Toxicology and Applied Pharmacology **183,** 153–159 (2002)
doi:10.1006/taap.2002.9482

Vesicular Catecholamine Release from Rat PC12 Cells on Acute and Subchronic Exposure to Polychlorinated Biphenyls

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Received January 15, 2002; accepted July 11, 2002

Vesicular Catecholamine Release from Rat PC12 Cells on Acute and Subchronic Exposure to Polychlorinated Biphenyls. Westerink, R. H. S., and Vijverberg, H. P. M. (2002). *Toxicol. Appl. Pharmacol.* **183, 153–159.**

Effects of selected polychlorinated biphenyls (PCBs) on vesicular catecholamine release from rat PC12 phaeochromocytoma cells have been measured using carbon fiber microelectrode amperometry. Exocytotic responses were evoked by superfusion of single PC12 cells with high K^+ saline. Subsequent exposure of the same cells to saline containing the nonplanar congener 2,2′-dichlorobiphenyl (PCB 4) and the coplanar congener 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) at concentrations between 5 and 25 μM for 15 min caused an enhancement of the frequency of basal vesicular catecholamine release at the lower concentrations but not at the high concentrations tested. The nonplanar congener 2,2′,3,3′,4,4′-hexachlorobiphenyl (PCB 128) did not affect basal release. The PCBs caused only marginal effects on the frequency of evoked events during high K^+ stimulation and did not affect vesicle contents. Prolonged exposure of PC12 cells to low concentrations of the same PCBs in the culture medium for a period of 3 days did not cause significant changes in vesicle contents. The results demonstrate that low concentrations of PCBs may cause acute vesicular catecholamine release but do not influence the contents of catecholamine-containing vesicles either on acute or after subchronic exposure. © 2002 Elsevier Science (USA)

Key Words: **PCB; quantal catecholamine release; rat PC12 phaeochromocytoma cells; carbon fiber microelectrode amperometry.**

The neurotoxic potential of specific polychlorinated biphenyls (PCBs) and related substances, many of which persist in the environment and may accumulate in biological tissues, is a matter of toxicological concern (Tilson and Kodavanti, 1997). Behavioral symptoms and brain neurotransmitter levels in exposed animals, as well as *in vitro* experimental data, indicate involvement of monoaminergic systems in PCB neurotoxicity and of the dopaminergic system in particular (for review see Seegal, 1995).

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Potential mechanisms underlying the effects of PCBs on the catecholaminergic system have been addressed using *in vitro* model systems. Several of the *ortho*-substituted, nonplanar PCB congeners reduce the dopamine contents of rat phaeochromocytoma (PC12) cells after 6 h of exposure with IC50 values < 100 μM, whereas coplanar congeners are inactive in this respect (Shain *et al.*, 1991). In an additional study of PC12 cells, the threshold concentrations of several PCB congeners to affect cellular dopamine contents and basal and evoked dopamine release were reported to be close to 10 μM (Angus and Contreras, 1996). In primary cultured bovine adrenal chromaffin cells, exposed to 50–100 μM of the nonplanar congener 2,2′,4,4′-tetrachlorobiphenyl for 1 and 5 days, cellular catecholamine contents and evoked catecholamine release were reduced, and basal catecholamine release was enhanced. The coplanar congener 3,3′,4,4′-tetrachlorobiphenyl did not cause any of these effects at concentrations up to 100 μM (Messeri *et al.*, 1997).

