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Calcium-Related Processes Involved in the Inhibition of Depolarization-Evoked Calcium Increase by Hydroxylated PBDEs in PC12 Cells

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In vitro **studies indicated that hydroxylated polybrominated diphenyl ethers (OH-PBDEs) have an increased toxic potential compared to their parent congeners. An example is the OH-PBDE–induced increase of basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by release of Ca^{2+} from endoplasmic reticulum (ER) and mitochondria and/or influx of extracellular Ca^{2+}. ER and mitochondria regulate Ca^{2+} homeostasis in close association with voltage-gated Ca^{2+} channels (VGCCs). Therefore, effects of (OH-)PBDEs on the depolarization-evoked (100mM K^+) net increase in $[Ca^{2+}]_i$ (depolarization-evoked $[Ca^{2+}]_i$) were measured in neuroendocrine pheochromocytoma cells using the Ca^{2+}-responsive dye Fura-2. OH-PBDEs dose dependently inhibited depolarization-evoked $[Ca^{2+}]_i$. This inhibition was potentiated by a preceding increase in basal $[Ca^{2+}]_i$. Especially at higher concentrations of OH-PBDEs (5–20μM), large increases in basal $[Ca^{2+}]_i$ strongly inhibited depolarization-evoked $[Ca^{2+}]_i$. The inhibition appeared more sensitive to increases in basal $[Ca^{2+}]_i$ by Ca^{2+} release from intracellular stores (by 3-OH-BDE-47 or 6′-OH-BDE-49) compared to those by influx of extracellular Ca^{2+} (by 6-OH-BDE-47 or 5-OH-BDE-47). The expected $[Ca^{2+}]_i$ difference close to the membrane suggests involvement of Ca^{2+}-dependent regulatory processes close to VGCCs. When coapplied with depolarization, some OH-PBDEs induced also moderate direct inhibition of depolarization-evoked $[Ca^{2+}]_i$. Polybrominated diphenyl ethers and methoxylated BDE-47 affected neither basal nor depolarization-evoked $[Ca^{2+}]_i$, except for BDE-47, which moderately increased fluctuations in basal $[Ca^{2+}]_i$ and depolarization-evoked $[Ca^{2+}]_i$. These findings demonstrate that OH-PBDEs inhibit depolarization-evoked $[Ca^{2+}]_i$ depending on preceding basal $[Ca^{2+}]_i$. Related environmental pollutants that affect Ca^{2+} homeostasis (e.g., polychlorinated biphenyls) may thus also inhibit depolarization-evoked $[Ca^{2+}]_i$, justifying further investigation of possible mixture effects of environmental pollutants on Ca^{2+} homeostasis.**

Key Words: **brominated flame retardant; calcium homeostasis; calcium signaling; calcium-induced VGCC inhibition; depolarization-evoked calcium influx; *in vitro* neurotoxicity.**

Polybrominated diphenyl ethers (PBDEs), a group of brominated flame retardants, have been shown to affect learning and spontaneous behavior in rodents (Eriksson *et al*., 2001; reviewed in Costa and Giordano, 2007; Fonnum and Mariussen, 2009). PBDEs have been detected, occasionally at high concentrations, in humans and particularly in young children (reviewed in Frederiksen *et al*., 2009).

In vitro neurotoxicity and endocrine studies have revealed that oxidative metabolism, resulting in hydroxylated polybrominated diphenyl ethers (OH-PBDEs), increases the potency of PBDEs (e.g., Cantón *et al*., 2008; Dingemans *et al*., 2008; Kojima *et al*., 2009). Recently, OH-PBDEs have also been detected in human serum at concentrations similar to those of parent PBDE congeners (Athanasiadou *et al*., 2008; Qiu *et al*., 2009), giving rise to concern about possible neurotoxic effects in humans.

Possible mechanisms underlying the neurobehavioral effects of PBDEs or their metabolites have been partly revealed at different biological levels, ranging from structural and functional effects in the brain to cellular and molecular effects measured *in vitro* (reviewed in Costa and Giordano, 2007; Fonnum and Mariussen, 2009). These include among others the effects of (OH-)PBDEs on Ca^{2+} homeostasis (Coburn *et al*., 2008; Dingemans *et al*., 2008; Kodavanti and Ward, 2005). During basal conditions, average cytosolic Ca^{2+} levels in chromaffin and pheochromocytoma (PC12) cells are maintained around 100nM by Ca^{2+} buffering and extrusion mechanisms (García *et al*., 2006). However, neuronal signaling also requires rapid transient increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), triggering various intracellular processes, including neurotransmitter release (Barclay *et al*., 2005; Clapham, 2007). In PC12 cells, rapid increases in $[Ca^{2+}]_i$ in response to depolarization mainly originate from influx of Ca^{2+} via voltage-gated Ca^{2+} channels (VGCCs) located in the cell membrane. VGCCs expressed in undifferentiated PC12 cells include L-, N-, P/Q-, R-, and T-type Ca^{2+} channels (Del Toro *et al*., 2003; Liu *et al*., 1996; Shafer and Atchison, 1991). High

TABLE 1
Full Names of the PBDEs and Hydroxylated/Methoxylated PBDEs Discussed in This Paper

