

Hexabromocyclododecane Inhibits Depolarization-Induced Increase in Intracellular Calcium Levels and Neurotransmitter Release in PC12 Cells

Milou M. L. Dingemans,^{*1,2} Harm J. Heusinkveld,^{*2} Aart de Groot,^{*1} Åke Bergman,[†] Martin van den Berg,^{*} and Remco H. S. Westerink^{*}

^{*}Institute for Risk Assessment Sciences (IRAS), Utrecht University, NL-3508 TD Utrecht, The Netherlands; and [†]Department of Environmental Chemistry, Stockholm University, SE-106 91 Stockholm, Sweden

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Environmental levels of the brominated flame retardant (BFR) hexabromocyclododecane (HBCD) have been increasing. HBCD has been shown to cause adverse effects on learning and behavior in mice, as well as on dopamine uptake in rat synaptosomes and synaptic vesicles. For other BFRs, alterations in the intracellular Ca^{2+} homeostasis have been observed. Therefore, the aim of this study was to investigate whether the technical HBCD mixture and individual stereoisomers affect the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in a neuroendocrine *in vitro* model (PC12 cells). $[\text{Ca}^{2+}]_i$ and vesicular catecholamine release were measured using respectively single-cell Fura-2 imaging and amperometry. Exposure of PC12 cells to the technical HBCD mixture or individual stereoisomers did neither affect basal $[\text{Ca}^{2+}]_i$, nor the frequency of basal neurotransmitter release. However, exposure to HBCD (0–20 μM) did cause a dose-dependent reduction of a subsequent depolarization-evoked increase in $[\text{Ca}^{2+}]_i$. This effect was apparent only when HBCD was applied at least 5 min before depolarization (maximum effect after 20 min exposure). The effects of α - and β -HBCD were comparable to that of the technical mixture, whereas the inhibitory effect of γ -HBCD was larger. Using specific blockers of L-, N- or P/Q-type voltage-gated Ca^{2+} channels (VGCCs) it was shown that the inhibitory effect of HBCD is not VGCC-specific. Additionally, the number of cells showing depolarization-evoked neurotransmitter release was markedly reduced following HBCD exposure. Summarizing, HBCD inhibits depolarization-evoked $[\text{Ca}^{2+}]_i$ and neurotransmitter release. As increasing HBCD levels should be anticipated, these findings justify additional efforts to establish an adequate exposure, hazard and risk assessment.

Key Words: brominated flame retardants; exocytosis; *in vitro*; ion channels; neurotoxicity; voltage-gated calcium channels.

Brominated flame retardants (BFRs) are a structurally diverse class of compounds added to consumer products for

fire prevention. Increasing concentrations of these compounds in the environment, human food chain and in human tissues raise concern about possible neurotoxic effects, particularly for the developing nervous system (for review, Costa and Giordano, 2007). This concern has led to voluntary action and legislative measures to eliminate the penta- and octabrominated diphenyl ethers from the market in Europe and North America. After cessation of their use in consumer products, a sharp rise in environmental levels of the BFR hexabromocyclododecane (HBCD) was recently observed (for review, Law *et al.*, 2008). This is possibly due to an increased use of HBCD as a replacement of the banned polybrominated diphenyl ethers (PBDEs), mainly in polystyrene products. The technical mixture of HBCD consists of three stereoisomers, denoted α -, β -, and γ -HBCD. HBCD has been shown to biomagnify in marine food chains (Jenssen *et al.*, 2007). Interestingly, biotic samples are in general dominated by the α -stereoisomer although the technical mixture consists for 70–90% of γ -HBCD (Covaci *et al.*, 2006). This is probably due to isomer composition changes during heating of the technical mixture during industrial application (Heeb *et al.*, 2008), and/or cytochrome P450 mediated biotransformation of β - and γ -HBCD in biota (Zegers *et al.*, 2005).

Acute *in vivo* toxicity of HBCD appears limited (Darnerud, 2003). So far, studies on the toxic potential of HBCD have mainly focused on endocrine disruption. Adverse effects of HBCD on the steroid and thyroid hormone system as well as antagonistic effects on the androgen, progesterone and aryl hydrocarbon receptors have been described (Ema *et al.*, 2008; Hamers *et al.*, 2006; Palace *et al.*, 2008; van der Ven *et al.*, 2006). Additionally, effects on hepatic gene expression and hepatic enzymes have been observed (Canton *et al.*, 2008; Germer *et al.*, 2006; Ronisz *et al.*, 2004; Zhang *et al.*, 2008b).

Although an adverse effect on learning and behavior in mice (1.4 $\mu\text{mol/kg}$ bw HBCD; Eriksson *et al.*, 2006), as well as on oxidative stress and the cellular antioxidant defense systems in fish brain (28 days exposure to 500 $\mu\text{g/l}$ HBCD; Zhang *et al.*,

¹ To whom correspondence should be addressed at Institute for Risk Assessment Sciences, (IRAS), Utrecht University, P.O. Box 80.177, NL-3508 TD Utrecht, The Netherlands. Fax: +31-30-253-5077. E-mail: m.dingemans@uu.nl.

² These authors have contributed equally.

2008b) have been observed, *in vitro* neurotoxicity data on HBCD are limited. So far, only inhibition of dopamine uptake in rat synaptosomes (IC₅₀: 4 μM) and synaptic vesicles (IC₅₀: 3 μM; Mariussen and Fonnum, 2003) and necrotic cell death in cerebellar granule cells after 24 h exposure (LC₅₀: 3 μM; Reistad *et al.*, 2006) have been reported.

Alterations in the intracellular Ca²⁺ homeostasis, as observed for PBDEs and *ortho*-substituted (noncoplanar) polychlorinated biphenyls (*ortho*-PCBs) (for review: Mariussen and Fonnum, 2006), may play a role in the observed cytotoxicity and oxidative stress (Orrenius *et al.*, 2003). In neuronal cell-types, the main influx route of Ca²⁺ is via voltage-gated Ca²⁺ channels (VGCCs). VGCCs are located in the cell membrane and open when the membrane depolarizes. The subsequent rapid influx of Ca²⁺ can trigger various intracellular processes including neurotransmitter release (Barclay *et al.*, 2005; Clapham, 2007). Therefore, the aim of this study was to investigate the possible effects of the technical HBCD mixture and the individual HBCD stereoisomers on basal and depolarization-evoked cytosolic Ca²⁺ levels as well as on vesicular catecholamine release in a neuroendocrine *in vitro* model (PC12 cells), using respectively Fura-2 imaging and amperometry.