Experiments with rat PC12 and mouse neuroblastoma N1E-115 cells (Seegal *et al.*, 1991) have shown that 30–200 μM 2,2′-dichlorobiphenyl (PCB 4) inhibits tyrosine hydroxylase. A less potent, partial inhibition of tyrosine hydroxylase activity by PCB 4 was observed in minced rat corpus striatum (Choksi *et al.*, 1997). Uptake of dopamine into rat brain synaptosomes and into rat brain synaptic vesicles are both inhibited by micromolar concentrations of PCBs. Highly chlorinated congeners appear to inhibit the plasma membrane dopamine transporter more potently than the vesicular monoamine transporter (Mariussen *et al.*, 1999; Mariussen and Fonnum, 2001). Exposure of primary cultured rat neocortical and cerebellar granule cells to micromolar concentrations of PCBs may lead to variety of early transient, sustained, and oscillatory elevations of the intracellular Ca^{2+} concentration (Kodavanti *et al.*, 1993; Carpenter *et al.*, 1997; Mundy *et al.*, 1999; Inglefield and Shafer, 2000; Inglefield *et al.*, 2001). Elevation of the intracellular Ca^{2+} concentration and of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) activation, i.e., common intracellular signaling pathways, appear to be involved in PCB-induced release of insulin from RINm5F rat insuloma cells (Fischer *et al.*, 1996, 1999). Acute exposure of clonal rat PC12 cells to *ortho*-substituted PCB congeners has also been demonstrated

0041-008X/02 $35.00

to cause an elevation of the intracellular Ca^{2+} concentration (Wong *et al.*, 2001).

Several of the alleged mechanisms of action of PCBs would be expected to lead to changes in the characteristics of vesicular catecholamine release from PC12 cells, which can be measured using carbon fiber microelectrode amperometry (Wightman *et al.*, 1991). An increase in the available amount of dopamine by supplementing L-DOPA causes an elevation of the contents of catecholamine-containing vesicles released from PC12 cells (Pothos and Sulzer, 1998; Westerink *et al.*, 2000). Conversely, a decrease of dopamine availability by inhibition of tyrosine hydroxylase (Pothos *et al.*, 1998) and by inhibition of dopamine transport across the plasma membrane (Pothos and Sulzer, 1998) is associated with a decrease in vesicle contents. In addition, a disturbance in Ca^{2+} homeostasis, e.g., induced by caffeine in PC12 cells (Taylor and Peers, 1999), may lead to changes in basal release.

The combined literature data indicate that PCBs will exert a general effect on the probability of vesicle exocytosis and a more specific effect on the contents of catecholamine-containing vesicles. The effects two *ortho*-substituted nonplanar congeners [PCB 4 and 2,2′,3,3′,4,4′-hexachlorobiphenyl (PCB 128)] and one coplanar congener [3,3′,4,4′,5-pentachlorobiphenyl (PCB 126)] on vesicular catecholamine release from single PC12 cells were investigated. PCB 126 and PCB 128 were selected for their abilities to differentially alter basal and evoked dopamine release and cellular dopamine content in populations of PC12 cells (Angus and Contreras, 1996). PCB 4 was chosen since, upon acute exposure, it causes an elevation of intracellular Ca^{2+} in PC12 cells (Wong *et al.*, 2001) and in rat cerebellar granule cells (Mundy *et al.*, 1999). In addition, PCB 4 is an inhibitor of vesicular dopamine uptake with an IC50 of ~20 μM (Mariussen *et al.*, 1999) and tyrosine hydroxylase (Seegal *et al.*, 1991). The use of single-cell carbon fiber microelectrode amperometry allows detailed examination of the effects of PCBs on presynaptic neurotransmission, i.e., the frequency of basal and evoked vesicular catecholamine release and the amount of catecholamine released per vesicle.

METHODS

Materials. NaCl, KCl, $Mg(NO_3)_2$, glucose, sucrose, Hepes, and NaOH (Aristar quality) were obtained from BDH Laboratory Supplies (Poole Dorset, UK). PCB 4 (purity ≥ 99%), PCB 126 (purity ≥ 99%), and PCB 128 (purity ≥ 99%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and DMSO was obtained from Sigma (St. Louis, MO). Saline solutions were prepared with deionized millipore water (Milli-Q; resistivity > 10 MΩ·cm). Immediately after preparation all saline solutions were filtered using a Millipore GSWP 0.22-μm filter (Millipore, Bedford, MA) and stored in thoroughly cleaned and rinsed glass bottles at −20°C until use. Stock solutions of 5 mM PCB 4, 5 mM PCB 126, and 5 mM PCB 128 were prepared by ultrasonication in DMSO, kept at room temperature, and diluted in external solution to obtain the desired concentrations immediately prior to the experiments. DMSO alone (0.5% v/v) has previously been shown not to affect release characteristics in acute experiments.

