EVIDENCE OF AN IMMUNOPHILIN-MEDIATED MECHANISM FOR *ORTHO*-SUBSTITUTED POLYCHLORINATED BIPHENYL NEUROTOXICITY

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ABSTRACT

Recently we reported that *ortho*-substituted polychlorinated biphenyls (PCBs) alter microsomal Ca^{2+} transport by selective interaction with ryanodine-sensitive Ca^{2+} channels (*i.e.*, ryanodine receptors; RyRs) localized within sarcoplasmic reticulum (SR) of muscle cells and endoplasmic reticulum (ER) of neurons (Wong and Pessah, *Molec. Pharmacol.* 49:740-51, 1996; Wong *et al. J. Biol. Chem.*, 272:). Isolated skeletal SR enriched in the major T-cell immunophilin in tight association with the skeletal isoform of RyR was used to understand the mechanism underlying the actions of PCBs on microsomal Ca^{2+} transport in more detail.. Disrupting the association between immunophilin FKBP12 and RyR with immunosupressive drug FK 506 eliminated PCB 95-enhanced binding of [^{3}H]ryanodine (IC_{50} = 40μM) to RyR and PCB 95-induced release of Ca^{2+} from actively loaded microsomal vesicles (IC_{50} = 10μM). FK 506 selectively eliminated PCB 95-induced Ca^{2+} release from SR since Ry_1Rs maintained responsiveness to caffeine and Ca^{2+}-induced Ca^{2+} release. These results demonstrated that PCB 95 mediated its actions on microsomal Ca^{2+} transport through an immunophilin-dependent mechanism. PCB 95 and FK 506 were also used to examine the relationship between ryanodine-sensitive Ca^{2+} channels and ryanodine-insensitive Ca^{2+} leak pathways present in SR vesicles. Micromolar ryanodine completely blocked ryanodine-sensitive Ca^{2+} efflux, but neither eliminated the ryanodine-insensitive Ca^{2+} leak unmasked by thapsigargin, SERCA pump blocker, nor enhanced the loading capacity of SR vesicles. PCB 95 alone enhanced thapsigargin evoked Ca^{2+} release and hence diminishes loading capacity of SR vesicles. However, in the presence of micromolar ryanodine which fully blocks sensitive channels, PCB 95 dose dependently eliminated the Ca^{2+} leak unmasked by thapsigargin and significantly enhanced loading capacity of SR vesicles. The actions of PCB 95 on SR loading capacity were additive with those of FK 506. Structural specificity for these novel actions are further demonstrated with co-planar PCB 126, which was inactive toward RyR, and lacked ability to eliminate the Ca^{2+} leak pathway and enhance the loading capacity of SR. The results reveal that FKBP12 relates ryanodine-insensitive Ca^{2+} "leak" and ryanodine-sensitive Ca^{2+} channel efflux pathways of SR by modulating distinct conformations Ry_1R complexes. Non-coplanar PCBs, like PCB 95, alter microsomal Ca^{2+} buffering by an FKBP12-mediated mechanism. An immunophilin based mechanism could account for many of the toxic actions attributed to certain non-coplanar PCB congeners.

INTRODUCTION

Polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans are halogenated aromatic hydrocarbons (HAHs), a family of persistent widely dispersed environmental contaminants. The unique chemical properties and low cost of producing PCBs have contributed to their extensive industrial use (1, 2). The high lipophilicity and chemical stability of PCBs have further resulted in widespread environmental contamination, and there is significant evidence of bio-accumulation of PCBs in biota (3). PCBs are found in extracts of virtually all environmental samples as well as in human tissue and breast milk (4, 5). Among the HAHs, the various congeners confer different ability to bind to a cytosolic receptor, the arylhydrocarbon (Ah) receptor. Many of the toxic responses followed by the exposure to HAHs are similar and appear to be related to the Ah receptor mediated pathway. 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-* but *meta-* or *para-* chlorine substitutions are able to confer the coplanar structure similar to TCDD and elicit similar toxicity. PCB congeners with single *ortho*-chlorine favor the non-coplanarity and behave as weak Ah receptor agonists. The PCB congeners with two or more *ortho*-chlorine favor the non-coplanarity structure, thus this group of congeners do not bind to the Ah receptor and exhibit different toxicity. Emerging evidences suggest 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 (6-8) and reduced in dopamine level in rat pheochromocytoma cells (PC12 cells) (9). Perinatal exposure of PCBs to monkeys and rodents, resulted in behavioral abnormality including delayed reflex development, altered activity patterns, learning deficits and impaired memory (10). 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 those PCB exposed monkeys (11). Epidemiological studies reveal that children with perinatal or prenatal exposure to PCBs and other HAHs developed long-lasting cognitive function deficits (12-15). Although the compounds that are responsible for the cognitive deficits are unknown, results from animal studies have revealed that *ortho*-substituted PCBs are probably responsible for the neurotoxicity observed.

