Inositol 1,4,5-Triphosphate Receptor-Sensitive Ca\(^{2+}\) Release, Store-Operated Ca\(^{2+}\) Entry, and cAMP Responsive Element Binding Protein Phosphorylation in Developing Cortical Cells following Exposure to Polychlorinated Biphenyls

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Received October 11, 2000; accepted December 19, 2000 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

The present study assessed intracellular Ca\(^{2+}\) signaling pathways sensitive to polychlorinated biphenyls (PCBs), xenobiotics that perturb neural development and plasticity. Mobilization of intracellular Ca\(^{2+}\) stores after acute exposure to a PCB mixture, Aroclor 1254 (A1254), as well as selected PCB congeners, was studied in P0 rat cortical neuronal culture using fluorescence microscopy. Ca\(^{2+}\) responses to A1254 progressed from a transient intracellular Ca\(^{2+}\) increase (lasting 3–5 min) at 1 to 2 µM (0.3–0.6 ppm) to a Ca\(^{2+}\) transient with store-operated Ca\(^{2+}\) influx and later disturbances of basal Ca\(^{2+}\) concentration; this latter pattern occurred more often with 10 to 20 µM (3–6 ppm) A1254. Thapsigargin, xestospongin C, and carbachol/Ca\(^{2+}\)-free buffer blocked significantly the PCB-induced Ca\(^{2+}\) transient, whereas both ryanodine (to deplete ryanodine-sensitive stores) and the L-type Ca\(^{2+}\) channel blocker nifedipine were without effect on the A1254 initial Ca\(^{2+}\) transient. Both thapsigargin and xestospongin also blocked latent elevations (at 0.5 h) in Ca\(^{2+}\), disturbances that depend upon extracellular Ca\(^{2+}\) entry via ion channels. Two possible consequences were explored. Phosphorylation of cAMP responsive element binding protein, a Ca\(^{2+}\)–activated nuclear transcription factor (CREB), occurred in an A1254 concentration-dependent manner and persisted at least 1 h. Cell viability following a 24-h exposure to A1254 (2–20 µM) was decreased at 20 µM, but only in cells cultured >6 days. This cell death did not occur via an apoptotic mechanism. These results indicate that Ca\(^{2+}\) disturbances following PCB exposure are associated with 1) discrete alterations in IP\(_3\) receptor-mediated signals and 2) activation of downstream events that impact developing cortical cells.

Polychlorinated biphenyls (PCBs) are a class of persistent pollutants that are prevalent in the environment, and there is increasing evidence from both human epidemiological studies and animal models that developmental exposure to low levels of PCBs can result in subtle changes in behavior and cognition (see review by Brouwer et al., 1999). Because there is an absence of overt pathological alterations in the human as well as in animal models (Brouwer et al., 1999), it presently appears that subtle rather than gross macroscopic changes in human and animal nervous systems underlie the altered neurologic function and/or impaired cognition that occur following developmental PCB exposure. The cellular and molecular basis for PCB-induced developmental neurotoxicity is unclear; but in vitro, PCBs have been shown to disrupt Ca\(^{2+}\) homeostasis and processes involved in Ca\(^{2+}\)-mediated signal transduction (reviewed in Tilson and Kodavanti, 1997).

Because Ca\(^{2+}\) signaling in developing and mature neurons can initiate and regulate a number of cellular responses,

AABBREVIATIONS: PCB, polychlorinated biphenyl; [Ca\(^{2+}\)], intracellular calcium concentration; IP\(_3\), inositol 1,4,5-triphosphate; A1254, Aroclor 1254; fura-2-AM, fura-2-acetoxymethyl ester; DCB, 2,2'-dichlorobiphenyl (PCB 4); PCB 15, 4,4'-dichlorobiphenyl; PCB 126, 3,3',4,4',5-penta-chlorobiphenyl; PCB 138, 2,2',3,4,4',5'-hexachlorobiphenyl; DMSO, dimethyl sulfoxide; DIV, days in vitro; TUNEL, terminal-deoxynucleotidyltransferase-mediated dATP biotin nick-end labeling; CREB, cAMP responsive element binding protein; pCREB, phospho-cAMP responsive element binding protein; SOC, store-operated channel; VGCC, voltage-gated Ca\(^{2+}\) channel; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; GABA\(_A_\), γ-aminobutyric acid receptor, type A; AFG, 7-amino-4-trifluoromethylcoumarin.
perturbations in temporal cellular Ca\textsuperscript{2+} signals may have important effects. Changes in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i) can lead to subtle or profound changes in neuronal function by regulating diverse processes, such as cell survival and death, or changes in cellular phenotype and synaptic plasticity (Curtis and Finkbeiner, 1999). Thus, the impact of Ca\textsuperscript{2+} signals in developing cells is far-reaching. Ca\textsuperscript{2+} signals in neurons may be stimulated by many factors, and sources of these signals include influx through plasma membrane bound ion channels and release from intracellular stores operated by inositol 1,4,5-triphosphate (IP\textsubscript{3}) receptors or ryanodine receptors (Berridge, 1998). In order to test the hypothesis that PCBs can affect Ca\textsuperscript{2+} signals in developing neurons, we have used an in vitro model system of developing neocortical cells that recapitulates many aspects of normal cortical neuron development, including transmitter pharmacology (Dichter, 1978; Inglefield and Shafer, 2000a). This model is appropriate to study the mechanisms of action of developmental neurotoxicants in view of the cognitive deficits, as well as functional changes in cortical (Altman et al., 1998) and hippocampal (Gilbert and Crofton, 1999) long-term potentiation following PCB exposure. In previous studies, we reported that exposure to Aroclor 1254 (A1254) is a PCB mixture, results in temporal alterations in [Ca\textsuperscript{2+}]i, and reductions in GABA\textsubscript{A} receptor-mediated responses (Inglefield and Shafer, 2000a,b). The present study expands on our earlier work and investigates the initial mechanism of PCBs to perturb [Ca\textsuperscript{2+}]i, as well as the role of the initial mechanism of PCB action in the subsequently prolonged Ca\textsuperscript{2+} disturbances reported previously. Finally, because perturbations in temporal cellular Ca\textsuperscript{2+} signals may have important effects, two potential downstream consequences of altered Ca\textsuperscript{2+} signaling, cell viability, and transcription factor activation were examined in this model system.