Cell culture. PC12 cells (Greene and Tischler, 1976; ATCC CRL-1721; cultured for 10 passages) were grown essentially as described previously (Westerink *et al.*, 2000), with some modifications. Cells were differentiated in culture medium (RPMI 1640; Gibco, Grand Island, NY) supplemented with 5 μM dexamethasone (Genfarma, Zaandam, The Netherlands) starting 2 days after subculturing. Culture dishes were coated with 5 $\mu g/cm^2$ poly-L-lysine (Sigma). The culture medium was refreshed every 2–3 days. Experiments were performed 7–10 days after subculturing, i.e., 5–8 days after initiating differentiation. For subchronic exposure, PCB congeners were added to culture medium during the last 3 days of culturing and the PCB-containing culture medium was refreshed daily.

Carbon fiber microelectrode amperometry and data analysis. Carbon fiber microelectrode (ϕ 10 μm) fabrication and data recording and analysis were as described previously (Westerink *et al.*, 2000). Before experiments cells were washed twice with saline solution containing (in mM): 125 NaCl, 5.5 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 Hepes, 24 glucose, and 36.5 sucrose at pH 7.3 adjusted with NaOH. The carbon fiber, polarized to 700 mV, was placed gently on the membrane surface of a PC12 cell. The cell under investigation was continuously superfused with saline solution through one barrel of a theta superfusion pipette (Clarke TGC150; pipette tip ϕ 80 μm) at a rate of ~150 μl/min. Exocytotic responses were evoked by switching the superfusate to high K^+ saline (KCl elevated to 125 mM and NaCl reduced to 5.5 mM) for a period of 5–15 s. Experiments were performed at room temperature (21–23°C). All reported values are mean ± SD of *n* cells and results are compared using the Student's *t*-test or the Mann–Whitney nonparametric test where appropriate.

RESULTS

Acute Effects

Vesicular catecholamine release was evoked from saline-superfused, single PC12 cells by switching the superfusate to high K^+ saline for a period of 5–15 s. Control experiments showed that exocytotic responses to high K^+ stimulation, recorded with a carbon fiber microelectrode on the cell surface, could be evoked repeatedly. High K^+ stimulation evoked vesicular catecholamine release in >80% of all cells tested, but the frequency of exocytotic events varied considerably between cells. Few very large events were often observed during the first stimulation and some rundown of the number of exocytotic events per stimulus occurred over time, as reported previously (Westerink and Vijverberg, 2002). After cessation of high K^+ stimulation, the frequency of exocytotic events rapidly declined to a low basal level of 1.2 ± 1.0 min^{-1} ($n = 10$).

In acute experiments cells were allowed to recover for 4 min from initial high K^+ stimulation and were then superfused with saline solution containing 10 and 25 μM PCB 4, 5 and 12.5 μM PCB 126, and 5 and 25 μM PCB 128 for a period of 15 min. Complete ~20-min recordings of raw data for a control cell and for cells exposed for 15 min to the selected congeners at the concentrations indicated above are shown in Fig. 1. Figure 1 shows a marked increase in the basal release frequency during the 15-min period of exposure to 10 μM PCB 4 and 5 μM PCB 126, but not during exposure to 25 μM PCB 4 and 12.5 μM PCB 126. In order to quantify the effects of PCBs on basal release, the basal release frequency was determined for each cell before exposure (i.e., during the 1- to 4-min

