HYDROXYLATION INCREASES THE NEUROTOXIC POTENTIAL OF THE BROMINATED FLAME RETARDANT BDE-47: THE STRUCTURE OF BDE-47 METABOLITES DETERMINES THE POTENCY TO AFFECT CALCIUM HOMEOSTASIS IN PC12 CELLS

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Introduction

Polybrominated diphenyl ethers (PBDEs) are present in the environment, human food chain, and human tissues. In most biotic samples, 2,2´,4,4´-tetrabromodiphenyl ether (BDE-47) is the predominant congener. Neonatal exposure to BDE-47 induces neurodevelopmental changes in behavior[1] and long-term potentiation (LTP) in mouse hippocampal slices[2]. Hippocampal LTP is generally used as an electrophysiological substrate for learning and memories and requires tetanic stimulation, strong depolarization, and a large increase in the intracellular calcium concentration ($[Ca^{2+}]_i$)[3]. Strict regulation of ($[Ca^{2+}]_i$) is not only a key regulator for induction and maintenance of LTP, but also for other neuronal processes, such as neurotransmitter release (exocytosis) at presynaptic terminals[4,5].

Similar to the structurally related polychlorinated biphenyls (PCBs), PBDEs have in recent years been shown to affect Ca^{2+} homeostasis in microsomes[6] and PC12 cells[2] and to reduce the Ca^{2+} uptake by brain microsomes and mitochondria[7]. Such xenobiotic-induced increases in $[Ca^{2+}]_i$ are of concern as they can trigger multiple physiological and pathological processes, including exocytosis. However, these acute toxic effects of BDE-47 were seen *in vitro* only at high concentrations (3 - 20 μM)[2,7], whereas the concentration of BDE-47 resulting in impaired learning and memory and reduced hippocampal LTP were estimated (using a distribution study[8]) to result in peak brain concentrations of approximately 1 μM.

This discrepancy may be explained by oxidative metabolism of PBDEs *in vivo*, resulting in the formation of more potent hydroxylated metabolites of BDE-47 (OH-BDEs). Indeed, the conversion of PBDEs to hydroxylated metabolites was confirmed by recent toxicokinetics studies[9-12]. Moreover, several hydroxylated metabolites of BDE-47 have been detected in human serum and cord blood[13,14]. Results of *in vitro* endocrine studies demonstrate that hydroxylated metabolites of PBDEs are more potent than the parent compounds[15,16]. However, the neurotoxic potential of hydroxylated PBDE metabolites and their ability to affect Ca^{2+} homeostasis is still unknown. We therefore investigated whether mono-hydroxylated metabolites of the abundant BDE-47 were more potent in affecting $[Ca^{2+}]_i$ in PC12 cells to compare its neurotoxic potential with that of the parent compound.

Materials and methods

Chemicals. BDE-47 as well as several metabolites of BDE-47 (6-OH-BDE-47, 6'-OH-BDE-49, 5-OH-BDE-47, 3-OH-BDE-47, 4'-OH-BDE-49 and methoxylated analogue 6-MeO-BDE-47) were synthesized and purified at the Department of Environmental Chemistry of Stockholm University.

Cell culture. Rat pheochromocytoma (PC12) cells[18] obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) were cultured as described previously[2,19]. 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[2]. For amperometric recordings, the cells were differentiated for 3 - 5 days with 5 μM dexamethasone to enhance exocytosis, as described previously[19].

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[2,19].

Intracellular Ca^{2+} imaging. Changes in $[Ca^{2+}]_i$ were measured using the Ca^{2+}-responsive fluorescent ratio dye Fura-2 as described previously[2]. 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 for 15 min to saline or saline containing (OH-)BDEs

(0.2 - 20 μM). A transient increase in $[Ca^{2+}]_i$ within 0 - 10 min from the start of exposure is referred to as an 'initial increase', while additional increases are referred to as 'late increases'. Where applicable, cells were washed with Ca^{2+}-free saline just before the imaging experiments and intracellular Ca^{2+} stores were emptied by incubation with 1 μM thapsigargin (TG) and 1 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP).

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 multifactorial ANOVA was performed using the mean increase in basal $[Ca^{2+}]_i$ at 20 μM as the dependent variable to investigate structure-activity relationships. Hydroxylation position (*ortho*, *meta* or *para*) and the presence of either one or two shielding atomic groups (phenyl ring and/or bromine atom) adjacent to the OH-group were used as fixed variables. A p-value < 0.05 was considered statistically significant.