Materials and Methods

**Chemicals and Solutions.** Fura-2 acetoxymethyl ester (fura-2-AM) and fura-2 free acid were obtained from Molecular Probes (Eugene, OR). Nifedipine, ionomycin, and EGTA were purchased from Sigma (St. Louis, MO). Thapsigargin, used to inhibit endoplasmic reticulum Ca\textsuperscript{2+}-ATPases, was obtained from Sigma. IP\textsubscript{3} receptors were stimulated with carbachol (Sigma) and blocked with xestospongin C (Calbiochem, San Diego, CA). Ryanodine receptors were probed with caffeine (Sigma) and carbachol (Sigma) and blocked with xestospongin C (Calbiochem, San Diego, CA). Ryanodine (Research Biochemicals International, Natick MA). A1254 (technical grade purity; lot no. NTO1022) was obtained from UltraScience (technical grade purity; lot no. NTO1022) was obtained from UltraScience (technical grade purity; lot no. NTO1022) was obtained from UltraScience (technical grade purity; lot no. NTO1022) was obtained from UltraScience (technical grade purity; lot no. 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NTO1022) was obtained from UltraScience (technical grade purity; lot no. NTO1022) was obtained from UltraScience (technical grade purity; lot no. NTO1022) was obtained from UltraScientific (North Kingston, RI). Molarity of the A1254 solutions was based (thereby converting the fura-2-AM to its Ca\textsuperscript{2+}-sensitive form, fura-2). Controls for PCB Specificity. A1254 is a PCB mixture that consists primarily of ortho-substituted (>95%), as well as non-ortho-substituted (dioxin-like), PCB congeners. Depending on the system and responses under study, a structure-activity relationship for the differing C1\textsuperscript{-} substitution patterns on the biphenyl ring has been demonstrated that distinguishes effects of ortho- and non-ortho substituted PCBs (reviewed in Tilson and Kodavanti, 1997). In this regard and to characterize the response, individual PCB congeners (5–10 \( \mu \)M) that are ortho-substituted (PCB 4 and PCB 138) or non-ortho-substituted (PCB 15, PCB 77, and PCB 126) were applied to the cortical cells, and [Ca\textsuperscript{2+}]i responses were monitored. There are no detectable levels of polychlorinated dibenzoxydioxins (PCDDs) and only very low abundance (0.0001%) dibenzofuran contaminants in the A1254 mixture (Kodavanti et al., 1999). Also, to control for the effects of DMSO on membrane integrity, 0.1\% DMSO was the comparator (baseline) for the A1254 concentration-response studies.

**Cytoplasmic Free [Ca\textsuperscript{2+}] Concentration Measurements.** [Ca\textsuperscript{2+}]i was measured with the Ca\textsuperscript{2+}-sensitive fluorescent dye, fura-2-AM as described previously (Inglefield and Shafer, 2000a). Cells cultured on coverslips were incubated in cell-permeable fura-2 AM (5 \( \mu \)M) for 40 min at 30°C diluted in 2 ml of HEPES-buffered Hank's balanced salt solution (refer to herein as HEPES buffer) consisting of: 135 mM NaCl, 4.2 mM KCl, 1.5 mM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}, 0.34 mM Na\textsubscript{2}PO\textsubscript{4}, 0.44 mM KH\textsubscript{2}PO\textsubscript{4}, 10 mM glucose, 20 mM sucrose, and 10 mM HEPES (pH 7.4; 290–300 mOsm). Cells were washed twice with fresh HEPES buffer then equilibrated >30 min in the dark at room temperature to remove extracellular dye and to complete the de-esterification process (thereby converting the fura-2-AM to its Ca\textsuperscript{2+}-sensitive form, fura-2). Coverslips containing fura-2-loaded cells were placed in a Leiden coverslip dish situated in a PDMI-2 microscope open incubator (23°C; Medical MicroSystems Corp., Greenvale, NY) that was mounted on the stage of a Nikon Diaphot inverted microscope with a Nikon Fluor40 objective (numerical aperture 1.3).

Cellular fura-2 fluorescence was obtained every 5 s using 340 and 380 nm excitation wavelengths with a DeltaScan dual excitation fluorescence imaging system from Photon Technology International (South Brunswick, NJ), and fluorescence emission at 510 nm was detected with a Hamamatsu C2400 STV videocamera (Hamamatsu, Bridgewater, NJ). Images were stored on a Dell Pentium II Dimension personal computer (Austin, TX) and the intracellular 340/380 ratio was determined off-line from stored images using Imagemaster 1.4 software (Photon Technology International). Ratio values were...
converted to the approximate free [Ca\textsuperscript{2+}], using the equation (Grynkiewicz et al., 1985):

\[
[\text{Ca}^{2+}] = K_d(R - R_{\text{max}})/(R_{\text{max}} - R)(F/F_0),
\]

in which \( R \) is the 340/380 ratio and \( K_d = 272 \) nM, the dissociation constant we determined for fura-2. Maximum ratio \( (R_{\text{max}} = 5.9) \), minimum ratio \( (R_{\text{min}} = 0.7) \), and \( F/F_0 \), [ratio of the fura-2 intensities at 380 nm in the Ca\textsuperscript{2+}-free (with 5 mM EGTA) and Ca\textsuperscript{2+} saturated buffers (with 10 \( \mu \)M nemonycin), respectively, 4:7] were determined from intracellular calibration because in vitro calibration may cause mis-estimation of Ca\textsuperscript{2+} values due to the difference in fura-2 properties in aqueous solution versus that of the cytoplasm. Estimated [Ca\textsuperscript{2+}] in the Ca\textsuperscript{2+}-free solution that was applied to cells was estimated to be 20 nM from a separate calibration in a cell-free system using fura-2 free acid and calcium standards obtained from Molecular Probes.

Following baseline recording, PCB exposures were initiated by pipetting manually an equal volume that contained two times the final PCB concentration into the chamber. Control experiments indicated that these volume changes did not alter [Ca\textsuperscript{2+}]). In some experiments, after collecting baseline measurements, the contributions of specific receptors to intracellular Ca\textsuperscript{2+} increases were determined by pre-exposing the cultures to receptor/store antagonists (typically 5–20 min) before A1254 exposure. After the pretreatment and when fura-2 fluorescence levels were stable, PCB was administered to the bath (in a solution containing the same buffer). Ca\textsuperscript{2+} responses were typically measured for 10 min after addition of toxicant, although extended Ca\textsuperscript{2+} recordings also were performed to monitor latent changes in [Ca\textsuperscript{2+}], in those studies where a pharmacologic inhibitor was effective at inhibiting/attenuating the initial transient. In all experiments, the final volume of the bathing solution was 2 ml. The absolute amplitude of Ca\textsuperscript{2+} transients and in some cases the time to decay to 10\% peak amplitude were determined using specialized software (Mini Analysis, Synaptosoft Software, Leonia, NJ). The peak [Ca\textsuperscript{2+}], obtained following agonist, A1254 or PCB congener application was determined for each “responding” neuronal-appearing cell in a group.

