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Non-coplanar 2,2′,3,5′,6-Pentachlorobiphenyl (PCB 95) Amplifies Ionotropic Glutamate Receptor Signaling in Embryonic Cerebellar Granule Neurons by a Mechanism Involving Ryanodine Receptors

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The mechanisms by which non-coplanar 2,2′,3,5′,6-pentachlorobiphenyl (PCB 95) and rapamycin interact with ryanodine receptor (RyR) complexes to alter Ca^{2+} signaling, were explored in intact cerebellar granule neurons. PCB 95 (10 μM, 20 min) significantly increased the number of neurons responding to caffeine. PCB 95 sensitization of RyR-mediated responses was further supported by the observations that ryanodine pretreatment blocked response to caffeine and coplanar 2,4,4′,5-tetrachlorobiphenyl (PCB 66), which lacks RyR activity, failed to sensitize neurons. PCB 95 did not significantly alter levels of resting cytosolic Ca^{2+} nor thapsigargin-sensitive Ca^{2+} stores, suggesting a more complex mechanism than sensitization from increased cytosolic Ca^{2+} or an increased endoplasmic reticulum/cytosolic Ca^{2+} gradient. The immunosuppressant, rapamycin, sensitized neurons to caffeine in a manner similar to PCB 95, suggesting a common mechanism. PCB 95 or rapamycin significantly enhanced Ca^{2+} responses following N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxasolepropiate (AMPA) receptor activation. Store depletion or direct block of RyR with ryanodine enhanced responses to NMDA. PCB 95 further enhanced these responses to NMDA. These results suggest that PCB 95 and rapamycin enhance NMDA- and AMPA-mediated Ca^{2+} signals by modifying a functional association of the FKBP12/RyR complex that results in amplification of glutamate signaling in cultured cerebellar granule neurons in culture.

Key Words: **polychlorinated biphenyls; cerebellar granule neurons; calcium signaling; glutamate excitotoxicity; ryanodine receptors.**

The complex spatial and temporal properties of intracellular Ca^{2+} signals encode a wide variety of neuronal processes including dendritic spine growth, synaptic plasticity, and sensory perception. While Ca^{2+} influx contributes to many of these signals, Ca^{2+} release from endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) stores defines the magnitude, time course, and spatial spread of the Ca^{2+} signal (Berridge, 1998; Delmas and Brown, 2002). The principal mechanisms affecting release of Ca^{2+} from ER/SR stores involves activation of inositol 1,4,5–trisphosphate receptors (IP_3R) and/or ryanodine receptors (RyR). Bidirectional signaling, mediated by a physical and/or functional interaction between voltage- or store-operated ion channels residing within the plasmallema and IP_3R, or RyR, has been established as a major physiological mechanism linking Ca^{2+} release from stores with activation of Ca^{2+} entry (refilling currents) in a broad variety of cell types (Delmas and Brown, 2002; Montell et al., 2002; Taylor, 2002; Venkatachalam *et al.*, 2002). Because of their central role in generating context-specific Ca^{2+} signals, neuronal pathways utilizing IP_3R- and RyR-mediated Ca^{2+} release are of great interest. Evidence for the role of RyRs in the regulation of specific aspects of neuronal plasticity, dendritic growth, and spatial learning has also been provided (Balschun *et al.*, 1999; Futatsugi *et al*., 1999; Korkotian and Segal, 1999; Sun *et al*., 2000; Zhao *et al*., 2000).

A major physiological ligand for activation of RyR is intracellular Ca^{2+} itself. Enhancement of RyR activity by intracellular Ca^{2+} amplifies signals through a process termed calcium-induced calcium release (CICR). Chemical probes that enhance or inhibit RyR channel activity, such as caffeine, ryanodine, and ruthenium red, have been demonstrated to influence Ca^{2+} signaling events in a number of neuronal cell types (Kano *et al.*, 1995; Llano *et al.*, 1994; Usachev and Thayer, 1997). In this regard, non-coplanar polychlorinated biphenyls (PCBs) are anthropogenic chemicals of concern to human health that have been shown to disrupt Ca^{2+} homeostasis in cultured primary neurons and a neuronal cell line (Kodavanti *et al*., 1993; Wong *et al*., 2001). One mechanism by which non-coplanar PCB congeners alter Ca^{2+} regulation is a result of their interaction with RyR complexes that alter the fidelity of Ca^{2+} release from ER/SR stores (Wong *et al*., 1996, 1997). One of the most active modulators of RyR identified to date, 2,2′,3,5′,6-pentachlorobiphenyl (PCB 95), has been shown to promote RyR-mediated Ca^{2+} release by dramatically enhancing channel sensitivity to

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CICR and diminishing the inhibitory potency of physiological negative modulators Ca^{2+} and Mg^{2+}. These actions of PCB 95 depend on the integrity of the complex formed between FKBP12 and RyR (Wong and Pessah 1997b). Immunosuppressants such as FK506 and rapamycin, which specifically bind to FKBP12 and promote its dissociation from RyR, selectively eliminate responses to non-coplanar PCBs and related compounds in subcellular preparations and in intact PC12 cells (Pessah and Wong, 2001).

Although the molecular mechanisms by which non-coplanar PCBs alter RyR function have been studied in subcellular preparations and PC12 cells, there are no studies to date that have investigated the consequence of these mechanisms on excitatory signaling events in primary neuronal cells. The objectives of the present study are to better understand how PCB 95 sensitizes Ca^{2+} signaling in primary cerebellar granule neuronal cultures, and to define their relationship to altered RyR function. PCB 95 at 1–10 μM is found to significantly enhance sensitivity of RyR towards caffeine, and these actions are similar to those of the immunosuppressant rapamycin, suggesting a common mechanism of sensitization. Furthermore, NMDA- and AMPA-elicited Ca^{2+} signals were significantly enhanced by PCB 95 or rapamycin, revealing a functional link between amplification of RyR signaling and sensitivity to excitatory amino acids. These results may explain, at least in part, the neurotoxicity of non-coplanar PCBs to neurons, including recently identified excitotoxicity that promotes apoptosis (Howard *et al.*, 2003).

MATERIALS AND METHODS

Cerebellar cultures. Cultures enriched in cerebellar granule cells were prepared using a standard trypsin dispersion protocol (Trenkner 1991; Qiu *et al.*, 1995). Cerebella from 7-day-old mice (C57BL/6; B&K Universal, Fremont, CA) were incubated in a Ca^{2}-Mg^{2+}-free PBS containing trypsin (0.5%) and DNase I (2400 U/ml) (15 min, RT). Following trituration of cerebellar tissue with Pasteur pipettes of decreasing diameter (1.0, 0.5, 0.25 mm), the cell suspension was filtered through a 210-μm nylon mesh and then centrifuged at 1000 rpm for 5.0 min. Neurons were resuspended in Dulbecco's modified Eagle's F-12 medium supplemented with 10% heat-inactivated horse serum, 20 mM KCl, 30 mM glucose, 2 mM L-glutamine, and penicillin (20 U/ml)-streptomycin (20 μg/ml) at a density of 1–4 $\times$ 10^6 cells/ml and plated onto poly-D-lysine-coated MatTek dishes. Specialized 35-mm culture dishes, each possessing a 13-mm hole to which a coverslip was fused (MatTek Corporation; Ashland, MA), were used. The resulting 250-ml depression yielded a larger number of dishes from each animal and permitted rapid fluid exchange during imaging experiments. Cultures were kept in a 37°C humidified chamber with 5% CO_2 up to day 10 in culture. Media was replaced with serum-free medium on day seven. Growth of astrocytes was minimized by treatment with 5-fluro-2′deoxyuridine (20 μg/ml) on the first and fifth days following plating. This method gave primarily cerebellar granule neurons interspersed with occasional Purkinje neurons, inhibitory interneurons, and glia (Trenkner, 1991).

