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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**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 IP3 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,
perturbations in temporal cellular Ca\(^{2+}\) signals may have important effects. Changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) 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\(^{2+}\) signals in developing cells is far-reaching. Ca\(^{2+}\) 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\(_3\)) receptors or ryanodine receptors (Berridge, 1998). In order to test the hypothesis that PCBs can affect Ca\(^{2+}\) 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 (Altmann 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), an environmentally relevant PCB mixture, results in temporal alterations in [Ca\(^{2+}\)]\(_i\), and reductions in GABA\(_A\) 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\(^{2+}\)]\(_i\), as well as the role of the initial mechanism of PCB action in the subsequently prolonged Ca\(^{2+}\) disturbances reported previously. Finally, because perturbations in temporal cellular Ca\(^{2+}\) signals may have important effects, two potential downstream consequences of altered Ca\(^{2+}\) 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\(^{2+}\)-ATPases, was obtained from Sigma. IP\(_3\) receptors were stimulated with carbacol (Sigma) and blocked with xestospongin C (Calbiochem, San Diego, CA). Ryanodine receptors were probed with caffeine (Sigma) and ryanodine (Research Biochemicals International, Natick MA).

Rat neocortical cultures were maintained for up to DIV 7, receiving fresh cortical medium (to limit replication of non-neuronal cells) in fresh cortical medium. Cultures were maintained for up to DIV 7, receiving fresh cortical medium upon removing cytosine arabinoside. All reagents were of the highest available grade from commercial sources. Cultures produced with these methods are enriched in neurons that have elaborate neurite processes, and culture wells at DIV 7 contain 70% neuron-specific enolase immunoreactive cells (neurons) that reside on a bed of glial fibrillary acidic protein-immunopositive glia (30%).

### 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 Cl\(^-\) 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 μM) 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\(^{2+}\)]\(_i\) responses were monitored. There are no detectable levels of polychlorinated dibenzodioxins (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\(^{2+}\)] Concentration Measurements

[Ca\(^{2+}\)]\(_i\) was measured with the Ca\(^{2+}\)-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 μM) for 40 min at 30°C diluted in 2 ml of HEPES-buffered Hanks' balanced salt solution (referred to herein as HEPES buffer) consisting of: 135 mM NaCl, 4.2 mM KCl, 1.5 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.34 mM Na\(_2\)PO\(_4\), 0.44 mM KH\(_2\)PO\(_4\), 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\(^{2+}\)-sensitive form, fura-2).

Coverslips containing fura-2-loaded cells were placed in a Leiden coverslip dish situated in a PDM2 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 Fluor 40X objective (numerical aperture 1.3).

Cytosolic 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 SIT videocamera (Hamamatsu, Bridgewater, NJ). Images were stored on a Dell Pentium II Dimensional 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\(^{2+}\)] using the equation (Gryniewicz et al., 1985):

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

in which \( R \) is the 340/380 ratio and \( K_d = 272 \text{nM} \), the dissociation constant we determined for fura-2. Maximum ratio \( (R_{\text{max}} = 5.9) \), minimum ratio \( (R_{\text{max}} = 0.7) \), and \( F/F_0 \) (ratio of the fura-2 intensities at 380 nm in the Ca\(^{2+}\)-free (with 5 mM EGTA) and Ca\(^{2+}\) saturated buffers (with 10 \( \mu \text{M} \) nonoyminic, respectively, 4.7) were determined from intracellular calibration because in vitro calibration may cause mis-estimation of Ca\(^{2+}\) values due to the difference in fura-2 properties in aqueous solution versus that of the cytoplasm. Estimated [Ca\(^{2+}\)] in the Ca\(^{2+}\)-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 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\(^{2+}\)]. In some experiments, after collecting baseline measurements, the contributions of specific receptors to intracellular Ca\(^{2+}\) 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 buffering). Ca\(^{2+}\) responses were typically measured for 10 min after addition of toxicant, although extended Ca\(^{2+}\) recordings also were performed to monitor latent changes in [Ca\(^{2+}\)], 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\(^{2+}\) transients and in some cases the time to decay to 10% peak amplitude were determined using specialized software (Mini Analysis, Synoptosoft, Leonia, NJ). The peak [Ca\(^{2+}\)], 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 \( \mu \text{M} \)), 0.4% Trypan blue was added to wells of cortical cultures that had been maintained in the incubator at 37°C (in 95% O\(_2\)/5% CO\(_2\)). Trypan blue excluding cells were counted using 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 4 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\(_3\)VO\(_4\), 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 (62.5 mM Tris (pH 6.8), 25% glycerol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue, and 5% 2-mercaptoethanol) and stored at ~80°C.

