NON-AH RECEPTOR MECHANISMS UNDERLYING IMMUNOTOXICITY AND NEUROTOXICITY

TEMPORAL DISTURBANCES OF INTRACELLULAR Ca^{2+} HOMEOSTASIS INDUCED BY AROCLOR 1254

Jon R. Inglefield, William R. Mundy and Timothy J. Shafer

Neurotoxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711

Introduction

Despite their well documented association with developmental neurotoxicity, the mechanisms of action of polychlorinated biphenyls (PCBs) on neurons are not well understood. While many different possible mechanisms have been proposed, disruption of Ca^{2+} homeostasis in neurons has been a recurring theme reported by a number of investigators[1-3]. For example, in cerebellar granule cells, PCB-induced disturbances of Ca^{2+} homeostasis are exemplified by slow, graded increases in intracellular Ca^{2+} concentration measured using fura 2[1].

In the nervous system, spatial and temporal Ca^{2+} signaling between neurons in developing networks is crucial for normal development, and regulates important events including gene transcription and neurite outgrowth. Thus, disruption of Ca^{2+} signaling in a network of neurons could alter differentiation of neurons without causing overt cell loss. We have used primary cultures of cortical neurons in order to examine the potential effects of PCBs on receptors responsible for generating Ca^{2+} signals in developing neonatal neurons. This *in vitro* model system forms simple networks of interconnected neurons over a period of days *in vitro* (DIV). In addition, these cortical cells have an ontogeny of $GABA_A$ receptor responses which recapitulates that observed *in vivo*, wherein $GABA_A$ receptor activation stimulates Ca^{2+} entry on early DIV (DIV 4-6), but on later DIV(>DIV6) is without stimulatory effect on Ca^{2+} entry[4]. Using this model system, we are examining the hypothesis that PCBs alter temporal Ca^{2+} signaling in networks of interconnected neurons, and, as a result, alters normal development in that system.

Materials and Methods

Cell culture. Primary cultures of cortical neurons were prepared from neocortices of newborn (<24 hr old) Long-Evans rat pups[4]. Briefly, the cortical neurons were isolated by standard tissue culture methods and plated onto 25 mm glass coverslips in Dulbeccos modified Eagles medium containing penicillin and streptomycin. After 3 days, cytosine arabinoside was added to limit proliferation of non-neuronal cells. The resulting culture consists of neuronal appearing cells on a monolayer of glial cells.

Imaging. All imaging experiments were conducted between DIV 4 and DIV 8. Coverslips were washed with standard physiological buffer solution and incubated for 30 min with fura 2AM in order to load cells with fura 2. At the end of this 30 min incubation, cells were washed with

physiological buffer solution and placed into an imaging chamber on the stage of a Nikon Diphot microscope. Measurements of intracellular Ca^{2+} responses were made using Photon Technology Internationals Deltascan system coupled to a Dell PC. Excitation wavelengthes were 340 and 380 nm, while emission wavelength was >510 nm. When fura 2 ratios were converted to intracellular Ca^{2+} concentration, the method of Grynkiewicz was used[4].

Results and Discussion

As demonstrated previously in other neuronal systems, $GABA_A$ receptor-mediated responses in cortical cells exhibited an ontogenic change from excitatory to inhibitory over several DIV. Excitatory responses, which occurred prior to DIV 7, were typified by depolarization coupled to Ca^{2+} entry through L-type voltage-sensitive Ca^{2+} channels, as nifedipine (1 μM) was capable of blocking this response[4]. Inhibitory $GABA_A$ responses, occurring on DIV7 and after, were typified by hyperpolarization and a lack of Ca^{2+} entry. Following a 1 hour exposure to Aroclor 1254 (A1254), excitatory and inhibitory responses to $GABA_A$ receptor activation were inhibited by concentrations of A1254 as low as 2 μM[4]. As shown in figure 1, stimulation of $GABA_A$ receptor responses with the $GABA_A$ agonist muscimol (25 μM) resulted in a large increase in DIV 4 control cells, but a blunted response in PCB-treated DIV 4 cells. At concentrations up to 20 μM, A1254 was without signs of impending cytotoxicity as measured by propidium iodide exclusion and cross-sectional surface area after 1 hour of exposure. These data suggested that $GABA_A$ receptor-mediated responses in developing cortical neurons are extremely sensitive to perturbation by PCBs.

