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Original Research

Acute Cytotoxicity Evoked by Tetrabromobisphenol A in Primary Cultures of Rat Cerebellar Granule Cells Outweighs the Effects of Polychlorinated Biphenyls

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Abstract

The aim of this study was to identify, among selected environmental toxins, the substance with the highest *in vitro* toxicity to neurons combined with the most marked induction of calcium imbalance, oxidative stress and mitochondrial dysfunction. Exposure of primary cultures of rat cerebellar granule cells for 30 min to polychlorinated biphenyls (PCBs) or brominated flame retardants (BFRs) at concentrations of 10-50 μM identified tetrabromobisphenol A as the compound with the highest toxicity. At a concentration of 25 μM, apart from the moderate activation of ^{45}Ca uptake, this BFR induced the most pronounced increase in intracellular Ca^{2+} concentration, depolarization of mitochondria, and activation of ROS production.

Keywords: brominated flame retardants, calcium, neurotoxicity, mitochondrial dysfunction, oxidative stress, TBBP-A

Introduction

Several polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs) used extensively in industry are well known environmental pollutants [1-4]. Many of these compounds are reported to be neurodevelopmental toxins, which might also induce damage to the adult nervous system [5-9]. The exact mechanisms causing the neurotoxic effects of these substances are not clear. PCBs that mimic the planar structure of dioxin (coplanar or dioxin-like PCBs) are agonists for the arylhydrocarbon hydroxylase receptor (AhR), and so may regulate the expression of dioxin-responsive genes [10]. However, non-dioxin-like PCB congeners with a non-coplanar structure are also highly toxic despite having no affinity for AhR [11]. Some reports suggest that, in this case, their neurotoxicity is due to excitotoxic mechanisms mediated by activation of NMDA receptors and excessive extracellular Ca^{2+} influx to neurons, as well as oxidative stress [12-18]. The results of other studies indicate that interference with intracellular Ca^{2+} buffering, either combined with the influx of extracellular calcium [19, 20], or just the release of intracellular

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Ca^{2+} from ryanodine-sensitive stores in the endoplasmic reticulum [21, 22], may play an important role in the toxic effects of some PCBs and also BFRs. The latter scenario to explain the neurotoxicity of these substances seems to be particularly novel and interesting. This effect has been well documented for non-coplanar PCBs [21], but only indirect evidence indicates that it is responsible for the neurotoxic effects of tetrabromobisphenol A (TBBP-A), a BFR [22, 23]. Although cytotoxic effects of this compound have been described [18], it is not clear whether TBBP-A toxicity to neuronal cultures is comparable with that caused by other BFRs or PCBs.

The role of calcium in cytotoxicity induced by PCBs and BFRs should not be considered in separation from the other important toxic mechanisms, including oxidative stress and mitochondrial dysfunction. Previous studies have demonstrated that PCBs and TBBP-A enhance the production of reactive oxygen species (ROS) [18, 24-26]. There are also data pointing to a role for mitochondrial dysfunction in the cytotoxicity of PCBs [27, 28]. To our knowledge, these toxic mechanisms have been examined in relation to TBBP-A-induced hepatotoxicity [29], but they have not been studied in cultured neurons treated with this compound.

The present *in vitro* study using primary cultures of rat cerebellar granule cells (CGC) was designed to examine the cytotoxicity and related biochemical endpoints induced by TBBP-A, in comparison with other bioactive BFRs and PCBs. The primary aim was to identify those substances with particularly high toxicity to neurons accompanied by the ability to disturb calcium homeostasis and mitochondrial membrane potential, and to induce ROS production.

Experimental Procedures

Five PCBs and BFRs were selected for examination: aroclor 1254 (PCB 1254), 2,2',3,5',6-pentachlorobiphenyl (PCB 95), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), hexabromocyclododecane (HBCD), and tetrabromobisphenol A (TBBP-A). Samples of these compounds (technical grade) were provided by LCG Standards Sp. z o.o. (Łomianki, Poland). L-glutamate (Glu), dimethyl sulphoxide (DMSO), fetal calf serum, and the materials for cell culture were purchased from Sigma Chemical Company (St. Louis, MO, USA). Fluo-3/AM, rhodamine 123, 6-carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA), and the Live/Dead® Viability/Cytotoxicity Kit for mammalian cells containing calcein and ethidium homodimer-1, were obtained from Molecular Probes Inc. (Eugene, OR, USA). All other chemicals were of analytical grade. ^{45}Ca was purchased from the Radioisotope Research Development Centre (Świerk, Poland).