6-MeO-BDE-47	6-methoxy-2,2′,4,4′-tetrabromodiphenyl ether
3-OH-BDE-47	3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether
4′-OH-BDE-49	4′-hydroxy-2,2′,4,5′-tetrabromodiphenyl ether
5-OH-BDE-47	5-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether
6-OH-BDE-47	6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether
6′-OH-BDE-49	6′-hydroxy-2,2′,4,5′-tetrabromodiphenyl ether
BDE-47	2,2′,4,4′-tetrabromodiphenyl ether
BDE-49	2,2′,4,5′-tetrabromodiphenyl ether
BDE-99	2,2′,4,4′,5-pentabromodiphenyl ether
BDE-100	2,2′,4,4′,6-pentabromodiphenyl ether
BDE-153	2,2′,4,4′,5,5′-hexabromodiphenyl ether

voltage–gated L-, N-, and P/Q-type VGCCs account for the majority of the depolarization-evoked increase in $[Ca^{2+}]_i$; it has been shown previously in PC12 cells that blocking L-, N-, and P/Q-type VGCCs reduces the depolarization-evoked increase in $[Ca^{2+}]_i$ to ~15% of control (Dingemans *et al.*, 2009b).

Previously, the effects of parent PBDE and OH-PBDEs as well as a methoxylated analog on basal $[Ca^{2+}]_i$ have been investigated in PC12 cells. While the parent PBDEs and methoxylated 6-MeO-BDE-47 do not affect the average or amplitude of basal $[Ca^{2+}]_i$ during 20-min exposure, all investigated OH-PBDEs increase basal $[Ca^{2+}]_i$ by release of Ca^{2+} from endoplasmic reticulum (ER), in some cases, combined with release of Ca^{2+} from mitochondria and/or influx of extracellular Ca^{2+}. The potency of OH-PBDEs for affecting basal $[Ca^{2+}]_i$ appears at least partly determined by shielding of the OH group by Br atoms or aromatic rings. OH-PBDEs in which the OH group was shielded on both sides by atomic groups (Br atoms or aromatic rings; 3-OH-BDE-47 and 6′-OH-BDE-49) had less effects on basal $[Ca^{2+}]_i$ than OH-PBDEs in which the OH group was shielded on only one side (6-OH-BDE-47 and 5-OH-BDE-47; Dingemans *et al.*, 2009a).

Because of the close functional associations of ER and mitochondria with VGCCs for regulating Ca^{2+} homeostasis (García *et al.*, 2006), the aim of this study was to investigate in a neuroendocrine *in vitro* model (PC12 cells) whether OH-PBDEs also affect depolarization-evoked $[Ca^{2+}]_i$.

METHODS

Chemicals. PBDEs (BDE-47, BDE-49, BDE-99, BDE-100, and BDE-153) and OH-PBDEs (3-OH-BDE-47, 4′-OH-BDE-49, 5-OH-BDE-47, 6′-OH-BDE-49, 6-OH-BDE-47, and methoxylated analog 6-MeO-BDE-47; full names in Table 1) were synthesized and purified (~99% purity) at the Department of Environmental Chemistry of Stockholm University as described earlier (Dingemans *et al.*, 2008, 2009a). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

PC12 cell culture. Rat PC12 cells were cultured as described previously (Dingemans *et al.*, 2008). See Supplementary methods for more detailed information.

Ca^{2+} imaging. Changes in $[Ca^{2+}]_i$ were measured using the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 as described previously (Dingemans *et al.*, 2008). Membrane depolarization by 100mM K^+ was used to investigate effects of the (MeO/OH-)PBDEs on the depolarization-evoked increase in $[Ca^{2+}]_i$. See Supplementary methods for more detailed information on experimental conditions and calculation of $[Ca^{2+}]_i$. The amplitude of $[Ca^{2+}]_i$ within a minute of the start of depolarization was determined per cell, and the net increase (amplitude $[Ca^{2+}]_i$ − preceding $[Ca^{2+}]_i$ last minute prior to depolarization) was used to investigate effects of PBDEs on depolarization-evoked $[Ca^{2+}]_i$, both following 20-min preexposure to (MeO/OH-)PBDEs (Fig. 1) and during coapplication of 100mM K^+ and (MeO/OH-)PBDE to investigate direct effects of the (MeO/OH-)PBDEs.

Data analysis and statistics. During exposure to OH-PBDEs, transient (initial) and/or late increases in basal $[Ca^{2+}]_i$ are observed (Dingemans *et al.*,