For experiments aimed at testing if the sensitizing actions of PCB 95 were dependent on the composition of the neuronal cultures (Fig. 3A–C), the method of Schilling and coworkers was utilized (Schilling *et al.*, 1991). Cerebella from 16-day-old embryos (C57BL/6) were incubated in 0.1% trypsin in PBS for 15 min at room temperature. Two volumes of 15% heat-inactivated fetal bovine serum was added to halt digestion, tissue was triturated with a plastic pipette, and the cell suspension was then filtered through a 210-μm nylon mesh. The cells were collected by centrifugation (500 $\times$ *g*, 15 min) and resuspended in basal Eagles Medium supplemented with 2 mM sodium pyruvate and hormones (Fischer′s growth medium). Neurons were plated at a density of approximately 2 $\times$ 10^5 cells per cm^2 on poly-D-lysine-coated MatTek culture dishes in Fischer's growth medium supplemented with 5% heat-inactivated horse serum. Two-thirds of the volume in each dish was replaced with serum-free Fischer's growth medium 24 h later, and cells were kept in a 37°C humidified chamber with 5% CO_2 up to day four in culture. These cultures were comprised of granule and Purkinje neurons and glia (Schilling *et al.*, 1991).

Calcium imaging. Cerebellar neurons were prepared for imaging experiments from culture days one to ten, with the majority of experiments performed on days two to four. Cells were loaded (30 min, room temperature) with fura-2 acetoxymethyl ester (1.5 mM; fura-2 AM) in a balanced salt solution (BSS; in mMs: 137 NaCl, 3.5 KCl, 0.4 KH_2PO_4, 0.33 Na_2HPO_4, 2.2 $CaCl_2$, 2.0 $MgSO_4$, 10 glucose, and 10 HEPES-NaOH, pH 7.3) supplemented with pluronic F-127 (0.02%). Following loading, cells were incubated in BSS for another 45 min to allow for cleavage of the AM-ester. Each dish was mounted on the stage of an inverted epifluorescence microscope for digital Ca^{2+} imaging of fura-2 (Qiu *et al.*, 1995). Cells were alternatively excited at 340 and 380 nm with a DeltaRam® light source (Photon Technology International, PTI, Princeton, NJ) and 510-nm emission image pairs were captured at 2-s intervals with an IC-300 digital camera (PTI). Images were digitized and saved for analysis. For each experiment, $R_{340/380}$ was reported as the quantitative measure of changes in cytosolic free Ca^{2+}. All recordings were made at room temperature.

Unless otherwise noted, preincubations of cells with PCB 95, PCB 66, rapamycin, thapsigargin, ryanodine, or control solvent (DMSO) were performed by replacing 250 μl of drug containing BSS into the culture dishes 20 min prior to commencing acquisition of images. In experiments designed to study the acute effect of PCBs and rapamycin, chemicals were introduced singly or in combination by manual perfusion using a glass pipette. Caffeine, NMDA, or AMPA was introduced by a gravity-driven, valve-controlled perfusion system (ALA Scientific, New York, NY) with the inlet placed a fixed distance from the cells imaged to reduce between-dish variability.

Analysis. Post acquisition analyses of temporal changes in fluorescence ratio ($R_{340/380}$ nm) from individual cell somas were calculated using Imagemaster software (PTI). $R_{340/380}$ were calculated using circular regions of interest placed within the somatic region of individual cells, based on the methods previously described (Grynkiewicz *et al*., 1985; also see below). In order to acquire data from granule neurons, imaging analysis was only performed on small-bodied (approximately 10-μm diameter soma) neurons. This eliminated acquisition of data from other cell types, since all other cells in the preparation (glia, Purkinje neurons, and inhibitory interneurons) contain larger somas (approximately 20-μm diameter). For experiments with mixed cerebellar cultures (Figs. 3A–3C), all cells in the field were imaged, regardless of cell diameter. Cells responding with a base-to-peak change $\geq$5 SD over the baseline recording were scored as responders and included for statistical analysis. Unless otherwise indicated, data obtained from individual neurons within each microscopic field were pooled from several dishes between days 2 and 4 in culture. In some experiments (e.g., responses to NMDA), the amplitude of each cell's response was expressed as a fraction of the mean control response obtained from all the cells analyzed within a microscopic field. For each treatment, data was collected from three or more dishes from at least two different culture sets. Differences in mean responses were tested for significance using Mann-Whitney *U* or Kruskal-Wallis statistical tests followed by a *post hoc* Dunn's test to determine significant differences between treatments. For the Dunn's test, the *p*-value for significant difference was determined by dividing 0.05 by the number of groups $\times$ the number of groups – 1. For experiments scoring percentage cells responding, data were fitted to a logit model, and differences between treatments and days in culture tested for significance by a *post hoc* χ^2 test of contrasts. Standard errors are not indicated

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for data analyzed using this statistical test since they are not symmetrical. The *p*-value for significance was calculated by dividing 0.05 by the number of comparisons and is indicated in each figure legend.

Materials. Cell culture reagents (media, serum, L-glutamine, penicillin-streptomycin, and trypsin) were purchased from Life Technologies (Grand Island, NY). DNase I was purchased from Worthington Biochemical Corporation (Lakewood, NJ), and poly-D-lysine was from Sigma (St Louis, MO). 5-Fluoro-2-deoxyuridine was purchased from Calbiochem (La Jolla, CA), Fura2-AM and pluronic F-127 were from Molecular Probes (Eugene, OR), PCB congeners were from Ultra Scientific (North Kensington, RI), ryanodine and NMDA were from Calbiochem (La Jolla, CA), rapamycin, AMPA, and caffeine were from Sigma (St Louis, MO), and thapsigargin was from Research Biochemicals (Natick, MA).