MATERIALS AND METHODS

Chemicals. The technical HBCD mixture was synthesized and the stereoisomers α-, β-, and γ-HBCD isolated and purified (~99%) at the Stockholm University (Sweden) as described previously (Fång, 2007). Unless otherwise noted, PC12 cells were exposed to the technical HBCD mixture. Before dilution to the desired final concentration, HBCD stock solutions in dimethyl sulfoxide (DMSO) were sonicated for at least 15 min. All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich, Zwijndrecht, The Netherlands.

PC12 cell culture. Rat pheochromocytoma (PC12) cells (Greene and Tischler, 1976) were cultured in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 5% fetal calf serum and 10% horse serum (ICN Biomedicals, Zoetermeer, The Netherlands). For Ca²⁺ imaging experiments, undifferentiated PC12 cells were subcultured in poly-L-lysine coated glass-bottom dishes (MatTek, Ashland, MA) as described previously (Dingemans *et al.*, 2007). For amperometric recordings, cells were differentiated for 3-5 days with 5 μM dexamethasone to enhance vesicular catecholamine release as described previously (Westerink and Vijverberg, 2002).

Cell viability assay. Effects of HBCD on cell viability were determined by measuring the capacity of undifferentiated PC12 cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Denizot and Lang, 1986). One day before the cell viability test, PC12 cells were seeded in a 96-wells plate at a density of 5 × 10⁴ cells per well. The cells were exposed to different concentrations of HBCD for 30 min, after which the cells were incubated for 30 min in 200 μl MTT (1 mg/ml) in serum-free medium. After washing the cells with phosphate buffered saline, the formazan was extracted in 100 μl of isopropanol. This isopropanol fraction was measured spectrophotometrically at 595 nm (FLUOstar Galaxy V4.30-0, BMG Lab-technologies, Offenburg, Germany) to determine the quantity of the blue formazan, which is used as a measure for cell viability.

Ca²⁺ imaging. Changes in the intracellular Ca²⁺ concentration ([Ca²⁺]_i) were measured using the Ca²⁺-sensitive fluorescent ratio dye Fura-2 as

described previously (Dingemans *et al.*, 2007). Briefly, cells were loaded with 5 μM Fura-2 AM (Molecular Probes; Invitrogen) in external saline (containing in mM: 1.8 CaCl₂, 24 glucose, 10 HEPES, 5.5 KCl, 0.8 MgCl₂, 125 NaCl, and 36.5 sucrose at pH 7.3, adjusted with NaOH) for 20 min at room temperature, followed by 15-min de-esterification in external saline. After de-esterification, cells were placed on the stage of an Axiovert 35M inverted microscope (Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (TILL Photonics GmbH, Gräfelfing, Germany). Fluorescence evoked by 340- and 380-nm excitation wavelengths (F₃₄₀ and F₃₈₀) was collected every 12 s at 510 nm with an Image SensiCam digital camera (TILL Photonics GmbH). The digital camera and polychromator were controlled by imaging software (TILLvisION, version 4.01), which was also used for data collection and processing. Changes in F₃₄₀/F₃₈₀ ratio (R), reflecting changes in [Ca²⁺]_i, were analyzed using custom-made MS-Excel macros. Following 5-min baseline recording, cells were exposed to DMSO, HBCD (0.2–20 μM), or α-, β-, or γ-HBCD (0.2–2 μM). Membrane depolarization by 100 mM K⁺ was used to investigate effects of HBCD on the depolarization-evoked increase in [Ca²⁺]_i, caused by influx of Ca²⁺ through VGCCs. Maximum and minimum ratios (R_{max} and R_{min}) were determined after the recording by addition of ionomycin (5 μM) and ethylenediamine tetraacetic acid (EDTA; 17 mM) as a control for experimental conditions. Free cytosolic [Ca²⁺]_i was calculated using Grynkiewicz's equation [Ca²⁺]_i = K_d* × (R – R_{min})/(R_{max} – R) (as described in Deitmer and Schild, 2000), where K_d* is the dissociation constant of Fura-2 determined in the experimental set-up used for the fluorescence measurements using Fura-2 Ca²⁺ imaging calibration buffers (Molecular Probes; Invitrogen).

Because the standard deviation during baseline [Ca²⁺]_i recording ranged from 1-21 nM (1-20% of average [Ca²⁺]_i), [Ca²⁺]_i levels > 125% of baseline during exposure were scored as spontaneous fluctuations. The average basal [Ca²⁺]_i and the number of cells showing spontaneous fluctuations in basal [Ca²⁺]_i, as well as amplitude and duration of these fluctuations, were determined to investigate possible effects of HBCD on basal [Ca²⁺]_i. The amplitude of [Ca²⁺]_i observed during depolarization was used to investigate possible effects of HBCD on the depolarization-induced increase in [Ca²⁺]_i. The involvement of specific VGCCs was investigated by selective pharmacological blocking using 2 μM nifedipine (L-type VGCCs), 2 μM ω-conotoxin GVIA (N-type VGCCs; Biotrend, Wangen, Switzerland), and 2 μM ω-conotoxin MVIIC (P/Q-type VGCCs; Biotrend).

