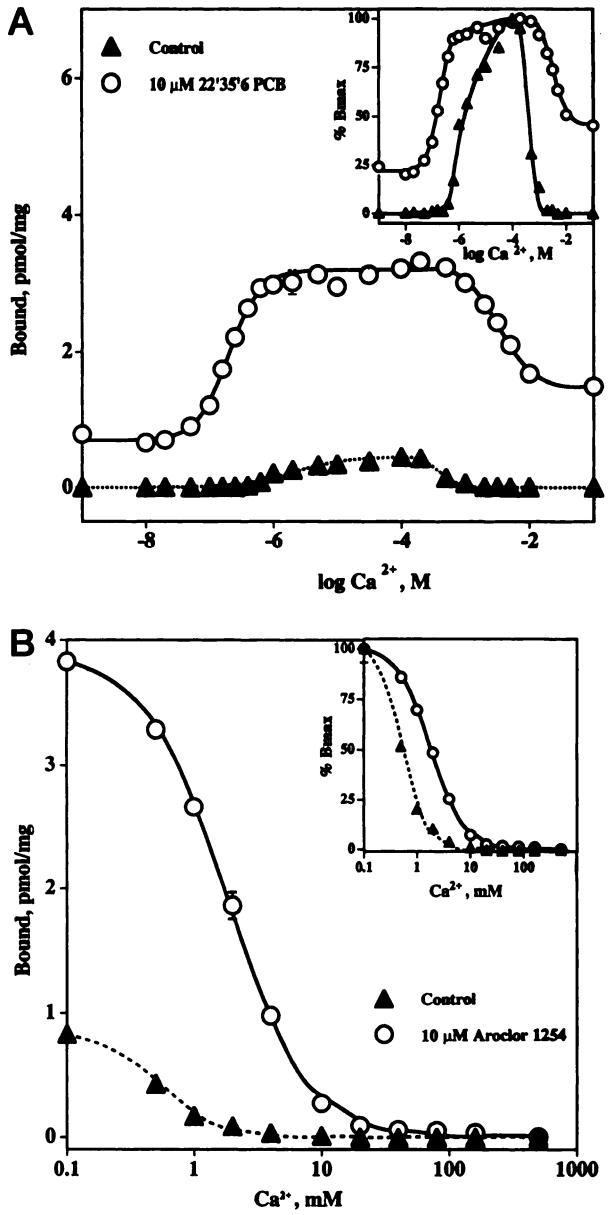

Fig. 8. *Ortho*-substituted 2,2',3,5',6-pentachlorobiphenyl alters the response of RyR1 to Ca^{2+}. Equilibrium binding of 1 nM [^{3}H]ryanodine to 12.5 μg of skeletal SR in 1 nM to 100 mM free Ca^{2+} was performed in buffer consisting of 20 mM HEPES, pH 7.1, 140 mM KCl, 15 mM NaCl, and 10% sucrose. A, Data are the mean ± standard error of three independent experiments, each performed in duplicate. *Inset*, normalized data, as percentage of maximal binding, showing that 2,2',3,5',6-pentachlorobiphenyl (10 μM) shifts the Ca^{2+} activation curve to the left and the inhibition curve to the right. B, Similar experiments show that Aroclor 1254 (10 μM) induces a similar shift in Ca^{2+} inhibition of [^{3}H]ryanodine occupancy as that seen with the pure congener. The Aroclor result represents the mean ± standard error of three experiments. The EC_{50} and IC_{50} values are summarized in Table 4.

