Arch Toxicol (1996) 70: 150–157 © Springer-Verlag 1996

ORIGINAL INVESTIGATION

P.R.S. Kodavanti · T.R. Ward · J.D. McKinney · H.A. Tilson

Inhibition of microsomal and mitochondrial Ca^{2+}-sequestration in rat cerebellum by polychlorinated biphenyl mixtures and congeners

Structure-activity relationships

Received: 10 March 1995/Accepted: 4 July 1995

Abstract Recent studies from our laboratory indicate that polychlorinated biphenyl (PCB) congeners in vitro perturbed signal transduction mechanisms including cellular Ca^{2+}-homeostasis and protein kinase C translocation. We have now investigated the structure-activity relationship (SAR) of three PCB mixtures, 24 PCB congeners and one dibenzofuran for their effects on microsomal and mitochondrial Ca^{2+}-sequestration in rat cerebellum. Ca^{2+}-sequestration by these intracellular organelles was determined using radioactive $^{45}CaCl_2$. All three mixtures studied, Aroclor 1016, Aroclor 1254 and Aroclor 1260, were equally potent in inhibiting microsomal and mitochondrial Ca^{2+}-sequestration with IC_{50} values of 6–8 μM. 1,2,3,7,8-Pentachlorodibenzofuran had no effect on Ca^{2+}-sequestration by these organelles. The SAR among the congeners revealed: (1) congeners with *ortho-/meta-* or *ortho-*, *para*-chlorine substitutions were the most potent in inhibiting microsomal and mitochondrial Ca^{2+}-sequestration (IC_{50} = 2.4–22.3 μM); (2) congeners with only *para-* but without *ortho*-substitutions were not effective in inhibiting Ca^{2+}-sequestration by microsomes and mitochondria; (3) increased chlorination was not related to the effectiveness of these congeners. The present SAR studies indicate that the effects of most PCB congeners in vitro may be related to an interaction at specific sites having preference for low lateral substitution or lateral content (*meta-* or *para*) in the presence of *ortho*-substitution.

Key words: Polychlorinated biphenyls · Calcium sequestration · Cerebellum · $^{45}Ca^{2+}$-uptake · In vitro · Structure-activity relationships

P.R.S. Kodavanti (✉) · T.R. Ward · H.A. Tilson
Cellular and Molecular Toxicology Branch,
Neurotoxicology Division, National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711, USA

J.D. McKinney
Experimental Toxicology Division, National Health and Environmental Effects Research Laboratory,
US Environmental Protection Agency,
Research Triangle Park, NC 27711, USA

These findings were presented at the 34th Annual Meeting of Society of Toxicology, Baltimore, MD [Toxicologist 15: 143 (1995)]. The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Introduction

The dynamic processes controlling the maintenance of intracellular calcium are important for a number of biological processes. The Ca^{2+} pool critical for the regulation of intracellular events is cytosolic free Ca^{2+}, which has a concentration of 0.1–0.3 μM (Carafoli 1987). There is about a 10 000-fold concentration gradient between intra- and extracellular concentrations (see reviews Borle 1981; Carafoli 1987; Farber 1990; Miller 1991). Ca^{2+} can enter the cell through voltage-dependent or receptor-mediated Ca^{2+}-sensitive channels (Siesjo 1990) and intracellular organelles such as the mitochondria and endoplasmic reticulum act to sequester increases in cytosolic free Ca^{2+} (Miller 1991). Cytoplasmic Ca^{2+} levels are also maintained by active extrusion through plasma membrane Ca^{2+}-ATPase and by the Na^+/Ca^{2+} exchanger (Carafoli 1987; Nicotera et al. 1992). It has also been reported that inhibition of endoplasmic reticulum Ca^{2+}-ATPase by agents such as thapsigargin can increase intracellular Ca^{2+} (Kwan et al. 1990). Thus, cytosolic free Ca^{2+} can be increased following influx of Ca^{2+} into the cell and/or failed sequestration or extrusion mechanisms inside the cell. If these homeostatic mechanisms fail to work in an optimal manner, increases in cytosolic free Ca^{2+}

can activate a number of intracellular Ca^{2+}-dependent enzymes, including protein kinase C, neutral proteases and nitric oxide synthase (Zimmerman and Schlaepfer 1982; Kiedrowski et al. 1992; Eboli et al. 1993). Neurotoxicity, including cell death, has been associated with many of these Ca^{2+}-dependent processes (Choi 1985; Nicotera et al. 1992). A close association between increased free Ca^{2+} levels measured in vitro, increased brain Ca^{2+} levels in vivo, and signs of neurotoxicity in rats has been reported for several chemicals (Johnson et al. 1986, 1987).