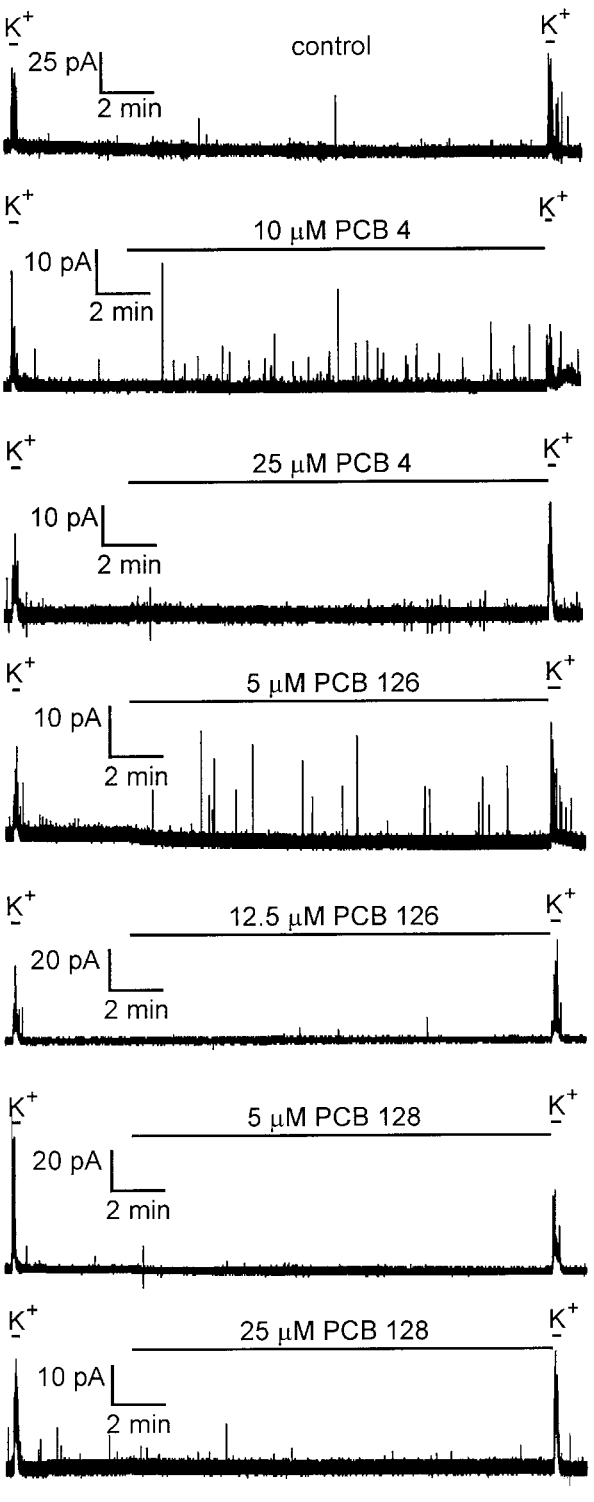

FIG. 1. Vesicular catecholamine release in PC12 cells measured by carbon fiber microelectrode amperometry. At the start and at the end of each experiment responses were evoked by superfusion of the cell with high K^+ saline. In between these responses, basal release frequency was very low when the cell was superfused with saline (control, top trace). All other traces are representative recordings from other cells, which were exposed to the PCB congeners at the concentrations indicated for a 15-min period in between the responses evoked with high K^+ saline. Only in cells exposed to 10 μM PCB 4 and in cells exposed to 5 μM PCB 126 is a marked increase of the basal frequency of exocytosis observed. Note that all biphasic deflections from baseline, as seen in some traces, are noise artifacts. Each trace was recorded from a different PC12 cell and periods of superfusion with high K^+ and PCB-containing saline are indicated by bars above the traces.

interval after the first high K^+ stimulus) and during exposure to PCB (i.e., during the 5- to 15-min interval after the start of superfusion with PCB-containing saline). The mean basal release frequencies and the results of a paired comparison of control and PCB values obtained from three to four cells for each PCB concentration are shown in Fig. 2. This summary of the results shows that a significant increase (paired *t*-test, $p < 0.05$) of the frequency of basal vesicular catecholamine release is observed only for the low concentrations of PCB 4 and PCB 126.