genic (40) responses in chronically treated animals. PCBs lacking chloro substituents in the *ortho*-position to the biphenyl bridge prefer coplanar conformation. Coplanar PCBs have been demonstrated to elicit the same spectrum of toxic and biochemical responses observed for TCDD (38). This is not surprising because coplanar PCBs effectively compete with TCDD for a common activator site on the cytosolic Ah receptor that effectively induces cytochrome P-450 1A1 monooxygenase. However, the commercial mixture Aroclor 1254, composed of coplanar PCBs, and congeners with multiple *ortho*-chloro substituents preferring nonplanar conformations, induces all five cytochrome P-450 isozymes in rats (41, 42), whereas nonplanar PCBs possessing two *ortho*- and two *para*-chloro substituents have been shown to induce only the phenobarbital type of cytochrome P-450 monooxygenases (isozymes 2B1 and 2B2) (42, 43). In general, PCBs possessing two or more *ortho* substituents do not effectively compete with TCDD at the Ah receptor and are poor inducers of cytochrome P-450 1A1 monooxygenase. It is the *ortho*-substituted PCB congeners that have been demonstrated to alter catecholamine in animal studies, both at the cellular level and *in vivo* (11–13). These results, taken together, reveal that the biological activity of *ortho*-substituted, nonplanar PCBs is mediated by Ah receptor-independent mechanisms that are poorly understood (40).

TABLE 4

PCBs alter the sensitivity of RyR1/Ca^{2+}-release channel complex to modulation by Mg^{2+} and Ca^{2+}

Condition[a]	EC_{50}[b]	IC_{50}[b]
	μM	mM
Calcium		
Control (three)	1.3 ± <0.1 (1.1 ± 0.1)	0.5 ± <0.1 (3.5 ± 0.7)
10 μM 2,2',3,5',6-pentachlorobiphenyl (three)	0.15 ± <0.01 (1.8 ± 0.1)	10.1 ± 0.5 (1.6 ± 0.1)
10 μM Aroclor 1254 (two)	n.d.[c]	1.8 ± 0.1
	IC_{50}	Hill coefficient
	mM	
Magnesium		
Control (three)	0.27 ± 0.01	1.3 ± 0.1
10 μM 2,2',3,5',6-pentachlorobiphenyl (five)	27.6 ± 0.3	0.8 ± <0.1

The data represent the mean ± standard error of the number of replicate experiments indicated in parentheses.

[a] Binding assays were performed in 20 mM HEPES, pH 7.1, 140 mM KCl, 15 mM NaCl, 10% sucrose, 1 nM [^{3}H]ryanodine, and 12.5 μg of skeletal SR protein. In calcium, 1 nM—100 mM free Ca^{2+} was varied in an EGTA buffer in the absence of Mg^{2+}. In magnesium, 5 μM—2 M free Mg^{2+} was varied in the presence of 50 μM Ca^{2+}.

[b] Hill coefficients of the dose-response curves are shown in parentheses.

[c] n.d. = not determined.

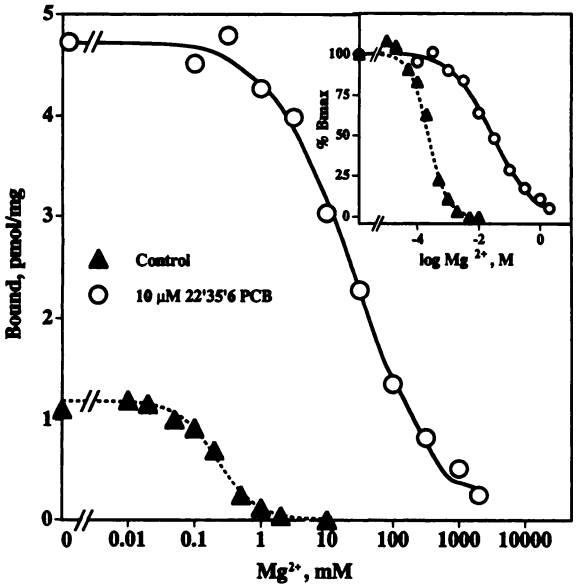

Fig. 9. *Ortho*-substituted 2,2',3,5',6-pentachlorobiphenyl alters the response of RyR1 to Mg^{2+}. Equilibrium binding of 1 nM [^{3}H]ryanodine to 12.5 μg of skeletal SR in the presence of 5 μM–2 M Mg^{2+} was performed in buffer consisting of 20 mM HEPES, pH 7.1, 140 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, and 10% sucrose. Data are the mean ± standard error of three (control) or five (PCB) experiments, each performed in duplicate. *Inset*, normalized data, as percentage of maximal binding. 2,2',3,5',6-Pentachlorobiphenyl (10 μM) dramatically shifts the Mg^{2+} inhibition curve to the right. IC_{50} values are summarized in Table 4.

