NON-DIOXIN-LIKE PCBS INCREASE BASAL INTRACELLULAR CALCIUM LEVELS BUT INHIBIT DEPOLAROZATION-EVOKED CALCIUM LEVELS IN RAT PC12 CELLS

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Introduction

Non-dioxin-like polychlorinated biphenyls (NDL-PCBs) are persistent environmental pollutants. Perinatal exposure to NDL-PCBs can result in neurodevelopmental and neurobehavioral effects in children[1-3]. Additionally, *in vivo* animal studies revealed that exposure to NDL-PCBs can result in a range of neurobehavioral effects, including changes in motor activity, learning, memory, and attention[4-6]. *In vitro* studies identified regulation of the intracellular calcium concentration ($[Ca^{2+}]_i$) as one of the critical parameters affected by NDL-PCBs[7-9].

Ca^{2+} plays a crucial role in numerous cellular processes, including neurotransmission and plasticity[10-11]. Neurons and neuroendocrine cells maintain low $[Ca^{2+}]_i$ at rest and tightly regulate the balance between influx, extrusion, sequestration, and buffering of calcium[10-11].

It is therefore not surprising that many neurotoxicological studies focused on the effects of NDL-PCBs on calcium homeostasis. These studies revealed that several NDL-PCBs can increase basal $[Ca^{2+}]_i$ [7-9], most likely via release of Ca^{2+} from the endoplasmic reticulum (ER) through activation of IP3 and ryanodine (Ry) receptors[8]. Despite the abundance of research, effects of many congeners, including PCB53, on calcium homeostasis are still largely unknown. Moreover, studies on the effects of NDL-PCBs on Ca^{2+} influx pathways, including depolarization-evoked Ca^{2+} influx, are poorly investigated. The aim of the present study was therefore to investigate the effects of 21 selected PCBs on basal and depolarization-evoked $[Ca^{2+}]_i$ in neuroendocrine PC12 cells.

Materials and methods

The 21 PCBs used in this study were PCB19, PCB28, PCB47, PCB51, PCB52, PCB53, PCB74, PCB95, PCB100, PCB101, PCB104, PCB118, PCB122, PCB126, PCB128, PCB136, PCB138, PCB153, PCB170, PCB180, and PCB190. These highly purified (> 99.2%) PCBs were purchased from Neosync Inc., and possible impurities, e.g., polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/Fs) and DL-PCBs, were removed by Drs. Stenberg and Andersson (Institute of Environmental Chemistry, Umea University, Sweden) as described previously[12]. PCBs were dissolved in purity-checked dimethyl sulfoxide (DMSO) to obtain 25mM PCB stock solutions, which were further diluted immediately before experiments to obtain final concentrations of 1 and 10 µM.

Undifferentiated rat pheochromocytoma (PC12) cells[13] (ATCC) were cultured as described previously[14-16]. Cells were subcultured in poly-L-lysine-coated glass bottom dishes at a density of $2x\ 10^5$ cells/dish for $[Ca^{2+}]_i$ imaging experiments, as described previously[14-16].

Changes in $[Ca^{2+}]_i$ were measured at room temperature on a single cell level using the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 (Molecular Probes Invitrogen, Breda, The Netherlands)) as described previously[14-16]. Fura-2-loaded cells were placed on the stage of an Axiovert 35M inverted microscope (Zeiss, Gottingen, Germany) equipped with a TILL Photonics Polychrome IV and an Image SensiCam digital camera (TILL Photonics GmBH, Grafelfing, Germany). Changes in fluorescence ratio (R), reflecting changes in $[Ca^{2+}]_i$, was measured every 3 s by 340- and 380-nm excitation wavelengths (F340 and F380).