Mixtures of polychlorinated biphenyls (PCBs) are distributed widely in the environment and are comprised of up to 209 highly stable congeners (Safe 1984; Erickson 1986; Safe et al. 1987). In 1968, accidental ingestion of PCBs and dibenzofurans caused significant adverse health effects in several people in Japan, including effects on the nervous system (Kuratsune et al. 1972; Erickson 1986). More recently, several studies have reported the possible neurotoxic effects of PCB mixtures and congeners (Seegal et al. 1986, 1991a,b). In vitro studies have demonstrated that PCB congeners decrease levels of neurotransmitters such as dopamine (Seegal et al. 1986, 1991a,b) and inhibit oxidative phosphorylation in both liver (Nishihara et al. 1985; Nishihara and Utsumi 1986) and brain (La Rocca and Carlson 1979; Maier et al. 1994). Subsequent studies found that *ortho*-substituted congeners were more potent in decreasing dopamine levels in vitro than *meta*- or *para*-substituted congeners (Shain et al. 1991). Initial studies from our laboratory found that the *ortho*-congener, 2,2'-dichlorobiphenyl (-DCB) altered intracellular Ca^{2+}-homeostasis by inhibiting the Ca^{2+}-buffering systems at concentrations where no cytotoxicity was observed, whereas the non-*ortho* congener, 3,3',4,4',5-pentachlorobiphenyl (-PeCB), was not effective in inhibiting Ca^{2+}-buffering systems, did not alter Ca^{2+}-homeostasis to a great extent, and was not cytotoxic (Kodavanti et al. 1993). Previously, a similar inhibition of Ca^{2+}-buffering by *ortho*-substituted PCB has been reported in liver mitochondria (Nishihara and Utsumi 1986). Rosin and Martin (1981) also reported an increase in the uptake of $^{45}Ca^{2+}$ by mouse brain synaptosomes by Aroclor 1254 (10–100 μM). Further studies generally support the structure-activity relationships (SAR) reported by Shain et al. (1991), and indicate that perturbations in intracellular signal transduction processes including Ca^{2+}-homeostasis play a crucial role in the in vitro effects of *ortho*-chlorinated PCB congeners (Kodavanti et al. 1993, 1994; Maier et al. 1994). The possible association between the effects of some PCB congeners on signal transduction processes in vitro to neurotoxicity in vivo remains to be fully elucidated. Since impairment of Ca^{2+}-buffering, which could lead to progressive increases in intracellular free Ca^{2+}, was well correlated with cytotoxicity, this aspect was chosen to study the SAR of a variety of PCB congeners.

We recently investigated the SAR of prototypic PCB congeners by studying their effects on indicators of intracellular Ca^{2+} disposition such as the translocation of protein kinase C (PKC) from cytosol to the membrane (Kodavanti et al. 1995). The present study extends that investigation of PCB SAR by assessing their effects on microsomal and mitochondrial Ca^{2+}-sequestration. It was postulated that PCB congeners reported previously to affect neurotransmitters (Shain et al. 1991) or PKC translocation (Kodavanti et al. 1995) will also interfere with intracellular Ca^{2+}-sequestration mechanisms. The PCBs chosen for study vary in pattern and degree of chlorination, and have been detected in human milk, human fat, fish, dairy products, meat products, and soil and water samples (WHO 1993). We report here that congeners with low lateral substitution, especially without *para*-substitution or *para*-substitution/high lateral content in the presence of *ortho*-substitution, possibly affect sequestration of intracellular Ca^{2+} by inhibiting Ca^{2+}-uptake into mitochondria and microsomes.

Materials and methods

Chemicals

3,5,-Dichlorobiphenyl, 2,2',6-trichlorobiphenyl, 2,2',4,6'-, and 3,3',5,5'-tetrachlorobiphenyls (purity >99%) were purchased from Accu-Standard (New Haven, Conn.). 2,2',3,3',4,4'-, 2,2',3,3',5,5'-, and 2,2',3,3',6,6'-hexachlorobiphenyls (purity >98%) were synthesized previously (McKinney et al. 1976; Kohli et al. 1979). PCB mixtures (Aroclors 1016, 1254 and 1260), all other PCB congeners and 1,2,3,7,8-pentachlorodibenzofuran (purity >99%) were purchased from Ultra Scientific (North Kingstown, R.I.). Radiolabeled $^{45}Ca^{2+}$ as $CaCl_2$ (34.12 mCi/mg) was purchased from Dupont New England Nuclear Corporation (Boston, Mass.). All the other chemicals used in assays and cell culture were obtained from commercial sources.

