MULTIPLE NEUROTOXIC EFFECTS OF TETRABROMOBISPHENOL-A (TBBPA)

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Introduction

Tetrabromobisphenol-A (TBBPA) is a widely used brominated flame retardant (BFR). Recent reports have shown its presence in wildlife and human samples[1-2]. Though information on the (neuro)toxicity of TBBPA is limited, it has been demonstrated that neonatal TBBPA exposure can affect the cholinergic system in neonates[3]. Moreover, *in vitro* studies demonstrated that TBBPA can increase the production of reactive oxygen species (ROS)[4-5] as well as the basal intracellular calcium concentration ($[Ca^{2+}]_i$) in human neutrophil granulocytes, cerebellar granule cells and Sertoli cells[4-6]. The TBBPA-induced increase in $[Ca^{2+}]_i$ is of importance as Ca^{2+} plays an essential role in multiple physiological and pathological processes, including neurotransmission[7].

Though most studies on the *in vitro* neurotoxic potential of TBBPA focussed on cytotoxicity or presynaptic effects of neurotransmission, recent studies indicate that persistent organic pollutants (POPs), such as PBDEs and polychlorinated biphenyls (PCBs), can also affect postsynaptic inhibitory human $GABA_A$ and excitatory α4β2 nicotinic acetylcholine (nACh) receptors[8-9]. Possible effects of TBBPA on these neurotransmitter receptors are of considerable interest as these receptors are critically involved in neurotransmission, synaptic plasticity and brain development[10-11]. We therefore investigated the acute effects of TBBPA on these neurotransmitter receptors as well as on Ca^{2+}-homeostasis, ROS production and cell viability.

Materials and methods

Stage V or VI *Xenopus* oocytes were injected with complementary DNA (cDNA) coding for the human α1, β2 and γ2 subunits of human $GABA_A$ receptors (Origene, Rockville, USA) or the human α4 and β2 subunits of the human neuronal nicotinic acetylcholine receptors (provided by Janssen Pharmaceutica N.V., Beerse, Belgium). Subsequent electrophysiological recordings of α1β2γ2 $GABA_A$ or α4β2 nicotinic ACh receptors function was performed as described previously[9,12]. The percentage of TBBPA-induced potentiation or inhibition of the GABA- or ACh-evoked ion current was calculated from the quotient of the maximum amplitude of the GABA- or ACh-TBBPA co-application response and the maximum amplitude of the control (GABA or ACh) response.

Rat B35 neuroblastoma cells and PC12 pheochromocytoma cells were grown for 10 passages on poly-L-lysine coated (50 µg/ml) culture flasks, dishes and plates as described previously[13].

One day prior to Ca^{2+} imaging experiments, undifferentiated cells ($1.4x10^6$ cells/dish) were subcultured in glass-bottom dishes (MatTek, Ashland, MA). Single cell $[Ca^{2+}]_i$ was measured in B35 and PC12 cells loaded with the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 AM (Invitrogen, Breda, The Netherlands) using a Polychrome IV (TILL Photonics GmBH, Grafelfing, Germany) as described previously[14]. The fluorescence ratio (R) evoked by 340 and 380 nm excitation was used to calculate $[Ca^{2+}]_i$ using a modified Grynkiewicz's equation. Following a 5min baseline recording, an increase in $[Ca^{2+}]_i$ was triggered by switching superfusion for 15s to saline containing 100 µM ACh (B35 cells) or 100 mM K^+ (PC12 cells) to measure stimulation-evoked $[Ca^{2+}]_i$. Next, cells recovered for ~5min, after which cells were exposed for 20min to saline containing DMSO (0.1%) or TBBPA (0.1-10 µM) to quantify the effects of TBBPA on basal $[Ca^{2+}]_i$. Next, the amplitude of the second K^+- or ACh-evoked increase in $[Ca^{2+}]_i$ was determined and expressed as % of the amplitude of the first stimulation per cell to obtain a 'treatment ratio' as described previously[15]. One day prior to measurements of cell viability or ROS production, undifferentiated B35 or PC12 cells were seeded in 96-wells plates (Greiner Bio-one, Solingen, Germany) at a density of $2x10^5$ respectively $1.5x10^5$ cells/well. As described previously[14], effects of TBBPA on cell viability or ROS production in B35 and PC12 cells were determined spectrophotometrically using a combined Alamar Blue (AB) and Neutral Red (NR) assay respectively using the fluorescent dye H2-DCFDA (Invitrogen).