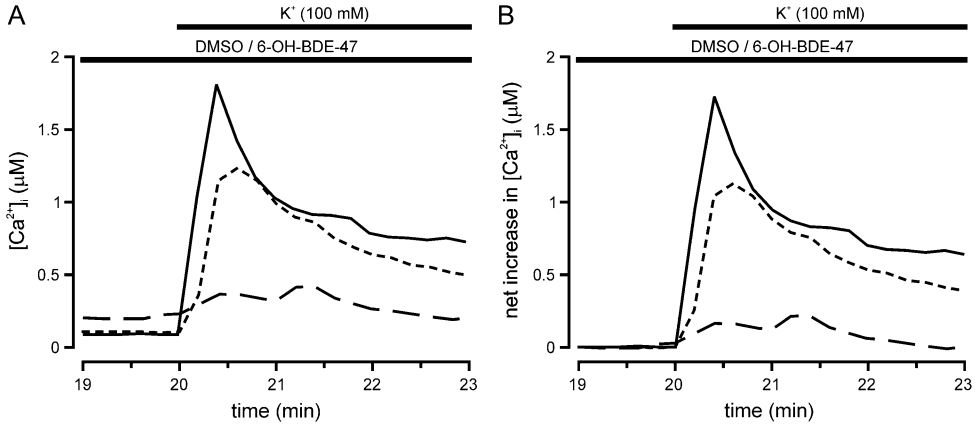

FIG. 1. Exposure of PC12 cells to OH-PBDEs inhibits the depolarization-evoked increase in $[Ca^{2+}]_i$. Representative traces of cytosolic $[Ca^{2+}]_i$ measurements of individual PC12 cells exposed to different concentrations of 6-OH-BDE-47 are shown, illustrating the reduction of the depolarization-evoked increase in $[Ca^{2+}]_i$ by exposure to OH-PBDEs (control: solid line, 2µM 6-OH-BDE-47: small dash, and 20µM 6-OH-BDE-47: large dash). During 20-min preexposure to OH-PBDEs, an increase in basal $[Ca^{2+}]_i$ is observed (A). The difference between the amplitude of $[Ca^{2+}]_i$ (measured within 1 min from the start of depolarization) and basal $[Ca^{2+}]_i$ (measured within 1 min before depolarization) is therefore used as the measure of depolarization-evoked $[Ca^{2+}]_i$ (depolarization-evoked net increase in $[Ca^{2+}]_i$; B).

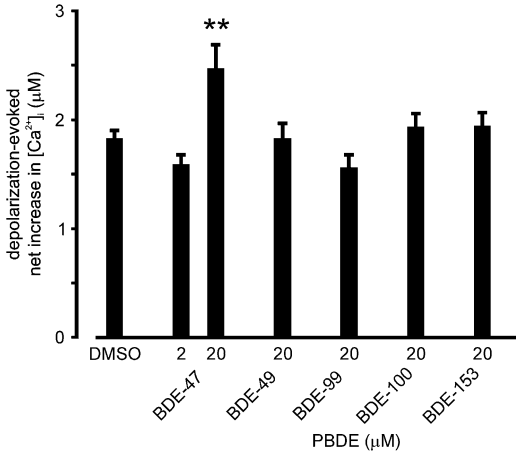

FIG. 2. BDE-47 (20μM) increases depolarization-evoked net increase in $[Ca^{2+}]_i$, while the other parent PBDEs have no effect. Bar graph shows the amplitudes of depolarization-evoked net increase in $[Ca^{2+}]_i$ in PC12 cells 20 min preexposed to PBDEs. Bars display data from 3 to 5 experiments per PBDE treatment (n = 37–62, average: 46) and 19 control experiments (n = 168). Difference from control: $^{**}p < 0.01$.

2009a; Supplementary fig. 1). When comparing the depolarization-evoked net increase in $[Ca^{2+}]_i$ in cells with only a transient or only a late increase in basal $[Ca^{2+}]_i$, the depolarization-evoked net increase in $[Ca^{2+}]_i$ in cells with a late increase is within the same range as in cells with a transient increase in basal $[Ca^{2+}]_i$ (data not shown). Therefore, the average net increase in basal $[Ca^{2+}]_i$ measured during 20-min preexposure was used as a measure for basal $[Ca^{2+}]_i$ disruption to investigate correlations between net increases in basal and depolarization-evoked $[Ca^{2+}]_i$.

All data are presented as mean ± SE from the number of cells (n) indicated. Statistical analyses were performed using SPSS 16 (SPSS, Chicago, IL). Categorical and continuous data were compared using, respectively, Fisher's exact test and Student's t-test, paired or unpaired where applicable. A $p < 0.05$ is considered statistically significant.

RESULTS

OH-PBDEs Dose Dependently Inhibit Depolarization-Evoked Net Increase in $[Ca^{2+}]_i$

In control cells (20 min exposed to 0.1% dimethyl sulfoxide [DMSO]), a robust depolarization-evoked net increase in $[Ca^{2+}]_i$ (1.82 ± 0.09μM, n = 168) was observed in the majority of cells. Exposing PC12 cells for 20 min to BDE-49, BDE-99, BDE-100, or BDE-153 or methoxylated PBDE 6-MeO-BDE-47 did affect neither the basal $[Ca^{2+}]_i$ nor the subsequent depolarization-evoked net increase in $[Ca^{2+}]_i$. However, cells exposed to 20μM BDE-47 showed more fluctuations in basal $[Ca^{2+}]_i$ as well as a larger depolarization-evoked net increase in $[Ca^{2+}]_i$ (2.46 ± 0.23μM, n = 37, $p < 0.01$) compared to control cells (Fig. 2).