RESULTS

Cultured Cerebellar Granule Neurons Exhibit Temporal Differences in Sensitivity to Caffeine

Caffeine, a known RyR agonist, was used to test functional expression of RyR in granule neuron cultures isolated from mouse cerebellum. The mean peak intracellular calcium concentration ($[Ca^{2+}]_i$), produced by infusion of a saturating concentration of caffeine (40 mM) into the granule cell medium, increased dramatically between days 1 and 3 in culture, and progressively waned through day 10 (Fig. 1). The attenuation of individual responses to caffeine was mirrored with a diminution in the fraction of cells detectably responding over the same time period from >80% on day 3 to 20% on day 10 ($p < 0.0025$). Pretreatment of granule cells with PCB 95 (10 μM, 20 min) consistently elevated the fraction of cells responding to caffeine and the corresponding mean $[Ca^{2+}]_i$ amplitude, although at this saturating concentration of caffeine, the difference was statistically significant only late in culture when the cells expressed lower levels of RyR. The response to caffeine was completely eliminated by pretreatment of the cells with a blocking concentration of ryanodine (500 μM, 20 min), even in cells exposed to PCB 95 (10 μM, 20 min; data not shown), underscoring the specificity of the response to caffeine. These results define the temporal pattern of functional RyR expression in granule neurons in culture used in the present study.

PCB 95 Enhances RyR Sensitivity to Caffeine in Cerebellar Granule Neurons

Cerebellar granule neurons 2 to 4 days in culture were used for subsequent studies, since expression of RyR responses were more robust during this time period. The $[Ca^{2+}]_i$ response of cerebellar granule neurons depended on the concentration of caffeine in the perfusion medium. PCB 95 (10 μM, 20 min) shifted the caffeine dose response relationship of cerebellar granule neurons to the left approximately 5-fold (EC_{50} from 19 mM to 4 mM caffeine; Fig. 2A). By contrast, pretreatment of cells under identical conditions with coplanar PCB 66 that lacks RyR activity (Wong *et al.*, 1997), closely matched results obtained with solvent control. The degree to which PCB 95 sensitized RyR responses to caffeine (5 mM) increased with increasing concentrations of PCB 95 after a 20-min preincubation (Fig. 2B). PCB 95 (5 μM) increased the fraction of cells responding to caffeine 2.8-fold ($p < 0.017$; Fig. 2B).

The characteristics of how PCB 95 influenced RyR-mediated responses were further studied in cultures of mixed cerebellar neurons. Figure 3 shows that the sensitizing actions of PCB 95 on RyR-mediated responses extended to mixed neuronal cultures. Twenty-seven percent of the cerebellar neurons tested ($n = 185$ cells) responded to 5 mM caffeine (Fig. 3A). Preincubation of the cells with PCB 95 (10 μM, 20 min) consistently and significantly amplified cellular responses to suboptimal caffeine and was primarily manifested by an increased fraction of cells responding to 5 mM caffeine (from 27 to 73%; $p < 0.025$). By contrast, preincubation of cells with coplanar PCB 66 under the same condition did not significantly

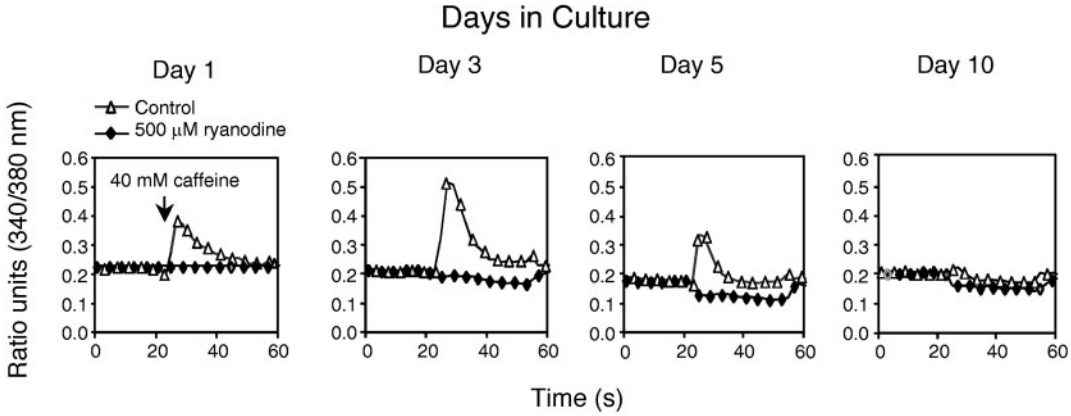

FIG. 1. Functional expression of caffeine-sensitive Ca^{2+}-stores in enriched cerebellar granule neurons exhibit temporal changes in culture. Fluorescence imaging of fura-2 responses to a 30-s challenge of saturating caffeine (40 mM) was measured on culture days 1–10, as described in Materials and Methods. Representative traces showing that the amplitude of the response to caffeine was greatest at day 3 in culture, and waned thereafter. Responses to caffeine were completely eliminated by preincubation of cells with ryanodine (500 μM) 20 min prior to caffeine challenge. Each representative trace is the mean from $n = 30–40$ cells on a single plate on each culture day. This experiment was repeated on three control and two ryanodine-treated dishes on each day in culture.

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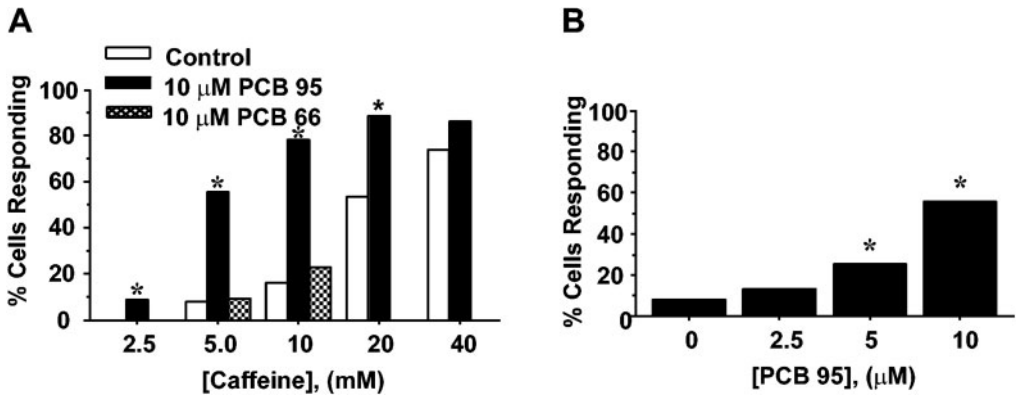

FIG. 2. PCB 95 sensitizes cerebellar granule neurons to caffeine in a dose-dependent manner. (A) PCB 95 (2,2′,3,5′,6-pentachlorobiphenyl) pretreatment (10 μM, 20 min) shifted the dose-response relationship for a 30-s exposure to caffeine (2.5–40 mM) significantly to the left, whereas PCB 66 (10 μM, 20 min) was ineffective;*$p < 0.005$, logit model followed by *post hoc* χ^2 test of contrasts. Each bar represents the % of cells responding to caffeine from at least three dishes from two culture sets. (B) Pretreatment of granule neurons with PCB 95 enhances the number of cells responding to caffeine (5 mM, 30 s) in a dose-dependent manner. Each bar represents the % of cells responding to caffeine from at least three dishes from two culture sets; *$p < 0.01$, logit model followed by *post hoc* χ^2 test of contrasts).

alter the responsiveness of neurons to caffeine (Fig. 3A). Cells exposed to PCB 95 also exhibited more vigorous responses to ryanodine; increasing the fraction of cells responding from 34 to 68% (Fig. 3B, $p < 0.05$), and significantly shortening the latency for attaining the peak response (from 14 to 69% of cells exhibiting a peak response within the first 100 s; Fig. 3C; $p < 0.004$). Taken together, these results indicate that the sensitizing actions of PCB 95 on RyR-mediated responses were not dependent on the methods used to culture cerebellar neurons.

