INHIBITION OF THE DEPOLARIZATION-EVOKED INCREASE IN INTRACELLULAR CALCIUM LEVELS AND CATECHOLAMINE EXOCYTOSIS BY HEXABROMOCYCLODODECANE (HBCD) IN PC12 CELLS

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Introduction

Environmental levels of the brominated flame retardant (BFR) hexabromocyclododecane (HBCD) have been increasing. This is possibly due to an increased use of HBCD as a replacement of the banned polybrominated diphenyl ethers (PBDEs)[1]. Industrially, HBCD is used as a technical mixture consisting of three stereoisomers, denoted α-, β-, and γ-HBCD. Although the technical mixture consists for 70 - 90% of γ-HBCD, biotic samples are in general dominated by the α-stereoisomer[2].

HBCD is known to be an endocrine disrupter. Additionally, HBCD has been shown to cause adverse effects on learning and behavior in mice[3], though acute *in vivo* toxicity of HBCD appears limited[4]. *In vitro* neurotoxicity data are largely limited to effects of HBCD on dopamine uptake in rat synaptosomes and synaptic vesicles[5] and necrotic cell death in cerebellar granule cells after 24 h exposure[6].

Other BFRs (PBDEs) and *ortho*-substituted PCBs are known to disrupt intracellular Ca^{2+} homeostasis, which may partly underlie their neurotoxic potential. Proper Ca^{2+} homeostasis is essential for many neurophysiological processes, including neurotransmitter release[7]. In neuronal cells, increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) are primarily due to influx of Ca^{2+} via voltage-gated Ca^{2+} channels (VGCCs) and release of Ca^{2+} from intracellular stores. PBDEs and *ortho*-substituted PCBs increase $[Ca^{2+}]_i$ mainly via release of Ca^{2+} from intracellular stores. It is however unclear if HBCD can also disrupt Ca^{2+} homeostasis and associated neuronal processes. The aim of this study was therefore to investigate the possible effects of the technical HBCD mixture and the individual HBCD stereoisomers on basal and depolarization-evoked changes in $[Ca^{2+}]_i$ and catecholamine exocytosis in a neuroendocrine *in vitro* model.

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[8]. Unless otherwise noted, PC12 cells were exposed to the technical HBCD mixture.

Cell culture. Rat pheochromocytoma (PC12) cells[9] obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) were cultured as described previously[10,11]. For Ca^{2+} imaging experiments, undifferentiated PC12 cells were subcultured in poly-L-lysine-coated glass-bottom dishes (MatTek, Ashland, MA, USA) as described previously[11]. For amperometric recordings, the cells were differentiated for 3 - 5 days with 5 μM dexamethasone to enhance exocytosis, as described previously[10].

Amperometry. Amperometric recordings of K^+-evoked and spontaneous vesicular catecholamine release from dexamethasone-differentiated PC12 cells using carbon fiber microelectrodes were made as described previously[10,11] to investigate effects of HBCD on the frequency of vesicular catecholamine release and vesicular release parameters.

Intracellular Ca^{2+} imaging. Changes in $[Ca^{2+}]_i$ were measured using the Ca^{2+}-responsive fluorescent ratio dye Fura-2 as described previously[11]. F_{340}/F_{380} ratio, reflecting changes in $[Ca^{2+}]_i$, was analyzed using custom-made Excel macros. After 5 min baseline recording, cells were exposed to DMSO, HBCD (0.2 - 20 μM), or α-, β-, or γ-HBCD (0.2 - 2 μM) for 20 min prior to a membrane depolarization evoked by 100 mM K^+. Where applicable, 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) and 2 μM ω-conotoxin MVIIC (P/Q-type VGCCs).

Data analysis and statistics. All data are presented as mean ± SE from the number of cells (*n*) indicated. Categorical and continuous data were compared using respectively Fisher's exact test and Student's *t*-test, paired or unpaired where applicable. Analysis of variance (ANOVA) and *post hoc t*-tests were performed to investigate possible concentration-response relationships. A p-value < 0.05 was considered statistically significant.

Results and discussion

Effects of HBCD on $[Ca^{2+}]_i$. PC12 cells exposed for 20 min to HBCD (0-20 μM) did not show any alterations in basal $[Ca^{2+}]_i$ compared to DMSO exposed cells. Similarly, exposure to the separate α-, β-, and γ-stereoisomers of HBCD did not change basal $[Ca^{2+}]_i$. 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. In control cells, depolarization rapidly increases $[Ca^{2+}]_i$ primarily via Ca^{2+} influx through L-, N-, and P/Q-type VGCCs. When cells were exposed to 20 μM HBCD immediately before the depolarization, no effects on the depolarization-evoked increase in $[Ca^{2+}]_i$ were observed, making direct effects of HBCD on VGCCs unlikely.

However, when the exposure time was prolonged to >5 min, a time and concentration-dependent inhibitory effect on the depolarization-evoked increase in $[Ca^{2+}]_i$ became visible (Figure 1A). 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. The underlying cause of this exposure-time dependency remains to be elucidated, but may involve targeting of voltage-independent intracellular signaling pathways or partitioning in the cell membrane.

Following 20 min exposure to HBCD at 20 μM, the depolarization-evoked increase in $[Ca^{2+}]_i$ was inhibited by approximately 40%, whereas 0.2 μM HBCD was ineffective (LOEC; Figure 1B). The highest concentration of HBCD in this study (20 μM) was thus insufficient to induce a complete inhibition of depolarization-evoked Ca^{2+} entry.

To investigate possible differences in activity between the different stereoisomers of HBCD, PC12 cells were exposed to 2 μM of the α-, β-, and γ-stereoisomers. 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, whereas the inhibitory effect of γ-HBCD was larger (Figure 1C).