**Cell Viability, Caspase 3 Activity, and Apoptosis.** Cell viability was assessed by determining the ability of cortical cells to exclude Trypan Blue following exposure to A1254. Following a 24-h exposure to control (0.1% DMSO) or A1254-containing buffer (2, 10, or 20 \( \mu \)M), 0.4% Trypan blue was added to wells of cortical cultures that had been maintained in the incubator at 37°C (in 95\% \( \text{O}_2/5\% \text{CO}_2 \)). Trypan blue excluding cells were counted using bright field microscopy (Olympus IMT2, 300 \( \times \); in each of two identically treated wells, two randomly selected microscopic fields of at least 100 cells were examined for each concentration.

The experimenter was blind with respect to the treatment. Neuronal cells were identified visually by their characteristic shape; but because experimentation was conducted in the absence of a specific stain for neurons, cell survival (as opposed to neuronal survival) is presented.

Activation of the cysteine protease, caspase 3, was assessed in DIV 6 cells to determine whether the A1254-induced Ca\textsuperscript{2+} disturbances were activating an apoptotic pathway. After 6 and 8 h of exposure, cells in 24-well plates were lysed and incubated at 37°C with 10 \( \mu \)M of the fluorogenic substrate zDEVD-AFC (Calbiochem, La Jolla, CA) in a buffer of 25 mM HEPES, 1 mM EDTA, 3 mM diithiothreitol, 0.1\% CHAPS (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonic acid), and 10\% sucrose (pH 7.5). After 45 min, the fluorogenic product AFC was measured using a fluorescent plate reader with excitation set at 395 nm and emission set at 508 nm (Armstrong et al., 1997).

Separate DIV 6 cells cultured on 12-mm poly(l-lysine)-coated coverslips were exposed for 24 h with A1254 or other reagents for determination of apoptotic cells using in situ terminal deoxynucleotidyl-transferase-mediated dATP biotin nick-end labeling (TUNEL) (DeadEnd apoptosis assay kit, Promega, Madison, WI). Coverslips, fixed in 4\% paraformaldehyde, were pretreated with 2\% H\textsubscript{2}O\textsubscript{2} to quench endogenous peroxidase before the addition of the terminal deoxynucleotidyl transferase. Positively stained (apoptotic) and negatively stained neurons were scored by cell counting under bright field microscopy in the same manner as for Trypan blue counting described above. However, for the TUNEL stain procedure, neuronal nuclei were clearly discerned from the larger astrocyte nuclei, thereby allowing the determination of apoptotic neurons.

**Cyclic AMP Responsive Element Binding Protein (CREB) Phosphorylation/Immunoblotting.** DIV 6 cultures were washed free of serum by incubation in HEPES buffer with 0.1% bovine serum albumin (Sigma) for 2 h at 37°C before stimulation with the specified agent. The exposure was terminated by washing with ice-cold phosphate-buffered saline and addition of lysis buffer [1% Triton X-100, 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10\% glycerol, 1 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, and 0.5\% protease inhibitor cocktail (Calbiochem, San Diego, CA)]. Cells were removed from the wells, vortexed gently, and allowed to sit on ice for 10 min. The lysed cells were then centrifuged at 10,000 g for 10 min at 4°C. An aliquot of the supernatant was taken for protein determination, and the remaining supernatant was added to an equal volume of sample buffer (2.5 mM Tris (pH 6.8), 25\% glycerol, 2\% sodium dodecyl sulfate, 0.01\% bromphenol blue, and 5\% \( \beta \)-mercaptoethanol) and stored at -80°C.

Cell lysates in sample buffer (15 \( \mu \)g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10\% polyacrylamide) before electrophoretic transfer onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The blots were blocked for 1 h with 5\% nonfat dried milk at room temperature. The blots were then incubated overnight at 4°C with commercially available polyclonal primary antibodies derived from rabbit [antiphospho-Ser\textsuperscript{133}-CREB (diluted 1:1000) or anti-CREB (diluted 1:2000); Upstate Biotechnology, Inc., Lake Placid, NY]. After three short washes, the blots were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The blots were then exposed to ECL substrate (Pierce, Rockford, IL), and chemiluminescence images were collected and analyzed using a Fluor-S Multilager (Bio-Rad). Phospho-CREB (pCREB) and CREB bands were detected at the 43 kDa standard. Relative activation was determined by normalization of the band density from the phosphorylated protein with that of the total (phosphorylated and nonphosphorylated) CREB protein from the same sample.

**Data Analysis.** Data are presented as means ± S.E.M. For the Ca\textsuperscript{2+} responses, the percentage of responding cells in treatment groups are also noted. For comparisons of peak Ca\textsuperscript{2+} responses, statistical significance was ascertained using one- or two-way ANOVAs followed by suitable post hoc tests. To determine whether pharmacological pretreatments were effective at preventing A1254-induced responses, the proportion of cellular Ca\textsuperscript{2+} responses among groups in an experiment was compared using the \( \chi^2 \)-test and Bonferroni-corrected \( p \) values for multiple comparisons. For the cell counts, a decrease in the cell density of control wells occurs with increasing DIV; thus, the experimental and statistical design took this into account, and data were analyzed with two-way ANOVA, followed by one-way ANOVA and Dunnnett’s t tests. Caspase 3 activity, TUNEL staining, and pCREB activation were assessed with one-way ANOVA and Dunnett’s test.

**Results**

**A1254-Initiated Changes in Cytoplasmic Free [Ca\textsuperscript{2+}]: Concentration Response.** Figure 1A illustrates the typical response observed during 1 h of imaging of Ca\textsuperscript{2+} in a cortical cell exposed continuously to 10 \( \mu \)M (3 ppm) A1254. In the continued presence of A1254, a Ca\textsuperscript{2+} transient is followed to 16 min later by disturbances in the basal Ca\textsuperscript{2+} level that often includes Ca\textsuperscript{2+} oscillations of ~200 to 700 nM in ampli-
to onset of the initial Ca\(^{2+}\) with the Ca\(^{2+}\)-intact P0 cortical cell maintained in culture for 5 days. Cells were loaded. The arrow indicates when the PCB mixture was

**Materials and Methods**

dent increase in the peak amplitude of [Ca\(^{2+}\)]\(_i\) upon A1254 (1–25 μM) stimulated at least a 7-fold increase of [Ca\(^{2+}\)]\(_i\), from baseline levels of 72 ± 5 nM in >70% of the cells. The characteristics of the initial Ca\(^{2+}\) transients stimulated by PCB 4 and PCB 138 were indistinguishable from that elicited by A1254 in that Ca\(^{2+}\) levels recovered toward basal levels over several minutes. Responses to the non-ortho-substituted PCBs occurred infrequently, although in those rare instances (in <10% of cells), the peak amplitude could be marked, reaching levels as high as 400 nM (Table 2).

