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Dual actions of lindane (γ-hexachlorocyclohexane) on calcium homeostasis and exocytosis in rat PC12 cells

Harm J. Heusinkveld [a,*], Gareth O. Thomas [a,b], Ischa Lamot [a], Martin van den Berg [a], Alfons B.A. Kroese [a,c], Remco H.S. Westerink [a]

[a] Neurotoxicology Research Group, Institute for Risk Assessment Sciences (IRAS), Utrecht University, P.O. Box 80.177, NL-3508 TD Utrecht, The Netherlands
[b] Department of Environmental Science, Lancaster University, Lancaster LA1 4YQ, UK
[c] Department of Surgery, University Medical Centre Utrecht, Utrecht, The Netherlands

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ABSTRACT

The persistent organochlorine pesticide lindane is still abundantly found in the environment and in human and animal tissue samples. Lindane induces a wide range of adverse health effects, which are at least partially mediated via the known inhibition of $GABA_A$ and glycine receptors. Additionally, lindane has been reported to increase the basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). As Ca^{2+} triggers many cellular processes, including cell death and vesicular neurotransmitter release (exocytosis), we investigated whether lindane affects exocytosis, Ca^{2+} homeostasis, production of reactive oxygen species (ROS) and cytotoxicity in neuroendocrine PC12 cells. Amperometric recordings and $[Ca^{2+}]_i$ imaging experiments with fura-2 demonstrated that lindane (≥10 μM) rapidly increases basal exocytosis and basal $[Ca^{2+}]_i$. Additional imaging and electrophysiological recordings revealed that this increase was largely due to a lindane-induced membrane depolarization and subsequent opening of N- and P/Q-type voltage-gated Ca^{2+} channels (VGCC). On the other hand, lindane (≥3 μM) induced a concentration-dependent but non-specific inhibition of VGCCs, thereby limiting the lindane-induced increase in basal $[Ca^{2+}]_i$ and exocytosis. Importantly, the non-specific inhibition of VGCCs also reduced stimulation-evoked exocytosis and Ca^{2+} influx. Though lindane exposure concentration-dependently increased ROS production, cell viability was not affected indicating that the used concentrations were not acute cytotoxic.

These combined findings indicate that lindane has two, partly counteracting effects. Lindane causes membrane depolarization, thereby increasing basal $[Ca^{2+}]_i$ and exocytosis. In parallel, lindane inhibits VGCCs, thereby limiting the basal effects and reducing stimulation-evoked $[Ca^{2+}]_i$ and exocytosis. This study further underlines the need to consider presynaptic, non-receptor-mediated effects in human risk assessment.

Introduction

Lindane (γ-hexachlorocyclohexane, γ-HCH, γ-benzene hexachloride, γ-BHC) is a persistent, lipophilic and bioaccumulative organochlorine pesticide. Lindane has been used for many years as a broad-range insecticide in agriculture and for the treatment of lice and scabies in humans. In some areas lindane is probably still in use, but its use has been banned or severely restricted in most countries for more than 15 years (Li et al., 1996). Lindane is mobile in the environment, accumulates in the food chain and lindane residues are ubiquitous in environmental samples (including biota) globally (Roche et al., 2008). Humans are exposed to lindane predominantly through the diet.

Despite the decline of lindane concentrations in environmental media (for review see: Hoferkamp et al., 2010), lindane levels in Europe reach up to hundreds of ng/g lipid weight (ng/g l.w.) in human tissues (Dirtu et al., 2006; Thomas et al., 2006), including (cord) serum, breast milk and amniotic fluid (Campoy et al., 2001; Jimenez-Torres et al., 2006; Luzardo et al., 2009). Breast milk samples from heavily polluted areas in India were reported to even contain up to 4500 ng/g l.w. (corresponding with approx 650 nM; calculated using average physiological values; Subramanian et al., 2007), whereas blood levels in Mexican school children are reported to reach 8200 ng/g l.w. (corresponding with a blood concentration of approximately 315 nM; calculated using average physiological values; Trejo-Acevedo et al., 2009). As lindane is reported to bioaccumulate, it is not unlikely that brain levels will reach even higher levels. Considering the known adverse effects of lindane on human health, these findings should be of major societal concern. Importantly, lindane exposure and elevated lindane levels in human brain have been associated with the

* Corresponding author. Fax: +31 30 2535077.
E-mail address: H.J.Heusinkveld@uu.nl (H.J. Heusinkveld).