Previously, it was shown that exposing PC12 cells for 20 min to OH-PBDEs resulted in increases in basal $[Ca^{2+}]_i$ by release of Ca^{2+} from intracellular stores and/or influx of extracellular Ca^{2+} (Dingemans *et al.*, 2009a). A dose-dependent increase in basal $[Ca^{2+}]_i$ was observed during exposure to 6-OH-BDE-47 or 5-OH-BDE-47 (lowest observed effect concentration [LOEC]: 1μM). 6-OH-BDE-47 and 5-OH-BDE-47 also dose dependently inhibited the subsequent depolarization-evoked net increase in $[Ca^{2+}]_i$, with a LOEC of, respectively, 2 and 5μM and near-complete inhibition at 20μM. During 20-min preexposure to 20μM 6′-OH-BDE-49, 3-OH-BDE-47, or 4′-OH-BDE-49, basal $[Ca^{2+}]_i$ increased and

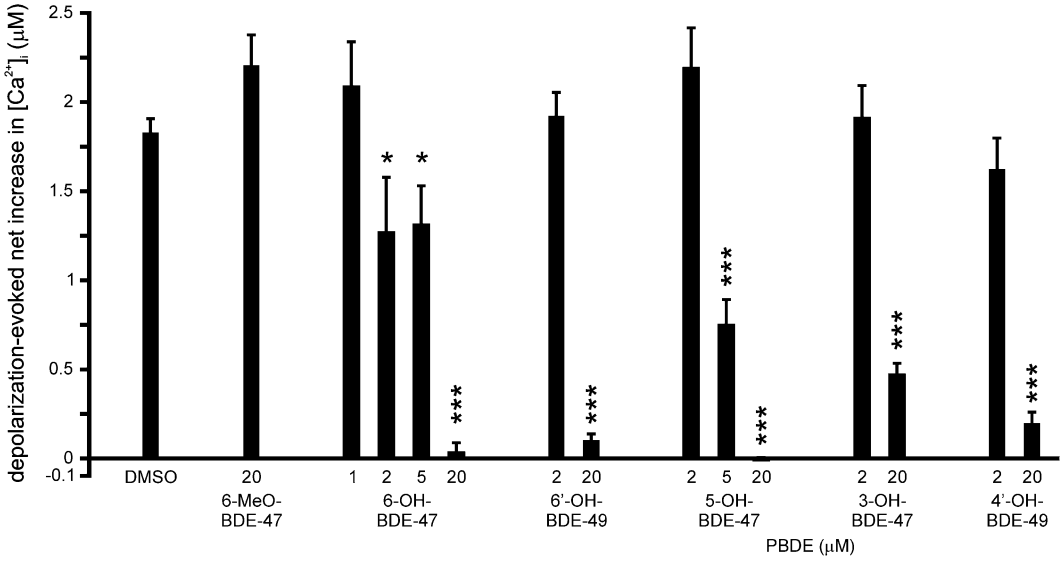

FIG. 3. OH-PBDEs inhibit depolarization-evoked net increase in $[Ca^{2+}]_i$ at similar LOECs as for increase in basal $[Ca^{2+}]_i$. Bar graph shows the dose-dependent reduction of the depolarization-evoked net increase in $[Ca^{2+}]_i$ by OH-PBDEs. Bars display data from 4 to 8 experiments per MeO/OH-PBDE treatment (n = 31–86; average 54) and 19 control experiments (n = 168). Difference from control: $^{*}p < 0.05$ and $^{***}p < 0.001$.