Cells lysates in sample buffer (15 \( \mu \text{g} \)) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacryl- amide) 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\(^{133}\)-CREB (diluted 1:1000) or anti-CREB (diluted 1:2000); Upstate Biotechnol- ogy, 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; Kirkeg- aard & Perry Laboratories, Inc., Gaithersburg, MD). The blots were then exposed to ECL substrate (Pierce, Rockford, IL), and chemilumi- nescence images were collected and analyzed using a Fluor-S MultiImager (Bio-Rad). Phospho-CREB (pCREB) and CREB bands were detected at the 43 kDa standard. Relative activation was de- termined by normalization of the band density from the phosphory- lated protein with that of the total (phosphorylated and nonphospho- rylated) CREB protein from the same sample.

Data Analysis. Data are presented as means ± S.E.M. For the Ca\(^{2+}\) responses, the percentage of responding cells in treatment groups are also noted. For comparisons of peak Ca\(^{2+}\) responses, statistical significance was ascertained using one- or two-way ANO- VAs followed by suitable post hoc tests. To determine whether phar- macological pretreatments were effective at preventing A1254-in- duced responses, the proportion of cellular Ca\(^{2+}\) 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 Dunnett’s test. Caspase 3 activity, TUNEL staining, and pCREB activation were assessed with one-way ANOVA and Dunnett’s test.

Results

A1254-Induced Changes in Cytoplasmic Free [Ca\(^{2+}\): Concentration Response. Figure 1A illustrates the typical response observed during 1 h of imaging of Ca\(^{2+}\) in a cortical cell exposed continuously to 10 \( \mu \text{M} \) (3 ppm) A1254. In the continued presence of A1254, a Ca\(^{2+}\) transient is followed 3 to 16 min later by disturbances in the basal Ca\(^{2+}\) level that often includes Ca\(^{2+}\) oscillations of ~200 to 700 nM in ampli-
PCB Effects on Ca²⁺ Signaling and CREB Phosphorylation

There were also concentration-dependent increases in the proportion of cells responding (Table 1).

**Specificity of Ca²⁺ Responses.** Addition of control buffer (0.1% DMSO) to cells from DIV 4 to 6 cortical cultures did not produce a [Ca²⁺]_i change (not shown) indicating that neither mechanical manipulation of the cells nor vehicle were responsible for the Ca²⁺ 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 Ca²⁺ 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 [Ca²⁺]_i from baseline levels of 72 ± 5 nM in >70% of the cells. The characteristics of the initial Ca²⁺ transients stimulated by PCB 4 and PCB 138 were indistinguishable from that elicited by A1254 in that Ca²⁺ 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>A1254 Exposure (μM)</th>
<th>Peak [Ca²⁺]_i (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>38</td>
</tr>
<tr>
<td>10</td>
<td>750 ± 150</td>
<td>79</td>
<td>28</td>
</tr>
<tr>
<td>20b</td>
<td>742 ± 72</td>
<td>90</td>
<td>60</td>
</tr>
<tr>
<td>25b</td>
<td>1200 ± 180</td>
<td>86</td>
<td>28</td>
</tr>
</tbody>
</table>

* Responses were studied by addition of A1254 to normal Ca²⁺-containing buffer (1.5 mM). Data are mean ± S.E.M. for the stated number of DIV 4 to 6 cortical cells cultured from two or more separate rat litters.

* During 0.5 h of imaging, 30% of cells exposed to A1254 (20 μM or higher) in Ca²⁺-containing buffer failed to have their stimulated Ca²⁺ levels return to below 20% of the peak amplitude. This delay/failure to return to baseline was termed a "type 3" response in an earlier report (Inglefield and Shafer, 2000b).
TABLE 2
Specificity test of PCBs involved in the A1254-induced [Ca\textsuperscript{2+}]
transient

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Peak [Ca\textsuperscript{2+}] \textsuperscript{a}</th>
<th>% Responding Cells</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1254 10 µM</td>
<td>750 ± 150</td>
<td>79</td>
<td>28</td>
</tr>
<tr>
<td>Ortho-substituted</td>
<td>700 ± 30</td>
<td>78</td>
<td>30</td>
</tr>
<tr>
<td>PCB 138 10 µM</td>
<td>501 ± 63</td>
<td>73</td>
<td>30</td>
</tr>
<tr>
<td>PCB 4 10 µM</td>
<td>474 ± 36</td>
<td>74</td>
<td>43</td>
</tr>
<tr>
<td>Non-ortho-substituted</td>
<td>406b</td>
<td>9*</td>
<td>22</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 χ\textsuperscript{2}; t-tests with Bonferroni’s correction followed.