**TABLE 1**

<table>
<thead>
<tr>
<th>PCB Exposure (μM)</th>
<th>Peak [Ca(^{2+})] (_i) Amplitude (nM)</th>
<th>% Responding Cells</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>170 ± 24</td>
<td>37</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>400 ± 200</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>750 ± 150</td>
<td>79</td>
<td>28</td>
</tr>
<tr>
<td>20(^b)</td>
<td>742 ± 72</td>
<td>90</td>
<td>60</td>
</tr>
<tr>
<td>25(^b)</td>
<td>1200 ± 180</td>
<td>86</td>
<td>28</td>
</tr>
</tbody>
</table>

\(^a\) Responses were studied by addition of A1254 to culture with A1254 addition occurring, is the focus of the data presented in B, as well as in Figs. 2 through 4 and Tables 1 through 3. B, representative examples of initial Ca\(^{2+}\) responses that occur following addition of A1254 from 1 to 25 μM. Each concentration was repeated in at least two different cultures with 27 to 60 cells per concentration.

**Fig. 1.** A, effect of A1254 (10 μM) to stimulate intracellular Ca\(^{2+}\) in an intact P0 cortical cell maintained in culture for 5 days. Cells were loaded with the Ca\(^{2+}\)-sensitive fluorescent indicator, fura-2, as described under Materials and Methods. The arrow indicates when the PCB mixture was

**PCB Effects on Ca\(^{2+}\) Signaling and CREB Phosphorylation**

Specificity of Ca\(^{2+}\) Responses. Addition of control buffer (0.1% DMSO) to cells from DIV 4 to 6 cortical cultures did not produce a [Ca\(^{2+}\)]\(_i\) change (not shown) indicating that neither mechanical manipulation of the cells nor vehicle were responsible for the Ca\(^{2+}\) disturbance. Moreover, separate exposures of cells to two main classes of PCB congeners that are dibenzofuran- and dioxin-free (personal communication, Michael Bolger, AccuStandard) elicited distinct responses. Initial Ca\(^{2+}\) transients were consistently stimulated by ortho-substituted PCB congeners (PCB 4 and PCB 138), but such responses were rare with non-ortho-substituted PCB congeners (PCB 15, PCB 77, and PCB 126) (Fig. 2 and Table 2), which are aryl hydrocarbon receptor agonists like the dioxins. Both of the ortho-substituted congeners (PCB 4 and PCB 138; 10 μM) stimulated at least a 7-fold increase of [Ca\(^{2+}\)]\(_i\) from baseline levels of 72 ± 5 nM in >70% of the cells.

<table>
<thead>
<tr>
<th>PCB Mixture</th>
<th>Ortho-substituted PCBs</th>
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<tbody>
<tr>
<td>Aroclor 1254</td>
<td>2,2',3,4,4',5-HeCB #138</td>
</tr>
<tr>
<td></td>
<td>2,2'-DCB #4</td>
</tr>
<tr>
<td>Non-ortho substituted PCBs</td>
<td>3,3',4,4',5-PcCB #126</td>
</tr>
<tr>
<td></td>
<td>4,4'-DCB #15</td>
</tr>
<tr>
<td></td>
<td>3,3',4,4'-TeCB #77</td>
</tr>
</tbody>
</table>

**Fig. 2.** Effect of A1254 and selected PCB congeners on [Ca\(^{2+}\)]\(_i\) in developing cortical cells. Ortho-substituted PCB congeners 138 (2,2',3,4,4',5'-hexachlorobiphenyl) and 4 (2,2'-dichlorobiphenyl) at 10 μM selectively induced an initial Ca\(^{2+}\) transient with characteristics similar to that induced by the PCB mixture, A1254 (10 μM). Changes in intracellular Ca\(^{2+}\) were blunted or absent when non-ortho-substituted congeners 15 (4,4'-dichlorobiphenyl), 77 (3,3',4,4'-tetrachlorobiphenyl), or 126 (3,3',4,4',5-pentachlorobiphenyl) were applied (all at 10 μM, except PCB 126, which was added at 5 μM due to solubility issues). Arrows denote the time of PCB addition, and the PCBs remained in the bath for the rest of the experiment. # refers to the IUPAC number of the PCB congener used.
A rapid, initial Ca\textsuperscript{2+} transient was largely due to release from intracellular Ca\textsuperscript{2+} stores, although Ca\textsuperscript{2+} influx from extracellular sources extends the period for return of intracellular Ca\textsuperscript{2+} to baseline.

Release by A1254 of Intracellular Ca\textsuperscript{2+} Stores and Stimulation of Store-Operated Entry of Ca\textsuperscript{2+}. PCB-induced disturbances in Ca\textsuperscript{2+} homeostasis have been reported to be due to both a mobilization of Ca\textsuperscript{2+} from an internal source(s) (Wong et al., 1997) and an influx of extracellular Ca\textsuperscript{2+} (Bae et al., 1999b; Mundy et al., 1999; Inglefield and Shafer, 2000b). Thus, we sought to determine the source of Ca\textsuperscript{2+} responsible for the initial Ca\textsuperscript{2+} transient induced by A1254. A rapid, initial Ca\textsuperscript{2+} transient was still present in Ca\textsuperscript{2+}-free extracellular buffer solution (having an estimated total free Ca\textsuperscript{2+} concentration of ~20 nM), compared with Ca\textsuperscript{2+} replete solution. Although the peak Ca\textsuperscript{2+} amplitude was not significantly attenuated after the cells’ exposure to a Ca\textsuperscript{2+}-free buffer (Fig. 3 and Table 3), there was an effect to shorten the duration of Ca\textsuperscript{2+} responses stimulated by A1254 in Ca\textsuperscript{2+}-free buffer (i.e., [Ca\textsuperscript{2+}]i exhibited a more rapid recovery to basal levels; Fig. 3A, inset). The Ca\textsuperscript{2+} responses stimulated by 20 \mu M A1254 in buffer containing the L-type Ca\textsuperscript{2+} channel blocker nifedipine (1 \mu M) also were not attenuated in terms of either the peak amplitude, percentage of cells responding (Table 3), or the decay to baseline (981 ± 187 s, N.S. p > 0.05 relative to A1254 + Ca\textsuperscript{2+}). This indicates that the Ca\textsuperscript{2+} transient is largely due to release from intracellular Ca\textsuperscript{2+} stores, although Ca\textsuperscript{2+} influx from the extracellular solution contributes to and prolongs the decay. Interestingly, when Ca\textsuperscript{2+}-containing buffer was reintroduced to cells exposed to A1254, the resting basal [Ca\textsuperscript{2+}]i became elevated rapidly in 59% of cells (Fig. 3B), suggestive of Ca\textsuperscript{2+} entry through plasma membrane-situated store-operated channels (SOCs) to refill A1254-activated intracellular Ca\textsuperscript{2+} stores (Fig. 3B). Figure 3C illustrates SOC-mediated Ca\textsuperscript{2+} entry stimulated with 1 nM of the muscarinic agonist, carbachol (79% of cells responded with store-operated Ca\textsuperscript{2+} entry); this has been shown to occur in developing neurons (Bouron, 2000). Overall, these results implicate the importance of intracellular Ca\textsuperscript{2+} pools in the peak initial Ca\textsuperscript{2+} transient stimulated by A1254 and demonstrate that a trigger of store-operated Ca\textsuperscript{2+} entry (also referred to as capacitative Ca\textsuperscript{2+} entry) from extracellular sources extends the period for return of intracellular Ca\textsuperscript{2+} to baseline.