The median values of vesicular contents of events recorded during the control response and during the response evoked after PCB exposure were also determined and were compared statistically. The frequency of evoked events during the response evoked after PCB exposure was normalized to that during the control response obtained from the same cell. The relative frequencies of events after PCB exposure were compared to that obtained from the second response in control cells. The results, summarized in Table 1, show that, except for a statistically significant increase of the frequency of evoked events after exposure to 5 μM PCB 128, the characteristics of evoked vesicular catecholamine release did not change significantly upon acute exposure to the PCBs.

Subchronic Effects

Since previously reported effects of PCBs on catecholamine release generally come from studies in which cells were exposed for periods of several hours up to several days, subchronic experiments were also performed. PC12 cell cultures were exposed for 3 days to 10 μM PCB 4, 5 μM PCB 126, or 10 μM PCB 128 added to the culture medium, which was refreshed daily. Exocytotic responses were evoked in PCB-exposed cells by superfusion with high K^+ saline. Representative responses to high K^+ stimulation recorded from a control cell, exposed to 0.2% DMSO only, and from cells exposed to the selected congeners are shown in Fig. 3A. Of the different PCB-exposed cells 13 to 16 of 16 cells tested responded to high K^+ stimulation with a robust exocytotic response compared to 14 DMSO-exposed cells. This indicates that the PCB exposure did not affect the proportion of responsive cells. The mean frequency of K^+-evoked exocytotic events in PCB-exposed cells (5.4, 5.6, and 4.5 s^{-1} for PCB 4, PCB 126, and PCB 128, respectively) did not differ significantly from that in DMSO-exposed cells (5.9 s^{-1}), but the intercellular variation was too large to detect subtle effects (Fig. 3B).

The contents of catecholamine-containing vesicles was determined from 13 to 16 cells for each experimental condition

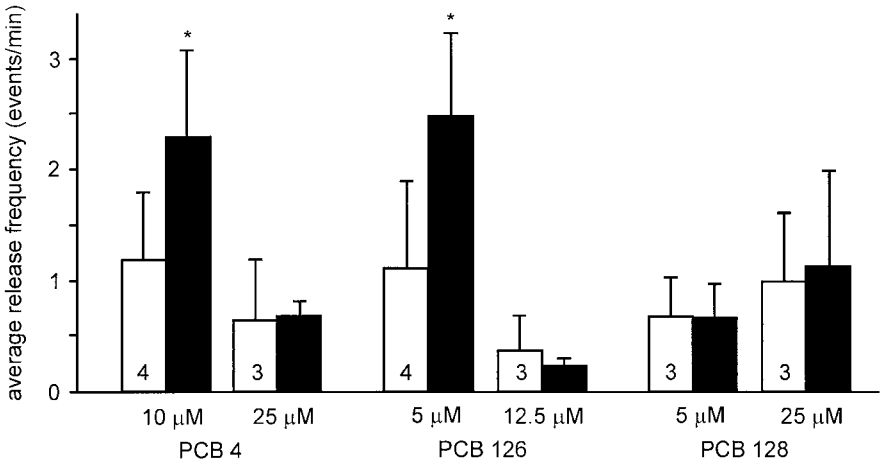

FIG. 2. Acute effects of PCB 4, PCB 126, and PCB 128 on the frequency of basal vesicular catecholamine release from PC12 cells. Control values of the basal release frequency (open bars) are compared with the values during acute exposure to the various PCB congeners at the concentrations indicated (solid bars). Bars are mean ± SD for the numbers of cells indicated and the statistical significance of the effects was determined by a paired *t*-test on the control and PCB values obtained from each cell. Note that 10 μM PCB 4 and 5 μM PCB 126, but not the higher concentrations of these PCBs, enhance the frequency of basal vesicular release ($*p < 0.05$).

and compared with that obtained from control cells from parallel cultures ($n = 14$). Analysis of the results did not reveal statistically significant changes in vesicle contents in cells exposed to the PCBs for a period of 3 days compared to the values obtained from control cells that were exposed for the same period to DMSO only (Fig. 4).