Relationship Between Rapamycin- and PCB 95-induced RyR Sensitization

Immunophilin FKBP12, a *cis/trans* peptidylprolyl isomerase, plays an important signaling function in neurons (Snyder *et al*., 1998), and has been shown to be a required component of PCB 95-mediated sensitization of RyR (Pessah and Wong, 2001; Wong and Pessah, 1997; Wong *et al.*, 2001). We investigated the influence of the immunosuppressant rapamycin, a high-affinity ligand for FKBP12, and its possible relationship

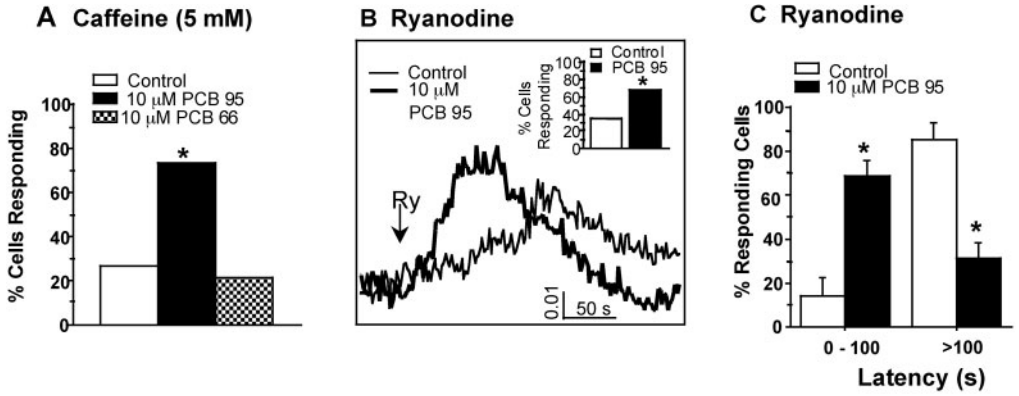

FIG. 3. PCB 95 amplifies RyR-mediated responses of cerebellar neurons. Fluorescence Ca^{2+} imaging was performed using 2–4 day-old cerebellar neurons cultured by the method of Schilling *et al.* (1991) as described in Materials and Methods. (A) Compared to the solvent controls (0.1% DMSO), 20 min preincubation with 10 μM PCB 95 significantly increased the number of cells responding to a 30-s challenge of 5 mM caffeine; *$p < 0.025$, logit model followed by a *post hoc* χ^2 test of contrasts. In comparison, preincubation with PCB 66, a coplanar PCB, under the same conditions, had no significant effect on responses to caffeine ($p > 0.025$). Each bar represents the % of cells responding to caffeine obtained from five independent dishes ($n = 26–48$ cells per dish). (B) Twenty minutes of preincubation with PCB 95 (10 μM) significantly increased the number of cells responding to a 10-s exposure of 250 μM ryanodine compared to DMSO controls; *$p < 0.05$, logit analysis followed by *post hoc* χ^2 test of contrasts. Each trace represents the mean response in a single dish (20–40 cells). Inset: each bar represents the % of cells responding to ryanodine compiled from four to six dishes (23–48 cells/dish) per treatment. (C) Pretreatment with PCB 95 (10 μM, 20 min) significantly reduces the latency to peak response elicited by 250 μM ryanodine compared to solvent control; *$p < 0.004$, Kruskal-Wallis test followed by a *post hoc* analysis using Dunn's test. Each bar represents the mean ± SEM % of responding cells in the time periods indicated. Data are obtained from the same dishes imaged in B.

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to sensitization of RyR by PCB 95 in intact granule neurons in culture. Pretreatment of cerebellar granule neurons with rapamycin (20 μM, 20 min) sensitized responses to 5 mM caffeine in a manner similar to that seen with PCB 95 (increased the fraction of cells responding over three-fold ($p < 0.008$; Fig. 4)). PCB 95 (10 μM) and rapamycin (50 μM) introduced in combination slightly elevated the number of neurons responding to caffeine compared to either treatment alone, suggesting a common mechanism of action (Fig. 4). The percentage of neurons responding to caffeine in the presence of PCB 95 and rapamycin in combination was not significantly different compared with responses obtained when the compounds were tested singly ($p > 0.008$; Fig. 4).

Since elevated $[Ca^{2+}]_i$ can enhance the open probability of RyR channels (Pessah *et al*., 1985, 1987; Zimanyi and Pessah, 1991), the immediate actions of DMSO (0.1%), PCB 95 (10 μM), or rapamycin (50 μM) on $[Ca^{2+}]_i$ were examined by capturing images during application of these agents and for 20 min thereafter (Fig. 5A). In contrast to previous reports with granule neurons isolated from rat cerebellum (Kodavanti *et al*, 1993, Mundy *et al*., 1999), no significant elevation in resting $[Ca^{2+}]_i$ of cerebellar granule cells were observed upon infusion of the maximum concentration of PCB 95 (10 μM) within the time frame (20 min) used in the present study. Rapamycin elicited a small but consistent decrease in the fluorescence ratio, which may represent enhanced buffering of cytosolic Ca^{2+} (Wong and Pessah, 1997). Since increases in the level of Ca^{2+} within the ER store has been shown to sensitize RyR-mediated Ca^{2+} release (Berridge, 1998; Verkhratsky and Petersen, 1998), more direct assessment of Ca^{2+}-store levels were undertaken following DMSO (0.1–0.5%), PCB 95 (10 μM), or rapamycin (50 μM) pretreatment. Thapsigargin, a SR/ER Ca^{2+}-ATPase-pump inhibitor, was infused onto the culture to induce Ca^{2+} leak from ER stores and provide a direct measurement of Ca^{2+}-store levels. Cerebellar granule cells pretreated with either PCB 95 or rapamycin exhibited a small (but not statistically significant) elevation in the amount of Ca^{2+} sequestered within the thapsigargin-sensitive ER store (Fig. 5B, inset) demonstrating that the increase in RyR-mediated Ca^{2+} release is not primarily due to increased Ca^{2+}-store capacity.

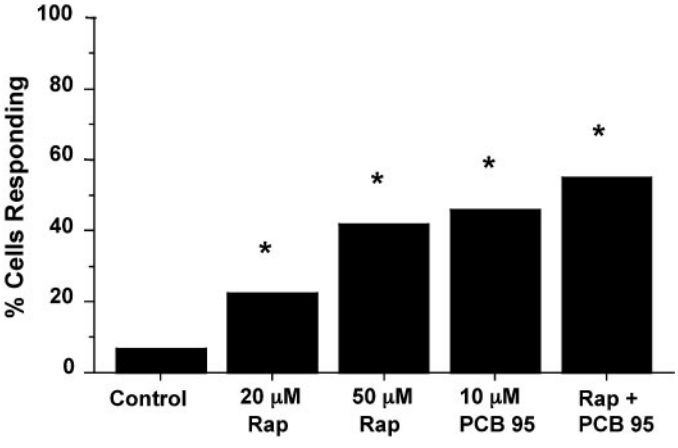

FIG. 4. Rapamycin enhances responses of granule neurons to caffeine in a manner similar to PCB 95. Rapamycin pretreatment (50 μM, 20 min) increased the fraction of neurons responding to caffeine (5 μM perfused for 30 s) in a manner similar to PCB 95 (10 μM). PCB 95 (10 μM) and rapamycin (50 μM) in combination slightly enhance the fraction of neurons responding to caffeine, suggesting a common mechanism for sensitization. Data were compiled from at least three dishes from two culture sets; *significant difference from control ($p < 0.008$, logit model followed by *post hoc* χ^2 test of contrasts.