To investigate the involvement of specific VGCCs 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 μM of, respectively, nifedipine, GVIA, or MVIIC ω-conotoxins. These experiments demonstrated that the majority of the increase in $[Ca^{2+}]_i$ is due to Ca^{2+} entry via L-type VGCCs and that exposure to HBCD (20 μM) while blocking L-type VGCCs with nifedipine resulted in a further decrease of the depolarization-evoked $[Ca^{2+}]_i$. Similarly, while blocking N-type or P/Q-type VGCCs with GVIA or MVIIC ω-conotoxins, HBCD exposure further decreased the depolarization-evoked $[Ca^{2+}]_i$. If HBCD would specifically block one subtype of VGCCs, a combined exposure to HBCD and a specific blocker should inhibit the depolarization-evoked increase in $[Ca^{2+}]_i$ to a similar extent as the specific blocker alone. These data thus indicate that the effect of HBCD is not VGCC-specific.

Effects of HBCD on exocytosis. As HBCD inhibits depolarization-evoked $[Ca^{2+}]_i$, possible effects of HBCD exposure on exocytosis were investigated. As expected, exposure to HBCD did not affect basal exocytosis. However, when cells were exposed to HBCD (20 μM) for 20 min prior to depolarization, depolarization-evoked release of catecholamines was completely inhibited in ~70% of the cells, whereas ~85% of the control cells (exposure to 0.1% DMSO) still displayed depolarization-evoked exocytosis. Somewhat surprising, the release frequencies of cells that still displayed exocytosis following exposure to HBCD were indistinguishable from those of control cells (data not shown). Thus, due to the HBCD-induced inhibition of the depolarization-evoked $[Ca^{2+}]_i$ a larger proportion of the cells probably does not reach the Ca^{2+} threshold for exocytosis. Exposure to HBCD did not affect vesicular release parameters (i.e., vesicle content, amplitude and t_{50}), indicating that exocytosis and its underlying processes are unaffected.

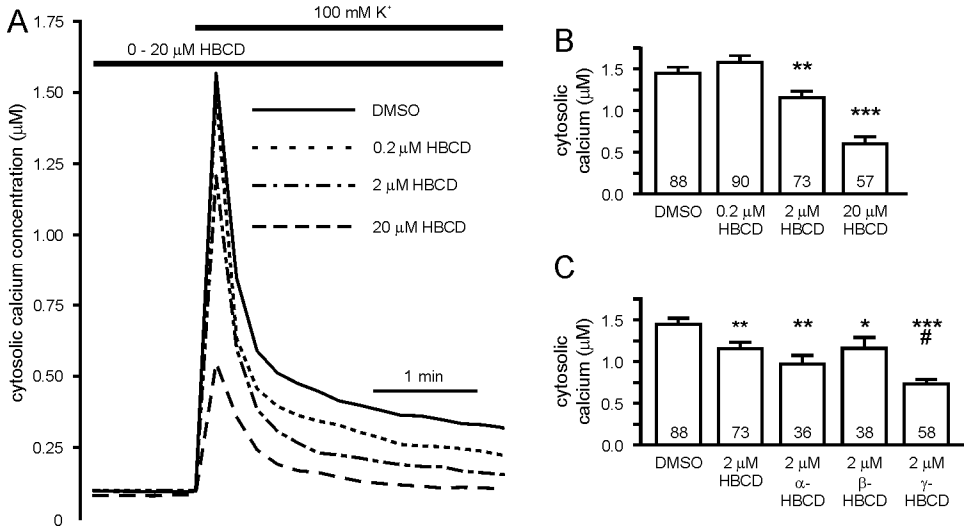

Figure 1. A) Representative traces of changes in $[Ca^{2+}]_i$ in individual PC12 cells, illustrating the reduction of the depolarization-evoked increase in $[Ca^{2+}]_i$ by exposure to 0-20 μM HBCD B) Bar graph shows the concentration-dependent reduction of the depolarization-evoked increase in $[Ca^{2+}]_i$ by HBCD. C) Bar graph shows the reduction of the depolarization-evoked $[Ca^{2+}]_i$ increase by the technical mixture and separate stereoisomers of HBCD. The number of cells used for data analysis is indicated inside each bar. Difference from control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. 2 μM γ-HBCD differs significantly from all other HBCD treatments (# $p < 0.05$)[12].

Risk assessment. The highest concentration of HBCD measured in human serum in an occupational setting amounts to ~15 nM[13], which is approximately two orders of magnitude below the lowest observed-effect concentration (LOEC) in this study. The highest background level of HBCD measured in human serum amounts to ~0.13 nM[14], which is approximately four orders of magnitude below the LOEC in this study.

Nevertheless, for risk assessment purposes, this difference is relatively small for several reasons, including scarcity of data on human HBCD serum levels, the lipophilic and bioaccumulative properties of HBCD, and the need for safety factors for species extrapolation and intraspecies variability. Additionally, *in vivo* HBCD exposure is expected to be of lifelong duration, whereas the LOEC obtained in this *in vitro* study was based on only 20 min exposure. Further, due to the cessation of the use of PBDEs it is not unlikely that the use of HBCD, and thus also human exposure levels, increase in the future.

Additional concern arises from the fact that organohalogen compounds, including PCBs, PBDEs and HBCD, transfer across the placenta[14]. Also, HBCD has been detected in human breast milk, up to 5 ng/g lipids[15]. As it has been shown that exposure to HBCD within the time frame of rapid brain development results in behavioral defects in mice[3], it is concerning that children are exposed to HBCD pre- as well as postnatally.

Considering the current findings on neuronal signaling, additional safety regulations in an occupational setting should be considered and additional efforts to establish an adequate exposure, hazard, and risk assessment are justified.

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