Responses with PCB congeners were studied at 10 µM (or 5 µM) and compared with 10 µM 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 rat litters were assessed.

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

Solubility limit reached before 10 µM.

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+}],) exhibited a more rapid recovery to basal levels (Fig. 3A, inset). The Ca\textsuperscript{2+} responses stimulated by 20 µM A1254 in buffer containing the L-type Ca\textsuperscript{2+} channel blocker nifedipine (1 µM) 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+}] 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 mM 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+}] transient. These mechanistic studies were performed with 20 µM 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 µM 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 preincubation was sufficient to suppress completely the intracellular Ca\textsuperscript{2+} responses to stimulation by carbachol (1 mM) (response duration of store-operated Ca\textsuperscript{2+})...
TABLE 3
Pharmacologic agents active at IP_3 receptors or IP_3-releasable stores inhibit PCB-induced initial Ca^{2+} transients

<table>
<thead>
<tr>
<th>Exposure ± Treatment</th>
<th>Peak [Ca^{2+}] (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 Ca^{2+} buffer</td>
<td>535 ± 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 + xestospong C</td>
<td>203 ± 37</td>
<td>11</td>
<td>37</td>
</tr>
<tr>
<td>A1254 after CCh/0 Ca^{2+} 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 + thapsigarin</td>
<td>285 ± 78</td>
<td>54</td>
<td>13</td>
</tr>
<tr>
<td>2.2'-DCB + xestospong C</td>
<td>156 ± 12</td>
<td>60</td>
<td>15</td>
</tr>
</tbody>
</table>

N.D., not detectable because Ca^{2+} levels remained at baseline upon A1254 addition.

*p < 0.05 compared with respective control response, whether A1254 alone or 2.2'-DCB. For Peak [Ca^{2+}], 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 was used.

A1254 (or 2.2'-DCB) was bath-applied in the imaging chamber in the absence or presence of Ca^{2+} signaling site-selective drugs, including nifedipine (1 μM), thapsigargin (10 μM), ryanodine (100 μM), xestospong C (1 μM), or carbachol (CCh; 1 mM). The carbachol prestimulation was done in Ca^{2+}-free buffer to prevent refilling of the released stores. Pretreatment was 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.

Twenty 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.

abolished in 16 of 18 cells). Further assessment of endoplasmic reticulum-activated mobilization of intracellular Ca^{2+} by A1254 was done using pharmacological agents active at IP_3 or ryanodine receptors. Both types of intracellular stores were functioning in these cells because applications of carbachol (1 mM), ryanodine (100 μM), or caffeine (20 mM) mobilized intracellular stores (Fig. 4). Although pretreatment with ryanodine (100 μM for 10 or 20 min) completely blocked the increase in [Ca^{2+}], induced by caffeine, it was without effect on the A1254-induced increases in [Ca^{2+}]; (Fig. 4 and Table 3). In contrast, block of the Ca^{2+} transient occurred with the specific IP_3 receptor antagonist, xestospong C (1 μM, 10 min preincubation). Only 11% of the 37 cells had a response to A1254 (p < 0.05 following significant treatment effect in the χ² analysis); for these remaining cells, the peak amplitude of the response was significantly reduced (Fig. 4 and Table 3). Xestospong C was a potent inhibitor of carbachol-stimulated [Ca^{2+}]_i increases (Fig. 4). Similar to results with A1254, xestospong C and thapsigargin also reduced Ca^{2+} transients initiated by 10 μM 2,2'-dichlorobiphenyl (PCB 4), although the percentage of responding cells was not attenuated (Table 3). As a final demonstration that A1254 was causing release of Ca^{2+} from an IP_3-sensitive Ca^{2+} pool, pretreatment of cells with 1 mM carbachol in Ca^{2+}-free buffer (to prevent replenishment of the IP_3-sensitive stores as they were emptied) led to a complete inhibition of the Ca^{2+} transient when A1254 was subsequently applied (Fig. 4 and Table 3). Overall, these results indicated that the initial Ca^{2+} signals stimulated by ortho-substituted PCBs in A1254 in DIV 4 to 6 cortical cells were due to release of IP_3-sensitive Ca^{2+} stores. These findings also distinguish the mechanism for this early Ca^{2+} disturbance induced by PCBs from that of the latent Ca^{2+} distu-
increases in basal Ca\(^{2+}\). The dependence of latent Ca\(^{2+}\) disturbances on the initial Ca\(^{2+}\) transient was investigated by pretreating cells with thapsigargin (10 \(\mu M\)) or xestospongin C (1 \(\mu M\)) and assessing changes in basal [Ca\(^{2+}\)] \(_i\) after a 30-min exposure to 20 \(\mu M\) A1254. As shown in Ca\(^{2+}\) traces (Fig. 5, A and B) and the bar graph (Fig. 5C), pretreatment with either thapsigargin or xestospongin before A1254 was effective at blocking the A1254-induced Ca\(^{2+}\) transient; both also significantly reduced the elevations in basal Ca\(^{2+}\) after a 0.5-h of A1254 exposure. These results suggested that the Ca\(^{2+}\) transient caused by A1254 is associated with later dysregulation of Ca\(^{2+}\) levels in the same cells.