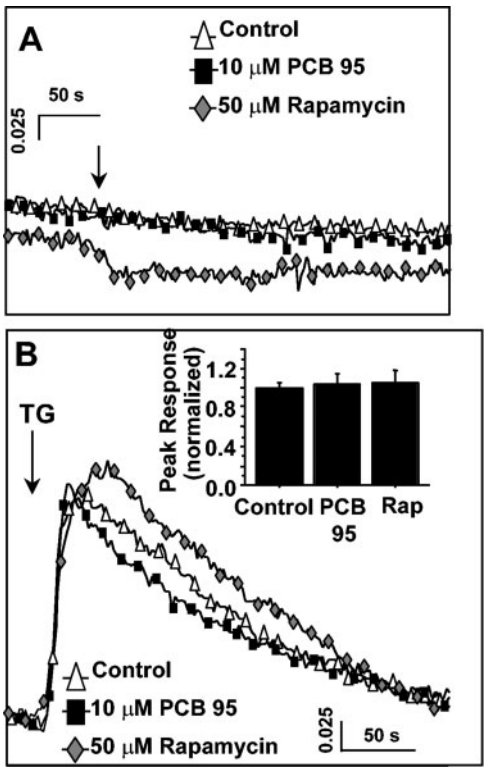

FIG. 5. Acute exposure to PCB 95 and rapamycin do not significantly alter Ca^{2+} levels in the cytosol or thapsigargin-sensitive Ca^{2+} stores of resting cerebellar granule neurons. (A) Introduction of PCB 95 (10 μM) or rapamycin (50 μM) did not elicit a rise in intracellular Ca^{2+} in resting cerebellar granule neurons. Each trace is the mean response from a single dish (22–32 cells). Similar results were obtained in two additional dishes from at least two culture sets. The arrow identifies the time point when DMSO (0.5%), PCB 95 (10 μM), or rapamycin (50 μM) was perfused onto the cells. The vertical axis is in ratio units (340/380 nm). (B) Pretreatment with PCB 95 (10 μM, 20 min) or rapamycin (50 μM, 20 min) did not significantly alter Ca^{2+} levels within the thapsigargin (TG)-sensitive store. Each trace is the mean response from a single dish (37–44 cells). Similar results were obtained in three to six additional dishes from two culture sets. The arrow identifies the time point when TG (1 μM) was perfused onto the cells. Vertical axis is in ratio units (340/380 nm). Inset: mean ± SEM peak response to TG. Data from each dish were normalized to the mean control response for each culture set and then data from both cultures combined. Each bar represents data from four to seven dishes of cells from two culture sets. No significant difference is observed between treatments; $p > 0.008$, Kruskal-Wallis test followed by a *post hoc* analysis using Dunn's test.

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PCB 95 Enhances NMDA- and AMPA-induced Ca^{2+} Signals in Cerebellar Granule Neurons

Studies in cerebellar granule neurons show that two glutamate-sensitive pathways (NMDA receptor and metabotropic glutamate receptor [mGluR]) modulate RyR-mediated Ca^{2+} release (Chavis *et al.*, 1996; Netzeband *et al.*, 1999; Simpson *et al.*, 1993). Experiments utilizing RyR modulators demonstrated that RyR-mediated Ca^{2+} release contributes to NMDA receptor-mediated Ca^{2+} signals in cerebellar granule neurons, and that activation of the mGluR alters RyR function. Taken together with the current findings, these results suggest that enhancement of RyR-mediated responses by PCB 95 could significantly influence glutamate-mediated Ca^{2+} signaling in cerebellar granule neurons.

Our attempts to measure Ca^{2+} responses mediated by the metabotropic glutamate receptor agonist, t-ACPD (400 μM) in granule cells in culture were unsuccessful (data not shown). Mixed success in eliciting t-ACPD-induced Ca^{2+} fluxes has been reported previously (del Río *et al.*, 1999). Since t-ACPD-elicited Ca^{2+} responses have been measured at 25°C following cellular depolarization (Irving *et al.*, 1992b), attempts were made to elicit t-ACPD-induced Ca^{2+} signals directly following a 10-s depolarization with 40 mM KCl with no added success. In addition, preincubation with PCB 95 (10 μM, 20 min) did not produce a measurable t-ACPD-induced Ca^{2+} response. In comparison, the responses to perfusion of NMDA or AMPA were robust. Cells pretreated with PCB 95 exhibited enhanced responses to a 10-s exposure of 5 μM NMDA or AMPA, with the peak Ca^{2+} response increasing by 40 and 87%, respectively, relative to control measurements (Figs. 6A–6D).

Additional experiments were undertaken to assess the mechanism by which PCB 95 sensitized responses to NMDA. Pretreatment of granule neurons with thapsigargin (1 μM, 65 min) caused complete depletion of RyR-sensitive stores, verified by the lack of response to subsequent caffeine challenge (data not shown). Depletion of ER stores with thapsigargin in granule neurons enhanced the amplitude of subsequent responses to NMDA 55% relative to control (Fig. 7A). Interestingly, PCB 95 elicited an additional 38% amplification of NMDA responses subsequent to store depletion, an effect not significantly different from that observed with PCB 95 alone (Fig. 6B) (a total of 110% enhancement relative to control). High concentrations of ryanodine have been shown to sequentially activate, then inhibit, RyR (Pessah and Zimanyi, 1991). As predicted by these mechanistic studies, ryanodine (250 μM, 65 min) inhibited caffeine-induced Ca^{2+} release and stores could be subsequently mobilized with thapsigargin (data not shown). RyR block by itself was sufficient to enhance responses to NMDA (14% enhancement relative to control) and, in combination with PCB 95, further enhanced responses to agonist 38% (total of 50% enhancement relative to control (Fig. 7B). These results showed that enhancement of neuronal responses to NMDA by PCB 95 were maintained and remained the same magnitude, either with (1) ER store depletion or (2) block of RyR.