Block of the A1254-Induced Initial Ca\textsuperscript{2+} Transient by Pharmacological Agents. Figure 4 shows the results of studies to determine the source of intracellular Ca\textsuperscript{2+} underlying the initial A1254-induced [Ca\textsuperscript{2+}]i transient. These mechanistic studies were performed with 20 \mu M A1254 since this concentration induced a nearly maximal response in the population of cortical cells. To determine the pool released by A1254, the effects of several pharmacological agents that alter Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores were examined. The intracellular Ca\textsuperscript{2+} source for the majority of the A1254-induced initial Ca\textsuperscript{2+} transient was the endoplasmic reticulum because pretreatment with thapsigargin (10 \mu M for 10 min), a specific inhibitor of the endoplasmic reticulum Ca\textsuperscript{2+}-ATPase pump, prevented detectable responses in 70% of the cells (Fig. 4). In those cells that did respond, the amplitude of the A1254 response was significantly attenuated (p < 0.05 following significant treatment effect in the one-way ANOVA) (Table 3). The same thapsigargin pretreatment was sufficient to suppress completely the intracellular Ca\textsuperscript{2+} responses to stimulation by carbachol (1 mM) (response to A1254 20 nM), compared with [Ca\textsuperscript{2+}]i exhibited a more rapid recovery of intracellular Ca\textsuperscript{2+} to baseline.

### Table 2

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Peak [Ca\textsuperscript{2+}]</th>
<th>% Responding Cells</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1254 10 \mu M</td>
<td>750 ± 150</td>
<td>79</td>
<td>28</td>
</tr>
<tr>
<td>Ortho-substituted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCB 138 10 \mu M</td>
<td>501 ± 63</td>
<td>73</td>
<td>30</td>
</tr>
<tr>
<td>PCB 4 10 \mu M</td>
<td>474 ± 36</td>
<td>74</td>
<td>43</td>
</tr>
<tr>
<td>Non-ortho-substituted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCB 77 10 \mu M</td>
<td>406*</td>
<td>9*</td>
<td>22</td>
</tr>
<tr>
<td>PCB 15 10 \mu M</td>
<td>209*</td>
<td>5*</td>
<td>42</td>
</tr>
<tr>
<td>PCB 126 5 \mu M</td>
<td>360*</td>
<td>6*</td>
<td>33</td>
</tr>
</tbody>
</table>

*p < 0.05 compared with the respective A1254 response. For peak [Ca\textsuperscript{2+}], the Kruskal-Wallis test followed by Dunn’s post hoc tests were used. Proportional responses gave a significant $\chi^2$; t-tests with Bonferroni’s correction followed.

*p Responses with PCB congeners were studied at 10 \mu M (or 5 \mu M) and compared with 10 \mu M A1254. Data are mean ± S.E.M. of DIV 4 to 6 cortical cells where, within each condition, the cultures from at least two separate rats litters were assessed.

*p This is the average peak response of the only two cells that responded in each group; S.E.M. was not calculated.

*p Solubility limit reached before 10 \mu M.
PCB Effects on Ca²⁺ Signaling and CREB Phosphorylation

abundance/oscillations identified previously where Ca²⁺ entry through L-type channels and excitatory glutamate receptors occurred (Inglefield and Shafer, 2000b).

Association of the Initial Release of Intracellular Ca²⁺ Stores Caused by PCBs with Latent Ca²⁺ Disturbances. Previous work has shown a significant increase in basal Ca²⁺ levels arising over the course of a 1 h exposure to 10 or 20 μM A1254 [often with recurring Ca²⁺ oscillations (see Fig. 1A)] (Inglefield and Shafer, 2000a). Following the initial Ca²⁺ transient in the cortical cells with A1254, ~70% of cells later exhibited disturbances of Ca²⁺ homeostasis, i.e.,

---

TABLE 3
Pharmacologic agents active at IP₃ receptors or IP₃ releasable stores inhibit PCB-induced initial Ca²⁺ transients

<table>
<thead>
<tr>
<th>Exposure → Treatment</th>
<th>Peak [Ca²⁺]i Amplitude (nM)</th>
<th>% Responding Cells</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1254</td>
<td>714 ± 64</td>
<td>90</td>
<td>52</td>
</tr>
<tr>
<td>A1254 + nifedipine</td>
<td>593 ± 84</td>
<td>83</td>
<td>40</td>
</tr>
<tr>
<td>A1254 + 0 Ca²⁺ buffer</td>
<td>553 ± 39</td>
<td>83</td>
<td>40</td>
</tr>
<tr>
<td>A1254 + thapsigargin</td>
<td>343 ± 51*</td>
<td>30*</td>
<td>47</td>
</tr>
<tr>
<td>A1254 + xestospongin C</td>
<td>203 ± 37*</td>
<td>11*</td>
<td>37</td>
</tr>
<tr>
<td>A1254 after CCh/0 Ca²⁺ buffer</td>
<td>N.D.</td>
<td>0*</td>
<td>25</td>
</tr>
<tr>
<td>A1254 + ryanodine</td>
<td>654 ± 74</td>
<td>97</td>
<td>65</td>
</tr>
<tr>
<td>2.2'-DCB (PCB 4)</td>
<td>474 ± 36</td>
<td>74</td>
<td>43</td>
</tr>
<tr>
<td>2.2'-DCB + thapsigain</td>
<td>285 ± 78*</td>
<td>54</td>
<td>13*</td>
</tr>
<tr>
<td>2.2'-DCB + xestospongin C</td>
<td>156 ± 12*</td>
<td>60</td>
<td>15*</td>
</tr>
</tbody>
</table>

N.D., not detectable because Ca²⁺ levels remained at baseline upon A1254 addition.

* p < 0.05 compared with respective control response, whether A1254 alone or 2.2'-DCB. For Peak [Ca²⁺]i, the Kruskal-Wallis test followed by Dunn’s t tests were used. For proportional responses, a χ² followed by t tests with Bonferroni’s correction were used.

A1254 (or 2.2'-DCB) was bath-applied in the imaging chamber in the absence or presence of Ca²⁺ signaling site-selective drugs, including nifedipine (1 μM), thapsigargin (10 μM), ryanodine (100 μM), xestospongin C (1 μM), or carbachol (CCh; 1 mM). The carbachol prestimulation was done in Ca²⁺-free buffer to prevent refilling of the released stores. Pretreatments were typically 10 min, although for ryanodine, the duration was 20 min and for carbachol the stimulus lasted 2 min followed by 3 min of washing off the carbachol. Data are mean ± S.E.M. for the number (n) of indicated DIV 4 to 6 cortical cells that were cultured from at least two separate rat litters, unless otherwise indicated.