During the 1 hr of exposure to A1254, temporal alterations in intracellular Ca^{2+} concentration were observed (Figure 1). In control cells, spontaneous increases in intracellular Ca^{2+} concentration were only rarely observed (<10 % of cells), nor did basal intracellular Ca^{2+} concentration (~70-90 nM) increase over this time period. By contrast, in A1254-treated cells, a dynamic response was observed which was typified by an initial, transient increase in intracellular Ca^{2+} concentration (referred to hereafter as a Ca^{2+} transient; immediately after the arrow in lower panel of figure 1). This transient was followed by a variable period (3-15 min) of relative quiescence, then by the initiation of repetitive oscillations in intracellular Ca^{2+} concentration (referred to hereafter as Ca^{2+} oscillations). Basal intracellular Ca^{2+}increased significantly in a concentration-dependent manner over this period[4] (not apparent in the examples in Figure 1). Both the A1254-induced Ca^{2+} transients and latent Ca^{2+} disturbances (for example, oscillations) also were concentration-dependent between 1 and 25 μM; including the occurrence (% of cells), amplitude, and frequency (oscillations/min).

Because of the importance of maintained Ca^{2+} homeostasis in developing neurons, we have examined the mechanisms underlying the A1254-induced Ca^{2+} transient and oscillations. In order to determine whether the source of Ca^{2+} for these events was intracellular or extracellular, experiments were conducted in nominally Ca^{2+} free buffer solutions. In the absence of added extracellular Ca^{2+}, A1254 exposure (1-20 μM) still resulted in a rapid Ca^{2+} transient, but no oscillations were observed. In addition, when cortical cells were exposed to A1254 in the presence of the L-type voltage-sensitive

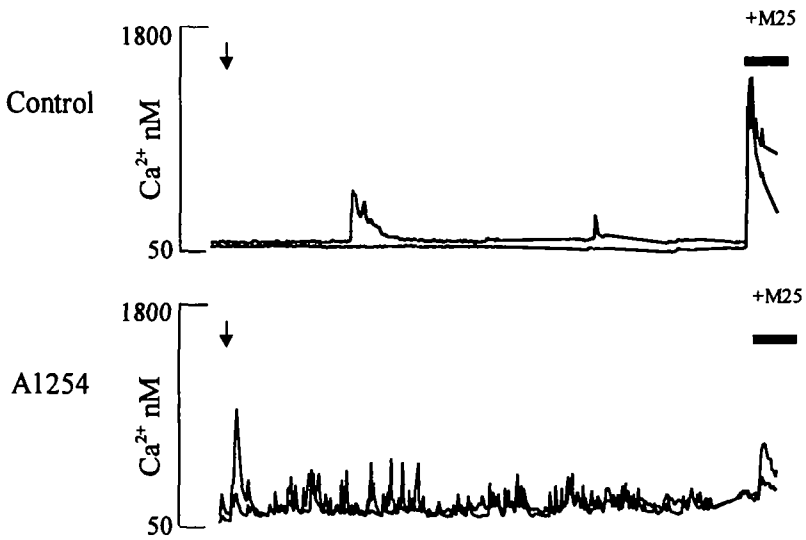

Figure 1. Intracellular Ca^{2+} responses in DIV 4 cells from 2 Control and 2 A1254 (20 μM)-treated cells (added at arrow) demonstrating the differences in Ca^{2+} signaling over 1 hr of exposure. Following the extended period of Ca^{2+} signaling with A1254-induced Ca^{2+} transients and oscillations, excitatory $GABA_A$ responses to $GABA_A$ receptor agonist, 25 μM muscimol (M25), were significantly blunted.

Ca^{2+} channel antagonist, nifedipine (1 μM), Ca^{2+} oscillations, but not the Ca^{2+} transient were eliminated. These data demonstrate that the sources of A1254-induced Ca^{2+} transients and oscillations differ; being dependent on intracellular and extracellular Ca^{2+} pools, respectively.