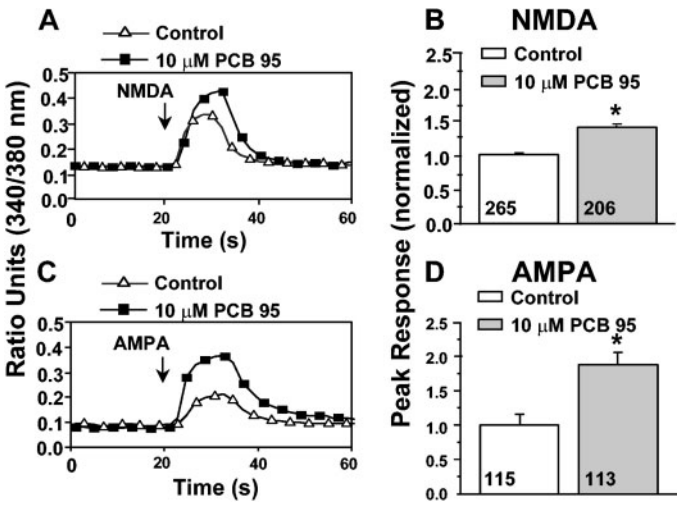

FIG. 6. PCB 95 significantly amplifies NMDA and AMPA-induced Ca^{2+} signals in cerebellar granule neurons. For the NMDA experiments, DMSO (0.1%) or PCB 95 (10 μM, 20 min preincubation) was added to the granule cells in BSS supplemented with the coagonist glycine (5 μM) and lacking Mg^{2+} to relieve the NMDA receptor block. Under these conditions, the NMDA receptor can be activated without concurrent depolarization. For the AMPA experiments, DMSO or PCB 95 was added to BSS containing HEPES (25 mM) to further ensure no change in pH of the solution upon addition of AMPA. Representative Ca^{2+} signals produced by NMDA are shown in A, and their corresponding mean ± SEM peak responses are shown in B. NMDA (5 μM) was perfused onto the cells for 10 s at the time indicated by the arrow. Each trace in A represents the mean response from a microscopic field of neurons (45–50 cells). The mean ± SEM values in B were calculated from six dishes per treatment from two culture sets. The peak Ca^{2+} response for each cell was normalized to the mean control response for each culture and then data from both cultures combined. Representative Ca^{2+} signals produced by addition of AMPA are shown in C and their corresponding mean ± SEM peak responses are shown in D. AMPA (5 μM) was perfused onto the cells for 10 s at the time indicated by the arrow. Each trace in C represents the mean response from a microscopic field of neurons (22–27 cells). The mean ± SEM in D was calculated from four dishes per treatment from two culture sets. The mean peak Ca^{2+} response for each dish was normalized to the mean peak control response for each culture, then data from both cultures combined. The numbers in the bar graphs (B and D) represent the number of neurons in each treatment group; *significant difference from control ($p < 0.05$, Mann-Whitney U test).

Response to NMDA was also enhanced with rapamycin pretreatment (50 μM, 20 min; Fig. 8), and was not surprising, since rapamycin amplified caffeine-induced release, a response clearly mediated by RyR. Since PCB 95, rapamycin, thapsigargin, and ryanodine all either directly or indirectly affect CICR, it is possible that all four reagents enhance NMDA-elicited Ca^{2+} signals through convergent mechanisms that modify RyR conformation. A potential model showing how PCB 95 and rapamycin affect ionotropic glutamate-receptor signaling through the RyR/FKBP12 complex is presented in Figure 9.

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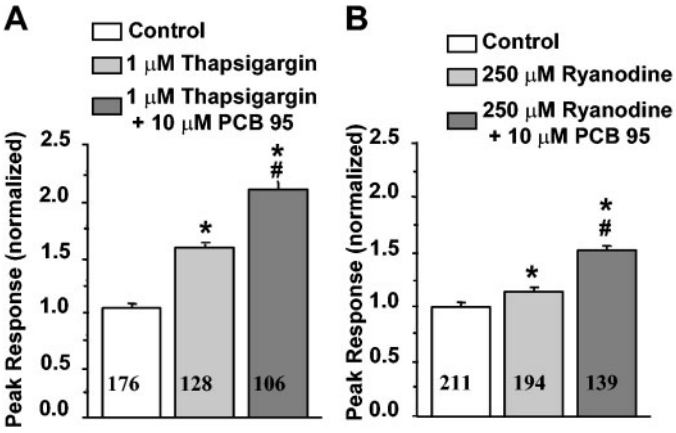

FIG. 7. The PCB 95-induced increase in the NMDA-elicited Ca^{2+} response is not due to enhanced Ca^{2+} release from thapsigargin-sensitive stores. (A) Depletion of Ca^{2+} stores with TG (1 μM, 65 min) did not eliminate the PCB 95-induced amplification of the Ca^{2+} response elicited by NMDA. Each bar represents the mean ± SEM peak Ca^{2+} response following a 10-s perfusion of NMDA (5 μM). The peak Ca^{2+} response for each cell was normalized to the mean control response for each culture and data from two cultures were combined. (B) Inhibition of the ryanodine receptor with ryanodine (250 μM, 65 min) did not eliminate the PCB 95-induced amplification of the NMDA Ca^{2+} response. As in A, each bar represents the mean ± SEM peak Ca^{2+} response following a 10-s perfusion of NMDA (5 μM). The peak Ca^{2+} response for each cell was normalized to the mean control response for each culture and data from two cultures combined. The numbers in the bar graphs represent the number of neurons in each treatment group; *significant difference from control; #significant difference when compared with treatment with respective pharmacological agent ($p < 0.008$, Kruskal-Wallis test followed by *post hoc* analysis using Dunn's test).

DISCUSSION

The present study revealed that non-coplanar PCB 95 reduced the threshold of RyR to caffeine in mouse cerebellar granule neurons. In good agreement with the findings of Irving and coworkers with granule cells cultured from rat cerebellum (Irving *et al.*, 1992a), mouse cerebellar granule neurons in the present study exhibited a transient responsiveness to a saturating concentration of caffeine from culture days 1 to 3, which abated almost completely by day 10. The temporal changes in caffeine sensitivity observed are likely to reflect changes in RyR protein expression and/or intracellular localization. Expression of RyR in the soma of cerebellar granule neurons has been shown to increase between culture days 1 and 3 (Mhyre *et al.*, 2000). By day 7, RyR expression decreased in the soma and increased in dendrites and axons.

In contrast to the temporal changes in caffeine sensitivity seen with cerebellar granule neurons in culture, PCB 95 enhanced the sensitivity to caffeine with short incubation times (20 min), suggesting a direct interaction with existing RyR complexes resulting in signal amplification. Interestingly the resting $[Ca^{2+}]_i$ and the Ca^{2+}-store levels were not measurably altered by PCB 95, indicating an allosteric mechanism altering the sensitivity to direct RyR activators. Also evident from the present studies is the similar nature by which rapamycin and PCB 95 enhanced the sensitivity of granule neurons to caffeine, indicating a common mechanism. Undifferentiated PC12 cells, perfused with nM to low mM PCB 95, elicited a transient rise of $[Ca^{2+}]_i$ that was not completely dependent on extracellular Ca^{2+} (Wong *et al.*, 2001). In addition, preincubation with FK506 completely eliminated PCB 95-induced rises in cytoplasmic Ca^{2+} in PC12 cells. The differences in eliciting direct responses to PCB 95 and the ability of immunosuppressant to eliminate the effects of PCB 95 in PC12 cells and granule neurons may reflect differential expression of RyR isoforms in the two cell types and/or how FKBP12 modulates RyR channel function. In support for this interpretation, mammalian type 1 (RyR1) and type 3 (RyR3) ryanodine receptors exhibit fundamental differences in pharmacology and gating behavior that may stem from differences in key protein-protein interactions (Fessenden *et al.*, 2000; Rousseau and Proteau, 2001). Although these results suggest a common primary mechanism mediated by the FKBP12/RyR-sensitive store, there appears to be a fundamental difference in how FKBP12 regulates RyR function in the two cell types, especially when probed with PCB 95 and immunosuppressant.