Twelve micromolar A1254 and 10 μM 2.2'-DCB were used for mechanistic studies because of the greater percentage of responding cells relative to when lower PCB concentrations were tested.

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Fig. 4. A1254 induces release of Ca²⁺ from IP₃-sensitive intracellular stores. The ability of A1254 to release Ca²⁺ from endoplasmic reticulum, IP₃-, and ryanodine-sensitive stores was examined using specific pharmacologic antagonists. In the top row, thapsigargin (10 μM) was used to deplete the endoplasmic reticulum of Ca²⁺ before addition of 20 μM A1254 (left, top row) or the positive control, carbachol (CCh; 1 mM; center, top row). In the middle row, the IP₃ receptor antagonist, xestospongin C (Xes C; 1 μM) was used to block the release of IP₃-sensitive Ca²⁺ stores from the endoplasmic reticulum before addition of 20 μM A1254 (left, middle row) or 1 mM carbachol (center, middle row). In the bottom row, ryanodine (100 μM) pretreatment was used to block the release of ryanodine-sensitive Ca²⁺ stores in the endoplasmic reticulum before addition of 20 μM A1254 (left, bottom row) or 20 mM caffeine (center, bottom row). The right panel in all three rows illustrates the effect of depleting carbachol-sensitive stores and preventing their refilling in Ca²⁺-free buffer before the addition of A1254. Thapsigargin and xestospongin C, but not ryanodine, treatments were effective at inhibiting the A1254-induced Ca²⁺ transient. Each panel illustrates the typical response to the indicated treatment(s) in an individual cell. All responses are taken from separate coverslips.
Consequences of PCB Actions: I. Delayed Cytotoxicity That Is Not Apoptotic. In previous studies, exposure (1-4 h) to PCBs in the range of 20 μM (Inglefield and Shafer, 2000a) to 50 μM (reviewed in Tilson and Kodavanti, 1997) have not been reported to cause acute cytotoxicity, with one notable exception (Carpenter et al., 1997). However, data on the status of cell viability after prolonged in vitro exposure to PCBs are lacking, yet important, based on the ability of identified alterations in Ca\(^{2+}\) homeostasis in the endoplasmic reticulum to contribute to neuronal apoptosis and excitotoxicity (Mattson et al., 2000). The present study, conducted using Trypan blue staining after 24 h of A1254 exposure, found a decrease in cell viability by A1254 that was maturation-sensitive; cytotoxicity became prevalent as the A1254 concentration and in vitro age of the culture increased (Fig. 6A). Compared with DMSO-containing controls, concentrations of A1254 up to 20 μM (24 h) were not associated with significant cytotoxicity when assessed on DIV 4, and on DIV 6 only the highest concentration of A1254 caused moderate (approximately 21%), but significant cytotoxicity. By contrast, significant cytotoxicity was observed in DIV 7 cultures treated with the 10 and 20 μM A1254 for the previous 24 h (~20 and ~50% for these concentrations, respectively).

Because dysregulation of Ca\(^{2+}\) homeostasis has been associated with activation of apoptotic pathways in neurons (Mattson et al., 2000), we examined whether A1254 cytotoxicity observed above was the result of apoptosis using two methods. No induction of caspase 3 activity occurred after a 6- to 8-h exposure to 2 to 20 μM A1254 (Fig. 6B). Moreover, TUNEL staining on DIV 7 (the day in vitro with greatest sensitivity to A1254 cytotoxicity) revealed no evidence for greater amounts of apoptotic cells after a 24-h A1254 exposure relative either to control cells that had been serum-depleted (24 h; Fig. 6, C and D) or control cells maintained in serum (not shown). These results are in contrast to that obtained with the positive-control staurosporine (1 μM). Therefore, in DIV 6/7 cortical cultures subacute exposure to the highest concentration, 20 μM A1254, causes significant cytotoxicity that occurs not via apoptosis, but probably via necrotic mechanisms.

Consequences of PCB Actions: II. CREB Phosphorylation. Because cell viability is not compromised in the majority (>80% with <20 μM A1254) of the population under the exposure conditions examined in these studies, ramifications other than cytotoxicity were also examined. CREB is an important transcription factor that is sensitive to Ca\(^{2+}\) signals (reviewed in Silva et al., 1998 and Curtis and Finkbeiner, 1999). Given the perturbations of Ca\(^{2+}\) homeostasis induced by A1254, studies were conducted to determine whether or not CREB phosphorylation (activation) was induced following A1254 exposure. Immunoblots using antibodies selective for the phosphorylated form of CREB (pCREB) and CREB (total expression) in DIV 6 cultures, demonstrated that pCREB was increased within 20 min of A1254 addition (Fig. 7A). Levels of pCREB reached maximal levels by 40 to 60 min of exposure to 10 μM A1254. A1254 did not induce de novo synthesis of CREB because the total amount of CREB did not increase as a result of the exposures (Fig. 7A). A1254 also led to concentration-dependent increases in pCREB activation, reaching a 2-fold induction with 10 and 20 μM (Fig. 7B). In comparison, glutamate (100 μM for 40 min) maximally stimulated pCREB —6-fold, whereas the ortho-substituted PCB congener 4 (2,2’-DCB, 10 μM) stimu-
lated pCREB 1.3 ± 0.1-fold (data not shown). In experiments conducted using DIV 4 cultures, A1254 induced CREB phosphorylation to levels similar to those observed using DIV 6 cultures (data not shown). These data demonstrate that CREB phosphorylation is not specifically linked to cytotoxicity because no cytotoxicity was observed in DIV 4 cultures.

Discussion

We have identified several novel actions of individual ortho-substituted PCBs and a PCB mixture, A1254, in intact cells cultured from neonatal rat cerebral cortex. The results, including immediate Ca\(^{2+}\) release from IP\(_3\)-sensitive stores with subsequent activation of store-operated Ca\(^{2+}\) influx (also referred to as capacitative Ca\(^{2+}\) entry), further perturbation of Ca\(^{2+}\) homeostasis, and activation of a nuclear transcription factor (Fig. 8), may aid understanding the mode of cellular action of persistent, bioaccumulating toxicants such as PCBs. These findings are consistent with other known cellular actions of PCBs, wherein changes in intracellular Ca\(^{2+}\) homeostasis is a recurring finding in a variety of intact cell types (Carpenter et al., 1997; Voie and Fonnum, 1998; Bae et al., 1999b; Fischer et al., 1999). However, further consequences as in the status of cell viability after subacute exposure has received less attention. The concentrations of 10 \(\mu M\) and lower used here for the subacute exposure of
cortical culture to A1254 are not unrealistic because rats
dosed perinatally with A1254 (with 6 mg/kg via the dam)
achieved levels in the frontal cortex on postnatal day 21 of 2.4
ppm (~7.2 μM) (Crofton et al., 2000). Previous in vitro in-
vestigations have used PCB concentrations higher than 20
μM. The demonstration of Ca^{2+} transients at PCB concen-
trations as low as 1 μM is among the lowest reported effects
in intact cells. Future mechanistic studies exploring sub-
acute exposure should take into account the present findings
of decreased cell viability in the continuous presence of 20
μM PCBs.