Animals

Male Long-Evans hooded rats (90–120 days of age; 300–400 g) were obtained from Charles River Laboratory (Raleigh, N.C.) and housed individually in American Association for Accreditation of Laboratory Animal Care (AAALAC) approved animal facilities. Food and water were provided ad libitum. The animal colony was maintained at 21 ± 2°C and 50 ± 10% relative humidity with a 12-h light/dark cycle (0700–1900 hours).

Preparation of test solutions

Stock solutions of PCB mixtures, PCB congeners and 1,2,3,7,8-pentachlorodibenzofuran were prepared by dissolving them in dimethyl sulfoxide (DMSO) and 1–3 μl aliquots of the stock solutions (different concentrations) were added to the medium to yield the desired final concentrations. For PCB mixtures, the approximate molecular weights were used as indicated by Erickson (1986). DMSO (3 μl/ml) had no significant effect (Kodavanti et al. 1993) on microsomal or mitochondrial $^{45}Ca^{2+}$-uptake (Microsomal $^{45}Ca^{2+}$-uptake: 42.5 ± 1.1 for control and 45.1 ± 1.3 for DMSO;

Mitochondrial $^{45}Ca^{2+}$-uptake: 4.0 ± 0.2 for control and 4.1 ± 0.1 for DMSO).

Isolation of mitochondria and microsomes

Adult rats were decapitated, the cerebella excised quickly, homogenized and fractionated according to Cottman and Matthews (1971). Cerebella were homogenized with a glass-Teflon homogenizer in 9 vol cold homogenizing buffer containing 0.32 M sucrose and 10 mM imidazole, pH 7.5. The homogenate was centrifuged at 750 *g* for 10 min, the pellet discarded, and the resulting supernatant centrifuged at 17 000 *g* for 20 min. The pellet was suspended in 10 ml 0.32 M sucrose and layered on a two-step discontinuous Ficoll-sucrose gradient, consisting of 13% and 7.5% (w/v) Ficoll in 0.32 M sucrose. After centrifugation at 65 000 *g* for 45 min in a Beckman model L8-70 centrifuge, the pellet containing mitochondria was collected and suspended in homogenizing buffer. The initial supernatant obtained after centrifugation at 17 000 *g*, was centrifuged at 105 000 *g* for 60 min to obtain a microsomal pellet and suspended in homogenizing buffer. Aliquots (1 ml) of each fraction were quick frozen in liquid nitrogen and stored at − 80°C. Protein content was determined by the method of Lowry et al. (1951).

$^{45}Ca^{2+}$-uptake by mitochondria and microsomes

Uptake of $^{45}Ca^{2+}$ was measured as outlined by Moore et al. (1975). The assay mixture of 1.5 ml contained 30 mM histidine-imidazole buffer (pH 6.8), 100 mM KCl, 5 mM $MgCl_2$, 5 mM sodium azide (only added for microsomal calcium uptake), 5 mM ammonium oxalate, mitochondrial (180 µg) or microsomal (40 µg) protein, and 0.1 µCi $^{45}CaCl_2$ containing 5 µM free Ca^{2+} in calcium-ethylene glycol-*bis*(*β*-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (Ca^{2+}-EGTA) buffered medium. The concentrations of free calcium were calculated according to Fabiato and Fabiato (1978). Test compounds (0–100 µM) were added immediately after the addition of enzyme protein and the tubes were preincubated at 37°C for 5 min. The uptake was initiated by the addition of ATP (5 mM final concentration) and incubated for 20 min at 37°C. After incubation, the samples were filtered through a 0.45-µm millipore filter. The filters were transferred after washing directly to vials containing 10 ml Ultima Gold and the amount of radioactivity was determined by liquid scintillation spectroscopy. Non-specific $^{45}Ca^{2+}$-binding was studied in the absence of ATP and subtracted from total uptake to get the specific uptake of $^{45}Ca^{2+}$ and calculated as pmol/mg protein/min. For clarity, the data were converted to percent of control in each experiment, mean and standard error values were calculated, and presented in Figures 1–7.

Statistics

The inhibition of microsomal $^{45}Ca^{2+}$-uptake by PCBs (Figs 1–7) was normalized to percent of control and was analyzed by a two-way analysis of variance (ANOVA) with compound (PCB mixtures or congeners) as one factor and concentration (0–100 µM) as the other. In the case of a significant interaction, step-down ANOVAs were used to test for the effects of each compound. Pairwise comparisons between groups were made by using Fisher's LSD (least significant difference) test (SAS 1989). IC_{50} values (Table 1) were calculated from the regression line fit to the linear portion of the curve using GraphPad InStat Software, and were analyzed by ANOVA with compound as one factor, and organelle (microsomes or mitochondria) as the other. The accepted level of significance was set at $p < 0.05$.