Previous studies with skeletal fibers and isolated SR have shown that preincubation with FK506 or rapamycin increased the sensitivity of RyR1 to caffeine (Ahern *et al.*, 1997; Brillantes *et al.*, 1994; Lamb and Stephenson, 1996; Mayrleitner *et al.*, 1994; Timerman *et al.*, 1993; Wong and Pessah, 1997). These actions have been attributed to the dissociation of

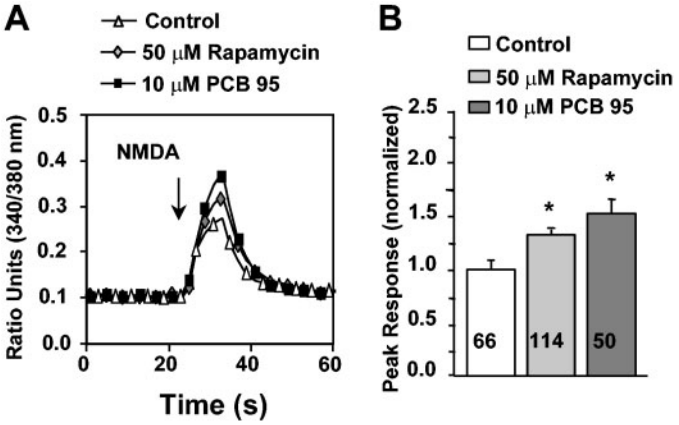

FIG. 8. Rapamycin amplifies the NMDA-induced Ca^{2+} response. Representative Ca^{2+} signals (A) and the mean ± SEM peak response of the Ca^{2+} signals (B) elicited by NMDA in control granule neurons (0.1% DMS0) and neurons pretreated with rapamycin (50 μM, 20 min) or PCB 95 (10 μM, 20 min). NMDA was perfused onto the cells for 10 s at the time indicated by the arrow. Each trace in A represents the mean response from a microscopic field of neurons (24–32 cells). The mean ± SEM values in B are calculated from at least two dishes per treatment from two culture sets. The peak Ca^{2+} response for each cell was normalized to the mean control response for each culture and then data from both cultures combined; *significant difference from control ($p < 0.008$ Kruskal-Wallis test followed by *post hoc* analysis using Dunn's test).

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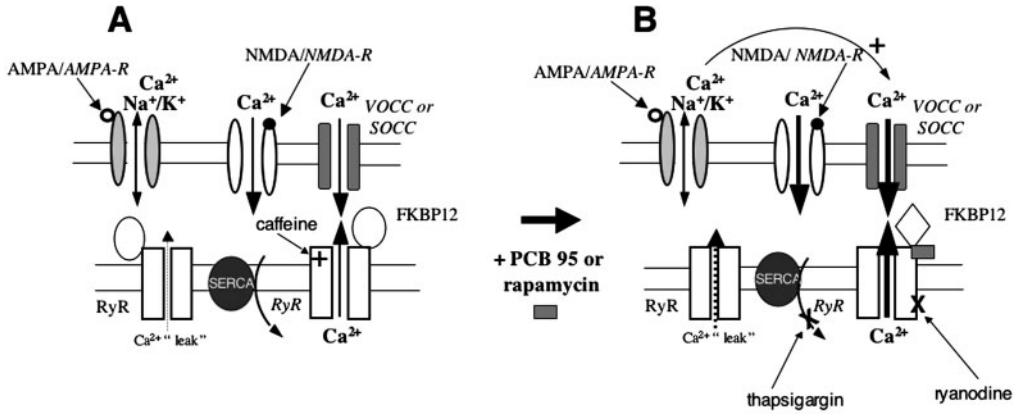

FIG. 9. Model for PCB 95 and rapamycin-induced enhancement of ionotropic glutamate receptor-mediated Ca^{2+} entry: under normal conditions (A), application of NMDA or AMPA results in Ca^{2+} influx through their respective receptors, triggering RyR-mediated Ca^{2+} release, as well as additional Ca^{2+} entry through VOCCs and SOCCs. Our data suggest that with PCB 95 or rapamycin pretreatment (B), there is an alteration in the RyR/FKBP12 complex leading to enhanced SOCC- and/or VOCC-mediated Ca^{2+} influx following NMDA or AMPA receptor activation. One hypothesis is that the communication between the RyR/FKBP12 complex and VOCCs or SOCCs is a physical one (as depicted here) and alterations in the RyR/FKBP12 complex leads to sensitization of VOCCs or SOCCs through direct physical interactions. An alternative hypothesis is that the communication from the RyR/FKBP12 complex to VOCCs or SOCCs is through more indirect chemical communication. It should also be noted that since we did not find a measurable decrease in the NMDA-induced Ca^{2+} response following RyR block (Ry) or store depletion (TG), this suggests that only a small portion of RyRs are activated during NMDA receptor stimulation in our experimental paradigm. Only a subset of RyRs may be activated due to differences in intracellular localization and/or protein/protein interactions.

FKBP12 from the RyR complex by the immunosuppressant. However, the actions of PCB 95 have been shown to require the integrity of the FKBP12/RyR1 complex, since both FK506 and rapamycin eliminated responses to non-coplanar PCB (Wong *et al.*, 1997b). Work with non-coplanar brominated tyrosine macrocyclic bastadins, isolated from the marine sponge *Ianthella basta*, have also been shown to possess activity that requires an intact FKBP12/RyR complex, but bastadin 5 itself failed to dissociate FKBP12 from skeletal SR membranes (Mack *et al.*, 1994). Moreover, bastadin 10 lacked RyR activity until the complex was reconstituted with recombinant FKBP12 (Chen *et al*., 1999). These results indicate that PCB 95 and bastadins interact with the FKBP12/RyR complex to enhance sensitivity to caffeine and RyR-mediated Ca^{2+} signaling. Whether PCB 95 sensitizes the RyR by weakening the FKBP12/RyR complex in culture granule neurons remains to be determined.