**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 \(\mu M\) (Inglefield and Shafer, 2000a) to 50 \(\mu M\) (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 \(\mu M\) (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 \(\mu M\) 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 \(\mu M\) 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 \(\mu M\)). Therefore, in DIV 6/7 cortical cultures subacute exposure to the highest concentration, 20 \(\mu M\) 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 \(\mu M\) 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 cortical cultures sensitive 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 \(\mu M\) A1254. A1254 did not induce de novo synthesis of CREB because the total amount of CREB did not increase as a result of the exposure (Fig. 7A). A1254 also led to concentration-dependent increases in pCREB activation, reaching a 2-fold induction with 10 and 20 \(\mu M\) (Fig. 7B). In comparison, glutamate (100 \(\mu M\) for 40 min) maximally stimulated pCREB 6-fold, whereas the ortho-substituted PCB congener 4 (2,2’-DCB, 10 \(\mu M\)) 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
of decreased cell viability in the continuous presence of 20 ppm (7.2 M) following 40-min exposure to A1254. For each A1254 concentration, values were normalized to the total amount of pCREB or CREB in the respective control. The pCREB/CREB ratio indicates a relative increase in phosphorylation of serine-133 of CREB in the present study. This reduction likely occurred as a consequence of either depletion of endoplasmic reticulum Ca^{2+} stores with a 20-min pre-exposure to a high concentration of ryanodine. The finding of nifedipine’s inability to attenuate 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+} transient, 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 xestospongin 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 xestospongin, 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 following 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\textsuperscript{2+} transient agrees with the failure by ryanodine to block the transient because the L-type voltage-sensitive Ca\textsuperscript{2+} channels are functionally coupled to ryanodine receptors (Chaviss et al., 1996). Thus, the absence of effect of two different modulators in the L-type voltage-gated Ca\textsuperscript{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\textsubscript{3}-releasable Ca\textsuperscript{2+} stores in intact cells are functionally important for the actions of this class of toxicant comes from the induction of store-operated Ca\textsuperscript{2+} entry, as indicated by the rebound increase of [Ca\textsuperscript{2+}], following the return of Ca\textsuperscript{2+}-containing buffer to those cells whose internal stores were mobilized by A1254 in Ca\textsuperscript{2+}-free buffer. Ca\textsuperscript{2+} release from IP\textsubscript{3} stores activates store-operated Ca\textsuperscript{2+} channels (also known as Ca\textsuperscript{2+}-release-activated Ca\textsuperscript{2+} channels, CRAC) on the plasma membrane (Hofer et al., 1998). A very recent study convincingly showed the physical and functional coupling of IP\textsubscript{3} receptor-mediated Ca\textsuperscript{2+} stores and SOCs (Ma et al., 2000). Store-operated Ca\textsuperscript{2+} entry is present in developing neurons (Bouron, 2000), and the preceding evidence is in accordance with store-operated Ca\textsuperscript{2+} entry in the A1254-induced stimulation of IP\textsubscript{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\textsubscript{3} releasable Ca\textsuperscript{2+} stores has been demonstrated. The present study confirms what those earlier investigators had proposed: that elevation of [Ca\textsuperscript{2+}], induced by ortho-substituted PCBs (also the mixture A1254 that consists primarily of ortho-substituted congeners), can be mediated by IP\textsubscript{3} receptor-sensitve stores probably via an increase in IP\textsubscript{3} levels of note, activation of IP\textsubscript{3}-releasable Ca\textsuperscript{2+} stores has also been demonstrated for another chlorinated hydrocarbon, γ-hexachlorocyclohexane (lindane), in smooth muscle cells (Criswell et al., 1994). Although Mundy et al. (1999) did report a significantly increased [H\textsuperscript{3}I] IP\textsubscript{3} receptor binding in cerebellar microsomes following a short exposure to PCB 4, a functional effect of PCB 4 on the IP\textsubscript{3}-releasable stores was not identified. In cortical microsomes from adult animals, there was a stimulatory action on ryanodine receptors to release Ca\textsuperscript{2+} by the ortho-substituted PCB 95, but no Ca\textsuperscript{2+} effect occurred in the larger population of vesicles harboring IP\textsubscript{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\textsubscript{3} receptors in Ca\textsuperscript{2+} responses induced by PCBs.