Neuronal cultures were prepared from Wistar rat pups (50 animals in total) by a procedure approved by the Fourth Local Ethical Committee in Warsaw. All efforts were made to reduce the number of animals used and to minimize suffering, in accordance with domestic and international regulations concerning experiments using animals (Journal of Law 1997, No. 111, item 724, Journal of Law 2005, No. 289, item 2143 and EC Directive 86/609/EEC of 24 November 1986).

Primary cerebellar granule cells (CGC) were prepared from 7-day-old rats and cultured according to the method of Schousboe et al. [30] with slight modifications as described previously [31, 32]. Briefly, the rat pups were decapitated, and after separation of the vessels the collected cerebella were cut into 400-μm cubes. The tissue was incubated for 15 min at 37ºC in ionic buffer containing 0.025% trypsin and 0.05% DNase 1. The incubation was terminated by the addition of trypsin inhibitor (0.04%) and centrifugation. The cells were then separated by trituration of the pellet and further centrifugation. A suspension of the cells in basal medium Eagle (BME) supplemented with 10% fetal calf serum (Sigma), 25 mM KCl, 4 mM glutamine, streptomycin (50 μg/ml) and penicillin (50 U/ml) was used to seed 6-, 12- or 24-well plates coated with poly-L-lysine (NUNC), at a density of 4, 2 or 1×10^6 cells per well, respectively. A high concentration of potassium ions was maintained in the growth medium to prevent the rapid development of apoptosis in the cultured neurons [33], while 7.5 μM cytosine arabinofuranoside was added to the cultures 36 h after plating to prevent the replication of non-neuronal cells. The CGC were used in experiments after 7 days *in vitro* (DIV).

In the experiments evaluating the acute neurotoxicity of PCBs and BFRs, and their effects on calcium homeostasis, mitochondrial membrane potential, and ROS production, Locke ionic media were used. Locke 5 buffer contained 154 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5 mM HEPES, pH 7.4, and 5 mM glucose, while in the Locke 25 buffer the concentration of KCl was increased to 25 mM and that of NaCl was reduced to 134 mM. To minimize changes in the incubation conditions, Locke 25 buffer containing the same concentration of potassium ions as the growth medium was used in the toxicity tests. Pilot experiments demonstrated that the application of high potassium incubation medium in the experiments evaluating calcium homeostasis promoted neuronal depolarization with the activation of NMDA receptor-mediated calcium transients. Since these effects masked the responses to the applied PCBs and BFRs, Locke 5 buffer was used in the mechanistic studies.

The toxicity of the selected PCBs and BFRs was tested by replacing the growth medium (BME) with Locke 25 buffer containing freshly prepared solutions of the compounds and the vehicle (DMSO, which never exceeded 0.5% in experiments), as required. After incubation at 37ºC for 30 min, acute exposure to the tested substances was terminated by two washes with Locke 25 buffer. The cells were then cultured in the original growth medium for a further 24 h under standard conditions. A two-color fluorescence cell viability test was used for the simultaneous staining of live cells with calcein (green fluorescence) and the dead cells with ethidium homodimer-1 (red fluorescence), as described previously [34]. The CGC were incubated for 10 min with ethidium homodimer-1 (2 μM) and calcein-AM (1 μM), then washed in 0.5 mL of Locke 25 buffer.

Fluorescence from calcein (ex/em 480/530 nm) and from ethidium homodimer-1 (ex/em 520/620 nm) was measured using a FLUOstar Omega multidetection microplate reader (BMG Labtech GmbH, Offenburg, Germany).

For measurements of ^{45}Ca uptake by neurons, the growth medium was replaced by Locke 5 buffer and the cells were preincubated for 10 min at 37ºC. Then $^{45}CaCl_2$ (1 μCi/well) together with the tested substance or 0.5% DMSO (vehicle control) were added, and the cells were incubated for 10 min. As a positive control, 100 μM Glu was applied instead of the tested compounds. The experiment was terminated by removing the medium and washing the cells three times with ice-cold glucose- and $CaCl_2$-free medium containing 2 mM EGTA. The cells were then solubilized by treatment with ice-cold 0.5 M NaOH for 30 min and the radioactive signal was measured by liquid scintillation spectrometry using a Wallac 1409 counter (Wallac, Turku, Finland).