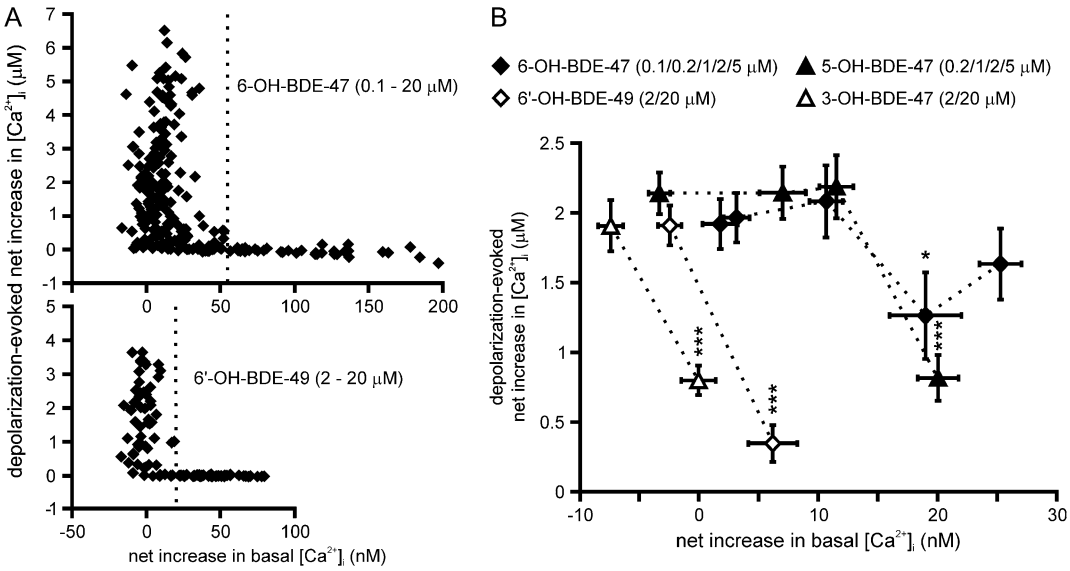

FIG. 4. Net increases in basal $[Ca^{2+}]_i$ higher than the full inhibition threshold completely inhibits depolarization-evoked net increase in $[Ca^{2+}]_i$. (A) Examples of associations of net increase in basal versus depolarization-evoked net increase in $[Ca^{2+}]_i$. Determined full inhibition thresholds are indicated by dashed lines. (B) When cells with net increases in basal $[Ca^{2+}]_i$ greater than the full inhibition threshold are removed, the inhibition of depolarization-evoked net increase in $[Ca^{2+}]_i$ is still dependent on net increases in basal $[Ca^{2+}]_i$. Different sensitivities of the depolarization-evoked net increase in $[Ca^{2+}]_i$ for net increases in basal $[Ca^{2+}]_i$ are observed for 6′-OH-BDE-49 and 3-OH-BDE-47 versus 6-OH-BDE-47 and 5-OH-BDE-47. Scatter displays data from 3 to 8 experiments per OH-PBDE treatment ($n = 21$–86; average 44) and 19 control experiments ($n = 168$). Each data point represents depolarization-evoked net increase in $[Ca^{2+}]_i$ in PC12 cells exposed to a OH-PBDE at a single exposure concentration (as indicated in figure, identifiable from left to right by increasing net increases in basal $[Ca^{2+}]_i$). Difference from control: $*p < 0.05$ and $***p < 0.001$.

the subsequent depolarization-evoked net increase in $[Ca^{2+}]_i$ was largely inhibited (Fig. 3; LOEC: 20μM).

Increase in Basal $[Ca^{2+}]_i$ Potentiates Inhibition of Depolarization-Evoked Net Increase in $[Ca^{2+}]_i$

A negative association exists between average net increases in basal $[Ca^{2+}]_i$ and the depolarization-evoked net increase in $[Ca^{2+}]_i$ when taking into account all different OH-PBDE treatments. Plotting the depolarization-evoked net increase in $[Ca^{2+}]_i$ only from individual cells exposed to 6-OH-BDE-47 (at different concentrations) against their preceding net increase in basal $[Ca^{2+}]_i$ revealed that in cells of which the average basal $[Ca^{2+}]_i$ increased with > 55nM, depolarization-evoked net increase in $[Ca^{2+}]_i$ was near completely inhibited (Fig. 4A). This was also observed for the other OH-PBDEs, although with varying threshold values of net increase in basal $[Ca^{2+}]_i$ for near-complete inhibition of the depolarization-evoked net increase in $[Ca^{2+}]_i$ (full inhibition thresholds): 6′-OH-BDE-49, 20nM; 5-OH-BDE-47, 55nM; 3-OH-BDE-47, 25nM; and 4′-OH-BDE-49, 25–50nM. These full inhibition threshold values were determined from frequency distribution of near-completely inhibited depolarization-evoked net increases in $[Ca^{2+}]_i$ (< 100nM) for net increase in basal $[Ca^{2+}]_i$ values (5nM intervals). The full inhibition threshold was identified by a rapid increase (to 100%) in the percentage of cells with inhibited depolarization-evoked net increases in $[Ca^{2+}]_i$. The full inhibition threshold for 4′-OH-BDE-49 (25–50nM) could not be specified further due to a lack of data points in this range of net increase in basal $[Ca^{2+}]_i$.

Moderate OH-PBDE–Induced Net Increases in Basal $[Ca^{2+}]_i$ Inhibit Depolarization-Evoked Net Increases in $[Ca^{2+}]_i$

In all cells with net increases in basal $[Ca^{2+}]_i$ above the full inhibition threshold, near-complete inhibition of depolarization-evoked net increase in $[Ca^{2+}]_i$ is observed (5μM 6-OH-BDE-47: 0.01 ± 0.01μM, $n = 19$; 20μM 6′-OH-BDE-49: 0.00 ± 0.00μM, $n = 46$; 5μM 5-OH-BDE-47: 0.21 ± 0.16μM, $n = 7$; and 20μM 3-OH-BDE-47: 0.10 ± 0.03μM, $n = 41$). To remove the influence of high net increase in basal $[Ca^{2+}]_i$, cells with a net increase in basal $[Ca^{2+}]_i$ higher than the full inhibition threshold were excluded from the following analysis. Subsets of cells with a net increase in basal $[Ca^{2+}]_i$ below the full inhibition threshold could only be identified within the groups of cells exposed to 5μM 6-OH-BDE-47, 20μM 6′-OH-BDE-49, 5μM 5-OH-BDE-47, or 20μM 3-OH-BDE-47. When cells with a high net increase in basal $[Ca^{2+}]_i$ are removed, the inhibition of depolarization-evoked net increases in $[Ca^{2+}]_i$ is still associated with preceding net increases in basal $[Ca^{2+}]_i$. In Figure 4B, a difference in sensitivity of inhibition of depolarization-evoked net increase in $[Ca^{2+}]_i$ for net increase in basal $[Ca^{2+}]_i$ can be seen for 6′-OH-BDE-49 and 3-OH-BDE-47 versus 6-OH-BDE-47 and 5-OH-BDE-47.