A1254-Mediated Release of IP_{3} Receptor-Linked
Intracellular Ca^{2+} Stores and Interaction with IP Signal-
ing. The initial Ca^{2+} transient induced by A1254 or ortho-
substituted PCB congeners (PCB 4 and PCB 138) in cortical
cells is analogous to that induced shortly after ortho-substi-
tuted PCB addition in granulocytes where a single transient
and slow decay to baseline also is seen (Voie and Fonnum,
1998). However, the findings of an early, transient change in
basal [Ca^{2+}]_i is not universal in investigations with intact
cells and exposure to ortho-substituted PCBs. The early-onset Ca^{2+} disturbances we observed for PCB 4 and PCB 138
in >70% of cortical cells occurred more rapidly than occurs in
cultured cerebellar granule cells upon exposure to PCB 4
(reviewed in Tilson and Kodavanti, 1997; Mundy et al.,
1999). In contrast to our finding that non-ortho-substituted
PCBs (15, 77, and 126) failed to induce an initial Ca^{2+} tran-
sient, a rapid Ca^{2+} increase has been reported in intact
hippocampal cells given dioxin (Hanneman et al., 1996),
which is structurally similar to non-ortho-substituted PCBs.
A number of factors could contribute to these differences,
including different cell types used, maturity of the cells at
time of testing, or different concentrations of the compound
under examination.

Inhibition of the initial A1254-induced [Ca^{2+}]_i increases by
pretreatment with compounds (thapsigargin and xestospon-
gin C) affecting select intracellular Ca^{2+} stores was observed in
the present study. This reduction likely occurred as a
consequence of either depletion of endoplasmic reticulum
Ca^{2+} stores in the case of thapsigargin or prevention of
release of the IP_{3}-mediated Ca^{2+} stores in the case of xesto-
spongin, based on the site of action of these pharmacons and
demonstration of their efficacy against appropriate agonists
(Fig. 4). In contrast to thapsigargin's efficacy against the
A1254-induced Ca^{2+} disturbance, the transient persisted fol-
lowing depletion of ryanodine-sensitive Ca^{2+} stores with a
20-min pre-exposure to a high concentration of ryanodine.
This strongly suggests that the Ca^{2+}-ATPase associated with
IP_{3} receptor stores was operative in the effect produced by
thapsigargin. The finding of nifedipine's inability to atten-
ate the A1254-induced Ca\(^{2+}\) transient agrees with the failure by ryanodine to block the transient because the L-type voltage-sensitive Ca\(^{2+}\) channels are functionally coupled to ryannodine receptors (Chavis et al., 1996). Thus, the absence of effect of two different modulators in the L-type voltage-gated Ca\(^{2+}\) channel (VGCC)/ryanodine receptor complex is internally consistent, but at odds with the earlier finding that A1254 enhances the binding of ryanodine to the ryanodine receptor subtype, RyR1 (Wong and Pessah, 1996).

Additional evidence that IP\(_3\)-releasable Ca\(^{2+}\) stores in intact cells are functionally important for the actions of this class of toxicant comes from the induction of store-operated Ca\(^{2+}\) entry, as indicated by the rebound increase of [Ca\(^{2+}\)], following the return of Ca\(^{2+}\)-containing buffer to those cells whose internal stores were mobilized by A1254 in Ca\(^{2+}\)-free buffer. Ca\(^{2+}\) release from IP\(_3\) stores activates store-operated Ca\(^{2+}\) channels (also known as Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels, CRAC) on the plasma membrane (Hofer et al., 1998). A very recent study convincingly showed the physical and functional coupling of IP\(_3\) receptor-mediated Ca\(^{2+}\) stores and SOCs (Ma et al., 2000). Store-operated Ca\(^{2+}\) entry is present in developing neurons (Bouron, 2000), and the preceding evidence is in accordance with store-operated Ca\(^{2+}\) entry in the A1254-induced stimulation of IP\(_3\) receptors.

For some time (Kodavanti et al., 1994; Tithof et al., 1995; Shafer et al., 1996; Voie and Fonnum, 1998), an effect of PCBs on immune cells or neurons to elevate inositol phosphate levels or activate inositol phosphate signaling has been known to occur, but no direct functional interaction with IP\(_3\) releasable Ca\(^{2+}\) stores has been demonstrated. The present study confirms what those earlier investigators had proposed: that elevation of [Ca\(^{2+}\)], induced by ortho-substituted PCBs (also the mixture A1254 that consists primarily of ortho-substituted congeners), can be mediated by IP\(_3\) receptor-sensitive stores probably via an increase in IP\(_3\) levels of note, activation of IP\(_3\)-releasable Ca\(^{2+}\) stores has also been demonstrated for another chlorinated hydrocarbon, \(\gamma\)-hexachlorocyclohexane (lindane), in smooth muscle cells (Criswell et al., 1994). Although Mundy et al. (1999) did report a significantly increased [\(\text{H}^2\)] IP\(_3\) receptor binding in cerebellar microsomes following a short exposure to PCB 4, a functional effect of PCB 4 on the IP\(_3\)-releasable stores was not identified. In cortical microsomes from adult animals, there was a stimulatory action on ryanodine receptors to release Ca\(^{2+}\) by the ortho-substituted PCB 95, but no Ca\(^{2+}\) effect occurred in the larger population of vesicles harboring IP\(_3\)-sensitive efflux pathways (Wong et al., 1997). Others have shown that a PCB mixture similar to A1254 does not stimulate release of inositol phosphate in smooth muscle cells (Bae et al., 1999a). Therefore, the phenotype of the cells (which tissue or even which cellular phenotype), as well as the age of the cells, may be important factors for the participation of IP\(_3\) receptors in Ca\(^{2+}\) responses induced by PCBs.