Stock solutions of 0.1-100 mM TBBPA (>99% pure, Sigma Aldrich) were prepared in DMSO and diluted in saline to obtain the desired concentrations just prior to the experiments. All experiments were performed at room temperature (20-22°C). Data are presented as mean ± standard error of the mean (SEM) from the number of wells, cells or oocytes (*n*) indicated, derived from 3-11 independent experiments (*N*).

Results and discussion

Effects of TBBPA on neurotransmitter receptors were investigated in Xenopus oocytes expressing human $GABA_A$ or nACh receptors. Superfusion of voltage-clamped (-60 mV) oocytes expressing α1β2γ2 $GABA_A$ receptors with saline containing 0.1-1 μM TBBPA did not result in significant activation of the $GABA_A$ receptor, whereas superfusion with 10 μM TBBPA resulted in a significant activation of the $GABA_A$ receptor (8 ± 1%; $p < 0.001$; normalized to the current observed at 1 mM GABA; Figure 1A and B). When TBBPA (≥ 0.1 μM) was co-applied with GABA (at EC_{20}), a concentration-dependent potentiation of the GABA-induced ion current was observed (Figure 1A and C). These findings demonstrate that TBBPA acts both as partial and full agonist of the $GABA_A$ receptor with lowest observed effect concentrations (LOECs) of 0.1 and 10 μM, respectively. On the other hand, TBBPA-induced receptor activation could not be observed in oocytes expressing human nACh receptors. On the contrary, co-application of TBBPA (≥ 10μM) with ACh (EC_{10}) resulted in strong inhibition of the ACh-evoked ion current (63 ± 4%; $p < 0.001$; Figure 1D and E), demonstrating that TBBPA acts as an antagonist of the human α4β2 nACh receptor. These opposite effects of TBBPA, i.e., activation or potentiation of inhibitory GABA-mediated signaling and reduced excitatory ACh-mediated signaling, are of concern as they may add up *in vivo*. Importantly, both GABAA and nACh receptors play an important role in long-term potentiation, synaptic plasticity and brain development[10-11]. Moreover, comparable, additive effects have been observed previously for other POPs[9].

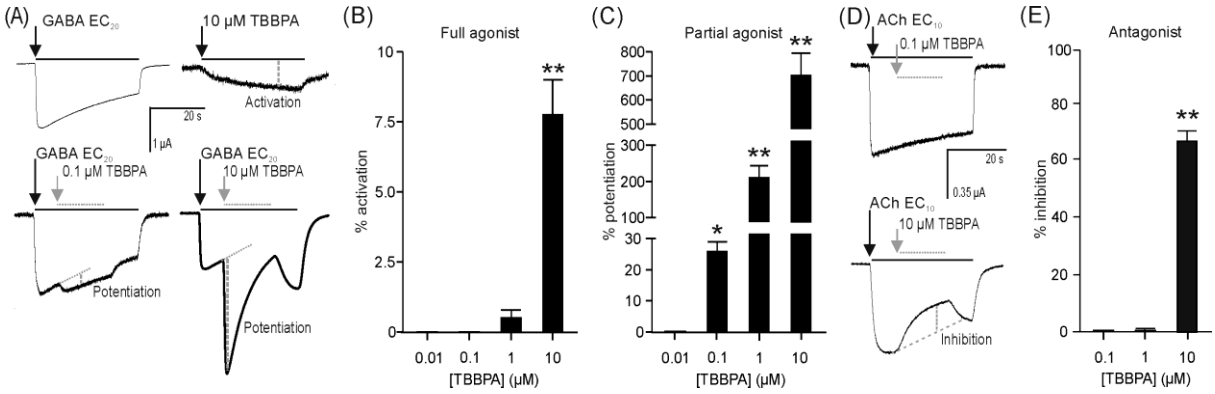

Figure 1. A). Example recordings demonstrating activation and potentiation of $GABA_A$ receptors expressed in oocytes by TBBPA. Scale bar applies to all traces (A). B). Bar graph demonstrating TBBPA-induced activation of the $GABA_A$ receptor, expressed as percentage of the maximum GABA-evoked response (1 mM). C). Bar graph demonstrating the concentration-dependent TBBPA-induced potentiation of GABA-induced ion currents expressed as percentage of the GABA-evoked response at EC_{20}. Bars represent mean ± SEM; n = 4-5 oocytes; * $p < 0.05$ versus control; ** $p < 0.001$ versus control. D). Example recordings demonstrating inhibition of nACh receptor expressed in oocytes by TBBPA. Scale bar applies to all traces. E). Bar graph demonstrating the TBBPA-induced inhibition of ACh-evoked (EC_{10}) responses. Bars represent mean inhibition ± SEM; n = 3-6 oocytes; ** $p < 0.001$ versus control.