To measure changes in the intracellular Ca^{2+} concentration, mitochondrial membrane potential and the generation of ROS, the CGC were pre-incubated for 30 min in Locke 5 buffer and loaded with the fluorescent probes 16 μM fluo-3/AM, 10 μM rhodamine123 or 100 μM 6-carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA), respectively, as described previously [31, 35, 36]. After washing with Locke 5 buffer, the cells were then incubated in this medium containing the tested BFRs and PCBs. In measurements of the intracellular Ca^{2+} concentration and ROS production, 100 μM Glu and 10 μM H_2O_2 were applied as positive controls, respectively. Over a 30-min period, changes in fluorescence were recorded every 60 s for fluo-3, every 90 s for DCF, or every 5 min for rhodamine 123, using a FLUOstar Omega multidetection microplate reader (ex/em 485/538 nm).

All results are presented as the mean±SD (n = 4-6, precise values given in the figure legends). One-way ANOVA followed by Dunnett's test was used for comparisons between groups. Significance was taken at $P<0.05$.

Results

The data presented in Fig. 1 indicate that the acute cytotoxicity of the selected PCBs and BFRs tested in this study was variable, although at a concentration of 50 μM, all of them significantly reduced CGC viability. Incubation with PCB 1254, PCB 95, and PCB 153 at 50 μM resulted in a decrease in the number of live cells to 63±12%, 65±6% and 53±11% of the original value, respectively; while the two BFRs, HBCD and TBBP-A, applied at the same concentration, reduced the viability of CGC to 48±4% and 17±2%, respectively. Moreover, the tendency of PCB 1254 to produce a toxic effect was greatly reduced at concentrations of 10 and 25 μM, whereas PCB 95, PCB 153, and HBCD applied at these concentrations produced only moderate toxicity. The most prominent concentration-dependent cytotoxicity was induced by TBBP-A, which showed a tendency to reduce the number of live cells at a concentration of 10 μM, but was significantly toxic at 25 and 50 μM (Fig. 1).

Disturbances in calcium homeostasis were studied using two different approaches. Enhanced ^{45}Ca uptake reflects stimulation of the influx of extracellular Ca^{2+} into neurons, which may result from the activation of various ionic channels, particularly the NMDA receptors and voltage-sensitive calcium channels. In turn, the increase in the intracellular concentration of Ca^{2+} may be the result of calcium influx into neurons and/or its release from intracellular stores. Both of these processes have been implicated in the mechanisms of neuronal damage induced by several toxins, including PCBs and BFRs. The results presented in Fig. 2A show the effects of the tested substances, applied at a concentration of 25 μM, on ^{45}Ca uptake by CGC. They demonstrate the lack of any effect of PCB 153, and moderate enhancements of calcium uptake evoked by PCB 95, PCB 1254, HBCD, and TBBP-A. However, the stimulatory effects on ^{45}Ca uptake induced by these PCBs and BFRs represented only a minor fraction of that evoked by 100 μM Glu in the positive control (Fig. 2A). Fig. 2B presents the results of the fluorimetric evaluation of changes in the intracellular Ca^{2+} level evoked by PCBs and BFRs applied at a concentration of 25 μM, in comparison with the effect of 100 μM Glu. Changes in fluo-3 fluorescence (Fig. 2 B) are indicative of alterations in the intracellular Ca^{2+} concentration. These data revealed the lack of any effect of PCB 153 and PCB 95 in comparison with the vehicle control (0.5% DMSO), which contrasted with the very rapid (within one minute) and prominent rise in fluorescence evoked by Glu, and less pronounced effects of TBBP-A, PCB 1254, and HBCD. After this initial increase in the intracellular Ca^{2+} concentration there was a slight decrease in the CGC treated with PCB 1254 and HBCD, whereas in the cells incubated with TBBP-A and Glu, the rise in fluorescence was sustained. It is also noteworthy that the increase in the intracellular Ca^{2+} level evoked by PCB 1254 was disturbed by

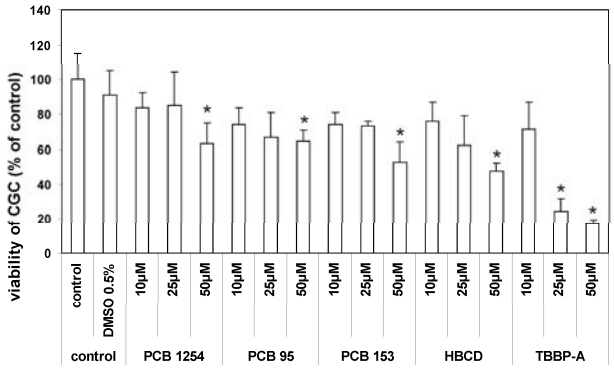

Fig. 1. Acute cytotoxic effect of select PCBs and BFRs in primary cultures of rat CGC. Cultured neurons were exposed for 30 min to PCB 1254, PCB 95, PCB 153, HBCD, and TBBP-A administered at concentrations as indicated, then the growth medium was restored and incubation continued for 24 h. The viability of neurons was assessed by calcein and ethidium homodimer-1 staining. Data represent the number of live cells presented as a percentage of the vehicle control value (means±S.D.; n = 6).