**On the Consequences of A1254-Induced Disturbances in Endoplasmic Reticulum Ca\(^{2+}\) Homeostasis.** Both excitotoxicity (which can lead to necrosis) and apoptosis are possible outcomes of alterations in neuronal Ca\(^{2+}\) homeostasis. Certain proteins from central nervous system infections, such as Tat (a human immunodeficiency virus type-1 protein), cause disturbances in endoplasmic reticulum Ca\(^{2+}\) homeostasis that are considered central to later apoptotic cell death (New et al., 1997; Krum an et al., 1998; Haughey et al., 1999). Using calcium imaging of cortical neurons that had either a thapsigargin or xestospongin C pretreatment to attenuate the A1254-induced initial Ca\(^{2+}\) transient, we identified a role of the initial mechanism of PCB action in the subsequent prolonged Ca\(^{2+}\) disturbances. This is in agreement with the recognition of an association of the two phases of Ca\(^{2+}\) disturbance induced by Tat in human embryonic neurons maintained in culture (Haughey et al., 1999). These latent Ca\(^{2+}\) changes are mediated by heightened Ca\(^{2+}\) influx across the plasma membrane, as seen after stimulation with PCBs (Inglefield and Shafer, 2000b) and with Tat (Haughey et al., 1999). From the relationship identified between the Ca\(^{2+}\) mobilization from intracellular stores induced by A1254 (or Tat) and the facilitation of later Ca\(^{2+}\) dysregulation, it would appear that mobilization of intracellular Ca\(^{2+}\) stores by a "stressor" serves to change the "gain" of signaling proteins at the plasma membrane. It is also possible that the replenishing of depleted IP\(_3\)-sensitive Ca\(^{2+}\) stores contributes in some way to latent increases in basal [Ca\(^{2+}\)], or to the activation of Ca\(^{2+}\)-sensitive second messengers that alter the responsiveness of plasma membrane receptors. In contrast to the prevalent cell death that is produced by Tat in vitro, 24 h of A1254 exposure led to cell death only in a subset of neurons under specific conditions; survival of the youngest cells (DIV 3–4) across all A1254 concentrations tested was not different from control cultures, whereas cytotoxicity from A1254 occurred in a subset of neurons as the neurons matured (i.e., DIV 7). However, despite the noted similarities in the Ca\(^{2+}\) disturbing mechanisms that occur with Tat and PCBs, under the conditions tested here, we obtained no data to support an apoptotic cascade induced by A1254.

**CREB Phosphorylation in Cortical Cells by A1254 and Implications.** CREB phosphorylation serves as a convergence of Ca\(^{2+}\) signaling pathways in neurons and is believed important for neuronal development, as well as learning and memory processes (reviewed in Silva et al., 1998 and Curtis and Finkbeiner, 1999). These are processes negatively impacted by PCBs (Altmann et al., 1998; Niemi et al., 1998; Gilbert and Crofton, 1999). Given the ability of A1254 to cause immediate (present data) and prolonged (present data; Inglefield and Shafer, 2000a,b) perturbations of intracellular Ca\(^{2+}\) homeostasis in cortical neurons, we hypothesized that these perturbations may cause activation of this transcription factor. The increase of pCREB levels in samples exposed to A1254, at concentrations that did not induce apoptotic activity nor appreciable cell death at 24 h, suggested that the effect was not a cytotoxic response. In agreement with our findings with DIV 6 cells on activation of pCREB by PCBs, CREB was also phosphorylated to the same degree when cells were given A1254 on earlier DIV (data not shown), when there was no cytotoxicity. At present, it is not known whether this CREB phosphorylation by PCBs necessarily leads to gene transcription.

The findings of CREB activation by A1254 are consistent with those showing that IP\(_3\)-depleted stores and subsequent store-operated/capacitative Ca\(^{2+}\) entry are a trigger of the signaling pathway leading to CREB activation (in cortical glial cells) (Pende et al., 1997). In addition to the likelihood (discussed above) that store-operated Ca\(^{2+}\) influx may have primed the cells to undergo a later Ca\(^{2+}\) disturbance, the activation of CREB by A1254 may also depend on both the...
intracellular Ca\textsuperscript{2+} release and ensuing transmembrane Ca\textsuperscript{2+} influx. Specifically, it has been shown that CREB activation following stimulation with carbachol (which mobilizes IP\textsubscript{3} stores) is prevented in Ca\textsuperscript{2+}-deficient buffer (Pende et al., 1997). The CREB activation seen with A1254 in a mixed cortical culture lasted longer than the transient CREB activation (<30 min) that occurs from carbachol-mediated Ca\textsuperscript{2+} influx (Pende et al., 1997), and this longer lasting CREB activation may be secondary to A1254's additional actions on Ca\textsuperscript{2+} signaling that include activation of excitatory amino acid receptors and L-type VGCCs (Inglefield and Shafer, 2000b). Transmembrane Ca\textsuperscript{2+} influx through these latter channels as a consequence of the release of synaptic glutamate is also tightly linked to CREB phosphorylation (reviewed in Silva et al., 1998 and Curtis and Finkbeiner, 1999). Thus, transmembrane Ca\textsuperscript{2+} influx may play an important role in the mechanistic link between PCB-induced Ca\textsuperscript{2+} dysregulation and the longer lasting CREB activation.

Finally, intracellular activators upstream from CREB are also regulated by PCBs because mitogen activated protein (MAP)/extracellular signal regulated kinase (ERK) (MAP/ERK) is activated both by A1254 and ortho-substituted PCBs in non-neuronal cells (Fischer et al., 1999). The MAP/ERK pathway, linked to the formation and storage of memory, is regulated by carbamyl-mediated increases in Ca\textsuperscript{2+} in cultured hippocampal and cortical cells (Rosenblum et al., 2000). Other kinase pathways in the cytosol are also altered as a consequence of PCB-mediated changes in Ca\textsuperscript{2+} (reviewed in Tilson and Kodavanti, 1997; Tithof et al., 1997).

**Conclusion.** A release of IP\textsubscript{3} receptor-linked intracellular Ca\textsuperscript{2+} stores in rat cortical neuronal culture was found to precede latent Ca\textsuperscript{2+} disturbances involving L-type Ca\textsuperscript{2+} channels/glutamate receptors that arise in the continued presence of this toxicant (Ba et al., 1999b; Inglefield and Shafer, 2000b). It is clear from the present study that apoptosis is not the message conveyed by this period of induced Ca\textsuperscript{2+} signals, as opposed to the proapoptotic effects of a neurotoxin (Tat) that elicits very similar Ca\textsuperscript{2+} signals. Instead, alteration of a nuclear transcription factor in cerebral neurons by 2,3,7,8-tetrachlorodibenzo-p-dioxin (Aroclor 1254) produces a persistent impairment in long-term potentiation in the rat dentate gyrus in vivo. Brain Res 807:207–209.


Bae J, Stuenkel EL and Loch-Caruso R (1999b) Stimulation of oscillatory uterine contractile activity by the PCB mixture Aroclor 1242 may be mediated by arachidonic acid release through activation of phospholipase A\textsubscript{2} enzymes. J Pharmacol Exp Ther 289:1112–1120.

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