The inhibitory effects of TBBPA on cholinergic signaling were further investigated using single cell fluorescent Ca^{2+}-imaging of Fura-2AM loaded B35 neuroblastoma cells, which highly express calcium permeable nACh receptors. The basal $[Ca^{2+}]_i$ in B35 cells is low (101 ± 2 nM (n = 88)) and not affected by exposure to DMSO or TBBPA (≤ 1 μM). On the other hand, exposure to 10 μM TBBPA resulted in a strong and rather persistent increase in basal $[Ca^{2+}]_i$ (1.4 ± 0.1 μM; Figure 2A and B). $[Ca^{2+}]_i$ increased rapidly and transiently when B35 cells were challenged with 100 μM ACh prior to and following a 20 min exposure to DMSO. The TR, i.e., the amplitude of the second ACh-evoked increase in $[Ca^{2+}]_i$ expressed as a percentage of the amplitude of the first stimulation, amounted to 135 ± 16% for DMSO and did not differ for cells exposed to 0.1 μM TBBPA. Exposure to 1 μM TBBPA resulted in a significant reduction of the net TR (44 ± 7% of control cells, $p < 0.001$; Figure 2A and C), whereas the second ACh-evoked increase in $[Ca^{2+}]_i$ was virtually absent in when cells were exposed to 10 μM TBBPA, thereby confirming that TBBPA acts as an antagonist of the nACh receptor.

Comparable with B35 cells, PC12 cells have low basal $[Ca^{2+}]_i$ (112 ± 5 nM; n = 77) that increases upon exposure to 10 µM TBBPA 2.2 ± 0.4 µM, whereas TBBPA ≤ 1 µM was without effects on basal $[Ca^{2+}]_i$ (Figure 2D and E). $[Ca^{2+}]_i$ increased rapidly and transiently when PC12 cells were depolarized with 100 mM K^+ prior to and following a 20 min exposure to DMSO. The TR, i.e., the amplitude of the second K^+-evoked increase in $[Ca^{2+}]_i$ expressed as a percentage of the amplitude of the first stimulation, amounted to 65 ± 3% for DMSO and did not differ for cells exposed to 0.1 µM TBBPA. Exposure to 1 µM TBBPA resulted in a significant reduction of the net TR (28 ± 4% of control cells, p < 0.001; Figure 2D and F), whereas the second ACh-evoked increase in $[Ca^{2+}]_i$ was virtually absent in when cells were exposed to 10 µM TBBPA, thereby demonstrating that TBBPA, comparable with PBDEs[16] and PCBs[15], acts as an antagonist of voltage-gated Ca^{2+} channels (VGCC).