Results

Inhibition of microsomal and mitochondrial $^{45}Ca^{2+}$-uptake by PCBs

The uptake of $^{45}Ca^{2+}$ by microsomes and mitochondria was studied to determine the effects of PCB mixtures, PCB congeners and a dibenzofuran on intracellular Ca^{2+}-buffering mechanisms in vitro. Microsomal and mitochondrial $^{45}Ca^{2+}$-uptake in DMSO controls were 41.4 ± 2.9 and 4.4 ± 0.3 pmol/mg protein/min ($n = 18$), respectively. Table 1 summarizes the IC_{50}s (concentration that inhibits 50% of the control activity) for both microsomal and mitochondrial data. The ANOVA of this data indicated an overall significant effect of compound ($F_{20,84} = 12.1$, $p < 0.0001$), organelle ($F_{1,84} = 39.2$, $p < 0.0001$), and interaction

Table 1 Inhibition of microsomal and mitochondrial $^{45}Ca^{2+}$-uptake in rat cerebellum by polychlorinated biphenyl (PCB) mixtures, congeners and a dibenzofuran. IC_{50} values were calculated from the regression line fit to the linear portion of the curve and represent the concentration that inhibits 50% of the control activity. Microsomal and mitochondrial $^{45}Ca^{2+}$-uptake in DMSO controls were 41.4 ± 2.9 and 4.4 ± 0.3 pmol/mg protein per min ($n = 18$), respectively. Each value is a mean ± SE of 3 experiments, assayed in triplicate. *NEO* no effect observed up to 100 µM

Chemical	IUPAC#	IC_{50} value (µM) Microsomes	Mitochondria
PCB mixtures			
Aroclor 1016		6.8 ± 0.6	6.5 ± 0.3
Aroclor 1254		6.3 ± 0.8	8.5 ± 0.8
Aroclor 1260		7.6 ± 1.2	7.1 ± 0.5
PCB congeners			
2,2′	4	8.0 ± 0.3	9.0 ± 0.5
3,3′	11	12.5 ± 3.4	13.1 ± 2.7
3,5	14	17.2 ± 1.5	22.3 ± 0.1
4,4′	15	NEO	NEO
2,2′,6	19	7.0 ± 0.4	14.8 ± 0.8
2,4,4′	28	6.9 ± 0.2	9.1 ± 1.1
2,2′,4,4′	47	5.8 ± 0.3	6.7 ± 0.3
2,2′,4,6	50	7.3 ± 0.5	9.6 ± 3.4
2,2′,4,6′	51	2.4 ± 0.1	9.1 ± 0.7
2,2′,5,5′	52	4.9 ± 0.2	5.8 ± 0.3
2,2′,6,6′	54	NEO	NEO
3,3′,4,4′	77	NEO	NEO
3,3′,5,5′	80	> 100	> 100
2,2′,4,6,6′	104	5.5 ± 0.2	4.7 ± 0.6
2,3,3′,4,4′	105	5.3 ± 0.4	6.6 ± 0.3
2,3′,4,4′,5	118	6.6 ± 0.1	9.1 ± 2.0
3,3′,4,4′,5	126	> 100	> 100
2,2′,3,3′,4,4′	128	4.9 ± 0.3	11.0 ± 0.3
2,2′,3,3′,5,5′	133	5.1 ± 0.1	15.2 ± 2.2
2,2′,3,3′,6,6′	136	6.3 ± 0.7	9.9 ± 0.3
2,2′,4,4′,5,5′	153	6.6 ± 0.3	5.8 ± 0.9
2,3,3′,4,4′,5	156	5.4 ± 0.1	7.3 ± 0.2
3,3′,4,4′,5,5′	169	NEO	NEO
2,2′,3,4,4′,5,5′	180	4.8 ± 0.1	6.6 ± 0.1
Polychlorinated dibenzofuran			
1,2,3,7,8		NEO	NEO

($F_{20,84} = 2.5$, $p < 0.0023$). It is obvious from Table 1 that not all the congeners were active. All the congeners that were active produced concentration-dependent decreases in both microsomal and mitochondrial $^{45}Ca^{2+}$-uptake. For some PCB congeners [2,2′,6-trichlorobiphenyl (-TCB), 2,2′,4,6′-tetrachlorobiphenyl (-TeCB), and 2,2′,3,3′,4,4′-, 2,2′,3,3′,5,5′-, 2,2′,3,3′,6,6′-hexachlorobiphenyls (-HCB)], the potency in inhibiting $^{45}Ca^{2+}$-uptake was less in mitochondria than microsomes (Table 1). For ease of reporting, only concentration-dependent effects of PCBs on microsomal $^{45}Ca^{2+}$-uptake are presented (Figs 1–7).