Amperometry. Amperometric recordings of spontaneous and K⁺-evoked vesicular catecholamine release from dexamethasone-differentiated PC12 cells using carbon fiber microelectrodes were made as described previously (Dingemans *et al.*, 2007; Westerink and Vijverberg, 2002). Following 150 s of baseline recording, PC12 cells were superfused for 15 s with high K⁺-containing saline (K⁺ increased to 125 mM and Na⁺ lowered to 5.5 mM) to determine their responsiveness. Cells were allowed to recover for 135 s prior to a 20-min exposure to HBCD to investigate acute effects on vesicular catecholamine release. Recordings were performed at room temperature. As described previously (Dingemans *et al.*, 2007, 2008), PC12 cells with high basal release (> 2 events/min), low evoked release (< 20 events/min) or poor recovery after depolarization ("recovered basal release"; > 14 events/min) were excluded from data analysis (5/34 cells). The remaining 29 cells were used to investigate effects of HBCD on the frequency of vesicular catecholamine release and vesicular release parameters, that is, amplitude, t_{1/2} (half-width) and time integral (Q, vesicular content) of the amperometric release events.

Data analysis and statistics. All data are presented as mean ± SEM from the number of cells indicated. Statistical analyses were performed using SPSS 12.0.1 (SPSS, Chicago, IL). Continuous data were compared using Student's *t*-test, paired or unpaired where applicable. ANOVA and *post hoc t*-tests were performed to investigate possible dose-response relationships. Categorical data were compared using Fisher's exact tests. A *p* value < 0.05 is considered statistically significant; n.s. indicates the absence of a statistically significant effect.

RESULTS

Cell Viability

PC12 cells, cultured in a 96-wells plate, were exposed to 0–20 μ M technical HBCD mixture or the individual stereoisomers for 30 min before incubation in 1 mg/ml MTT. Exposure to 0–2 μ M HBCD (30 min) had no effects on cell viability, whereas exposure to 20 μ M HBCD (30 min) resulted in a limited reduction of cell viability to $88 \pm 3\%$ of control ($p < 0.05$). After 30 min exposure to 0–20 μ M α -, β -, or γ -HBCD, no effects on cell viability were observed (Fig. 1).

Cytosolic $[Ca^{2+}]_i$

To investigate whether HBCD affects basal $[Ca^{2+}]_i$, Fura-2 loaded PC12 cells were exposed for 20 min to HBCD (0–20 μ M). Basal $[Ca^{2+}]_i$ before exposure amounted to 106 ± 1 nM ($n = 440$). In control experiments, 30% of the cells showed spontaneous fluctuation in $[Ca^{2+}]_i$. The average basal $[Ca^{2+}]_i$ (20 μ M HBCD: 100 ± 3 nM; n.s.) as well as the percentage of cells showing spontaneous fluctuations in $[Ca^{2+}]_i$ (20 μ M HBCD: 35%; n.s.) were not affected by exposure to HBCD (Fig. 2). The amplitude and duration of these spontaneous fluctuations in $[Ca^{2+}]_i$ were also not affected during exposure to 20 μ M HBCD (data not shown). Exposure to the separate α -, β -, or γ -stereoisomers (0–2 μ M) did neither affect the average basal $[Ca^{2+}]_i$ nor the percentage of cells showing spontaneous fluctuations in $[Ca^{2+}]_i$, during exposure (data not shown).

VGCCs expressed in undifferentiated PC12 cells include L-, N-, P/Q-, R-, and T-type Ca^{2+} channels (Del Toro *et al.*, 2003; Greene and Tischler, 1976; Liu *et al.*, 1996; Shafer and Atchison, 1991), with L-, N-, and P/Q-type VGCCs accounting for the majority of the depolarization-induced increase in $[Ca^{2+}]_i$ (blocking L-, N-, and P/Q-type VGCCs reduced the depolarization-evoked increase in $[Ca^{2+}]_i$ to $\sim 15\%$ of control, data not shown). To investigate whether HBCD has an effect on the depolarization-evoked increase in $[Ca^{2+}]_i$, PC12 cells were exposed to different concentrations of HBCD before and during depolarization by a high K^+ -containing saline.

When applying 20 μ M HBCD immediately before the depolarization, no effects were observed on the depolarization-evoked increase in $[Ca^{2+}]_i$, making direct effects of HBCD on VGCCs less likely. When the exposure time is prolonged, the inhibitory effect on the depolarization-evoked increase in $[Ca^{2+}]_i$ aggravated. Increasing the exposure time from 20 to 40 min did not further reduce the depolarization-evoked increase in $[Ca^{2+}]_i$ (data not shown). Therefore, in all following $[Ca^{2+}]_i$ measurements, cells were exposed to HBCD for 20 min prior to depolarization.

Exposing PC12 cells for 20 min to HBCD technical mixture (0–20 μ M) caused a dose-dependent reduction of the subsequent depolarization-evoked increase in $[Ca^{2+}]_i$ (ANOVA: $p < 0.0001$; Fig. 2). In control cells, $[Ca^{2+}]_i$ increased to 1.45 ± 0.07 μ M ($n = 88$) during depolarization (basal $[Ca^{2+}]_i$: 107 ± 3 nM). No effects on evoked $[Ca^{2+}]_i$ were observed when

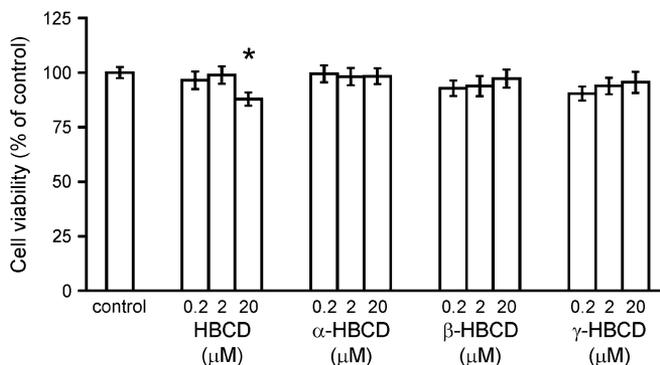


FIG. 1. Exposure of PC12 cells to the technical HBCD mixture has a small effect on cell viability only at 20 μ M, whereas no significant effects were observed after exposure to the individual stereoisomers. Bars represent average cell viability as measured by mitochondrial MTT reduction, normalized to DMSO control wells per plate. Bars display data from four experiments, number of wells used for data analysis: control: $n = 36$; all HBCD treatments: $n = 12$. * $p < 0.05$.

exposing the cells to 0.2 μ M HBCD. Increasing the concentration of HBCD resulted in a reduction of evoked $[Ca^{2+}]_i$ with a lowest observed effect level (LOEL) of 2 μ M HBCD (1.15 ± 0.08 μ M, $n = 73$; $p < 0.01$). Exposing the cells to 20 μ M HBCD inhibited the evoked $[Ca^{2+}]_i$ to 0.61 ± 0.08 μ M ($n = 57$; $p < 0.0001$), which is approximately 40% of the control value.