Results and discussion

Effects of 6-OH-BDE-47 on exocytosis. Exposure of PC12 cells to a high concentration (20 μM) of BDE-47 was previously shown to induce exocytosis[2]. To investigate whether oxidative metabolism changes the ability of PBDEs to affect exocytosis, cells were exposed to 6-OH-BDE-47, a hydroxylated metabolite of BDE-47. Amperometric recordings demonstrated that in cells exposed to 5 μM 6-OH-BDE-47 (n = 9), the release frequency was enhanced from 1.0 ± 0.3 to 13 ± 5.3 events/min ($p < 0.05$). After an initial burst of exocytotic activity, the release frequency in 6-OH-BDE-47-exposed cells declined to a value not significantly different from controls (Figure 1A).

Effects of 6-OH-BDE-47 on $[Ca^{2+}]_i$. Exposure of PC12 cells to 6-OH-BDE-47 (2 μM) resulted in a dose-dependent increase in $[Ca^{2+}]_i$ in PC12 cells, whereas exposure to similar concentrations of the parent compound had no effects on $[Ca^{2+}]_i$ (Figure 1B). The parent compound BDE-47 caused a gradual increase of $[Ca^{2+}]_i$ only at 20 μM[2], whereas the hydroxylated metabolite increased $[Ca^{2+}]_i$ already at concentrations ≥ 1 μM. These findings clearly indicate that the hydroxylated metabolite is much more potent in disrupting calcium homeostasis than the parent compound. The increase in $[Ca^{2+}]_i$ by 6-OH-BDE-47 consists of an initial transient increase as well as an additional late increase (Figure 1B).

Mechanisms of 6-OH-BDE-47-induced increase in $[Ca^{2+}]_i$. To investigate the mechanisms underlying the observed increase in $[Ca^{2+}]_i$, experiments were performed under Ca^{2+}-free conditions to reveal the role of extracellular Ca^{2+}. Under these conditions, both the initial transient and the additional late increase in $[Ca^{2+}]_i$ were somewhat reduced but still present (not shown). These findings thus indicate that the 6-OH-BDE-47-induced increase in $[Ca^{2+}]_i$ largely relies on the release of Ca^{2+} from intracellular stores. To identify the intracellular stores responsible for the observed increase in $[Ca^{2+}]_i$, the endoplasmic reticulum (ER) was depleted from Ca^{2+} by pretreatment with TG under Ca^{2+}-free conditions. After depletion of ER, 6-OH-BDE-47 (5 μM) no longer evoked the initial transient increase in $[Ca^{2+}]_i$, but the late increase was still present (Figure 1C). When, in addition to ER, mitochondrial Ca^{2+} stores were emptied by pretreatment with FCCP, both the initial transient and the late increase in $[Ca^{2+}]_i$ were completely absent. These data thus indicate that the initial transient increase in $[Ca^{2+}]_i$ is due to Ca^{2+} release from ER, whereas the late increase in $[Ca^{2+}]_i$ is due to Ca^{2+} release from mitochondria[19].

Structure-activity relationship of hydroxylated metabolites to increase $[Ca^{2+}]_i$. To establish a structure-activity relationship (SAR), we investigate the potency of other metabolites of the environmentally relevant BDE-47 to increase $[Ca^{2+}]_i$. To that aim, cells were exposed to 6-OH-BDE-47 (and its methoxylated analogue 6-MeO-BDE-47), 6'-OH-BDE-49, 5-OH-BDE-47, 3-OH-BDE-47 and 4'-OH-BDE-49. These metabolites of BDE-47 differ in location and degree of shielding of the OH-group (Table 1). The methoxylated analogue 6-MeO-BDE-47 was ineffective in increasing $[Ca^{2+}]_i$. The hydroxylated metabolites, however, all evoked dose-dependent initial increases in $[Ca^{2+}]_i$. LOECs were determined for the amplitude of the increase in $[Ca^{2+}]_i$ as well as for the percentage of cells showing initial and late increases in $[Ca^{2+}]_i$. For 6'-OH-BDE-49, 3-OH-BDE-47 and 4'-OH-BDE-49, the LOEC for increased $[Ca^{2+}]_i$ is 20 μM (Table 1). For 6-OH-BDE-47 and 5-OH-BDE-47 the LOEC amounted to 1 μM for initial increases in $[Ca^{2+}]_i$. Late increases were observed only at concentrations ≥ 2 μM. At 20 μM, the late increase induced by 6-OH-BDE-47 or 5-OH-BDE-47 was much larger, as was also observed for 20 μM 4'-OH-BDE-49.