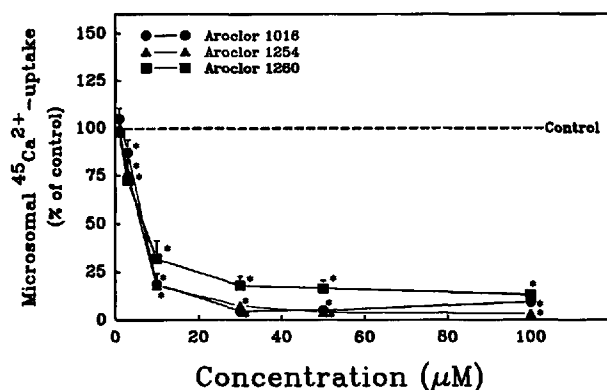

Fig. 1 Effects of different polychlorinated biphenyl mixtures on microsomal $^{45}Ca^{2+}$-uptake in rat cerebellum. Microsomes, isolated from adult rat cerebellum, were incubated in 30 mM histidine-imidazole buffer with different concentrations of chemicals and 0.1 μCi $^{45}CaCl_2$ (5 μM). After incubation, the samples were filtered rapidly through a 0.45-μm Millipore filter and the amount of radioactivity in filters was determined by liquid scintillation spectroscopy. Microsomal $^{45}Ca^{2+}$-uptake in DMSO controls was 41.4 ± 2.9 pmol/mg protein per min ($n = 18$), respectively. Values are mean ± SEM of three preparations, assayed in triplicate. *Significantly different from control ($p < 0.05$, Fisher's LSD after significant main effect of concentration)

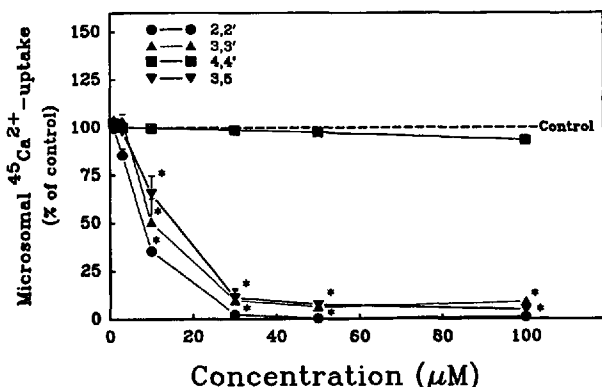

Fig. 2 Effects of dichlorobiphenyls with *ortho* (2,2′-), *meta* (3,3′- and 3,5-) or *para* (4,4′-) substitutions on microsomal $^{45}Ca^{2+}$-uptake in rat cerebellum. All other details are mentioned in the caption to Fig. 1. Values are mean ± SEM of three preparations, assayed in triplicate. *Significantly different from control ($p < 0.05$, Fisher's LSD after significant interaction)

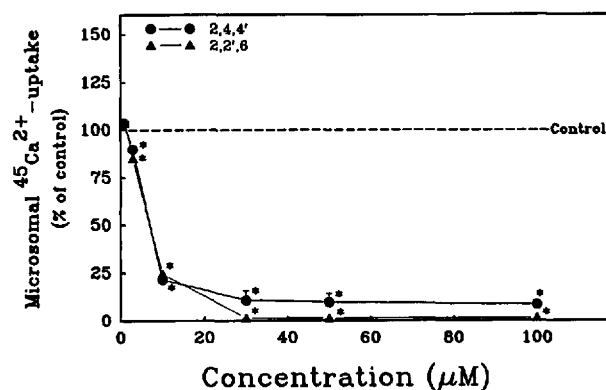

Fig. 3 Effect of trichlorobiphenyls (2,4,4′- and 2,2′,6-) on microsomal $^{45}Ca^{2+}$-uptake in rat cerebellum. All other details are mentioned in the caption to Fig. 1. Values are mean ± SEM of three preparations, assayed in triplicate. *Significantly different from control ($p < 0.05$, Fisher's LSD after significant main effect of concentration)

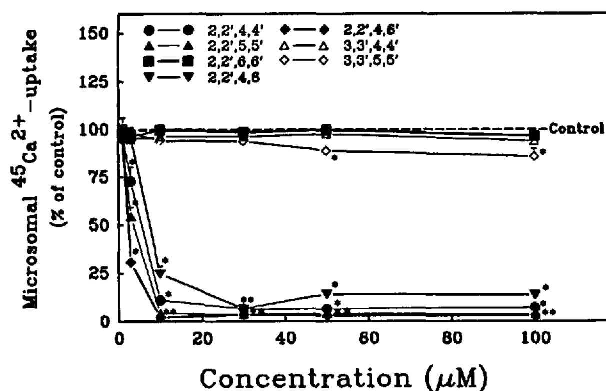

Fig. 4 Effects of tetrachlorobiphenyls on microsomal $^{45}Ca^{2+}$-uptake in rat cerebellum. All other details are mentioned in the caption to Fig. 1. Values are mean ± SEM of three preparations, assayed in triplicate. *Significantly different from control ($p < 0.05$, Fisher's LSD after significant interaction)