* Values significantly different from the vehicle control (0.5 % DMSO; $p<0.05$).

occasional fluctuations. In summary, the results of these experiments demonstrated that TBBP-A, at a concentration of 25 μM in comparison to other PCBs and BFRs tested, induces the most pronounced increase in intracellular Ca^{2+}, which may result from its influx from the extracellular space and the mobilization of intracellular stores.

To detect changes in the mitochondrial membrane potential, the fluorescent probe rhodamine 123 was used. This indicator accumulates in polarized mitochondria, while its efflux into the cytosol, which results in increased fluorescence, reflects the depolarization of mitochondria. As presented in Fig. 3A, the application of different PCBs and BFRs induced distinct changes in rhodamine 123 fluorescence. A rapid and highly pronounced increase was induced by 25 μM TBBP-A, and this was followed by a plateau of fluorescence after 10 min. Following application of 25 μM PCB 1254 there was an initial decrease in fluorescence, while the early responses induced by PCB 95,

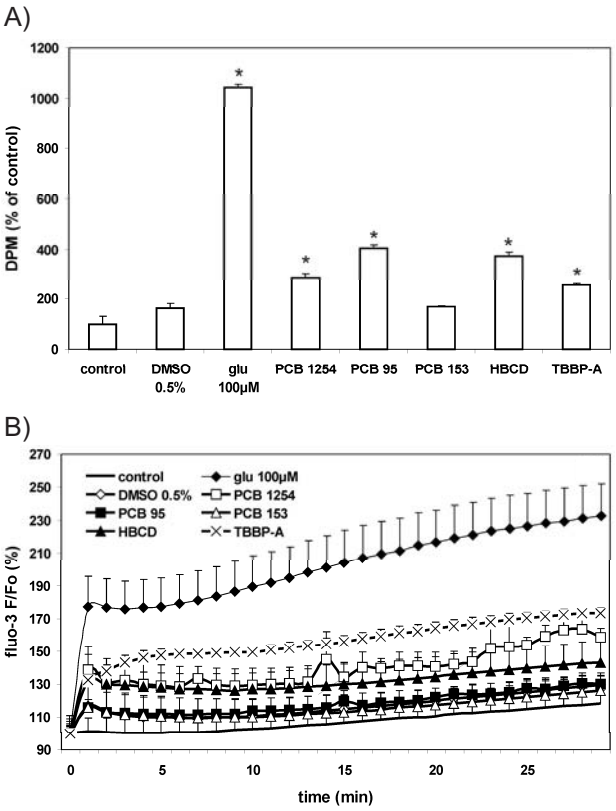

Fig. 2. Disturbances of calcium homeostasis in CGC induced by selected PCBs and BFRs. Effects of 25 μM PCB 1254, PCB 95, PCB 153, HBCD, and TBBP-A in comparison with 100 μM glutamate (glu) on ^{45}Ca uptake, expressed as dpm/culture (A), and on intracellular Ca^{2+} concentration evaluated by measuring fluo-3 fluorescence and expressed as the percentage of the basal level (B). Data represent mean values±S.D. (n = 6 in panel A, n = 4 in panel B).

* Values presented in graph A are significantly different from the vehicle control (0.5% DMSO; $p<0.05$).

Values in graph B representing the effects of glu (from the 1st minute) and TBBP-A (from the 5th minute) to the end of the experiment, are significantly different from the corresponding controls ($p<0.05$) and the other substances tested. The data representing the effects of HBCD and PCB 1254 are significantly different from the vehicle control.