Although the technical mixture mainly consists of γ -HBCD, it has been observed that in biotic samples, α -HBCD is most abundant. To investigate possible differences in activity between the different stereoisomers of HBCD, PC12 cells were exposed to 2 μ M of the different stereoisomers (α -, β -, and γ -HBCD). All three stereoisomers inhibited the depolarization-evoked increase in $[Ca^{2+}]_i$. The inhibitory effects of α - and β -HBCD were comparable with that of the technical mixture (HBCD mixture: 1.15 ± 0.08 μ M; α -HBCD: 0.97 ± 0.10 μ M, $n = 36$; β -HBCD: 1.16 ± 0.13 μ M, $n = 38$), whereas the inhibitory effect of γ -HBCD was larger (0.73 ± 0.06 μ M, $n = 58$; Fig. 2).

To investigate whether specific VGCCs were involved in the inhibitory effects of HBCD on the depolarization-evoked increase in $[Ca^{2+}]_i$, L-, N-, or P/Q-type VGCCs were pharmacologically blocked using 2 μ M of, respectively, nifedipine, GVIA, or MVIIC ω -conotoxins (Fig. 3). Though all VGCC blockers decreased the depolarization-induced increase in $[Ca^{2+}]_i$, nifedipine had the largest effect (0.80 ± 0.05 μ M). Exposure to HBCD (20 μ M) while blocking L-type VGCCs with nifedipine, resulted in a further decrease of the depolarization-induced $[Ca^{2+}]_i$ (0.64 ± 0.07 μ M). Comparable results were seen when blocking either N- or P/Q-type VGCCs (Fig. 3).

Vesicular Neurotransmitter Release

To investigate whether the observed robust reduction in depolarization-evoked $[Ca^{2+}]_i$ after exposure to 20 μ M HBCD has functional consequences for neurotransmitter release,

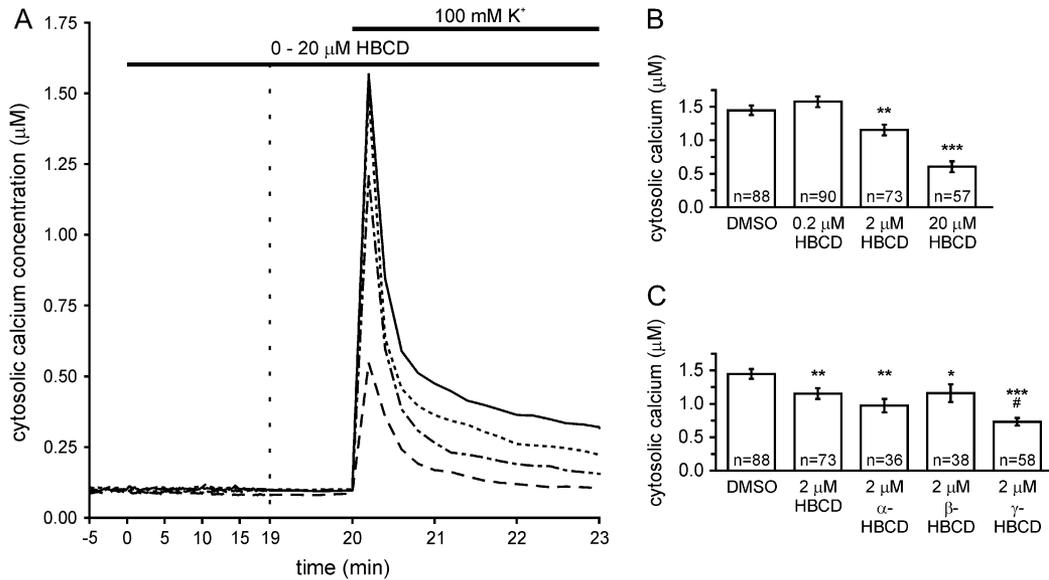


FIG. 2. Exposure of PC12 cells to HBCD has no effects on basal [Ca²⁺]_i, but inhibits the depolarization-evoked increase in [Ca²⁺]_i. (A) Representative traces of cytosolic [Ca²⁺]_i measurements of individual PC12 cells are shown, illustrating the reduction of the depolarization-evoked increase in [Ca²⁺]_i by exposure to 0–20 μM HBCD (control: solid line; 0.2 μM HBCD: small dash; 2 μM HBCD: dash-dot-dash; 20 μM HBCD: large dash; note the difference in time scaling from *t* = 19 min). (B) Bar graph shows the dose-dependent reduction of the depolarization-evoked increase in [Ca²⁺]_i by HBCD. (C) Bar graph shows the reduction of the evoked [Ca²⁺]_i by the technical mixture and separate stereoisomers of HBCD. 2 μM γ-HBCD significantly differs from all other HBCD treatments (#*p* < 0.05). Bars display data from three to nine experiments per treatment. The number of cells used for data analysis is indicated inside each bar. Difference from control: **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

depolarization-evoked release of catecholamines was measured following 20-min exposure to 20 μM HBCD. Analysis of the release frequencies showed that the average basal release frequency amounted to 0.4 ± 0.1 events/min (*n* = 29). Upon depolarization the release frequency increased to 97 ± 12 events/min (*n* = 29), which slowly declined to basal release

levels (recovered basal release: 2.5 ± 0.5 events/min, *n* = 29) after cessation of depolarization. Basal release during exposure to 20 μM HBCD (1.1 ± 0.3 events/min, *n* = 14) did not significantly differ from basal release in control cells (0.5 ± 0.1 events/min, *n* = 15; Fig. 4). However, following 20 min exposure to HBCD, ~70% of the cells (10 out of 14) no longer showed evoked release (> 20 events/min), whereas in control experiments, ~85% of the cells (13 out of 15) still showed evoked release following 20-min exposure to 0.1% DMSO (*p* < 0.01). Release frequencies of the four cells that still show evoked release following exposure to HBCD were within the range of evoked release in control cells (20–216 events/min) and the vesicular release parameters (i.e., vesicle content, amplitude and *t*_{1/2}) were unaffected (data not shown).