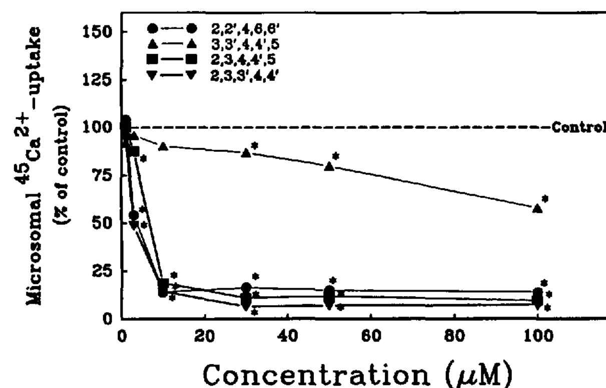

Fig. 5 Effect of pentachlorobiphenyls on microsomal $^{45}Ca^{2+}$-uptake in rat cerebellum. All other details were mentioned in the caption to Fig. 1. Values are mean ± SEM of three preparations, assayed in triplicate. *Significantly different from control ($p < 0.05$, Fisher's LSD after significant interaction)

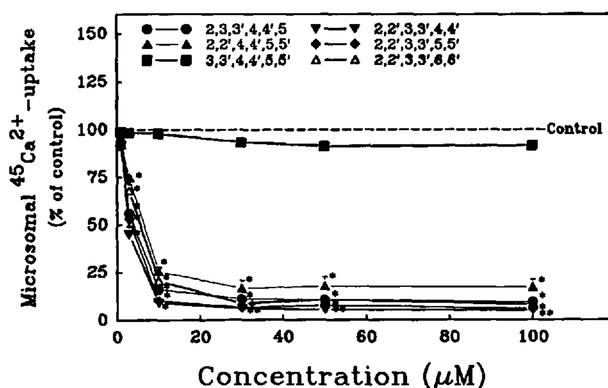

Fig. 6 Effect of hexachlorobiphenyls on microsomal $^{45}Ca^{2+}$-uptake in rat cerebellum. All other details are mentioned in the caption to Fig. 1. Values are mean ± SEM of three preparations, assayed in triplicate. *Significantly different from control ($p < 0.05$, Fisher's LSD after significant interaction)

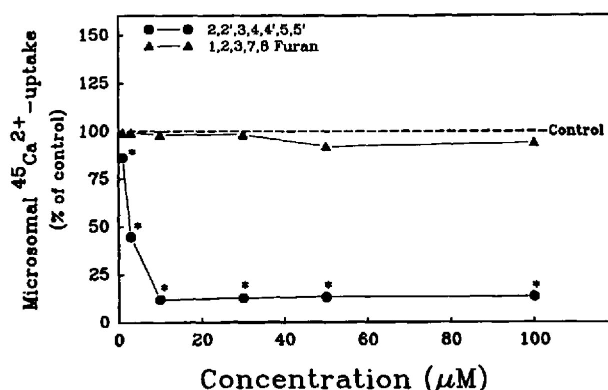

Fig. 7 Effect of a heptachlorobiphenyl (2,2′,3,4,4′,5,5′-), and a dibenzofuran (1,2,3,7,8-) on microsomal $^{45}Ca^{2+}$-uptake in rat cerebellum. All other details are mentioned in the caption to Fig. 1. Values are mean ± SEM of three preparations, assayed in triplicate. *Significantly different from control at $p < 0.05$