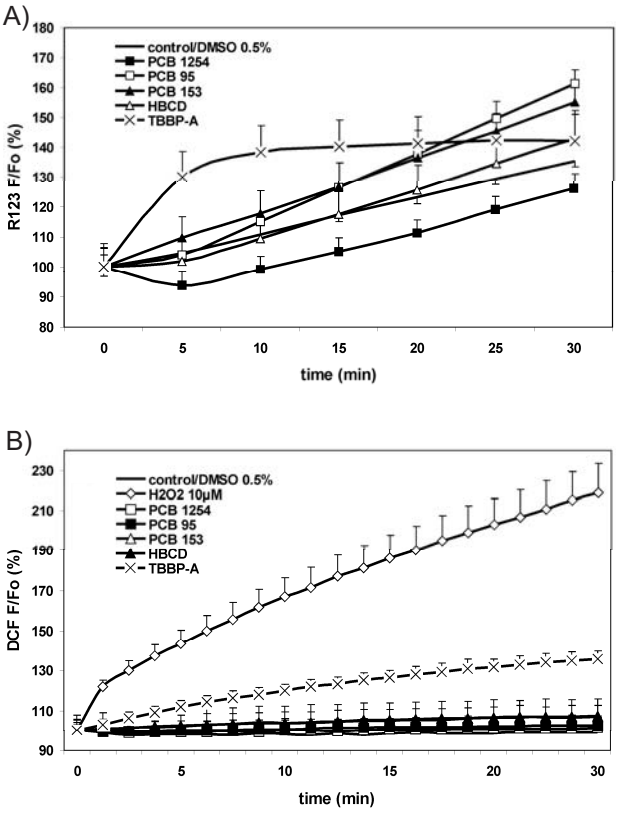

Fig. 3. Effects of selected PCBs and BFRs on mitochondrial membrane potential (A) and ROS production (B), assessed with the fluorescent probes rhodamine 123 and DCFH-DA, respectively. The tested substances were 25 μM PCB 1254, PCB 95, PCB 153, HBCD, and TBBP-A. Hydrogen peroxide (10 μM) served as a positive control for ROS production (graph B). The data, expressed as a percentage of the basal level, represent mean values±S.D. (n=4). The values for TBBP-A in graph A are significantly different from the vehicle control (0.5 % DMSO; $p<0.05$) and the other substances tested, up to the 10th min of incubation. Also, the data representing PCB 1254 are significantly different from the vehicle control and the other substances tested. Data for H_2O_2 and TBBP-A on graph B are significantly different from the corresponding controls and the other substances tested ($p<0.05$).

PCB 153, and HBCD, administered at the same concentration, were negligible. However, after 5 min of incubation with these compounds, a linear increase in fluorescence was observed in all cases, but this also occurred with the vehicle control (Fig. 3A). Thus, rapid and highly pronounced depolarization of CGC mitochondria appears to be a characteristic feature of TBBP-A-induced cytotoxicity.

ROS production induced by application of the selected PCBs and BFRs at a concentration of 25 μM was evaluated using the dye DCFH-DA. After its accumulation and oxidation inside CGC, fluorescent DCF is released. Treatment of the cells with 10 μM hydrogen peroxide, which was used as a positive control, induced a rapid rise in ROS production. The application of TBBP-A resulted in an immediate, but significantly less pronounced rise in DCF fluorescence. This effect continued to the end of the observation period. The changes in fluorescence induced by the

other compounds investigated in this study were negligible (Fig. 3 B). These results demonstrated the particularly high oxidative stress-inducing potential of TBBP-A.

In order to identify possible causal relationships between the events observed in this study, the correlation between the parameters representing them was examined for all groups. Data from experiments with the 25 μM concentration of PCBs and BFRs, and the vehicle and naïve controls were analyzed. Significant correlations (at $p<0.05$; $n=42$) were detected as follows. For the percentage of live cells and:

(a) the increase in intracellular calcium level, r = -0.52
(b) the rise in rhodamine 123 fluorescence r = -0.57
(c) the rise in DCF fluorescence r = -0.56

For the increase in ^{45}Ca uptake and the increase in intracellular calcium level, r = 0.46. For the increase in intracellular calcium level and:

(a) the rise in rhodamine 123 fluorescence, r = 0.55
(b) the rise in DCF fluorescence, r = 0.81

For the rise in rhodamine 123 fluorescence and the rise in DCF fluorescence, r = 0.89.

Discussion of Results

The results of these *in vitro* experiments using primary rat CGC cultures demonstrated that cytotoxicity and the ability of TBBP-A to induce disturbances in calcium homeostasis were far greater than those of the other BFRs and PCBs that were tested. In other words, TBBP-A appeared to be particularly cytotoxic, and this toxicity was accompanied by a large increase in the intracellular calcium level, which may be ascribed to the influx of extracellular calcium into neurons and to its release from intracellular stores. TBBP-A was also the compound inducing by far the most substantial depolarization of mitochondria and the greatest enhancement of ROS production.

In these experiments we employed primary cultures of rat CGC as the *in vitro* model. These cells have previously been used in a number of studies concerning general neurobiological and neurotoxicological subjects [30, 37], including the toxicity of PCBs and BFRs [16, 18, 27, 38]. Our previous studies have confirmed that CGC is a suitable model for mechanistic studies on toxicity in neuronal cells, excitotoxic challenges, calcium imbalance, and mitochondrial dysfunction [31, 32, 39].