**On the Consequences of A1254-Induced Disturbances in Endoplasmic Reticulum Ca\textsuperscript{2+} Homeostasis.** Both excitotoxicity (which can lead to necrosis) and apoptosis are possible outcomes of alterations in neuronal Ca\textsuperscript{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\textsuperscript{2+} homeostasis that are considered central to later apoptotic cell death (New et al., 1997; Krumann 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\textsuperscript{2+} transient, we identified a role of the initial mechanism of PCB action in the subsequent prolonged Ca\textsuperscript{2+} disturbances. This is in agreement with the recognition of an association of the two phases of Ca\textsuperscript{2+} disturbance induced by Tat in human embryonic neurons maintained in culture (Haughey et al., 1999). These latent Ca\textsuperscript{2+} changes are mediated by heightened Ca\textsuperscript{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\textsuperscript{2+} mobilization from intracellular stores induced by A1254 (or Tat) and the facilitation of later Ca\textsuperscript{2+} dysregulation, it would appear that mobilization of intracellular Ca\textsuperscript{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\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores contributes in some way to latent increases in basal [Ca\textsuperscript{2+}], or to the activation of Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsubscript{3}-depleted stores and subsequent store-operated/capacitative Ca\textsuperscript{2+} entry are a trigger of the signaling pathway leading to CREB activation (in cortical glial cells) (Penede et al., 1997). In addition to the likelihood (discussed above) that store-operated Ca\textsuperscript{2+} influx may have primed the cells to undergo a later Ca\textsuperscript{2+} disturbance, the activation of CREB by A1254 may also depend on both the...
intracellular Ca\(^{2+}\) release and ensuing transmembrane Ca\(^{2+}\) influx. Specifically, it has been shown that CREB activation following stimulation with carbacol (which mobilizes IP\(_3\) stores) is prevented in Ca\(^{2+}\)-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 carbacol-mediated Ca\(^{2+}\) influx (Pende et al., 1997), and this longer lasting CREB activation may be secondary to A1254’s additional actions on Ca\(^{2+}\) signaling that include activation of excitatory acid receptors and L-type VGCCs (Inglefield and Shafer, 2000b). Transmembrane Ca\(^{2+}\) 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\(^{2+}\) influx may play an important role in the mechanistic link between PCB-induced Ca\(^{2+}\) 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 carbacol-mediated increases in Ca\(^{2+}\) 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\(^{2+}\)](E) (reviewed in Tilson and Kodavanti, 1997; Tithof et al., 1997).

**Conclusion.** A release of IP\(_3\) receptor-linked intracellular Ca\(^{2+}\) stores in rat cortical neuronal culture was found to precede latent Ca\(^{2+}\) disturbances involving L-type Ca\(^{2+}\) channels/glutamate receptors that arise in the continued presence of this toxicant (Bae 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\(^{2+}\) signals, as opposed to the proapoptotic effects of a neurotoxin (Tat) that elicits very similar Ca\(^{2+}\) signals. Instead, alteration of a nuclear transcription factor in cerebral cortical cells is prevented in Ca\(^{2+}\) stores) is a study using simultaneous measurements of IP\(_{3}\) and intramembranous [Ca\(^{2+}\)](E). J Cell Biol 140:325–334.

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PCB Effects on Ca$^{2+}$ Signaling and CREB Phosphorylation

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PCB Effects on Ca$^{2+}$ Signaling and CREB Phosphorylation


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