PCB 95 and Rapamycin Amplify Signaling through Ionotropic Glutamate Receptors

In addition to sensitizing RyR-mediated Ca^{2+} release, a new finding was that PCB 95 and rapamycin significantly enhanced responses to agonists (NMDA and AMPA) of ionotropic glutamate receptors. In addition to typical Ca^{2+} influx mediated through NMDA receptor activation, AMPA receptors expressed in cerebellar granule neurons have been reported to also conduct Ca^{2+} (Jones *et al.*, 2000). Our results indicated that PCB 95 sensitizes both receptor subtypes to their respective agonists. Unexpectedly, these actions of PCB 95 were not significantly altered by store depletion (thapsigargin) and RyR block (Ry). In fact, thapsigargin or Ry alone enhanced NMDA responses and were seen in addition to the enhancement produced by PCB 95. Since PCB 95, rapamycin, thapsigargin, and ryanodine all either directly or indirectly affect CICR, it is possible that all four reagents enhance NMDA-elicited Ca^{2+} signals through convergent mechanisms that modify RyR conformation. Ca^{2+} influx through store- and voltage-operated Ca^{2+} influx channels (SOCCs and VOCCs) have been shown to amplify Ca^{2+} signals mediated by NMDA (Netzeband *et al*., 1999; Qiu *et al*., 1995, 1998; Simpson *et al*., 1993). Furthermore, SOCCs and VOCCs have been shown to physically interact with RyRs in a manner that enhances Ca^{2+} entry in response to RyR activation (Chavis *et al.*, 1996; Kiselyov *et al.*, 2000; Nakai *et al*., 1996). These results indicate that the amplification mechanism may be mediated through conformational states of RyR resulting from direct binding of SOCCs or VOCCs to the FKBP12/RyR complex (caffeine, ryanodine, rapamycin, or PCB 95) or indirectly as a consequence of depletion of Ca^{2+} stores (thapsigargin). Considering that PCB 95 amplifies NMDA-mediated signaling, even when the RyR channels are blocked by ryanodine, the possibility is raised that conformational coupling between RyR and SOCCs or VOCCs could amplify excitatory signals in cerebellar granule neurons. Therefore, ligands that alter the interaction between RyR and FKBP12 may in turn modulate functional interactions with plasma membrane Ca^{2+} entry channels responsible for signal amplification and refilling of stores. In support of this interpretation, rapamycin has been shown to alter functional communication between the RyR and L-type VOCC (Lamb and Stephenson, 1996).

While there is evidence in the literature that non-coplanar PCBs may affect Ca^{2+} regulation through RyR-independent

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mechanisms (Kodavanti *et al*., 1993; Mundy *et al*., 1999), it is unlikely these interactions are responsible for the PCB 95 enhancement of the NMDA and AMPA receptor-mediated Ca^{2+} signal observed in the present study. Kodavanti *et al*. (1993) originally reported that non-coplanar 2,2′-dichlorobiphenyl (PCB 4; 30–50 μM) caused an increase in $[Ca^{2+}]_i$ when perfused onto cerebellar granule neurons, and Mundy *et al.* (1999) determined this intracellular Ca^{2+} rise to be due to an increase in Ca^{2+} influx, likely due to diminished microsomal and mitochondrial Ca^{2+} buffering. While this is a plausible explanation for their data, it should be noted that in our studies, we did not observe an increase in $[Ca^{2+}]_i$ upon PCB 95 addition (Fig. 5A). Several experimental differences should be noted in the present study, including the age of the neurons in culture on experimental challenge (days 2–4 vs. days 6–8), and the substantially higher levels (30–50 μM) of a different non-coplanar PCB congener (2,2′-dichlorobiphenyl) needed to alter resting $[Ca^{2+}]_i$ in the previous studies. More recently, Inglefield and coworkers tested the actions of A1254 and non-coplanar *ortho*-substituted PCBs (10 μM) on cortical neurons in culture (Inglefield *et al.*, 2001, 2002). Aroclor 1254 and the non-coplanar PCBs tested (but not those having coplanar structures) elicited intracellular Ca^{2+} transients that were not altered either by ryanodine or the L-type Ca^{2+} channel blocker, nifedipine. Rather, these effects seemed to be mediated through activation of IP_3Rs. It is interesting to note that Howard and coworkers recently found that Aroclor 1254 and non-coplanar PCB 47 (1–10 μM) significantly enhanced caspase-mediated apoptosis in hippocampal, but not cortical, neurons in culture (Howard *et al*., 2003). Although inhibitors of Ca^{2+} entry and IP_3R-mediated Ca^{2+} release failed to ameliorate PCB 47-induced apoptosis, benzeneethanamine 2,6-dichloro-4-(dimethylamino)-α-methyl-9-chloride, FLA 365, a selective cell-permeable blocker of RyRs (Calviello and Chiesi, 1989; Mack *et al.*, 1992), completely prevented excitotoxicity mediated by this non-coplanar PCB (Howard *et al*., 2003). Collectively, these data indicate that the initial molecular target(s) of non-coplanar PCBs are likely to reside within the endoplasmic reticulum (structurally and functionally related RyR and IP_3R channel complexes). How these compounds alter Ca^{2+} signaling depends not only on the pattern of expression of these channels within cells but the context in which they are expressed. Considering that both RyR and IP_3R channels are highly regulated by both small ligands and accessory proteins, it is not surprising that the net actions of non-coplanar PCBs on Ca^{2+} signaling are highly dependent on cellular context.

Implications for Non-coplanar Ortho-substituted PCB Neurotoxicity

Multiple lines of evidence support the preferential neurotoxicity of non-coplanar, *ortho*-substituted PCBs. Decreased catecholamine levels in certain brain regions in mammals (Seegal *et al*., 1990, 1991a,b) and reduced dopamine levels in rat pheochromocytoma cells (Shain *et al.*, 1991) have been attributed to certain *ortho*-substituted PCB congeners. Perinatal exposure of monkeys and rodents to PCBs induced behavioral abnormalities consisting of delayed reflex development, altered activity patterns, learning deficits, and impaired memory, problems currently of concern to human exposure (Schantz, *et al.*, 1995, 1997, 2003). Kodavanti *et al*. (1993) first linked non-coplanar PCB toxicity to its ability to disrupt Ca^{2+} regulation in cerebellar granule cell studies. Evidence for a direct interaction of *ortho*-substituted PCBs with the RyR (Wong *et al*., 1996, 1997), suggests this may be a selective mechanism for the disruption of Ca^{2+} homeostasis and toxicity in neurons.

The finding that low levels of PCB 95 significantly enhanced Ca^{2+} signals elicited by NMDA and AMPA glutamate receptor activation, without altering basal Ca^{2+} levels, lends support for a selective, receptor-mediated mechanism of neurotoxicity. An alteration in the tightly regulated Ca^{2+} signaling pathways may induce profound changes in cellular functioning that eventually leads to the neurotoxicity described in numerous experimental and epidemiological studies. In conclusion, *ortho*-substituted PCBs are shown for the first time to directly interact with the RyR to sensitize RyR-mediated Ca^{2+} release in cerebellar granule neurons. Sensitization of responses to caffeine by rapamycin was similar to that of PCB 95, supporting a common mechanism of sensitization that could involve weakening of the RyR/FKBP12 complex. In addition, PCB 95 was shown to enhance NMDA- and AMPA-elicited Ca^{2+} signals, suggesting that the PCB 95/RyR/FKBP12 complex may be responsible for directly or indirectly altering Ca^{2+} influx through VOCCs or SOCCs. The PCB 95-induced alterations in Ca^{2+} signaling presented here provide insight into the mechanism of non-coplanar PCB toxicity.

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