One of the main aims of this study was to test a few different PCBs and BFRs to select the most promising compounds for subsequent mechanistic studies on the role of calcium imbalance in neurotoxicity. Based on previous circumstantial evidence [18, 22, 23], we assumed that TBBP-A may be a likely candidate. The potential mechanisms responsible for the disturbances in calcium homeostasis induced by PCBs and BFRs may be complex. On the one hand, there are data indicating that both PCBs and BFRs induce excitotoxicity and oxidative stress as a result of excessive activation of the excitatory amino acid receptors and influx of calcium from the extracellular space. These results were mainly obtained in *in vitro* studies using primary cultures of rat CGC. Using this experimental model, Mariussen et al. [24] demonstrated that accumulation of ROS and neuronal death induced by a mixture of PCBs (aroclor) was significantly reduced in the presence of the non-competitive NMDA receptor antagonist MK-801, thus indicating a role for these receptors and Ca^{2+} influx in the neurotoxic effects of these substances (see also the review [25]). Also using the CGC model, Reistad et al. [18] showed that TBBP-A could induce neurotoxicity combined with an MK-801-sensitive increase in the intracellular calcium level and cytotoxicity. On the other hand, the role of calcium release from intracellular stores in the neurotoxic effects of many non-coplanar PCBs has been repeatedly demonstrated (for a recent review see Pessah et al. [21]). It should be noted that there has been some controversy concerning the involvement of ryanodine and/or IP3 receptors in PCB-induced intracellular calcium release [40, 41]. Until recently, no such data were available regarding the mobilization of Ca^{2+} in neurons by BFRs, although intracellular calcium release evoked by TBBP-A was demonstrated in the TM4 Sertoli cell line [22].

Previous studies have found variations in the concentration dependence of the toxic effects of TBBP-A in different cell types. In rat hepatocytes, TBBP-A toxicity was observed at concentrations of 0.25-1.0 mM, while an uncoupling effect on isolated hepatic mitochondria was observed in the range 5-25 μM [29]. Ogunbayo et al. [22], using a cultured Sertoli cell line, observed increases in the intracellular Ca^{2+} level at TBBP-A concentrations of 5-60 μM, and cell death at an LC_{50} of 18 μM. Reistad et al. [18] found that TBBP-A at low micromolar concentrations induced cell death, calcium imbalance, and oxidative stress in CGC. In another study by Reistad et al. [42], another BFR, HBCD, also induced death of CGC at low micromolar concentrations, but failed to affect ROS formation or to elevate the intracellular Ca^{2+} level. In the present study, we compared the effects of TBBP-A and three non-coplanar PCB congeners, which were selected because they are known to have different effects on cell viability, calcium homeostasis and cellular signaling. PCB 1254 was previously shown to induce cytotoxicity and intracellular calcium release in dopaminergic cells when applied at a concentration of about 30 μM [43]. PCB 95 applied to CGC at low micromolar concentrations interfered with ryanodine receptors and enhanced their calcium responses to caffeine, but did not alter the resting calcium level in the cytosol [41]. PCB 153 at an EC_{50} of 120 μM was found to be cytotoxic and impaired the function of the glutamate-NO-cGMP signaling pathway in cultured cerebellar neurons [15], and at a concentration of 30 μM it decreased the mitochondrial membrane potential in kidney cell cultures [28]. Bearing in mind the significant differences in the concentrations of particular PCBs and BFRs used in previous studies, we applied them at concentrations of 10, 25, and 50 μM for the viability tests and a concentration of 25 μM was used in mechanistic studies. We anticipated that this strategy would ensure that any effects of the studied substances would not be missed because the concentrations used were too low.

In previous studies in which TBBP-A toxicity was associated with increases in the intracellular calcium concentration, the effects of this compound were investigated by applying it to cells over a prolonged 18-24 h incubation, while the intracellular calcium level was measured in the course of acute exposure to TBBP-A lasting several minutes [18, 22]. This protocol may have produced discrepancies between the results of the cytotoxicity tests and the mechanistic experiments. In fact, it was previously reported [18] that TBBP-A-evoked glutamate release from neurons to the incubation medium – which may be responsible for the excitotoxic component of TBBP-A cytotoxicity in neurons – develops after prolonged incubation of CGC with this toxin (at least 90 min). Thus, to standardize the experimental conditions employed in our study, the evaluation of PCB and BFR toxicity, as well as measurements of calcium and other biochemical indicators, were performed using an acute exposure of neuronal cultures to these toxins, lasting 30 min. However, there is no ideal solution, since secondary mechanisms influencing the survival of neurons might develop during the 24-h delay in evaluating neuronal viability after acute exposure of the cells to the tested substances.