Each experiment consisted of a 5-min baseline recording to measure basal $[Ca^{2+}]_i$, after which an increase in $[Ca^{2+}]_i$ was triggered by changing superfusion to 100mM K^+ for 20 s. Following this first depolarization and an 8-min recovery period, cells were exposed to saline containing DMSO (control) or PCB (1 or 10 µM) for 15 min prior to a second depolarization with 100mM K^+- (control) or 100mM K^+- and PCB-containing saline (see Fig. 1

example recordings). For specific experiments, cells were maintained in Ca^{2+}-free saline or pretreated with thapsigargin (TG, 1μM) to determine the involvement of Ca^{2+} influx pathways intracellular Ca^{2+} stores.
Free cytosolic $[Ca^{2+}]_i$ was calculated according to a modified Grynkiewicz's equation as described previously[14-16] and cells that showed $[Ca^{2+}]_i$ 2x SD above or below average, either during basal recording or during depolarization, were excluded from further analysis (~17%).
The 15 min PCB exposure was subdivided in an early (0-3 min), mid (4-7 min), and late (8-15 min) phase of exposure to temporally characterize the change in basal $[Ca^{2+}]_i$. The amplitude of the second K^+-evoked increase in $[Ca^{2+}]_i$ (after 15 min of PCB exposure) was expressed as a percentage of the amplitude of the first K^+-evoked increase in $[Ca^{2+}]_i$ per cell to obtain a ''net treatment ratio'' (TR) as described previously[15-16]; for illustration, see Fig. 1A).
Data are presented as mean ± SD of n cells obtained from N independent experiments. As the SD for basal $[Ca^{2+}]_i$ and TR amounted to 17 and 21%, respectively, effects < 25% were considered irrelevant; all relevant effects are statistically significant ($p < 0.05$; Student's t-test, paired or unpaired where applicable).

Results and discussion
Control cells display low $[Ca^{2+}]_i$ (99 ± 17nM), that rapidly and transiently increases (1.80 ± 0.64μM) upon depolarization with K^+-containing saline. Following a 8-min recovery, a 15-min DMSO exposure did not affect $[Ca^{2+}]_i$ (Fig. 1). However, when cells were exposed to PCB-containing saline several different effects on $[Ca^{2+}]_i$ could be observed (Figs. 1 and 2A). A number of NDL-PCBs, including all tested hexa- and heptachlorobiphenyls (with the exception of PCB136) as well as some pentachlorinated (PCB104, PCB118, PCB122, and the DL-PCB126) and one tetrachlorinated biphenyl (PCB74), did not induce clear effects on basal $[Ca^{2+}]_i$ either at 1μM or at 10μM (Fig. 2A). However, at 10μM, the other tested tri- and tetrachlorobiphenyls increased basal $[Ca^{2+}]_i$, with PCB52, PCB53, and PCB95 already strongly increasing basal $[Ca^{2+}]_i$ at 1μM. The increase in basal $[Ca^{2+}]_i$, which differed in kinetics for the different congeners, depended partly on influx of extracellular calcium and calcium release from the endoplasmic reticulum (not shown), as also demonstrated previously[7-9].