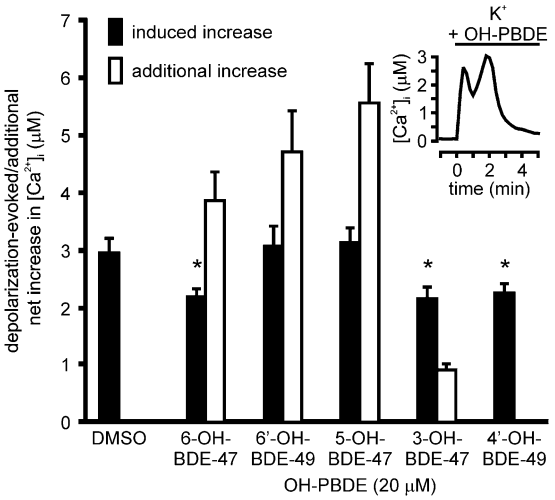

FIG. 5. Several OH-PBDEs directly inhibit depolarization-evoked net increase in $[Ca^{2+}]_i$. An additional increase (representative trace in inset) is also observed in all cells during exposure to 20μM 6-OH-BDE-47, 6′-OH-BDE-49, and 5-OH-BDE-47 and in 76% of cells exposed to 20μM 3-OH-BDE-47 ($n = 19$). Bars display depolarization-evoked (closed bars) and additional (open bars) net increases in $[Ca^{2+}]_i$ data from three to four experiments per treatment (DMSO: $n = 31$, 20μM 6-OH-BDE-47: $n = 39$, 20μM 6′-OH-BDE-49: $n = 26$, 20μM 5-OH-BDE-47: $n = 32$, 20μM 3-OH-BDE-47: $n = 25$, and 20μM 4′-OH-BDE-49: $n = 31$). Difference from control: $^*p < 0.05$.

OH-PBDE Directly Inhibit Depolarization-Evoked Net Increase in $[Ca^{2+}]_i$

To investigate whether the effects of the OH-PBDEs depend on preexposure, in separate experiments, 20μM of OH-PBDE was applied only during depolarization (Fig. 5). In control cells, depolarization-evoked net increase in $[Ca^{2+}]_i$ increased with 2.95 ± 0.28μM. During exposure to 20μM 6-OH-BDE-47, 3-OH-BDE-47, or 4′-OH-BDE-49, the depolarization-evoked net increase in $[Ca^{2+}]_i$ was inhibited to ~75% of control. Interestingly, during exposure to 6-OH-BDE-47, 6′-OH-BDE-49, 5-OH-BDE-47, and 3-OH-BDE-47, an additional increase is observed (Fig. 5, inset). For 6-OH-BDE-47, 6′-OH-BDE-49, and 5-OH-BDE-47, the amplitude of this additional increase is larger than the depolarization-evoked net increase in $[Ca^{2+}]_i$ (~4 to 6μM; Fig. 5). During exposure to 3-OH-BDE-47, the additional increase in $[Ca^{2+}]_i$ is smaller than the depolarization-evoked net increase in $[Ca^{2+}]_i$ and is observed in only 76% of the cells. Additional increases last ~5 min from the start of depolarization, and amplitudes of the additional increase in $[Ca^{2+}]_i$ are reached 1–4 min after the start of depolarization (data not shown). During exposure to 4′-OH-BDE-49, an additional increase is not observed.

DISCUSSION

OH-PBDEs dose dependently inhibit the depolarization-evoked net increase in $[Ca^{2+}]_i$ after 20-min preexposure. This inhibition at least partly depends on net increase in basal $[Ca^{2+}]_i$ during preexposure to OH-PBDEs. Especially at high concentrations, OH-PBDEs induce high net increases in basal $[Ca^{2+}]_i$ that near completely inhibit depolarization-evoked net increases in $[Ca^{2+}]_i$ (Ca^{2+}-induced inhibition, see below). When cells with a high net increase in basal $[Ca^{2+}]_i$ are excluded from data analysis, inhibition associated with a net increase in basal $[Ca^{2+}]_i$ is still observed (Ca^{2+}-mediated inhibition, see below). Moderate inhibition of the depolarization-evoked net increase in $[Ca^{2+}]_i$ was also observed for some OH-PBDEs when coapplied with depolarization (direct inhibition, see below). No or subtle effects were observed on basal and depolarization-evoked net increases in $[Ca^{2+}]_i$ during exposure to parent PBDEs and 6-MeO-BDE-47.