The data recorded in the present study demonstrated that although all of the tested PCBs and BFRs induced a pronounced cytotoxicity when applied at the high concentration of 50 μM, only TBBP-A produced very pronounced neurotoxicity at lower concentrations. In general, our results are consistent with those of previous studies demonstrating the toxic effects of different PCBs and BFRs [14, 15, 42, 43], and highlighting the particularly high cytotoxicity of TBBP-A [12, 18]. In the present study, we intended to evaluate the role of calcium imbalance, mitochondrial dysfunction, and oxidative stress in the cytotoxicity of the studied substances. Our finding that enhanced toxicity of TBBP-A was accompanied by a significantly increased intracellular Ca^{2+} level is in agreement with previously published data concerning neuronal and non-neuronal cells [12, 18]. The authors of these studies suggested different explanations for this observation. According to Reistad et al. [18], the stimulation of glutamate receptors in TBBP-A-treated cells induces the influx of extracellular calcium, whereas Ogunbayo et al. [12] proposed that TBBP-A induces Ca^{2+} release from ryanodine-sensitive stores. In our experiments, TBBP-A activated ^{45}Ca uptake, thus the increased calcium influx from the extracellular space may participate in the mechanism underlying the observed phenomenon. However, based on the corresponding effects of 100 μM Glu, which produced much more pronounced ^{45}Ca uptake than TBBP-A, but caused a comparable rise in the intracellular calcium concentration, it may be speculated that the participation of the release of intracellular Ca^{2+} in TBBP-A-induced calcium transients is substantial.

Apart from TBBP-A, two other substances, namely PCB 1254 and HBCD, also significantly increased the intracellular calcium concentration in CGC. Moreover, these substances simultaneously stimulated ^{45}Ca uptake. The effects of PCB 1254 appear to be particularly interesting. Both Mariussen et al. [24] and Lee and Opanashuk [44] presented data showing that oxidative stress plays a key role in its cytotoxic effects, whereas we found that ROS production in CGC induced by this substance hardly differed from the vehicle control. Possibly the stabilization of the mitochondrial membrane potential by PCB 1254 observed in our study could be responsible for low ROS production, which contrasts with its high toxicity and the significant accumulation of ^{45}Ca it evokes. Indeed, inhibition of the mitochondrial permeability transition by PCB 1254 has been described previously [45], although its mechanism and relationship to neurotoxicity remain unclear. Another intriguing feature of PCB 1254 that we observed, is its ability to induce oscillations of the intracellular Ca^{2+} level in CGC. A similar effect of this compound was described previously in developing neuronal cells [17], and it may be speculated that these oscillations might reflect cyclic accumulation of Ca^{2+} by mitochondria and then its release to avoid the induction of mitochondrial permeability pores. Our data demonstrating a significant increase in the intracellular calcium level in CGC challenged with 25 μM HBCD are at odds with those of Reistad et al. [42], who failed to detect the same effect using this compound at low micromolar concentrations. Most probably this discrepancy arose from the use of different concentrations of the tested substance. The non-coplanar PCBs, PCB 95 and PCB 153, which, according to the literature, interfere with ryanodine receptor proteins in different ways, or have no effect on the steady-state calcium level in neurons [15, 21, 41], appeared not to interfere with intracellular Ca^{2+} levels in our experiments. Data from previous studies suggest that these compounds disturb neuronal calcium homeostasis only under certain conditions. PCB 153 appeared not to be involved in disturbances of neurobehavioral development [46], and the neurotoxic effects of this compound were altered in experiments where neurons were challenged with a combination of this PCB and methyl mercury [47, 48]. PCB 95 was found to induce Ca^{2+} release *via* ryanodine receptors under specific conditions, i.e. in the embryonic CGC, after stimulation of these receptors by caffeine or in the malignant hypothermia mutation [41, 49].

The potential of TBBP-A to activate the formation of ROS, which was demonstrated in the present study, has previously been described in *in vitro* studies using human neutrophil granulocytes and CGC [18, 50]. It was also demonstrated *in vivo* in animal models [51, 52]. These previous studies showed that excitotoxicity and enhanced calcium uptake evoked by NMDA activation are not critically involved in this phenomenon, since TBBP-A-evoked enhanced ROS production in CGC appeared to be insensitive to MK-801, an antagonist of these receptors [18]. The lack of significant activation of ROS production in CGC by HBCD or PCB 95, that we observed, is consistent with the literature [42]. Both PCB 153 and PCB 1254, which in our study exhibited an oxidative stress-inducing potential significantly weaker than TBBP-A, are known to enhance ROS production in different cell types [24, 53].