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development of neurodegenerative diseases, including idiopathic Parkinson's disease (PD; Corrigan et al., 2000; Corrigan et al., 1996), whereas perinatal exposure to lindane has been associated with the development of PD later in life (Barlow et al., 2007).

The nervous system is among the most vulnerable targets for lindane exposure (Mariussen and Fonnum, 2006), which holds in particular for the developing nervous system (Singal and Thami, 2006). Already decades ago, it was shown that lindane exposure affects learning behaviour as well as motor activity (for review see: Mariussen and Fonnum, 2006). Although the primary working mechanism of lindane was reported to be inhibition of postsynaptic $GABA_A$ and glycine receptors in the nervous system (Anand et al., 1998; Vale et al., 2003), lindane was also reported to exert presynaptic effects. At high concentrations (50 μM), lindane increases the frequency of basal neurotransmitter release at frog neuromuscular junctions (Publicover and Duncan, 1979) as well as depolarization-evoked noradrenaline release in the rat hippocampus (Cristofol and Rodriguez-Farré, 1994). Noteworthy, effects of lindane on the intracellular calcium concentration ($[Ca^{2+}]_i$) were reported to occur in the low μM range. Lindane (1–50 μM) increases basal $[Ca^{2+}]_i$ in a range of cell types within seconds or minutes of application (Criswell et al., 1994; Hawkinson et al., 1989; Lu et al., 2000; Rosa et al., 1997). This effect depends, at least partly, on the presence of extracellular Ca^{2+} and appears to be related to different mechanisms in different cell types, including Ca^{2+} release from intracellular ionositol-1,4,5-triphosphate (IP_3)-sensitive Ca^{2+} stores followed by capacitive Ca^{2+} entry (Lu et al., 2000). Also, non-GABA-mediated, Ca^{2+}-sensitive effects of lindane on transcriptional parameters of neuronal development *in vitro* have been observed at concentrations as low as 100 nM (Ferguson and Audesirk, 1995). Furthermore, it has been suggested from subchronic *in vivo* studies that lindane-induced changes in $[Ca^{2+}]_i$ might be related to increased oxidative stress (for review see: Mariussen and Fonnum, 2006).

Exocytosis is driven by an increase in $[Ca^{2+}]_i$ and is regulated by a variety of cytoplasmic, vesicle- and membrane-associated proteins (for review see: Catterall and Few, 2008; Garcia et al., 2006; Westerink, 2006). Hence, in view of the reported lindane-induced effects on basal $[Ca^{2+}]_i$ and considering the importance of depolarization-evoked Ca^{2+} influx for exocytosis, it is surprising that effects of lindane on depolarization-evoked changes in $[Ca^{2+}]_i$ and exocytosis are thus far not reported.

The finding that lindane, while its primary neurotoxic mechanism appears to be inhibition of GABA and glycine receptors, affects $[Ca^{2+}]_i$ and is linked to selective degeneration of dopaminergic brain areas, has led to the hypothesis that lindane affects critical parameters of dopaminergic neurotransmission.

Therefore, the aim of the present study was to investigate effects of acute lindane exposure on basal as well as depolarization-evoked dopamine exocytosis, Ca^{2+} homeostasis and cytotoxicity in PC12 cells. Dopaminergic PC12 cells are extensively characterized as an *in vitro* model to study changes in Ca^{2+} homeostasis and exocytosis (Westerink and Ewing, 2008). Additionally, PC12 cells lack functional GABA receptors (Hales and Tyndale, 1994), thereby providing an appropriate model for identification of non-GABA receptor-mediated neurotoxicity of lindane. Using these neuroendocrine PC12 cells, it is now demonstrated that lindane differentially affects $[Ca^{2+}]_i$, resulting in disturbed dopamine exocytosis.