From the combined data, it can be concluded that when the average net increase in basal $[Ca^{2+}]_i$ is greater than a certain value (full inhibition threshold), the depolarization-evoked net increase in $[Ca^{2+}]_i$ is near completely inhibited (Ca^{2+}-induced inhibition). During depolarization, Ca^{2+}- and voltage-dependent processes desensitize VGCCs (Catterall, 2000). Membrane depolarization should have been similar in all experimental exposure conditions as no evidence exists for direct depolarization induced by (OH-)PBDEs. Therefore, the inhibitory effect on depolarization-evoked net increase in $[Ca^{2+}]_i$ observed in cells with high net increases in basal $[Ca^{2+}]_i$ (Ca^{2+}-induced inhibition) is likely mediated by Ca^{2+}-induced desensitization of VGCCs (reviewed by Budde *et al.*, 2002).

Interestingly, the full inhibition threshold for Ca^{2+}-induced desensitization of VGCCs varied between OH-PBDEs. When considering different sources of Ca^{2+} responsible for preceding net increases in basal $[Ca^{2+}]_i$ (Dingemans *et al.*, 2009a), it is apparent that for those OH-PBDEs that induce influx of extracellular Ca^{2+} besides Ca^{2+} release from intracellular stores (6-OH-BDE-47 and 5-OH-BDE-47), the threshold for Ca^{2+}-induced desensitization of VGCCs is higher compared to that observed for those OH-PBDEs that mainly induce Ca^{2+} release from intracellular stores (6′-OH-BDE-49 and 3-OH-BDE-47; Dingemans *et al.*, 2009a). Because of spatial differences between these processes, higher concentrations of $[Ca^{2+}]_i$ are expected in local membrane–associated Ca^{2+} microdomains during preexposure to 6-OH-BDE-47 and 5-OH-BDE-47 compared to 3-OH-BDE-47 and 6′-OH-BDE-49. It is noteworthy that this difference is larger than when comparing measured increases in basal $[Ca^{2+}]_i$ because a cytosolic average is measured. Therefore, increases in $[Ca^{2+}]_i$ close to the membrane are overestimated when release of Ca^{2+} from intracellular stores is involved but underestimated when influx of extracellular Ca^{2+} is involved. The presumed difference in membrane-associated $[Ca^{2+}]_i$ suggests Ca^{2+}-dependent regulation of the observed Ca^{2+}-induced desensitization of VGCCs (higher full inhibition thresholds). As Ca^{2+}-induced desensitization involves calmodulin as Ca^{2+} sensor in complex with VGCCs (Kim *et al.*, 2004), this is

a potential target for the regulatory processes caused by preceding increase in basal $[Ca^{2+}]_i$ induced by OH-PBDEs.

LOECs for inhibition of depolarization-evoked net increase in $[Ca^{2+}]_i$ are comparable with LOECs for preceding increases in basal $[Ca^{2+}]_i$ (Dingemans *et al*., 2009a). However, the observed effects on depolarization-evoked net increase in $[Ca^{2+}]_i$ are confounded by large preceding net increases in basal $[Ca^{2+}]_i$ at high concentrations of OH-PBDEs. When cells with a high net increase in basal $[Ca^{2+}]_i$ are excluded, inhibition of depolarization-evoked net increase $[Ca^{2+}]_i$ still depends on preceding net increases in basal $[Ca^{2+}]_i$ (Ca^{2+}-mediated inhibition; Fig. 4B). Interestingly, inhibition of depolarization-evoked net increases in $[Ca^{2+}]_i$ appeared more sensitive to increases in basal $[Ca^{2+}]_i$ by 3-OH-BDE-47 and 6′-OH-BDE-49 than to those in basal $[Ca^{2+}]_i$ by 6-OH-BDE-47 and 5-OH-BDE-47. Since higher sensitivities to increases in basal $[Ca^{2+}]_i$ coincide (Fig. 4B) with lower thresholds for Ca^{2+}-induced desensitization (Fig. 4A), these processes appear associated and dependent on membrane-associated $[Ca^{2+}]_i$ (as discussed above).

When coapplied with depolarization, 20μM 6-OH-BDE-47, 3-OH-BDE-47, and 4′-OH-BDE-49 inhibit the depolarization-evoked net increase in $[Ca^{2+}]_i$, suggesting direct inhibition of VGCCs by these OH-PBDEs (direct inhibition; Fig. 5). For 6-OH-BDE-49, 5-OH-BDE-47, and 3-OH-BDE-47, the relative inhibition of the depolarization-evoked net increase in $[Ca^{2+}]_i$ is larger after 20-min preexposure compared to coapplication, indicating preexposure-dependent effects, which affirms the hypothesis on regulatory processes.

Only after exposure to BDE-47, the depolarization-evoked net increase in $[Ca^{2+}]_i$ is enhanced to ~135% of control. At concentrations of OH-PBDEs that induce an increase in the frequency, amplitude, or duration of fluctuations in $[Ca^{2+}]_i$ (0.2μM 6-OH-BDE-47, 1μM 5-OH-BDE-47, and 2μM 4′-OH-BDE-49; Dingemans *et al*., 2009a), a small (not significant) increase in the depolarization-evoked net increase in $[Ca^{2+}]_i$ is also observed (not for 2μM 4′-OH-BDE-49). This may be due to facilitation of VGCC by the increased number of preceding fluctuations in basal $[Ca^{2+}]_i$. Also, release of Ca^{2+} from intracellular stores is involved in the depolarization-evoked increase in $[Ca^{2+}]_i$ by Ca^{2+}-induced Ca^{2+} release (reviewed in García *et al*., 2006). Possibly, intracellular stores have compartmentalized more Ca^{2+} because of the increase in fluctuations by BDE-47. This could moderately increase the depolarization-evoked net increase in $[Ca^{2+}]_i$.