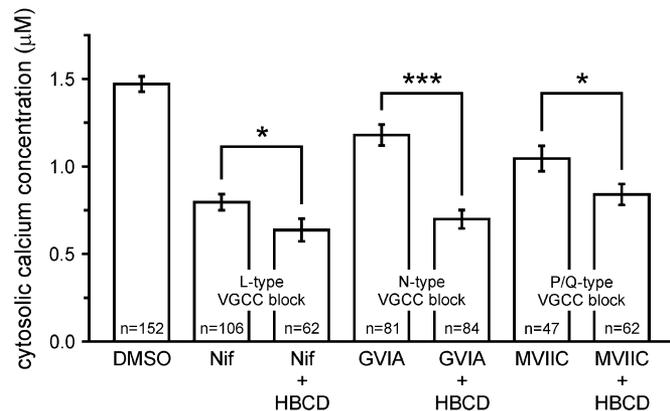


FIG. 3. HBCD a-specifically inhibits Ca²⁺ influx through all major VGCC-types in PC12 cells. Nifedipine (Nif; 2 μM), ω-conotoxin GVIA (2 μM), and ω-conotoxin MVIIC (2 μM) were used to block L-, N-, and P/Q-type VGCCs, respectively. All treatments significantly differ from the DMSO control. Coexposure to HBCD (20 μM) results in a further reduction of the depolarization-evoked increase in [Ca²⁺]_i compared with the corresponding “blocker alone” treatments. Bars display data from 4 to 13 experiments per treatment. The number of cells used for data analysis is indicated inside each bar. **p* < 0.05; ****p* < 0.001.

DISCUSSION

The results presented here demonstrate that the BFR HBCD does not affect the basal [Ca²⁺]_i, which is in accordance with previous observations in rat cerebellar granule cells (Reistad *et al.*, 2006). Additionally, HBCD exposure does not affect the frequency of basal catecholamine release (Fig. 4) or the parameters of the vesicular release events. However, HBCD exposure does exert an inhibitory effect on the depolarization-evoked increase in [Ca²⁺]_i via VGCCs with a LOEL of 2 μM (Figs. 2 and 3) as well as on depolarization-evoked vesicular catecholamine release (Fig. 4).

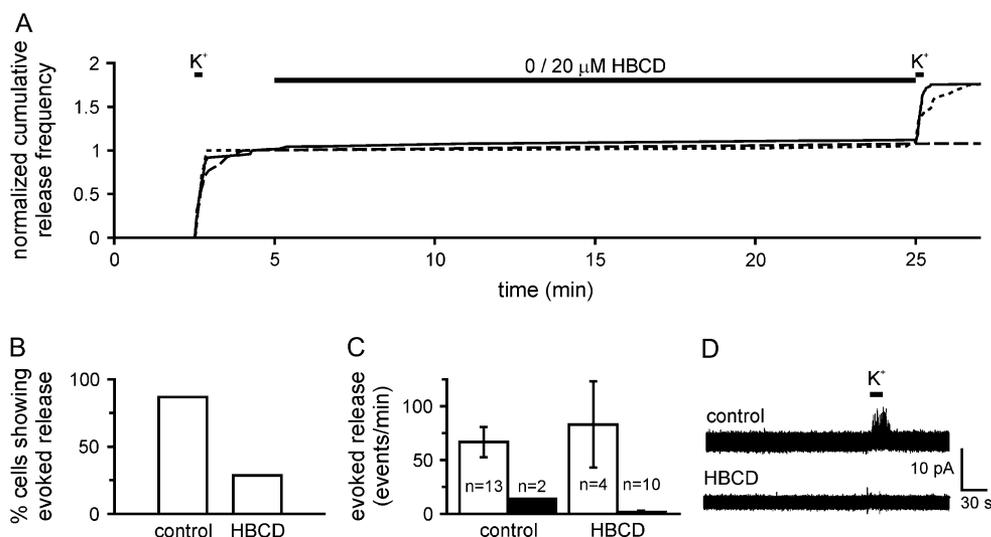


FIG. 4. Exposure to HBCD inhibits depolarization-evoked catecholamine release. (A) Representative cumulative event frequencies, normalized to the number of events observed before the onset of exposure, of a control cell (solid line) and two cells exposed to 20 μ M HBCD, one of which shows evoked release after exposure (small dash) and one which does not (large dash). (B) Percentage of cells showing evoked release after exposure to vehicle (13 out of 15) or 20 μ M HBCD (4 out of 14). (C) The average evoked release frequencies of control and HBCD-exposed cells. Although the number of cells lacking evoked release (closed bars) was strongly increased after exposure to HBCD, release frequencies in cells that still exhibit vesicular catecholamine release after exposure to HBCD (open bars) are within the range of control, DMSO-exposed cells. (D) Representative amperometric traces recorded from individual PC12 cells. The majority of cells show evoked release after exposure to vehicle (control; top), whereas the majority of cells lacks evoked release after exposure to 20 μ M HBCD (bottom).

A small cytotoxic effect (measured via mitochondrial activity) was observed after exposing PC12 cells to 20 μ M technical HBCD mixture for only 30 min, whereas exposure to the individual stereoisomers or lower concentrations of HBCD did not result in reduction of cell viability (Fig. 1). In cerebellar granule cells, 24 h exposure to 10 μ M HBCD resulted in ~90% cell death, whereas 24 h exposure to 2 μ M HBCD (the LOEL in the current study) caused ~35% cell death (Reistad *et al.*, 2006). A recent study (Zhang *et al.*, 2008a) describes stereoisomer- and enantiomer-specific cytotoxicity of HBCD in HepG2 cells, showing that γ -HBCD has the strongest cytotoxic effect, and that (+)-enantiomers are significantly more toxic than the corresponding (–)-enantiomers. From the current cell viability results and other cell viability studies it can be concluded that the LOEL for effects in Ca^{2+} homeostasis in the current study (2 μ M HBCD) is not confounded by cytotoxicity.