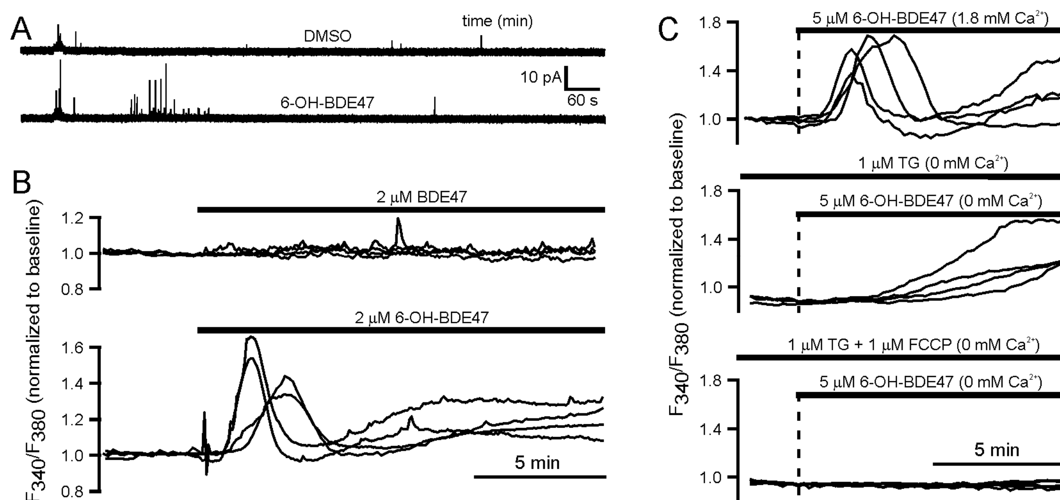

Figure 1. A) Representative amperometric traces of catecholamine exocytosis recorded from cells exposed to DMSO or 5 μM 6-OH-BDE-47. B) Representative traces of changes in $[Ca^{2+}]_i$ from individual PC12 cells, depicted as normalized F_{340}/F_{380}, demonstrating a biphasic increase in $[Ca^{2+}]_i$ in cells exposed to 2 μM 6-OH-BDE-47 (lower trace), but not in cells exposed to 2 μM BDE-47 (top trace). C) Representative traces of changes in $[Ca^{2+}]_i$ demonstrating a biphasic increase in $[Ca^{2+}]_i$ in cells exposed to 5 μM 6-OH-BDE-47 (top trace). The initial transient increase is smaller under Ca^{2+}-free conditions (not shown) and absent in cells pretreated with TG (middle trace). In cells pretreated with TG and FCCP, the 6-OH-BDE-47-induced late increase is also absent (lower trace).

	Molecular structure	Position OH-group	Shielding	Calcium influx (extracellular)	Calcium release (ER)	Calcium release (mitochondria)
6-OH-BDE-47		*ortho*	phenyl ether	1 μM	1 μM	2 μM
6'-OH-BDE-49		*ortho*	phenyl ether + bromine atom	-	20 μM	-
5-OH-BDE-47		*meta*	bromine atom	1 μM	1 μM	-
3-OH-BDE-47		*meta*	two bromine atoms	-	20 μM	20 μM
4'-OH-BDE-49		*para*	bromine atom	20 μM	20 μM	20 μM

Table 1. Molecular structures of the metabolites of BDE-47 investigated in this study. The position of the OH group and its degree of shielding are indicated. Right columns indicate LOECs (μM) of BDE-47 for different parameters of $[Ca^{2+}]_i$ in PC12 cells.

The mean amplitude of $[Ca^{2+}]_i$ during exposure to 20 μM of the hydroxylated metabolites was independent of the position (*ortho*, *meta* or *para*) of the OH-group on the PBDE-molecule (ANOVA: n.s.). However, OH-PBDEs in which the OH-group was shielded on only one side, with either the other phenyl ring or a bromine atom, induced significantly larger increases in $[Ca^{2+}]_i$ compared to OH-PBDEs in which the OH-group was shielded on both sides (ANOVA: $p < 0.01$). Moreover, the influence of shielding of the OH-group (on one compared to two sides) is independent of its position (*ortho*, *meta* or *para*) on the PBDE-molecule (ANOVA: n.s.).
Additional experiments to reveal the origin of the increase in $[Ca^{2+}]_i$, using Ca^{2+}-free conditions and pretreatment with TG and FCCP, revealed that increases in $[Ca^{2+}]_i$ largely originated from intracellular stores, though in case of 6-OH-BDE-47, 5-OH-BDE-47 and 4'-OH-BDE-49 influx of extracellular Ca^{2+} also plays a role[20].
These combined findings demonstrate that hydroxylation increases the potency of BDE-47 to increase $[Ca^{2+}]_i$. The OH-BDE-induced increases in $[Ca^{2+}]_i$ are in general biphasic, consisting of an initial transient increase in $[Ca^{2+}]_i$ and an additional late, persistent increase in $[Ca^{2+}]_i$. Release of Ca^{2+} from intracellular stores, mainly ER and mitochondria, is the pre-dominant pathway underlying the increase in $[Ca^{2+}]_i$., though small differences between the different metabolites exist. Importantly, shielding of the OH-group on both sides with Br-atoms and/or the ether-bond to the other phenyl ring lowers the potency of hydroxylated PBDE-metabolites.

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