Based on the temporal characteristics of the additional increase in $[Ca^{2+}]_i$ observed during coapplication of K^+ and 20μM 6-OH-BDE-47, 6′-OH-BDE-49, 5-OH-BDE-47, and to a lesser extent 3-OH-BDE-47, this is likely caused by the previously described release of Ca^{2+} from intracellular Ca^{2+} stores (mainly ER; Dingemans *et al*., 2009a), although with a much higher amplitude. Possibly, this higher amplitude occurs because involved Ca^{2+} stores are charged by Ca^{2+} originating from influx through VGCCs.

As discussed above, thresholds for Ca^{2+}-induced desensitization of VGCCs and sensitivity of depolarization-evoked net increase in $[Ca^{2+}]_i$ to preceding net increase in basal $[Ca^{2+}]_i$ appear associated with OH-PBDE–induced changes in membrane-associated $[Ca^{2+}]_i$, i.e., in close proximity to VGCCs. Ca^{2+} currents through VGCCs are regulated by protein kinases, mainly protein kinase C (PKC), protein kinase A (PKA), and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and phosphatases (reviewed in Catterall, 2000; Dai *et al*., 2009). These kinases and phosphatases are also involved in regulation of inositol triphosphate (IP_3) receptor– and ryanodine receptor–mediated Ca^{2+} release from ER (reviewed in Vanderheyden *et al*., 2009; Zalk *et al*., 2007).

Some evidence exists for interaction of PBDEs with PKC. Influx of extracellular Ca^{2+} by PBDEs and polychlorinated biphenyls (PCBs) was caused by the release of arachonidic acid, a second messenger involved in, among other things, PKA and PKC activation (Kodavanti and Derr-Yellin, 2002). BDE-99 activates PKC (Madia *et al*., 2004), while PCBs and PBDEs also increase PKC translocation (Dorn and Mochly-Rosen, 2002) and affect its binding to IP_3 receptors (Kodavanti *et al*., 1994; Kodavanti and Ward, 2005; Shafer *et al*., 1996). PKC is also known to phosphorylate IP_3 receptors causing increased IP_3-mediated Ca^{2+} release from ER (Matter *et al*., 1993). PCBs and PBDEs have also been demonstrated to activate IP_3 kinase (Reistad and Mariussen, 2005; Voie and Fonnum, 2000). Additionally, several studies revealed effects of PBDEs on (phosphorylated-activated–)CaMKII levels in mice exposed to PBDEs (Dingemans *et al*., 2007; Viberg, 2009; Viberg *et al*., 2008). Phosphorylation of VGCCs by CaMKII facilitates Ca^{2+} currents (Lee *et al*., 2006). Like PKC, CaMKII also phosphorylates IP_3 receptors, thereby controlling IP_3-mediated Ca^{2+} release from ER (Zhang *et al*., 1993). Therefore, effects of OH-PBDEs on PKC- and/or CamKII-mediated processes are possibly involved in the observed release of Ca^{2+} from intracellular stores by OH-PBDEs (Dingemans *et al*., 2009a).

For the first time, it is demonstrated here that OH-PBDEs not only increase basal $[Ca^{2+}]_i$ but also inhibit depolarization-evoked $[Ca^{2+}]_i$. Because of the above-mentioned similar effects of PBDEs and PCBs on $[Ca^{2+}]_i$ and related protein kinases, the observed inhibition of depolarization-evoked $[Ca^{2+}]_i$ as well as the potentiation of inhibition of depolarization-evoked $[Ca^{2+}]_i$ by preceding increases in basal $[Ca^{2+}]_i$ might be not specific for OH-PBDEs. It is not unlikely that the observed inhibition also has functional consequences for depolarization-evoked neurotransmitter release in neuronal cells but possibly also for other more general Ca^{2+}-related processes, such as apoptosis and gene transcription (Clapham, 2007).

In summary, OH-PBDEs inhibit the depolarization-evoked net increase in $[Ca^{2+}]_i$. Regulatory mechanisms, possibly related to protein kinases, likely play a role in the basal $[Ca^{2+}]_i$-dependent inhibition of depolarization-evoked net increase in $[Ca^{2+}]_i$ (Ca^{2+}-induced inhibition and Ca^{2+}-mediated

inhibition) but also moderate direct inhibition is observed. The observed inhibition might also occur in the *in vivo* situation in which resting and stimulated states of neurons alternate, with varying temporal characteristics. As (spontaneous) neuronal activity is essential in early brain development and brain function (Moody and Bosma, 2005; Spitzer, 2006), an imbalance in neuronal activity by the observed increase in basal $[Ca^{2+}]_i$ and inhibition of depolarization-evoked $[Ca^{2+}]_i$ could play a role in the observed neurobehavioral effects of PBDEs and possibly PCBs. Further investigation of possible mixture effects of environmental pollutants is therefore justified.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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