To investigate whether HBCD specifically blocks one subtype of VGCCs, the depolarization-induced increase in $[\text{Ca}^{2+}]_i$ was investigated in the presence of specific blockers of the different VGCC-subtypes. Though all VGCC blockers decrease the depolarization-induced increase in $[\text{Ca}^{2+}]_i$, nifedipine has the largest effect indicating that L-type VGCCs are the most abundant VGCC-subtype in undifferentiated PC12 cells. If HBCD would specifically block one subtype of VGCCs, a combined exposure to HBCD and a specific blocker should inhibit the depolarization-induced increase in $[\text{Ca}^{2+}]_i$ to a similar extent as the specific blocker alone. A further decrease of the depolarization-induced $[\text{Ca}^{2+}]_i$ was observed after

exposure to HBCD (20 μ M) while blocking either the L-, N-, or P/Q-type VGCCs (Fig. 3). These combined data therefore indicate that the effect of HBCD is not VGCC-specific. Additionally, effects on other Ca^{2+} -influx pathways involved in the depolarization-evoked increase in $[\text{Ca}^{2+}]_i$ cannot be excluded.

Exposing the PC12 cells to HBCD immediately before depolarization did not affect the depolarization-evoked increase in $[\text{Ca}^{2+}]_i$. Hence, HBCD apparently has no direct effect on the VGCCs. Interestingly, the inhibitory effect of HBCD aggravates when the exposure is prolonged. The maximal effect is reached after 20 min of exposure and increasing the exposure time to 40 min did not further increase the inhibitory effect of HBCD. Although the underlying cause of the exposure-time dependency of the inhibitory effect of HBCD remains as yet unclear, a possible explanation could be the targeting of voltage-independent intracellular signaling pathways by HBCD which need some minutes to take effect. VGCCs are regulated by a range of signal transduction pathways, including phosphorylation and G-protein modulation. Nevertheless, few signaling pathways inhibit L-, N-, and P/Q-type VGCCs, and potentiation has been observed as well (for review, Westerink 2006). Another possible explanation could reside in the physico-chemical properties of HBCD. The high *n*-octanol-water-partition coefficient ($\log K_{ow} \approx 5$; Hayward *et al.*, 2006) indicates the potential for HBCD to accumulate in cell membranes. Possibly, the inhibitory effect of HBCD depends on the amount of HBCD partitioned into the cell membrane, and is limited (as the effect no longer aggravated after 20 min

exposure) by the available quantity of HBCD and the absolute quantity of cell membrane. If so, effects on other membrane-anchored proteins, including ion channels, cannot be excluded.

The percentage of cells that do not show evoked release following HBCD exposure is significantly increased. However, cells that still exhibit depolarization-evoked release following HBCD exposure display a release frequency that is comparable to control cells, suggesting that the inhibition of the depolarization-evoked release is related to the inhibition of depolarization-evoked increase in $[Ca^{2+}]_i$, whereas exocytosis and its underlying processes appear unaffected. Thus, due to the inhibition of the depolarization-evoked $[Ca^{2+}]_i$ a larger proportion of the cells probably does not reach the Ca^{2+} threshold for release after exposure to HBCD.

HBCD has previously been shown to inhibit the plasma membrane uptake of dopamine (IC_{50} : $4\mu M$, measured after 15 min exposure to HBCD), as well as the vesicular uptake of dopamine (IC_{50} : $3\mu M$, measured after 15 min exposure to HBCD; Mariussen and Fonnum, 2003). Inhibition of uptake of dopamine in rat brain synaptic vesicles has also been demonstrated for the commercial penta-PBDE mixture DE-71 and *ortho*-PCBs (for review, Mariussen and Fonnum, 2006). However, changes in vesicle content were not observed following exposure to HBCD. This indicates that the functional consequences for quantal release per se are rather limited, or are obscured because of the relatively short time span of the amperometric experiments compared with the slow rate of vesicle cycling in PC12 cells (Westerink *et al.*, 2000).

Human HBCD serum levels are based on only a few observations, which so far have been limited to European countries and the United States. HBCD intake related to oily fish consumption was estimated to be 0.3 ng/kg bw/day (Knutsen *et al.*, 2008). The highest concentration of HBCD measured in human serum in an occupational setting (856 ng/g lipids ; Thomsen *et al.*, 2007) corresponds to $\sim 15\text{ nM}$ HBCD in blood (calculated using average physiologic values), which is approximately two orders of magnitude lower than the LOEL in this study. For risk assessment this difference is relatively small, especially considering safety factors for species extrapolation and intraspecies variability, as well as the lipophilic and bioaccumulative properties of HBCD. Considering the current findings on neuronal signaling, additional safety regulations in an occupational setting should be considered.

The highest background level of HBCD measured in humans is 7.4 ng/g lipids (Meijer *et al.*, 2008), which corresponds to $\sim 0.13\text{ nM}$ HBCD in blood (calculated using average physiologic values). This is approximately four orders of magnitude lower than the LOEL in this study, thus well below the concentrations at which adverse effects have been observed. Nevertheless, *in vivo* HBCD exposure is expected to be of a longer or maybe even lifelong duration, whereas the LOEL obtained in this *in vitro* study was based on only 20-min exposure. Additional concern arises from the fact that organohalogen compounds, including PCBs, PBDEs and HBCD,

transfer across the placenta (Meijer *et al.*, 2008). Also, HBCD has been detected in human breast milk, up to 5 ng/g lipids (Antignac *et al.*, 2008). As it has been shown that exposure to HBCD within the time frame of rapid brain development results in behavioral defects in mice (Eriksson *et al.*, 2006), it is concerning that children are exposed to HBCD pre- as well as postnatally.