Aroclors 1016, 1254 and 1260 inhibited $^{45}Ca^{2+}$-uptake in a concentration-dependent manner (significant effect of concentration $F_{6,42} = 54.3$, $p < 0.0001$) (Fig. 1). The potency of Aroclor mixtures to inhibit $^{45}Ca^{2+}$-uptake was similar (no significant effect of compound) and IC_{50} values ranged from 6 to 9 μM (Table 1). 1,2,3,7,8-Pentachlorodibenzofuran, a known contaminant of PCB mixtures (Erickson 1986), did not inhibit $^{45}Ca^{2+}$-uptake at concentrations up to 100 μM (Fig. 7; Table 1). PCB congeners, however, exhibited differential effects on Ca^{2+}-sequestration. Analysis of data from the DCBs indicated a significant interaction ($F_{18,56} = 12.8$, $p < 0.0001$). 2,2′-, 3,3′-, and 3,5-DCBs inhibited $^{45}Ca^{2+}$-uptake in a concentration-dependent manner while 4,4′-DCB did not (Fig. 2), indicating that *ortho-* or *meta-* chlorine substitution is necessary for this activity in this preparation whereas *para*-substitution is not. Both TCBs (2,2′,6- and 2,4,4′-) inhibited the Ca^{2+}-buffering systems in a concentration dependent manner (significant effect of concentration, $F_{6,28} = 214.2$, $p < 0.0001$) with IC_{50} values of 6–14 μM (Fig. 3; Table 1). Among the TeCBs, there was a significant interaction ($F_{36,98} = 45.6$, $p < 0.0001$) with 2,2′,4,4′-, 2,2′,4,6-, 2,2′,4,6′-, and 2,2′,5,5′- being very potent ($IC_{50} = 2$–9 μM) in inhibiting microsomal and mitochondrial Ca^{2+}-sequestration, whereas 2,2′,6,6′-, 3,3′,4,4′-, and 3,3′,5,5′- were not (Fig. 4; Table 1). Of the PeCBs, 2,2′,4,6,6′-, 2,3,4,4′,5-, and 2,3,3′,4,4′- were equally potent in inhibiting Ca^{2+}-sequestration, while 3,3′,4,4′,5- was less effective (significant interaction, $F_{18,56} = 20.6$, $p < 0.0001$) (Fig. 5; Table 1). Of the HCBs studied, 2,2′,3,3′,4,4′-, 2,2′,3,3′,5,5′-, 2,2′,3,3′,6,6′-, 2,2′,4,4′,5,5′-, and 2,3,3′,4,4′,5- were all potent inhibitors of microsomal and mitochondrial Ca^{2+}-sequestration, while 3,3′,4,4′,5,5′- was not effective up to 100 μM (significant interaction, ($F_{30,84} = 10.5$, $p < 0.0001$) (Fig. 6; Table 1). The heptachlorobiphenyl (HeCB) studied, 2,2′,3,4,4′,5,5′-, was also active and had a potency similar to other congeners with an *ortho-* or *meta*-chlorine substitution (Fig. 7; Table 1).

Structure-activity relationship (SAR) of PCB congeners

Assessment of the SAR suggests that congeners with chlorine substitutions at *ortho-* or *ortho*-lateral (*meta*, *para*) were the most potent in inhibiting microsomal and mitochondrial Ca^{2+}-sequestration (Table 1). On the other hand, congeners with only *para*-substitution (4,4′-DCB) or high lateral content in the absence of *ortho*-substitution (3,3′,4,4′,5-PeCB and 3,3′,4,4′,5,5′-HCB) were not effective in inhibiting Ca^{2+}-sequestration by microsomes and mitochondria (Table 1). Substitution of chlorine at *ortho-* positions on congeners with only *para-* or with high lateral content (*meta-* and *para-*) resulted in a congener that inhibited Ca^{2+}-sequestration phenomenon, e.g., 2,4,4′-TCB was active, i.e., having a significant effect on Ca^{2+}-buffering, while 4,4′-DCB was not (Figs 2 and 3; Table 1). Likewise, 3,3′,4,4′-TeCB and 3,3′,4,4′,5-PeCB were not very active whereas 2,3,3′,4,4′-PeCB and 2,3,3′,4,4′,5-HCB were very active (Figs 4–6; Table 1). It is interesting to note that all congeners with *ortho* substitutions were active except 2,2′,6,6′-TeCB, which is a fully *ortho*-substituted congener (Table 1). However, chlorine substitutions at lateral positions (*para-* in case of 2,2′,4,6,6′-PeCB; *meta-* in case of 2,2′,3,3′,6,6′-HCB) on the fully *ortho*-substituted congener resulted in congeners that are very active (Figs 5–6; Table 1). Also, when the chlorine was transferred from an *ortho-* position to the *para*-position irrespective of the benzene ring in the fully *ortho*-substituted congener resulted in congeners that were active (Fig. 4; Table 1). 2,2′,6,6′-TeCB was not active whereas 2,2′,4,6- and 2,2′,4,6′-TeCBs were very active in inhibiting Ca^{2+}-sequestration by microsomes and mitochondria (Table 1). It is evident from the present SAR that increased chlorination was not

related to the potency of these congeners to inhibit Ca^{2+}-buffering by microsomes and mitochondria (Table 1).