DISCUSSION

The results demonstrate that acute exposure to low concentrations of PCB 4 and PCB 126, but not exposure to PCB 128, leads to an enhancement of the basal frequency of vesicular catecholamine release from PC12 cells. In addition, acute and subchronic exposure fail to cause changes in the contents of catecholamine-containing vesicles.

In rat cerebellar granule cells, PCB 4 has been reported to cause a sustained rise in the intracellular Ca^{2+} concentration following exposure to 30–50 μM (Kodavanti *et al.*, 1993; Mundy *et al.*, 1999). A concentration of 10 μM PCB 4, which enhanced basal release in the present experiments (Figs. 1 and 2), has also been shown to cause an acute, slow transient elevation of the intracellular Ca^{2+} concentration in cultured rat neocortical neurons (Inglefield *et al.*, 2001). In PC12 cells, the transient increase in the intracellular Ca^{2+} concentration following acute exposure to PCB 4 was observed at concentrations as low as 5 μM (Wong *et al.*, 2001). PCB 126 appeared ineffective in changing the intracellular Ca^{2+} concentration in rat cerebellar granule cells (Mundy *et al.*, 1999) and in PC12 cells (Wong *et al.*, 2001), but a dose-dependent increase at high concentrations ($\geq$25 μM) in rat cerebellar granule cells has also been reported (Kodavanti *et al.*, 1993). In addition, 5 μM PCB 126 increased the intracellular Ca^{2+} concentration in a small fraction of cultured rat neocortical cells (Inglefield *et al.*, 2001). The combined results suggest that the enhancement of basal catecholamine release by PCB 4 (Figs. 1 and 2) is caused by an increase in the intracellular Ca^{2+} concentration. However, a similar conclusion for 5 μM PCB 126, which caused a similar enhancement of basal release frequency in PC12 cells as PCB 4 (Figs. 1 and 2), is complicated by the fact that reported effects of PCB 126 on intracellular Ca^{2+} concentration vary. Therefore, a straightforward relation between reported effects of low concentrations of PCBs on intracellular Ca^{2+} and the presently observed enhancement of the basal frequency of exocytosis in PC12 cells is not evident.

TABLE 1
Characteristics of Vesicular Catecholamine Release in PC12 Cells after Acute Exposure to PCB 4, PCB 126, and PCB 128

	Median vesicle contents (% of matched controls)	Evoked event frequency (% of matched controls)
Control	108 ± 31 (10)	72 ± 31 (12)
PCB 4		
10 μM	70 ± 24 (4)	48 ± 15 (4)
25 μM	98 ± 28 (3)	106 ± 42 (3)
PCB 126		
5 μM	82 ± 17 (4)	79 ± 58 (4)
12.5 μM	127 ± 22 (3)	62 ± 35 (3)
PCB 128		
5 μM	75 ± 25 (4)	166 ± 93 (3)*
25 μM	105 ± 11 (2)	79 ± 44 (3)

Note. Values are the mean ± SD of the response evoked after 15 min of exposure expressed as a percentage of the control response from the same cell with the number of cells in parentheses.

* $p < 0.05$ compared to control cells (Mann–Whitney).

