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Research Report

Protein kinase C is a target for diverse developmental neurotoxicants: Transcriptional responses to chlorpyrifos, diazinon, dieldrin and divalent nickel in PC12 cells

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ABSTRACT

Unrelated developmental neurotoxicants can elicit similar functional outcomes, whereas agents in the same class may differ. We compared two organophosphate insecticides (chlorpyrifos, diazinon) with an organochlorine (dieldrin) and a metal (Ni^{2+}) for similarities and differences in their effects on gene expression encoding subtypes of protein kinase C and their modulators, a cell signaling cascade that integrates the actions of neurotrophic factors involved in brain development. We conducted evaluations in PC12 cells, a model for neuronal development, with each agent introduced at 30 μM for 24 or 72 h, treatments devoid of cytotoxicity. Chlorpyrifos evoked by far the largest effect, with widespread upregulation of multiple genes; the effects were greater during neurodifferentiation than when cells were exposed prior to differentiation. Diazinon had smaller and less widespread effects, consistent with its lesser long-term impact on synaptic function and behavior noted for in vivo exposures in developing rats. Surprisingly, the effects of diazinon, dieldrin and Ni^{2+} showed basic similarities despite the fact that all three come from different classes of toxicants. Our findings provide some of the first evidence for a specific mechanistic cascade contributing to the cholinesterase-independent developmental neurotoxicant actions of chlorpyrifos and its differences from diazinon, while at the same time identifying mechanistic convergence between otherwise unrelated toxicants that provides predictions about common neurodevelopmental outcomes. These results further show how combined use of cell cultures and microarray technology can guide future in vivo work on diverse developmental neurotoxicants.

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1. Introduction

Of the tens of thousands of chemicals in active production that are thought to be developmental neurotoxicants, only a small percentage have ever been evaluated for such activity (Boyes, 2001; Grandjean and Landrigan, 2006). The combined effects of these agents are likely contributors to what has been called a "silent pandemic" of neurobehavioral dysfunction in children (Grandjean and Landrigan, 2006), including learning disabilities, cognitive impairment and autism spectrum

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Abbreviations: ANOVA, analysis of variance; *fgf*, fibroblast growth factor gene family; *fzd*, frizzled gene family; NGF, nerve growth factor; PKA, protein kinase A; PKC, protein kinase C; *wnt*, wingless gene family

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disorders (Eriksson, 1997; Grandjean and Landrigan, 2006; Landrigan et al., 1994, 1999; Szpir, 2006a,b; Weiss et al., 2004). Although basic research tends to focus on specific mechanisms of action of individual agents or classes of neurotoxicants, there are surprising similarities in outcomes among apparently unrelated agents, as well as disparities between chemicals within the same class (Barone et al., 2000; Monnet-Tschudi et al., 2007; Slotkin, 2004; Slotkin et al., 2006, 2007c, 2008a,b, in press; Slotkin and Seidler, 2007, 2008; Szpir, 2006a; Yanai et al., 2002, 2004). In a series of recent studies, we examined how chlorpyrifos and diazinon, two organophosphates, show similarities but also major differences in their impact on the expression of neurotrophic factors that coordinate neuronal cell differentiation and brain assembly (Slotkin et al., 2007c, 2008c, in press). The disparities are likely to explain divergent outcomes in terms of synaptic function of specific neurotransmitter pathways and their dependent behaviors (Aldridge et al., 2004, 2005a; Levin et al., 2001; Roegge et al., 2008; Slotkin et al., 2001, 2008a,b; Slotkin and Seidler, 2005; Timofeeva et al., 2008).

At the same time, we found surprising similarities in some of the effects of unrelated agents, such as diazinon and the organochlorine insecticide, dieldrin, as well as a metal, divalent nickel (Slotkin et al., 2007b, in press; Slotkin and Seidler, 2009), suggesting that a wide variety of agents may all converge on common sets of pathways that govern neurodevelopment. Several studies point to cell signaling cascades that transduce the actions of numerous neurotransmitters and hormones as likely points of crosstalk for the integration of diverse trophic signals toward common events in neurodifferentiation, notably the inputs converging on protein kinases such as PKA, PKC and tyrosine kinases (Aldridge et al., 2005b; Kapfhammer, 2004; Meyer et al., 2004, 2005; Nakagawara, 2001; Reuss and von Bohlen und Halbach, 2003; Slikker et al., 2005; Slotkin et al., 2003, 2008c, in press; Slotkin and Seidler, 2007; Yanai et al., 2002, 2004, 2006). The proof of the importance of these pathways has been reinforced by demonstrating amelioration or reversal of neurotoxicant effects by agents that offset the actions at the level of PKA or PKC (Beer et al., 2005; Slotkin et al., 2007a; Steingart et al., 2000; Yanai et al., 2006).