Structural specificity of PCBs toward RyR1 and RyR2. In the current study, well-characterized microsomal membrane preparations isolated from junctional regions of skeletal and cardiac muscles SR were used to test the hypothesis that a receptor-mediated mechanism alters microsomal Ca^{2+} transport and could account for PCB-induced changes in cellular Ca^{2+} regulation. Direct evidence is presented for the first time showing that certain *ortho*-substituted PCB congeners are extremely potent inducers of Ca^{2+} release from SR membrane vesicles by a selective interaction with ryanodine-sensitive Ca^{2+}-release channel complexes. Mammalian skeletal and cardiac junctional SR membrane fractions possess predominantly one isoform, RyR1 and RyR2, respectively, and were used to study the structure-activity relationship and mechanism by which PCB congeners alter microsomal Ca^{2+} transport. SR Ca^{2+}-release channels mediate Ca^{2+} efflux during excitation-contraction coupling of skeletal and cardiac muscles. In addition, RyR1 is highly expressed in neurons of the cerebral cortex, pyramidal cells of CA regions of the hippocampus, and Purkinje cells of the cerebellum. The "cardiac" isoform RyR2 is also widespread in the brain, whereas the brain isoform of the ryanodine receptor can be found generally distributed in brain, lung, kidney, liver, testis, and many other tissues (44, 45). The exact role of RyR isoforms in Ca^{2+} signaling in nonmuscle cells is unclear. However, on stimulation, both excitable and nonexcitable cells increase their cytosolic Ca^{2+} through (i) influx of Ca^{2+} through voltage- or receptor-operated Ca^{2+} channels within the plasma membranes (46, 47) and (ii) release of Ca^{2+} from intracellular stores through the inositol-1,4,5-trisphosphate receptors and/or ryanodine receptor/Ca^{2+}-release channel complexes (RyRs) (48, 49). Both pathways are important for the transient, spatiotemporal rise of Ca^{2+} in the cytosol, particularly in neurons. The intracellular Ca^{2+} level in neurons plays a major role in regulating both the amount of neurotransmitter released by the nerve terminal and the levels of neurotransmitters in the neuron (50, 51).

In recent studies, Kodavanti *et al.* (15, 16) suggested that selected *ortho*-substituted PCB congeners disrupt Ca^{2+} homeostasis in rat cerebellar granule cells *in vitro*. Inhibition of synaptosomal Ca^{2+}-ATPase and mitochondrial Ca^{2+} accumulation were proposed to form the molecular mechanism that perturbs Ca^{2+} homeostasis in neuronal cells in culture. The current study provides evidence for an alternative mechanism. Aroclor mixtures with intermediate degrees of chlorination on PCBs are optimal for activation of microsomal Ca^{2+}-release channels. Neither high nor low chlorine composition PCB mixtures show significant activity toward the Ca^{2+}-release channel. Studies on the physical properties of all 209 PCB congeners have shown that congeners with a higher degree of chlorination have longer retention time on gas chromatography, suggesting that, in general, hydrophobicity of the PCB mixtures increases with their degree of chlorination (52). The increase in average molecular weight leads to an increase in Van der Waals interaction between the PCB molecules. Aroclors consisting of PCB congeners with high (1268) or low (1221) hydrophobicity are inactive toward the channel, whereas Aroclors consisting of PCB congeners with intermediate hydrophobicity (1248, 1254, and 1260) enhance the channel activity significantly. Because the ability of PCB mixtures to modulate SR Ca^{2+}-release channel activity does not strictly follow the hydrophobicity of these mixtures, positions of chlorine on the biphenyls, instead of the hydrophobicity, seem to be critical for channel activation and point toward a specific interaction between certain PCBs and ryanodine-sensitive Ca^{2+}-release channels. All of the active mixtures contain two common constituents, pentachlorinated and hexachlorinated biphenyls, in the following abundance, respectively: Aroclor 1248, 36% and