Recently we provided evidence for a stringent structure-activity relationship among PCBs possessing two or more chlorine substitutions in the *ortho* positions for activation of ryanodine-sensitive Ca^{2+} channels of mammalian striated muscle (skeletal and cardiac) and the central nervous system, revealing an Ah receptor-independent mechanism through which PCBs disrupt Ca^{2+} signaling (16, 17). The most potent congener at the receptor yet identified, PCB 95 (2,2',3,5',6-pentachlorobiphenyl), was found to alter Ca^{2+} transport across neuronal microsomal membrane vesicles by a ryanodine receptor-mediated pathway, instead of an IP_3 receptor-mediated pathway. These actions of PCB 95 at ryanodine receptors may underlie its ability to alter neuronal excitability in the rat hippocampal slices *in vitro* (18) and both locomotor activity and spatial learning in an *in vivo* rat model (19). More generally, a ryanodine-receptor mediated mechanism could account for the ability of non-coplanar PCBs to alter protein kinase C translocation and phosphoinositide metabolism in primary cerebellar granular cell cultures (20, 21).

Results from more detailed mechanistic studies have revealed that the actions of PCB 95 on microsomal Ca^{2+} transport and RyRs are mediated through the major T-cell immunophilin FKBP12 which is tightly associated with RyRs in muscle and brain (22). The toxicological significance of an immunophilin mediated mechanisms by which *ortho*-substituted PCBs alter microsomal Ca^{2+} signaling and Ca^{2+}-dependent cascades will be discussed.

METHODS

Membrane preparations. Junctional sarcoplasmic reticulum (SR) membrane vesicles enriched in the skeletal isoform of ryanodine receptor, RyR_1, were prepared from fast-twitch skeletal muscle obtained from 3 - 4 kg male New Zealand White rabbits according to the method previously reported (14).

[^{3}H]Ryanodine binding assays. High-affinity binding of [^{3}H]ryanodine to microsomes was used as a biochemical indicator of changes in RyR conformation induced by PCB 95 using the method previously reported (14). The ability of FK 506 to eliminate PCB 95-induced changes in RyR conformation were determined by incubating FK 506 in an assay buffer containing 12.5 µg microsomal protein and 0, 0.6 or 1 µM PCB 95, in a final volume of 250 µl. The reaction mixtures were allowed to equilibrate at 37°C for 3.5 hr with constant shaking. Values of IC_{50} and Hill coefficients were calculated by sigmoidal curve fitting.

Ca^{2+} transport measurements. Net Ca^{2+} flux across the SR membrane vesicles was monitored by metallochromic dye antipyrylazo III (APIII), according to the method previously reported (14). Vesicles were actively loaded to capacity by serial additions of $CaCl_2$ in the presence of ATP and a

regenerating system. The ability of FK 506 to inhibit PCB 95-induced Ca^{2+} release from loaded membrane vesicles were determined by pre-incubating the vesicles for with immunosuppressant before Ca^{2+} loading, or after Ca^{2+} loading was complete 3 min prior to introduction of PCB 95.

How PCB 95 and coplanar PCBs 66 or 126 altered thapsigargin-evoked Ca^{2+} efflux from membrane vesicles actively loaded with Ca^{2+} was examined in the presence or absence of channel-blocking concentration of ryanodine. Membrane vesicles were loaded to near capacity as described above. Once the loading phase was complete, thapsigargin was introduced to inhibit the SERCA pump and hence block active Ca^{2+} uptake immediately after the addition of PCB 95 or PCB 126. The ability of PCBs to modulate the ryanodine-insensitive Ca^{2+} efflux was studied by pre-incubating the vesicles for 3 min with PCB 95 or PCB 126 in the presence of ryanodine to block all ryanodine-sensitive Ca^{2+} channels before initiating Ca^{2+} loading. Ryanodine-insensitive Ca^{2+} efflux was then evoked by addition thapsigargin.

The ability of FK 506 to alter thapsigargin evoked Ca^{2+} efflux from SR vesicles was also studied. FK 506 or DMSO (control) was added to actively loaded SR vesicles and the reaction mixture was allowed to incubate for about 3 min before addition of thapsigargin to induce Ca^{2+} efflux. The ability of FK 506 to alter ryanodine-insensitive Ca^{2+} efflux was examined by performing the measurements with ryanodine pretreated SR vesicles. In these experiments, SR vesicles were pre-treated for 3 min with ryanodine prior to loading with Ca^{2+}. Once the loading was complete, FK 506 or DMSO (control) was introduced and the reaction mixture was allowed to incubate for 3 min before addition of thapsigargin to induce Ca^{2+} efflux.