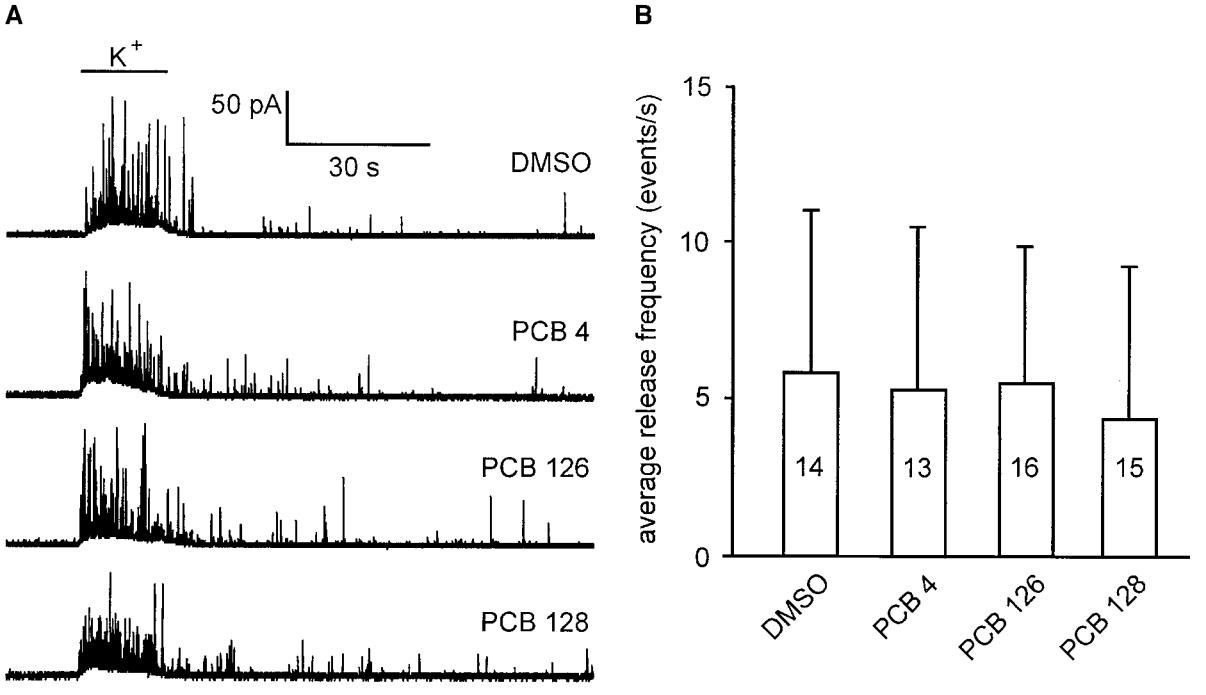

FIG. 3. Exocytotic responses evoked by high K^+ stimulation from PC12 cells after subchronic exposure to PCBs. (A) Representative amperometric recordings displaying high K^+-evoked catecholamine release from a control cell cultured for 3 days in medium containing 0.2% DMSO (upper trace) and from cells cultured for 3 days in medium containing 10 μM PCB 4, 5 μM PCB 126, and 10 μM PCB 128. Note that vesicular catecholamine release was not observed in any of the cells tested before stimulation with high K^+ saline. (B) Bar diagram of the average release frequency during high K^+ stimulation of PC12 cells exposed to DMSO (control) and to 10 μM PCB 4, 5 μM PCB 126, and 10 μM PCB 128 for a period of 3 days. The bars represent the mean $\pm$ SD of the average release frequency calculated from the numbers of cells indicated. None of the PCB treatments caused a significant change in average release frequency compared to the average release frequency of cells exposed to DMSO only (Mann–Whitney; all p values > 0.3).

At the higher concentrations tested, acute effects of PCB 4 and PCB 126 on basal release frequency are no longer observed and an inverted U-shaped dose–response relationship becomes apparent. Although a comparable inverted U-shaped dose–response relationship was observed previously following subchronic exposure of PC12 cell populations to PCB 126 (Angus and Contreras, 1999), there is no evidence for a similar relationship between PCB exposure and intracellular Ca^{2+} (Kodavanti *et al.*, 1993; Mundy *et al.*, 1999; Inglefield *et al.*, 2001; Wong *et al.*, 2001). Thus, it appears that low concentrations of the PCBs may enhance vesicular catecholamine release from PC12 cells, whereas higher concentrations are without effect on both basal and evoked vesicular catecholamine release. This would indicate that the effects of the low concentrations are counteracted by additional effects of the high concentrations of the PCBs. However, inhibitory effects of high concentrations of PCBs are not observed as a change in the exocytotic response evoked by stimulation with high K^+ saline (Table 1). In the absence of more detailed information on the effects of PCBs on intracellular Ca^{2+} concentration and on vesicular catecholamine release and on the concentration dependence of these effects in particular, the underlying mechanisms remain unclear. As PCB 4 and PCB 128 are *ortho*-substituted, nonplanar congeners and PCB 126 is a coplanar congener, the acute effects observed do not appear to obey the pro-