4%; Aroclor 1254, 49% and 34%; and Aroclor 1260, 12% and 38% (53). Pentachlorinated and hexachlorinated biphenyls are in extremely low abundance or absent in inactive mixtures tested (Aroclors 1221, 1232, and 1268). Aroclor 1248 contains only 4% hexachlorinated congeners yet maintains similar potency and efficacy toward activation of the microsomal Ca^{2+}-release channels compared with other active mixtures. Because all active mixtures possess a similar abundance of pentachlorinated biphenyl congeners, our initial structure-activity study on pure congeners was directed at selected pentachlorobiphenyls.

Coplanar 3,3',4,4',5-pentachlorobiphenyl is most widely studied for its ability to bind to the Ah receptor and to induce hepatic microsomal enzymes (38). However, this congener does not alter Ca^{2+} transport across isolated microsomes. Coplanar 3,3',4,4',5-pentachlorobiphenyl exhibits neither activity toward RyR/Ca^{2+}-release channel complexes nor SERCA pumps in skeletal and cardiac SR. In contrast, nonplanar 2,2',3,5',6-pentachlorobiphenyl induces Ca^{2+} release from both skeletal and cardiac microsomes. Ca^{2+} release is completely blocked by the prior addition of inhibitory concentration of ryanodine, suggesting that 2,2',3,5',6-pentachlorobiphenyl induces Ca^{2+} release from SR through selective activation of the ryanodine-sensitive Ca^{2+}-release channel. The similar Ca^{2+}-uptake rate seen on the addition of ryanodine, whether in the presence or absence of PCB, further suggests that 2,2',3,5',6-pentachlorobiphenyl does not affect the SERCA pump activity. An increase in the number of chloro substituents in the *ortho*-position of PCBs increases their activities toward ryanodine receptors. Although nonplanar conformations of the biphenyl structures are critical for high potency, a certain degree of rotation of the phenyl groups along the principal molecular axis also seems to be important to produce maximal activation of the Ca^{2+}-release channel, as the congener with four chloro substituents in the *ortho*-positions is significantly less active. The ranked order of potency and efficacy toward RyR1 is 3,3',4,4',5- < 2,3,3',4,4'- < 2,2',3,3',4- ≤ 2,2',4,6,6'- < 2,2',3,5',6-pentachlorobiphenyl, which parallels the order of potency with which these congeners decrease dopamine levels in PC12 cells *in vitro* and brain tissue *in vivo* (11, 13). The differences in efficacy toward RyR1 activation suggest that the position of chlorine substituents among pentachlorobiphenyl reflects either (i) lower intrinsic activity imparting partial agonist qualities or 2) that certain pentachlorobiphenyls can inactivate the Ca^{2+}-release channels subsequent to activation, especially with the prolong exposure needed to equilibrate the [^{3}H]ryanodine binding assay. An expanded structure-activity study with cerebellar and hippocampal microsomal preparations from rat brains revealed that 2,2'-dichlorobiphenyl, a congener shown to enhance [^{3}H]phorbol ester binding in rat cerebellar granule cells (16), also has extremely high activity toward ryanodine receptors found in these tissues.[1] In addition to the three chloro substituents in the *ortho*-positions, substituents at the *meta*- and *para*-positions are also critical for optimal activity at ryanodine-sensitive Ca^{2+}-release channels. The most active congener tested, 2,2',3,5',6-pentachlorobiphenyl, exhibits nanomolar potency toward activating the binding of [^{3}H]ryanodine to RyR1. Its potency at RyR2 in the binding assay, however, is in the low micromolar range. The difference could reflect inherent differences in the affinity of two isoforms for PCB or differences in the amount of SR membrane lipid present in the skeletal and cardiac assays. The latter interpretation is favored considering the lipophilic nature of PCBs and the observation that the EC_{50} values for activation of the two isoforms differ (7-fold) identically with the mass of protein (and lipid) in the respective standard assays (7-fold higher for cardiac preparations). Similarly, at a saturating concentration of 2,2',3,5',6-pentachlorobiphenyl, the congener enhances [^{3}H]ryanodine binding to RyR1 by ~11-fold, whereas binding to RyR2 is only ~2-fold. The apparent difference in efficacies toward RyR isoforms could be accounted by two factors. First, the typical receptor density in the cardiac SR preparation is ~20–25% of that found in the purified skeletal junctional SR preparation (19). Second, under the respective control assay conditions, ~30% the cardiac channels are occupied, whereas only ~6% of the skeletal channels are occupied with [^{3}H]ryanodine. The combination of lower [^{3}H]ryanodine binding density and a higher fractional occupancy under control conditions seen with cardiac preparations is likely to account for the lower apparent efficacy (enhanced occupancy) seen with RyR2 relative to RyR1 at saturating PCB concentrations. The parallel structural requirements for PCB activity at ryanodine-sensitive Ca^{2+}-release channels and their activities toward dopamine in neurogenic cells in culture suggest that microsomal Ca^{2+}-release channels may represent a major target by which these environmental contaminants alter neuronal function(s). The remarkable selectivity of active PCB congeners toward the Ca^{2+}-release channel complexes over SERCA pumps further supports a ryanodine receptor-mediated mechanism.