RESULTS AND DISCUSSION

FK 506 completely eliminated PCB 95-induced Ca^{2+} channel activation. The level of high-affinity binding of [^{3}H]ryanodine (1nM) to microsomal preparations was low when assayed in the presence of physiological concentration of monovalent cations. Incubation with 1μM of the non-coplanar congener PCB 95 (2,2',3,5',6-pentachlorobiphenyl) enhanced the specific occupancy of [^{3}H]ryanodine to RyR dose dependently (Fig. 1A). Although FK 506 did not significantly alter the high affinity binding of [^{3}H]ryanodine to RyR, FK 506 did eliminate PCB 95-enhanced [^{3}H]ryanodine occupancy with an IC_{50} of 40μM. These results demonstrated that at concentrations known to dissociate FKBP12 from RyR, FK 506 eliminated PCB 95-enhanced binding of [^{3}H]ryanodine to RyR, suggesting an immunophilin dependent mechanism. Ca^{2+} transport measurements revealed that addition of 1μM PCB 95 induced a net Ca^{2+} efflux from actively loaded SR vesicles (Fig. 1B, trace a). FK 506 (50 μM) introduced approximately 3 min prior to addition of 1μM PCB 95 completely eliminated the response to PCB 95 (Fig. 1B, trace b). FK 506 selectively eliminated PCB 95-induced Ca^{2+} release from SR since Ry_1Rs maintained responsiveness to caffeine and Ca^{2+}-induced Ca^{2+} release (not shown).

Marks and coworkers (23) have shown that the high affinity interaction between the RyR oligomer and FKBP12 is essential for stabilizing the native full conductance gating behavior of the SR Ca^{2+} release channel, since RyR expressed heterologously in the absence of FKBP12 exhibits several channel subconductances when reconstituted in bilayer lipid membranes. Further support of the functional importance of the immunophilin in stabilizing the RyR channel complex comes from pharmacological studies with immunosuppressant FK 506 and its analogs. Studies from several laboratories (24) have revealed that FK 506 is sufficient to dissociate FKBP12 from RyR, although it is not clear whether complete dissociation of the immunophilin is achieved. In the present study, FK 506 completely eliminated PCB 95-induced Ca^{2+} release and PCB 95-enhanced binding of [^{3}H]ryanodine to RyR in the same concentration range required to dissociate FKBP12 from RyR,

indicating a strong correlation between the activity of PCB 95 towards RyR and the integrity of the FKBP12/RyR_1 complex.

PCB 95 eliminated a ryanodine-insensitive Ca^{2+} leak pathway from microsomes. Recent studies with brominated macrocyclic bastadins isolated from the marine sponge *Ianthella basta* have indicated that bastadin 5 enhances SR loading capacity by modulating the FKBP12/RyR complex and converting a ryanodine-insensitive efflux pathway ("leak") into a ryanodine-sensitive efflux pathway ("channel") which recognizes ryanodine with high affinity (25). PCB 95 was shown to modulate [^{3}H]ryanodine binding sites in a manner very similar to bastadin 5 (16, 17). Both PCB 95 and bastadin 5 increased the affinity and capacity of high affinity [^{3}H]ryanodine binding to RyR, as well as significantly altered modulation of RyR by Ca^{2+} and Mg^{2+}.