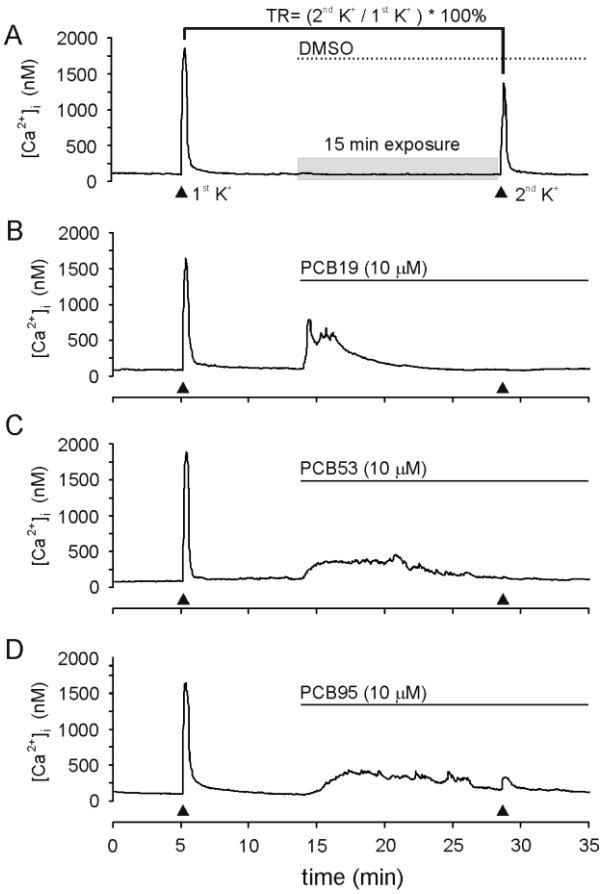

Figure 1. Representative example recordings of $[Ca^{2+}]_i$ from individual PC12 cell exposed for 15 min to DMSO-containing saline (A), 10μM PCB19 (B), 10μM PCB53 (C) or 10μM PCB95 (D) or as indicated by the dotted line in the recording. The cell is depolarized with 100mM K^+-containing saline (indicated by the arrowheads below the traces) before and at the end of the exposure period. This allows for calculation of a TR[15-16] to investigate the effects of exposure to the different congeners on depolarization-evoked Ca^{2+} influx. PCB-induced effects on basal and depolarization-evoked $[Ca^{2+}]_i$.

Following 15-min exposure to DMSO- or PCB-containing saline, cells were challenged with a second depolarization. Control cells, exposed to DMSO, showed a fast transient increase in $[Ca^{2+}]_i$ that amounted to 73 ± 21% (1.39 ± 0.62μM) of the first depolarization (Fig. 1). A number of NDL-PCBs, including all tested hexa- and heptachlorobiphenyls (with the exception of PCB136) but also some pentachlorobiphenyls (PCB101, PCB118, PCB122, and the DL-PCB126), did not affect depolarization-evoked Ca^{2+} influx, neither at 1μM nor at 10μM (Fig. 2B). However, at 10μM, all tested tri- and tetrachlorobiphenyls as well as some pentachlorobiphenyls (PCB95, PCB100, and PCB104) inhibited depolarization-evoked Ca^{2+} influx, with PCB47, PCB51, PCB53, and PCB104 already strongly inhibiting Ca^{2+} influx at 1μM (Fig. 2B). This inhibition of voltage-gated calcium channels is a novel and sensitive mode of action for NDL-PCBs that contributes to the disturbances in calcium homeostasis and likely is related to NDL-PCB-induced (developmental) neurotoxicity.

The NDL-PCB-induced reduction in depolarization-evoked Ca^{2+} influx appeared independent on the foregoing PCB-induced increase in basal $[Ca^{2+}]_i$ since some NDL-PCBs (PCB74 and PCB104 [10μM]) inhibited depolarization-evoked Ca^{2+} influx in the absence of effects on basal $[Ca^{2+}]_i$ (Fig. 2). However, when cells were exposed to 10μM PCB74 or PCB104 only during the second depolarization, Ca^{2+} influx was not different from control, indicating that the reduction in depolarization-evoked Ca^{2+} influx depended on exposure duration.