Administration of developmental neurotoxicants in vivo elicits actions that reflect both the inherent neurotoxicity of the agent as well as indirect effects mediated through actions on the maternal-fetal or maternal-neonatal unit. In the current study, we wanted to compare and contrast the direct effects of chlorpyrifos, diazinon, dieldrin and Ni^{2+} on the expression of PKC isoforms and PKC regulators, especially given the key role of PKC in neuritic outgrowth and synaptic connectivity (Kapfhammer, 2004), effects that are known targets for the organophosphates (Slotkin, 1999, 2004, 2005). Furthermore, PKC is a collecting point for expression of the genes encoding the neurotrophic factors of the *fgf* family (Reuss and von Bohlen und Halbach, 2003) and the *wnt/fzd* pathway (Li et al., 2005), both of which are targeted by chlorpyrifos, diazinon, dieldrin, and Ni^{2+} (Slotkin et al., 2007c, 2008c, in press). PKC also mediates neurotoxic effects of metals, environmental tobacco smoke, pesticides and neuroactive drugs in the developing brain (Hasan et al., 2001; Haykal-Coates et al., 1998; Hilliard et al., 1999; Yanai et al., 2002), and therefore represents a likely point where disparate agents may lead to convergent neurodevelopmental outcomes.

Our evaluations were conducted with a widely-used in vitro model for neuronal development, PC12 cells (Teng and Greene, 1994). This model reproduces the mechanisms and outcomes of in vivo organophosphate exposures of developing rats (Bagchi et al., 1995, 1996; Crumpton et al., 2000a,b; Das and Barone, 1999; Flaskos et al., 1994; Jameson et al., 2006, 2007; Li and Casida, 1998; Nagata et al., 1997; Qiao et al., 2001, 2005; Slotkin et al., 2007a,b; 2008c, in press; Song et al., 1998; Tuler et al., 1989), and has already been characterized for comparative effects of the four agents on differentiation outcomes and on expression of the trophic factors encoded by the *fgf* and *wnt/fzd* gene families (Jameson et al., 2006; Slotkin et al., 2007b, 2008c, in press; Slotkin and Seidler, 2008, 2009). In the PC12 model, nerve growth factor (NGF) triggers differentiation into neurotransmitter phenotypes, with formation of neuritic projections and acquisition of electrical excitability (Fujita et al., 1989; Song et al., 1998; Teng and Greene, 1994). For chlorpyrifos, we contrasted the effects in the undifferentiated vs. differentiating state, and then we compared the effects during differentiation for all four agents. Gene expression profiles were assessed with microarrays, using a "planned comparisons" approach (Slotkin and Seidler, 2007, 2009; Slotkin et al., 2007c, 2008c, in press).

2. Results

The microarrays detected 11 PKC isoforms (*prkca*, *prkcb1*, *prkcc*, *prkcd*, *prkce*, *prkch*, *prkci*, *prkcm*, *prkcn*, *prkcq*, *prkcz*) and 3 regulatory binding proteins (*prkcbp1*, *prkcabp*, *prkcdbp*) that passed the quality control procedures. Because only one agent (chlorpyrifos) was tested in both undifferentiated and differentiating cells, we conducted two sets of global statistical tests. For chlorpyrifos, the ANOVA factors were treatment, differentiation state, time and gene, and we found a main treatment effect ($p<0.02$) as well as interactions of treatment × gene ($p<0.0002$), treatment × differentiation state × time ($p<0.02$), treatment × time × gene ($p<0.05$) and treatment × state × gene ($p<0.05$). Accordingly, we subdivided the results according to differentiation state and then evaluated main treatment effects and treatment × time interactions for each gene. Chlorpyrifos exposure evoked significant changes in the expression of 10 of the total of 14 genes, as compared to an expected false positive rate of only <1 gene ($p<0.0007$), and the same was true for the separate analyses of undifferentiated cells (7 out of 14 genes, $p<0.02$) and differentiating cells (9 out of 14 genes, $p<0.003$). Diazinon, dieldrin and Ni^{2+} were studied only in differentiating cells, so the ANOVA factors for these agents were treatment, gene and time. The global test identified significant interactions of treatment × gene ($p<0.0001$) and treatment × gene × time ($p<0.003$), so we subdivided the data into the individual treatments and then evaluated each gene for main treatment effects and the interaction of treatment × time. Of the 14 total genes, we identified significant differences for 8 ($p<0.007$ vs. the false positive rate of <1 gene).

In undifferentiated cells, chlorpyrifos exposure evoked significant upregulation of three PKC isoforms, *prkcb1*, *prkce*

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