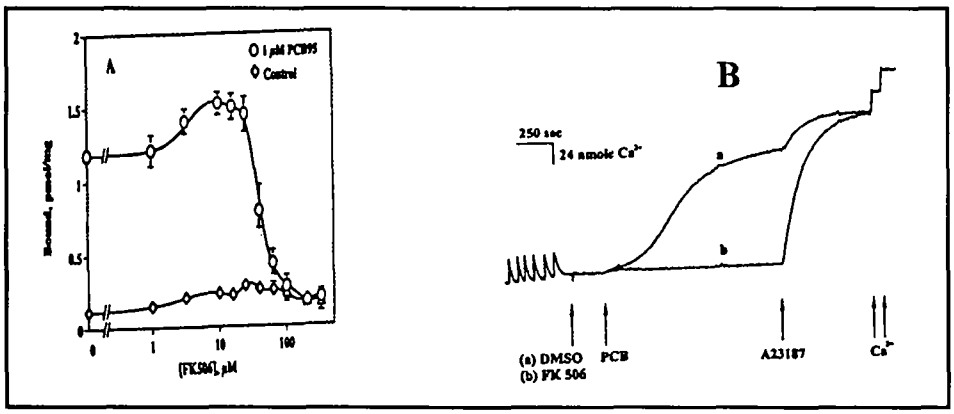

Figure 1

To test the hypothesis that PCB 95, like bastadin 5, alters SR Ca^{2+} loading capacity by converting a ryanodine-insensitive Ca^{2+} efflux pathway (leak) normally present in SR into a ryanodine-sensitive efflux (channel), the SERCA pump inhibitor thapsigargin was employed. In the absence of ryanodine, addition of thapsigargin blocked SERCA pump activity which would be expected to evoke Ca^{2+} efflux from actively loaded SR vesicles through both ryanodine-sensitive and insensitive pathways. In contrast, pump blockade on actively loaded SR vesicles pretreated with high micromolar ryanodine should only unmask Ca^{2+} efflux through a ryanodine-insensitive pathway. Figure 2A (trace a) demonstrated that after completion of active Ca^{2+} loading under the control condition, addition of thapsigargin evoked release of accumulated Ca^{2+} even though extravesicular Ca^{2+} level was initially below threshold to activate CICR. Pre-treating vesicles with 500 μM ryanodine has been shown to completely block caffeine-induced Ca^{2+} release or CICR under conditions identical to those used here (25). Figure 2B (trace a) revealed that addition of thapsigargin after completion of Ca^{2+} loading to SR vesicles pre-treated with 500 μM ryanodine unmasked a ryanodine-insensitive Ca^{2+} efflux pathway, consistent with previous findings (25). Coplanar PCB 126 (3,3',4,4',5-pentachlorobiphenyl; 5 μM), a PCB congener lacking activity toward RyRs and SR/ER Ca^{2+} transport, did not alter thapsigargin-evoked Ca^{2+} efflux regardless of whether the vesicles are pretreated with micromolar ryanodine (Fig. 2A&B, traces labeled b). In marked contrast, 5 μM PCB 95 dramatically (386% of control, $p < 0.025$) enhanced the initial rate of Ca^{2+} efflux evoked by the addition of 375 nM thapsigargin (Fig. 2A, trace c). Importantly, in the presence of channel-blocking concentration of ryanodine, the ryanodine-insensitive component of Ca^{2+} efflux unmasked by addition of thapsigargin was greatly reduced by the presence of PCB 95 in a dose dependent manner (Fig. 2B, trace c; Fig. 4C). The IC_{50} for the elimination of ryanodine-insensitive Ca^{2+} leak by PCB 95 was 3.5 ± 0.2 μM.

These results suggested that PCB 95 through its actions on the FKBP12/RyR complex, related ryanodine-insensitive and ryanodine-sensitive Ca^{2+} efflux pathways in native microsomes. Micromolar ryanodine eliminated CICR and caffeine-induced Ca^{2+} release, but not a major ryanodine-insensitive Ca^{2+} leak unmasked by SERCA pump blockade. In contrast, PCB 95; 1) enhanced net Ca^{2+} efflux from SR with the presence or absence of pump activity; and 2) eliminated the ryanodine-insensitive component of Ca^{2+} efflux (leak) unmasked by thapsigargin. The most direct interpretation of these results is that PCB 95 enhanced the proportion of channel to leak states of RyR on the SR membrane. Coplanar PCB 126 altered neither the ryanodine-sensitive Ca^{2+} efflux nor the ryanodine-insensitive Ca^{2+} leak unmasked by thapsigargin, demonstrating the structural specificity of the non-coplanar PCB 95 for eliciting the unique actions on microsomal Ca^{2+} transport.

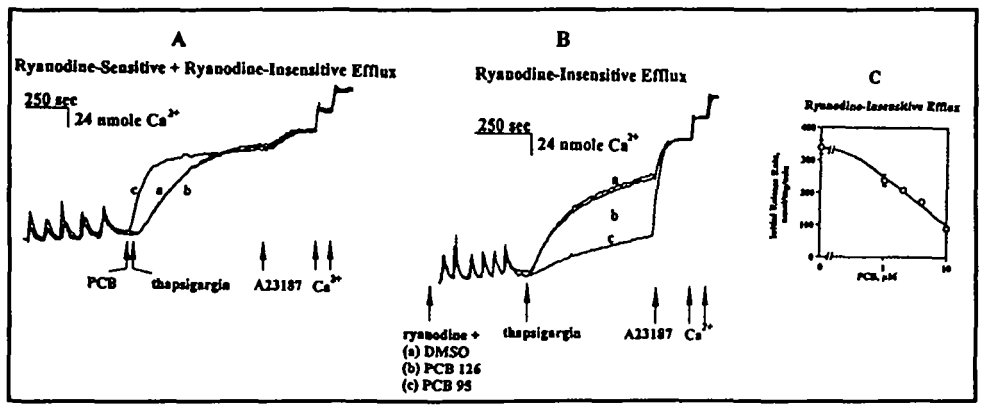

FIGURE 2

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