Novel molecular mechanism for *ortho*-substituted PCBs at RyR1. Several aspects of PCB-induced Ca^{2+} release implicate a novel mechanism at the RyR1 complex. First, 2,2',3,5',6-pentachlorobiphenyl stabilizes a conformation of RyR1 that recognizes [^{3}H]ryanodine with high affinity, as indicated by its ability to significantly enhance both maximal binding capacity (2-fold) and affinity (nearly 4-fold) of the SR membranes, despite the absence of Mg^{2+} and in the presence of physiological levels of intracellular K^+, Na^+, and optimal Ca^{2+}. The increase in binding affinity can be due to increase in the association rate and/or decrease in the dissociation rate of ryanodine binding to the receptor. Because most of the RyR channel agonists (e.g., doxorubicin) increase ryanodine binding affinity by increasing the association rate of ryanodine to the receptor (54), 2,2',3,5',6-pentachlorobiphenyl is likely to enhance the binding affinity by affecting the association kinetic of ryanodine. Second, 2,2',3,5',6-pentachlorobiphenyl dramatically decreases the inhibitory potency of mM Ca^{2+} (22-fold) and Mg^{2+} (>100-fold). In the presence of 10 μM 2,2',3,5',6-pentachlorobiphenyl, Hill coefficients of Ca^{2+} and Mg^{2+} inhibition shift from 3.5 to 1.6 and 1.3 to 0.7, respectively. Taken together, these data indicate that the major actions of active PCBs are to alter the responsiveness of physiologically important inhibitory sites for Ca^{2+} and Mg^{2+} on the RyR1 oligomer. In addition, 2,2',3,5',6-pentachlorobiphenyl enhances the apparent affinity of the channel activator sites for Ca^{2+} by nearly 9-fold for

[1] S. L. Schantz, P. W. Wong, B. W. Seo, R. M. Joy, T. E. Albertson, and I. N. Pessah. Non-coplanar polychlorinated biphenyls alter rat brain ryanodine receptors and neuroplasticity *in vitro* and *in vivo* neurodevelopmental behavior. Evidence for altered hippocampal function and learning behavior, submitted for publication.