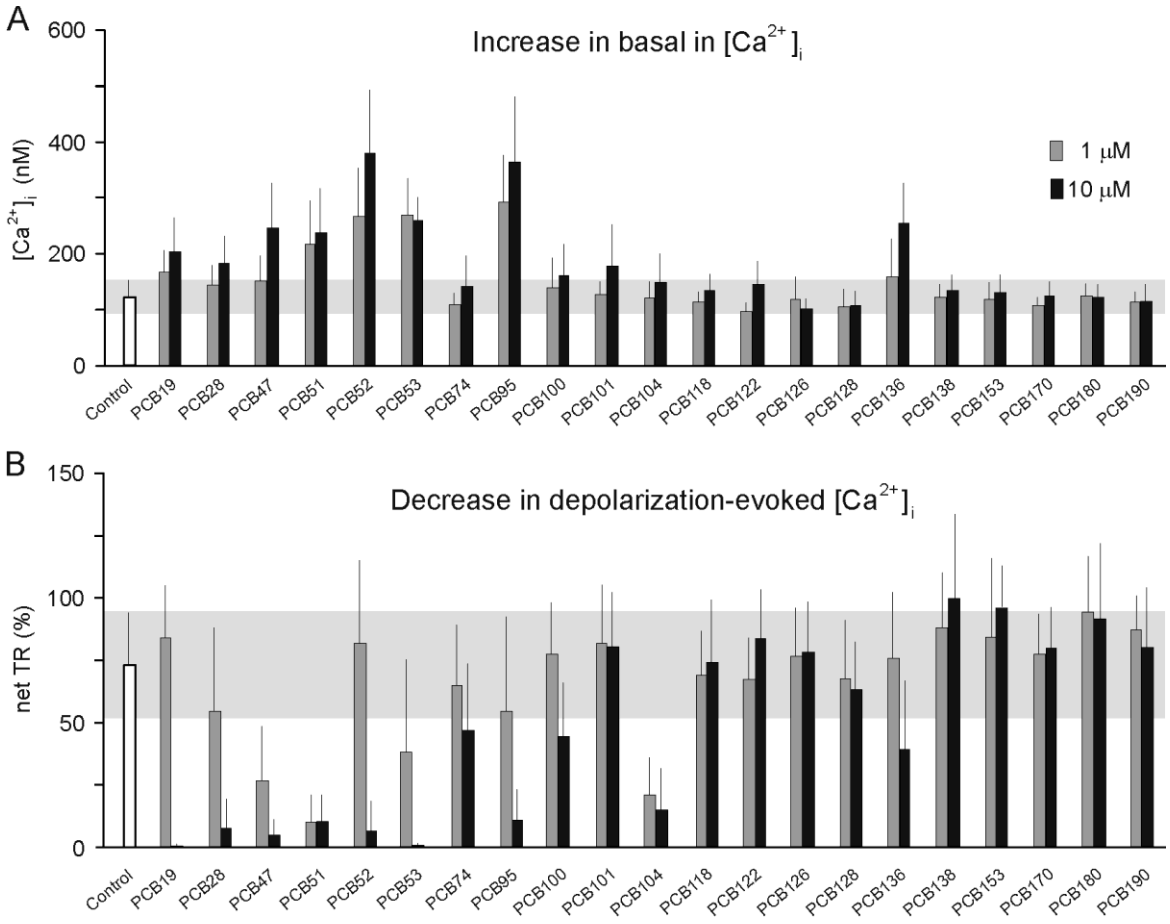

Figure 2. Summary graphs of PCB-induced effects on basal (A) and depolarization-evoked (B) $[Ca^{2+}]_i$ in PC12 cells. (A) Average amplitude of basal $[Ca^{2+}]_i$ during 15 min exposure (in between the 2 depolarizations) to DMSO (control) or one of the 21 congeners at 1 or 10μM. (B) Average change in net TR[15-16], which is used as a measure for inhibition of depolarization-evoked Ca^{2+} influx. Effects smaller than control ± SD (indicated by the shaded area) are considered irrelevant. Bars indicate mean ± SD of 317 control cells (N=47) or of 25-50 congener-exposed cells (N=3-8).

Human exposure to PCBs occurs mainly via the diet[17] and, especially for lower-chlorinated PCBs, via inhalation of (indoor) air and house dust[18-19]. This is concerning since our study demonstrates that most penta- and hexachlorinated NDL-PCBs are ineffective in changing basal and depolarization-evoked $[Ca^{2+}]_i$, whereas all tested tri- and tetrachlorobiphenyls (except PCB74) increased basal $[Ca^{2+}]_i$ to some extent. Similarly, the inhibition of depolarization-evoked Ca^{2+} influx is also mainly limited to di- and tri-ortho-substituted trichlorobiphenyls and tetrachlorobiphenyls. Additionally, the levels of individual PCBs in human blood are generally in the low nanomolar range[20-21]. The concentrations used in the present study are thus ~2 to 3 orders of magnitude higher than those found in humans, though some congeners, including PCB47, PCB51, PCB52, PCB53, PCB95, and PCB104, induce robust increases in basal $[Ca^{2+}]_i$ and inhibition of VGCCs already at 1μM. The LOECs for these more potent PCBs are thus expected to be in the nanomolar range. As such, the current findings are likely to be of particular relevance for developing children as the developing nervous system is more vulnerable and young children have a relatively high exposure, despite the ongoing decline in environmental and human serum levels of PCBs.

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