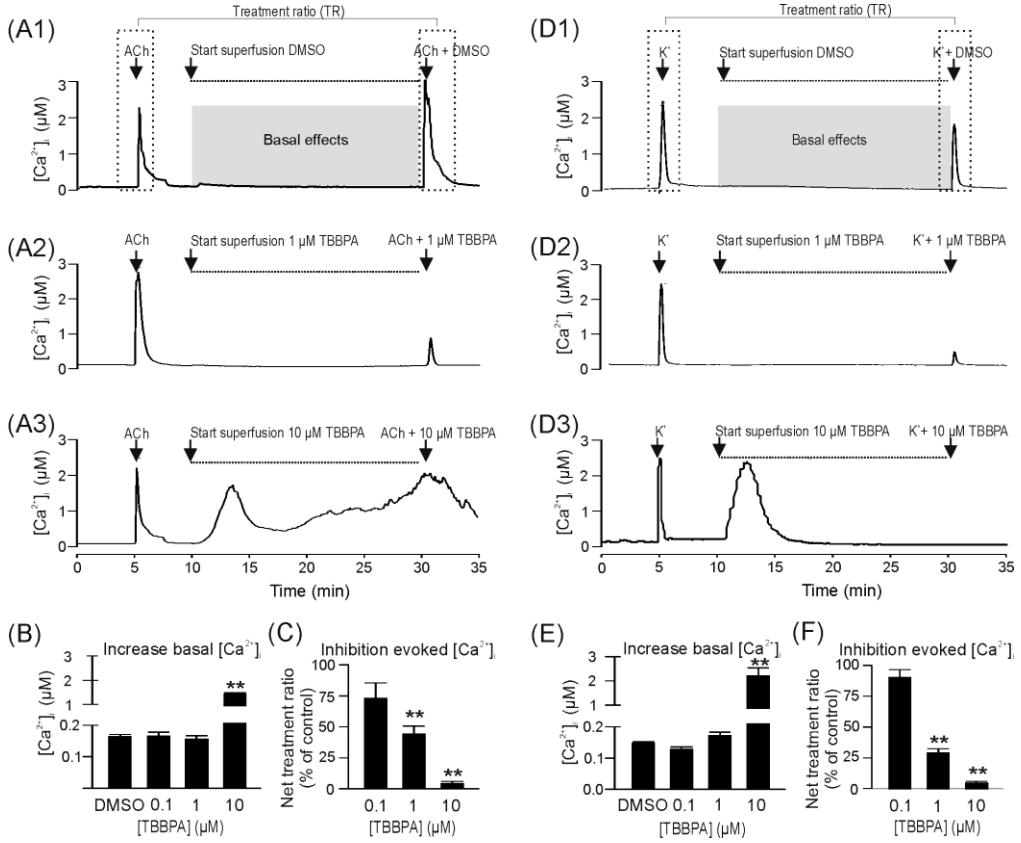

Figure 2. Example recordings of single cell $[Ca^{2+}]_i$ imaging from B35 (A) and PC12 (D) cells exposed to DMSO or 1-10 µM TBBPA in between two 15s stimulations. Bar graphs illustrate the concentration-dependent TBBPA-induced increase in basal $[Ca^{2+}]_i$ (B and E) and inhibition of the ACh- (C) or K^+-evoked (F) increase in $[Ca^{2+}]_i$ expressed as TR normalized to DMSO-exposed control cells. n = 29-88 cells, ** p < 0.001 versus control.

The TBBPA-induced increase in basal $[Ca^{2+}]_i$ in B35 and PC12 cells, was reduced under Ca^{2+}-free conditions (not shown), indicating that the TBBPA-induced increase in basal $[Ca^{2+}]_i$ depends to a large degree on influx of extracellular Ca^{2+}, but likely originates from the release of Ca^{2+} from intracellular stores, such as endoplasmic reticulum (ER) and mitochondria. Following depletion of both the ER and mitochondria by combined exposure to TG and FCCP prior to exposure to 10 µM TBBPA, almost completely attenuated the TBBPA-induced increase in basal $[Ca^{2+}]_i$ (not shown), suggesting that the small, remaining increase in basal $[Ca^{2+}]_i$ is probably due to release of Ca^{2+} from other intracellular stores, such as the nucleus and secretory vesicles. The TBBPA-induced (≥10 µM) increase in basal $[Ca^{2+}]_i$ in both B35 and PC12 cells (Figure 2A, B, D and E) is in line with previous studies on cerebellar granule cells, granulocytes and Sertoli cells[4-6] and indicates this is a general mechanism of action of TBBPA. Noteworthy, comparable store-mediated Ca^{2+} release was observed following exposure to PCBs and PBDEs[16-17].

To ensure that these effects are not confounded by acute cytotoxicity, B35 and PC12 cells were exposed to 1-100 μM TBBPA for 24 h to determine effects on cell viability using a combined AB and NR assay. Cell viability was reduced only at 100 μM (not shown), though at ≥10 μM, TBBPA also increased apoptosis-related caspase-3 activity at 24 h (not shown). Similarly, ROS production in B35 or PC12 cells was increased by TBBPA only at ≥10 μM after 24h (not shown). These findings thus indicate that the observed acute TBBPA-induced neurotoxic effects *in vitro* at concentrations up to 10 μM are not confounded by acute cytotoxicity.

The margin of safety between the LOECs derived from our *in vitro* study and concentrations found in human serum of non-occupational exposed adults ~3 orders of magnitude. However, High concentrations of TBBPA have been found in breast milk (up to 4.1 ng/g lw) and cord serum (up to 103.5 ng/g lw)[18-20], which corresponds with ~2 nM in blood. These cord serum values are less than two orders of magnitude below the LOECs of the most sensitive endpoint observed in this study (partial agonistic effect on the $GABA_A$ receptor, LOEC 100 nM), indicating a possible risk for newborns and their developing brain. Moreover, our *in vitro* experiments focused on acute exposure, while human exposure is in general chronic with concentrations that may increase over time. Finally, TBBPA has several modes of action (potentiation and activation of $GABA_A$ receptors, inhibition of nACh receptors and VGCCs, ER-mediated Ca^{2+} release) that are shared by PCBs and PBDEs. Since several studies suggested that POPs, including PCBs and PBDEs, may exert additive neurotoxic effects[8-9,21-22], TBBPA and other POPs may consequently interact at these targets and potentially cause additive effects *in vivo*.

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