A unique property of TBBP-A that was identified in the present study was its ability to induce a prominent decrease in the mitochondrial membrane potential when applied at a

concentration of 25 μM. Based only on these preliminary data, it is difficult to speculate if and to what extent this effect may be secondary to the increase in the intracellular calcium level resulting in mitochondrial calcium overload. This finding begs the question, what is the mutual relationship between TBBP-A-induced mitochondrial depolarization and enhanced ROS production? Oxidative stress and/or calcium accumulation in the mitochondrial matrix may result in fatal mitochondrial dysfunction [54, 55]. However, primary mitochondrial dysfunction leads to enhanced ROS production and oxidative stress [56]. Nakagawa et al. [29] observed TBBP-A-induced dysfunction of isolated rat liver mitochondria, which could be the underlying cause of subsequent lipid peroxidation. Consequently, the depolarization of mitochondria in CGC may represent the earliest step in TBBP-A toxicity. Oxidants, as well as proteins formed in mitochondria, perform the role of mediators of the molecular signaling leading to apoptosis [57]. These data are consistent with the previous finding that CGC challenged with TBBP-A exhibited morphological and molecular hallmarks of an atypical form of apoptosis [18].

An analysis of the relationships between the most important parameters measured in this study revealed only relatively weak correlations between ^{45}Ca uptake and the increase in intracellular calcium level. This result seems not to support an important role for calcium influx into neurons in the toxic effects of PCBs and BFRs. Cytotoxicity was correlated with the increase in intracellular calcium level, ROS production and mitochondrial dysfunction, although r values vere close to 0.5. However, we identified a rather strong correlation ($r>0.8$) between the increase in intracellular calcium level and ROS production and between ROS production and the biochemical indicator of mitochondrial dysfunction. There are well known mutual connections between intracellular calcium, mitochondrial dysfunction and oxidative stress [54-57], and our results highlight the role of this interrelationship in the cytotoxicity of PCBs and BFRs. Still, caution should be exercised in drawing conclusions concerning a common primary mechanism of their toxicity. There are differences between individual compounds in their targets and mechanisms of cytotoxic activity.

Although PCBs have been banned from production and use in the majority of developed countries because of their recognized toxicity, these compounds continue to pollute the environment, and this is the incentive for numerous ongoing neurotoxicological and behavioral studies. It is known that prenatal exposure to PCBs can result in neurodevelopmental deficits [58, 59]. Other studies have demonstrated cognitive deficits in adult animals treated with PCBs [60]. For example, we recently found that the administration of PCB 1254 to young adult rats at doses of 10 mg/kg b.w. per day for 2 weeks resulted in the development of neurochemical and morphological indicators of a very pronounced hippocampal lesion [61]. The toxic effects of BFRs have also previously been underestimated. Data on the no observed adverse effect level (NOAEL) of TBBP-A presented by ICPS/WHO indicated that for i.p. administration of this compound for 4 weeks or even 3 months, the value was 50-100 mg/kg b.w. for rats, and 700 mg/kg b.w. for mice [52]. This finding subsequently discouraged *in vivo* behavioral and neurotoxicological studies on TBBP-A. However, the annual global production of TBBP-A is very high (approximately 170 million kg), and the contamination of water and soil with this substance is increasing considerably, which has raised concerns about the environmental and occupational risks it causes [53, 62]. It was recently reported that chronic exposure of fish to environmentally relevant concentrations of TBBP-A and HBCD induced endocrine disturbances and accumulation of these BFRs in muscle [63]. Other data have demonstrated various behavioral deficits in mice acutely treated with TBBP-A at doses of 0.1 or 5 mg/kg b.w., which were accompanied by selective accumulation of TBBP-A in the striatum [64]. Other reports showing *in vitro* TBBP-A cytotoxicity [12, 18] and the results of the present study indicate the need for further *in vivo* behavioral and neurotoxicological studies to characterize the activities of BFRs, and particularly of TBBP-A, as potentially dangerous environmental neurotoxins.

Conclusions

The screening tests reported here identified TBBP-A as a substance of particularly strong and interesting toxic capacity, which outweighed the cytotoxic potential of the other PCBs and BFRs tested. The mechanism of TBBP-A-induced toxicity appears to be complex. Our results demonstrated calcium imbalance resulting from the influx of Ca^{2+} into neurons and its release from intracellular stores, plus the depolarization of mitochondria and enhanced ROS production in CGC treated with TBBP-A. Further investigations are required to identify any causative relationships between these phenomena and to identify the primary mechanism of TBBP-A cytotoxicity in CGC.

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