The major constituent of the technical mixture, γ -HBCD, has a larger effect on the depolarization-evoked increase in $[Ca^{2+}]_i$ than α - and β -HBCD, which have a comparable effect as seen with the technical mixture. Presently, very few toxicity data is available to compare the activity of the three stereoisomers present in the technical HBCD mixture. All three stereoisomers affect the thyroid hormone system, but γ -HBCD to the largest extent (Palace *et al.*, 2008). Nevertheless, biotic samples are generally dominated by α -HBCD (Covaci *et al.*, 2006), although for humans this has been demonstrated only once (Antignac *et al.*, 2008). This shortage in knowledge on the potency of the individual stereoisomers and (tissue-specific) stereoisomer distribution in humans hampers proper effect and risk assessment. In this light it is noteworthy that as α -HBCD is less potent but more common in biological samples, neurotoxic effects might be overestimated when the technical mixture, which mostly contains γ -HBCD, is used in experimental studies. In view of the differences observed in response between the individual stereoisomers, future toxicity studies should include these stereoisomers to improve the relevance of experimental studies for the human exposure situation.

The cessation of the use of the penta- and octa-PBDE, and in the EU also the use of the deca-BDE, could result in increased use of HBCD in polystyrene materials as an alternative for the banned PBDEs. Consequently, it is not unlikely that human exposure levels increase in the future. Considering this potential increase as well as the current findings on neuroendocrine functioning, additional efforts to establish an adequate exposure, hazard, and risk assessment are justified.

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REFERENCES

- Antignac, J. P., Cariou, R., Maume, D., Marchand, P., Monteau, F., Zalko, D., Berrebi, A., Cravedi, J. P., Andre, F., and Le Bizec, B. (2008). Exposure