To determine the intracellular pool of Ca^{2+} responsible for the A1254-induced Ca^{2+} transient, experiments were conducted in the presence of antagonists of various intracellular Ca^{2+} stores. Pretreatment of cortical cells with ryanodine (100 μM) was without effect on the A1254-induced Ca^{2+} transient. However, antagonism of IP_3-sensitive Ca^{2+} pools with 10 μM thapsigargin or 1 μM xestospongin C completely eliminated A1254-induced Ca^{2+} transients (Figure 2). These data demonstrate that the Ca^{2+} transient observed immediately following exposure to A1254 is the result of release of IP_3-sensitive stores in cortical cells.

As stated above, oscillations were absent in nominally Ca^{2+}-free solutions and in the presence of 1 μM nifedipine, demonstrating that they were dependent on entry of Ca^{2+} from the extracellular space through L-type voltage-sensitive Ca^{2+} channels. Because other investigators have demonstrated that Ca^{2+} oscillations in cortical neurons grown in culture were dependent on excitatory

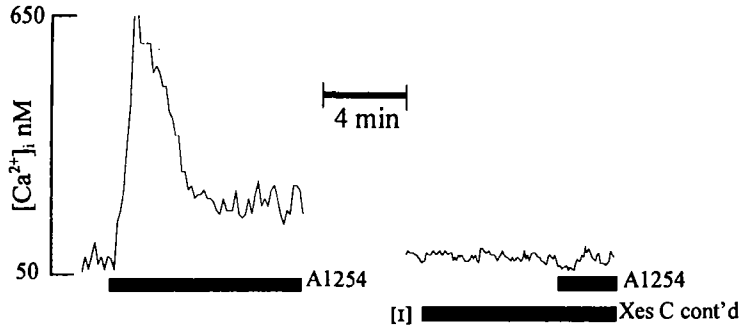

Figure 2. Initial Ca^{2+} transient induced by exposure to 20 μM A1254 (left) is eliminated by prior incubation of a different cell in the presence of 1 μM xestospongin C (right) for 15 min. The peak $[Ca^{2+}]_i$ amplitude was 714 ± 64 nM vs. 203 ± 37 nM in A1254 or A1254 + xestospongin treatment, respectively (n = 52 and 37 cells).

amino acid neurotransmission and could be blocked by tetrodotoxin[5], we examined the sensitivity of A1254-induced oscillations to amino-acid transmitter receptor antagonists and tetrodotoxin. A1254-induced Ca^{2+} oscillations were completely blocked by 2 μM tetrodotoxin, suggesting that increased neuronal excitability was responsible for activation of L-type Ca^{2+} channels. In addition, oscillations could be blocked by addition of antagonists to excitatory amino acid receptors, including glutamate receptors. These data suggest that A1254-induced Ca^{2+} oscillations in cortical cells are mediated by activation of L-type voltage-sensitive Ca^{2+} channels secondary to activation of excitatory amino acid receptors.

Temporal alterations in Ca^{2+} signaling in developing neuronal networks is important for regulation of gene expression and differentiation[6]. The consequences of PCB-induced alterations in Ca^{2+} homeostasis in developing cortical cells may then have ramifications which includes induction of inappropriate gene expression leading to altered differentiation of these cells. The potential ramifications of A1254-induced disturbances of Ca^{2+} homeostasis in primary cultures of cortical neurons are presently being examined.

Acknowledgments. We wish to thank Ms. Theresa Freudenrich and Ms. Connie Meacham for preparation of the cultures of cortical neurons.

References.

1. Kodavanti, P.R.S., Shin, D-S., Tilson, H.A., and Harry, G.J. (1993) Toxicol. Appl. Pharmacol. 123, 97-106.
2. Wong, P.W. and Pessah, I.N. (1997) Mol. Pharmacol. 51, 693-702.
3. Mundy, W.R., Shafer, T.J., Tilson, H.A., and Kodvanti, P.R.S. (1999). Toxicology 136, 27-39.
4. Inglefield J.R. and Shafer, T.J. (2000) Toxicol. Appl. Pharmacol. 164, 184-195.
5. Shen, M., Piser, T.M., Seybold, V.S., and Thayer, S.A. (1996) J. Neurosci. 16, 4322-4334.
6. Mermelstein, P. G., Bito, H., Deisseroth, K., and Tsien, R.W. (2000) J. Neurosci. 20, 266-273.