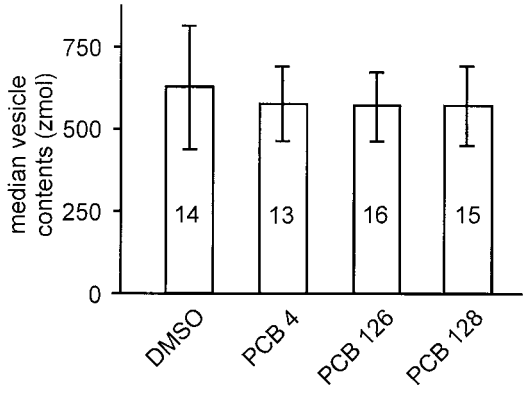

FIG. 4. Median contents released from vesicles during high K^+ stimulation of PC12 cells exposed to DMSO (control) and to 10 μM PCB 4, 5 μM PCB 126, and 10 μM PCB 128 for a period of 3 days. Bars represent mean $\pm$ SD calculated from the median contents for the numbers of cells indicated. None of the PCB treatments caused a significant change in vesicle contents compared to the vesicle contents of cells exposed to DMSO only (*t*-test; all p values > 0.3).

posed relation between PCB structure and neurotoxic potential (Kodavanti and Tilson, 1997). The observed effects following acute exposure to the coplanar congener PCB 126 (Figs. 1 and 2) are not unique, since PCB 126 has previously been shown to affect neuronal functioning (Kodavanti *et al.*, 1993; Angus and Contreras, 1996; Inglefield *et al.*, 2001). Effects on vesicle contents may not be expected in acute experiments on PC12 cells, since previous studies have shown that vesicle cycling in these cells is slow and the total number of vesicles that can be released from a single PC12 cells in an acute experiment is limited (Westerink *et al.*, 2000; Westerink and Vijverberg, 2002). Establishing a reduction of vesicle contents may require exposure to PCBs for more than 15 min, which was the exposure period in the acute experiments.

A subchronic exposure study of PC12 cells showed that basal catecholamine release was selectively enhanced by 50% by 5 μM PCB 126 and K^+-evoked release was selectively inhibited by 50% by 10 μM PCB 128 after 3 days of exposure. Significant cytotoxicity did not occur at these PCB concentrations (Angus and Contreras, 1996). The present results (Fig. 4) show that these effects cannot be accounted for on the basis of changes in the available amount of catecholamines. In PC12 cells the amount of catecholamines available in the cytoplasm is closely linked to vesicle contents (see Introduction). The concentration of PCB 4 tested is rather low compared to that which has been reported to inhibit tyrosine hydroxylase (Seegal *et al.*, 1991) and is also below the IC50 values for inhibition of dopamine uptake into rat brain synaptosomes and into rat brain synaptic vesicles (Mariussen *et al.*, 1999; Mariussen and Fonnum, 2001). Therefore, an effect on vesicle contents may not have been expected. Nonetheless, the same low concentration of PCB 4 is able to enhance the basal frequency of vesicular catecholamine release from PC12 cells. These combined results indicate that the toxicological focus may need to be redirected from the turnover of catecholamines to other effects, including the mechanisms underlying the subtle changes in basal neurotransmitter release and the functional consequences thereof for the developing nervous system.

ACKNOWLEDGMENTS

We thank Dr. L. Tertoolen (Hubrecht Laboratory, Utrecht, The Netherlands) for donation of PC12 cells; Dr. S. Agulian (Yale University, West Haven, CT) for donation of carbon fibers; T. Sinnige for help with preparation of PCB stock solutions; Prof. M. van den Berg for initial discussions; and Ing. A. de Groot for excellent technical assistance.

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