~75% of the measurable high affinity sites. Binding of nM [^{3}H]ryanodine to the remaining 25% of the sites becomes essentially Ca^{2+} independent. These properties of 2,2',3,5',6-pentachlorobiphenyl at RyR1 parallel those recently reported for bastadins isolated from the marine sponge *Ianthella basta* (55). Bastadins are macrocyclic bromotyrosine derivatives that stabilize the high affinity, full conductance state of RyR1 and shift the inhibitory potency of Ca^{2+} and Mg^{2+} in much the same way as 2,2',3,5',6-pentachlorobiphenyl. Thus, the actions of 2,2',3,5',6-pentachlorobiphenyl, like bastadin 5, seem to be mediated by interacting with a novel modulator site(s) on the RyR complexes, which stabilizes the high affinity state of RyR complexes and increases maximal occupancy. This could be brought about by converting low affinity sites to high affinity sites and/or preventing the loss of high affinity sites to low affinity sites. The actions of bastadin 5 are antagonized in a dose-dependent manner by FK506, but unlike FK506, the activity of bastadin 5 is not associated with its ability to directly dissociate the FKBP12/RyR1 heterocomplex (55). Rather, bastadin 5 synergizes FK506-induced release of FKBP12 from the RyR1 complex. Unlike bastadin 5, other active bastadin structures that have recently been isolated and characterized (e.g., bastadin 10) modulate the FKBP12/RyR1 complex in a Ca^{2+}-independent manner.[2] These data indicate a common mechanism by which bastadins and 2,2',3,5',6-pentachlorobiphenyl modulate Ca^{2+}-release channel behavior, which may be mediated through the immunophilin and alter critical protein/protein interactions of the FKBP12/RyR1 complex. Initial results from our laboratory definitively demonstrate that FK506 and rapamycin completely inhibit the actions of 2,2',3,5',6-pentachlorobiphenyl at RyR1, without inhibiting the responsiveness of RyR1 to caffeine (56).[3]

In conclusion, the present results demonstrate that certain *ortho*-substituted pentachlorobiphenyls have direct and potent activity on two isoforms of ryanodine-sensitive Ca^{2+}-release channels. The mechanism responsible may involve the major T cell immunophilin FKBP12, which is known to be tightly associated with ryanodine receptor and modulates channel gating behavior (24, 25, 55). Thus, PCBs provide important new probes for studying the structure and function of ryanodine-sensitive Ca^{2+}-release channels. Equally important, the current study reveals a new molecular mechanism by which *ortho*-substituted PCBs perturb Ca^{2+} homeostasis by targeting FKBP12/RyR complexes. The mechanism may be of fundamental importance for changes in intracellular Ca^{2+} signaling in excitable and nonexcitable cells that have recently been attributed to *ortho*-substituted PCBs. Because both ryanodine receptors and FKBP12 are expressed in a wide variety of cell types, including neurons and immune cells, this mechanism may also provide a general explanation for the toxicities of *ortho*-substituted PCBs in different tissues.

Acknowledgments

We thank Dr. M. M. Mack and Dr. E. D. Buck for their help in the preliminary studies. We also thank Dr. S. L. Schantz for her editorial assistance and Mr. T. H. Lam for his technical assistance in the transport assays. We dedicate this article to Prof. Robert M. Joy.

[2] I. N. Pessah and T. F. Molinski, unpublished observations.

[3] P. W. Wong and I. N. Pessah. *Ortho*-substituted PCB modulates ryanodine-sensitive Ca^{2+} channels and microsomal Ca^{2+} release by an immunophilin FKBP12-dependent mechanism, manuscript in preparation.

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Send reprint requests to: Dr. Isaac N. Pessah, Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, CA 95616. E-mail: inpessah@ucdavis.edu