- assessment of fetus and newborn to brominated flame retardants in France: Preliminary data. *Mol. Nutr. Food Res.* **52**, 258–265.
- Barclay, J. W., Morgan, A., and Burgoyne, R. D. (2005). Calcium-dependent regulation of exocytosis. *Cell Calcium* **38**, 343–353.
- Cantón, R. F., Peijnenburg, A. A., Hoogenboom, R. L., Piersma, A. H., van der Ven, L. T., van den Berg, M., and Heneweer, M. (2008). Subacute effects of hexabromocyclododecane (HBCD) on hepatic gene expression profiles in rats. *Toxicol. Appl. Pharmacol.* **231**, 267–272.
- Clapham, D. E. (2007). Calcium signaling. *Cell* **131**, 1047–1058.
- Costa, L. G., and Giordano, G. (2007). Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. *Neurotoxicology* **28**, 1047–1067.
- Covaci, A., Gerecke, A. C., Law, R. J., Voorspoels, S., Kohler, M., Heeb, N. V., Leslie, H., Allchin, C. R., and De Boer, J. (2006). Hexabromocyclododecanes (HBCDs) in the environment and humans: A review. *Environ. Sci. Technol.* **40**, 3679–3688.
- Darnerud, P. O. (2003). Toxic effects of brominated flame retardants in man and in wildlife. *Environ. Int.* **29**, 841–853.
- Deitmer, J. W., and Schild, D. (2000). Calcium—Imaging, Protocoll und Ergebnisse. In *Ca²⁺ und pH, Ionenmessungen in Zellen und Geweben* (J. W. Deitmer and D. Schild, Eds.), pp. 77–99. Spektrum Akademischer Verleger, Heidelberg.
- Del Toro, R., Levitsky, K. L., López-Barneo, J., and Chiara, M. D. (2003). Induction of T-type calcium channel gene expression by chronic hypoxia. *J. Biol. Chem.* **278**, 22316–22324.
- Denizot, F., and Lang, R. (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **89**, 271–277.
- Dingemans, M. M. L., de Groot, A., van Kleef, R. G. D. M., Bergman, Å., van den Berg, M., Vijverberg, H. P. M., and Westerink, R. H. S. (2008). Hydroxylation increases the neurotoxic potential of BDE-47 to affect exocytosis and calcium homeostasis in PC12 cells. *Environ. Health Perspect.* **116**, 637–643.
- Dingemans, M. M. L., Ramakers, G. M. J., Gardoni, F., van Kleef, R. G. D. M., Bergman, Å., Di Luca, M., van den Berg, M., Westerink, R. H. S., and Vijverberg, H. P. M. (2007). Neonatal exposure to brominated flame retardant BDE-47 reduces long-term potentiation and postsynaptic protein levels in mouse hippocampus. *Environ. Health Perspect.* **115**, 865–870.
- Ema, M., Fujii, S., Hirata-Koizumi, M., and Matsumoto, M. (2008). Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats. *Reprod. Toxicol.* **25**, 335–351.
- Eriksson, P., Fischer, C., Wallin, M., Jakobsson, E., and Fredriksson, A. (2006). Impaired behaviour, learning and memory in adult mice neonatally exposed to hexabromocyclododecane (HBCDD). *Environ. Toxicol. Pharmacol.* **21**, 317–322.
- Fång, J. (2007). In *Separation and NMR-spectroscopy characterization of technical grade hexabromocyclododecane isomers*. Master Degree Thesis Stockholm University, Sweden.
- Germer, S., Piersma, A. H., van der Ven, L., Kamyschnikow, A., Fery, Y., Schmitz, H. J., and Schrenk, D. (2006). Subacute effects of the brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on hepatic cytochrome P450 levels in rats. *Toxicology* **218**, 229–36.
- Greene, L. A., and Tischler, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 2424–2428.
- Hamers, T., Kamstra, J. H., Sonneveld, E., Murk, A. J., Kester, M. H., Andersson, P. L., Legler, J., and Brouwer, A. (2006). *In vitro* profiling of the endocrine-disrupting potency of brominated flame retardants. *Toxicol. Sci.* **92**, 157–173.
- Hayward, S. J., Lei, Y. D., and Wania, F. (2006). Comparative evaluation of three high-performance liquid chromatography-based K_{ow} estimation methods for highly hydrophobic organic compounds: Polybrominated diphenyl ethers and hexabromocyclododecane. *Environ. Toxicol. Chem.* **25**, 2018–2027.
- Heeb, N. V., Schweizer, W. B., Mattrel, P., Haag, R., Kohler, M., Schmid, P., Zennegg, M., and Wolfensberger, M. (2008). Regio- and stereoselective isomerization of hexabromocyclododecanes (HBCDs): Kinetics and mechanism of β -HBCD racemization. *Chemosphere* **71**, 1547–1556.
- Jenssen, B. M., Sørmo, E. G., Bæk, K., Bytingsvik, J., Gaustad, H., Ruus, A., and Skaare, J. U. (2007). Brominated flame retardants in North-East Atlantic marine ecosystems. *Environ. Health Perspect.* **115**(Suppl. 1), 35–41.
- Knutsen, H. K., Kvale, H. E., Thomsen, C., Frøshaug, M., Haugen, M., Becher, G., Alexander, J., and Meltzer, H. M. (2008). Dietary exposure to brominated flame retardants correlates with male blood levels in a selected group of Norwegians with a wide range of seafood consumption. *Mol. Nutr. Food Res.* **52**, 217–227.
- Law, R. J., Herzke, D., Harrad, S., Morris, S., Bersuder, P., and Allchin, C. R. (2008). Levels and trends of HBCD and BDEs in the European and Asian environments, with some information for other BFRs. *Chemosphere* **73**, 223–241.
- Liu, H., Felix, R., Gurnett, C. A., De Waard, M., Witcher, D. R., and Campbell, K. P. (1996). Expression and subunit interaction of voltage-dependent Ca^{2+} channels in PC12 cells. *J. Neurosci.* **16**, 7557–7565.
- Mariussen, E., and Fonnum, F. (2003). The effect of brominated flame retardants on neurotransmitter uptake into rat brain synaptosomes and vesicles. *Neurochem. Int.* **43**, 533–542.
- Mariussen, E., and Fonnum, F. (2006). Neurochemical targets and behavioral effects of organohalogen compounds: An update. *Crit. Rev. Toxicol.* **36**, 253–289.
- Meijer, L., Weiss, J., Van Velzen, M., Brouwer, A., Bergman, Å., and Sauer, P. J. (2008). Serum concentrations of neutral and phenolic organohalogenes in pregnant women and some of their infants in The Netherlands. *Environ. Sci. Technol.* **42**, 3428–3433.
- Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003). Regulation of cell death: The calcium-apoptosis link. *Nat. Rev. Mol. Cell Biol.* **4**, 552–565.
- Palace, V. P., Pleskach, K., Halldorson, T., Danell, R., Wautier, K., Evans, B., Alae, M., Marvin, C., and Tomy, G. T. (2008). Biotransformation enzymes and thyroid axis disruption in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to hexabromocyclododecane diastereoisomers. *Environ. Sci. Technol.* **42**, 1967–1972.
- Reistad, T., Fonnum, F., and Mariussen, E. (2006). Neurotoxicity of the pentabrominated diphenyl ether mixture, DE-71, and hexabromocyclododecane (HBCD) in rat cerebellar granule cells *in vitro*. *Arch. Toxicol.* **80**, 785–796.
- Ronisz, D., Finne, E. F., Karlsson, H., and Förlin, L. (2004). Effects of the brominated flame retardants hexabromocyclododecane (HBCDD), and tetrabromobisphenol A (TBBPA), on hepatic enzymes and other biomarkers in juvenile rainbow trout and feral eelpout. *Aquat. Toxicol.* **69**, 229–245.
- Shafer, T. J., and Atchison, W. D. (1991). Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: A model for neurotoxicological studies. *Neurotoxicology* **12**, 473–492.
- Thomsen, C., Molander, P., Daae, H. L., Janák, K., Frøshaug, M., Liane, V. H., Thorud, S., Becher, G., and Dybing, E. (2007). Occupational exposure to hexabromocyclododecane at an industrial plant. *Environ. Sci. Technol.* **41**, 5210–5216.
- van der Ven, L. T., Verhoef, A., van de Kuil, T., Slob, W., Leonards, P. E., Visser, T. J., Hamers, T., Herlin, M., Håkansson, H., Olausson, H., et al. (2006). A 28-day oral dose toxicity study enhanced to detect endocrine effects of hexabromocyclododecane in Wistar rats. *Toxicol. Sci.* **94**, 281–292.
- Westerink, R. H. S. (2006). Targeting exocytosis: Ins and outs of the modulation of quantal dopamine release. *CNS Neurol. Disord. Drug Targets* **5**, 57–77.

- Westerink, R. H. S., de Groot, A., and Vijverberg, H. P. M. (2000). Heterogeneity of catecholamine-containing vesicles in PC12 cells. *Biochem. Biophys. Res. Commun.* **270**, 625–630.
- Westerink, R. H. S., and Vijverberg, H. P. M. (2002). Ca^{2+} -independent vesicular catecholamine release in PC12 cells by nanomolar concentrations of Pb^{2+} . *J. Neurochem.* **80**, 861–873.
- Zegers, B. N., Mets, A., Van Bommel, R., Minkenberg, C., Hamers, T., Kamstra, J. H., Pierce, G. J., and Boon, J. P. (2005). Levels of hexabromocyclododecane in harbor porpoises and common dolphins from western European seas, with evidence for stereoisomer-specific biotransformation by cytochrome p450. *Environ. Sci. Technol.* **39**, 2095–2100.
- Zhang, X., Yang, F., Xu, C., Liu, W., Wen, S., and Xu, Y. (2008a). Cytotoxicity evaluation of three pairs of hexabromocyclododecane (HBCD) enantiomers on Hep G2 cell. *Toxicol. In Vitro* **22**, 1520–1527.
- Zhang, X., Yang, F., Zhang, X., Xu, Y., Liao, T., Song, S., and Wang, J. (2008b). Induction of hepatic enzymes and oxidative stress in Chinese rare minnow (*Gobiocypris rarus*) exposed to waterborne hexabromocyclododecane (HBCDD). *Aquat. Toxicol.* **86**, 4–11.