Materials and methods

Chemicals. NaCl, KCl and HEPES were obtained from Merck (Whitehouse Station, NJ, USA); $MgCl_2$, $CaCl_2$, glucose, sucrose and NaOH were obtained from BDH Laboratory Supplies (Poole, UK); ω-conotoxin GVIA and ω-conotoxin MVIIC were obtained from Biotrend Chemicals AG (Zürich, Switzerland); Fura-2AM and 2,7-dichlorofluorescein diacetate (H_2-DCFDA) were obtained from Molecular Probes (Invitrogen, Breda, The Netherlands). All other chemicals were obtained from Sigma (St. Louis MO, USA), unless otherwise noted. Saline solutions were prepared with de-ionized water (Milli-Q®; resistivity >10 MΩ.cm). Stock solutions of 2 mM ionomycin in DMSO were kept at −20 °C. Stock solutions of 1–100 mM lindane (Pestanal® grade, 99.8% pure, Riedel de Haën, Seelze, Germany) and 2 mM nifedipine were prepared in DMSO and diluted in saline solution to obtain the desired concentrations just prior to the experiments (all solutions used in experiments, including control experiments, contained 1 μl DMSO/ml). Stock solutions of 100 μM ω-conotoxin GVIA and MVIIC were prepared in de-ionized water and stored at 4 °C.

Cell culture. Rat pheochromocytoma (PC12) cells (Greene and Tischler, 1976) were grown for 10 passages in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 5% fetal calf serum and 10% horse serum (ICN Biomedicals, Zoetermeer, The Netherlands) as described previously (Dingemans et al., 2009). For Ca^{2+} imaging and electrophysiological experiments, undifferentiated PC12 cells were subcultured in poly-l-lysine coated glass-bottom dishes (MatTek, Ashland, MA) as described previously (Dingemans et al., 2009). As exocytosis is limited in undifferentiated PC12 cells, cells were differentiated for 3–5 days with 5 μM dexamethasone for amperometric recordings as described previously (Dingemans et al., 2009). Dexamethasone differentiation is known to enhance exocytosis by increasing the number of releasable vesicles as well as the total amount of dopamine per vesicle (Westerink and Vijverberg, 2002). Control experiments (not shown) demonstrated that intracellular calcium homeostasis was not qualitatively affected by differentiation with dexamethasone, neither under basal conditions nor during lindane exposure.

Cell viability assay. To exclude that results are confounded by acute lindane-induced cytotoxicity, effects of lindane on cell viability were determined using a combined alamar Blue (aB) and Neutral Red (NR) assay in undifferentiated PC12 cells. One day before the cell viability test, cells were seeded in (poly-l-lysine coated) 96-wells plates (Greiner Bio-one, Solingen, Germany) at a density of 10^5 cells/well. Cells were exposed in serum-free medium to concentrations up to 100 μM for 6 h. Mitochondrial activity of the cells was recorded as a measure of cell viability with the aB assay, which is based on the ability of the cells to reduce resazurin to resorufin (protocol adapted from Magnani and Bettini, 2000). Membrane integrity and lysosomal activity were subsequently determined as a measure of cell viability using the NR assay (protocol adapted from Repetto et al., 2008). Briefly, cells were incubated for 30 min with 200 μl resazurin solution (12 μM in phosphate buffered saline; PBS, Invitrogen, Breda, The Netherlands). Resorufin was measured spectrophotometrically at 530/590 nm (FLUOstar Galaxy V4.30-0, BMG Labtechnologies, Offenburg, Germany). After removal of the aB solution, cells were incubated for 1 h with 200 μl NR solution (12 μM in PBS). Following the incubation, cells were rinsed with warm (37 °C) PBS and 100 μl extraction solution (1% glacial acetic acid, 50% ethanol and 49% H_2O) was added to the wells. After 30 min extraction fluorescence was measured at 430/480 nm.

ROS measurement using H_2-DCFDA. ROS production was assessed using the fluorescent dye H_2-DCFDA (protocol adapted from Lee et al., 2009). Briefly, undifferentiated PC12 cells were seeded in black, glass-bottom, 96-wells plates (Greiner Bio-one, Solingen, Germany) at a density of 1.5×10^5 cells/well. Cells were loaded with 5 μM H2-DCFDA for 30 min at 37 °C. Subsequently, cells were exposed for up to 6 h to 1, 10 or 100 μM lindane and fluorescence was measured spectrophotometrically at 480/530 nm (FLUOstar Galaxy V4.30-0, BMG Labtechnologies, Offenburg, Germany). As control cells show a basal ROS production over time, data is expressed as average percentage compared to the time-matched control values.