Discussion

The results of the present study indicate differential effects of PCB mixtures, PCB congeners and a dibenzofuran on microsomal and mitochondrial $^{45}Ca^{2+}$-uptake in rat cerebellum. Although the shapes of the concentration-response curves for the inhibition of $^{45}Ca^{2+}$-uptake in these two organelles were similar, ANOVA of the IC_{50} values indicated an overall significant effect of organelle (Table 1). This could be due to the fact that $^{45}Ca^{2+}$-uptake by these organelles is mediated by different mechanisms. That is, microsomal $^{45}Ca^{2+}$-uptake is an active process involving ATP hydrolysis by Ca^{2+}-ATPase, while mitochondrial $^{45}Ca^{2+}$-uptake is an electrophoretic uniport process driven by the potential difference established across the mitochondrial inner membrane by a proton pump energized by ATP hydrolysis (Borle 1981; Carafoli 1987). Thus, the mechanism by which PCBs inhibit $^{45}Ca^{2+}$-uptake in these two organelles could be different. In general, microsomal $^{45}Ca^{2+}$-uptake seems to be more sensitive to the effects of PCB congeners than mitochondrial $^{45}Ca^{2+}$-uptake (Table 1). Under normal conditions, mitochondria stores more Ca^{2+} than endoplasmic reticulum (ER; microsomes); however, ER is more efficient in sequestering cytosolic Ca^{2+} than mitochondria (Carafoli 1987). Since microsomal $^{45}Ca^{2+}$-uptake is more sensitive to some PCB congeners, the inhibition of microsomal Ca^{2+}-sequestration might play a significant role in PCB-induced perturbations in Ca^{2+} homeostasis in rat cerebellum.

In agreement with previous studies on PKC translocation in cerebellar granule cells (Kodavanti et al. 1995) and dopamine levels in PC12 cells (Shain et al. 1991), results from the present SAR study indicate that the substitution pattern of chlorine on the biphenyl rings plays a crucial role in the activity of PCB congeners. The relative importance of the various positions (*ortho, meta or para*) is difficult to assign at the present time, although *ortho*-substitution seems to be an important factor in the activity. Chlorines in the *ortho*-position hinder coplanarity of the two phenyl groups, while lateral (*meta* and *para*) substitution is thought to facilitate certain protein binding events (McKinney and Waller 1994). Of the three *ortho*-only substituted PCB congeners tested, both 2,2′-DCB and 2,2′,6-TCB inhibited Ca^{2+}-buffering systems, while the fully substituted congener, 2,2′,6,6′-TeCB was not active. This is in agreement with previous studies on translocation of PKC (Kodavanti et al. 1995). These data indicate that except for 2,2′,6,6′-TeCB, *ortho*-substitution appears sufficient to convey activity in vitro. The two *meta*-only substituted PCBs (3,3′- and 3,5-DCBs) were active, while the *para*-only substituted congener (4,4′-DCB) was not. Although *para*-substitution alone is not sufficient for the effects on $^{45}Ca^{2+}$-uptake, it appears to facilitate the activity of other congeners. For example, when a *para*-substitution is made on 2,2′,6,6′-TeCB resulting in 2,2′,4,6,6′-PeCB, the activity was increased (Table 1). Similarly, when the chlorine is transferred from *ortho*- to *para*- regardless of the benzene ring on 2,2′,6,6′-TeCB, resulting in 2,2′,4,6- or 2,2′,4,6′-TeCB, activity increased significantly (Table 1). Some *para*-substitutions in the absence of *ortho*-substitution, however, appear to weaken activity in this preparation. For example, when *para*-substitution was made to 3,3′-DCB resulting in 3,3′,4,4′-TeCB, activity was essentially eliminated (Table 1). It is suggested from the present SAR data that lateral substitution may be equally necessary for most PCB congeners to be active in vitro, although *ortho*-substitution seems to play the most important role. This is further strengthened by the fact that chlorine substitution at the *ortho*-position increased the activity of congeners with only *para*-or with high lateral content. When *ortho*-substitution was made on 4,4′-DCB resulting in 2,4,4′-TCB, the activity was increased. When *ortho*-substitution was made on 3,3′,4,4′-TeCB or 3,3′,4,4′,5-PeCB resulting in 2,3,3′,4,4′-PeCB and 2,3,3′,4,4′,5-HCB, the activity was also increased (Table 1).

There were two exceptions in which the activity of the congeners in the present study was not consistent with our earlier work on PKC translocation (Kodavanti et al. 1995). The first was 3,3′,5,5′-TeCB, which stimulated PKC translocation in previous studies, had a very small effect on microsomal or mitochondrial $^{45}Ca^{2+}$-uptake in the present study. The second was 2,2′,3,4,4′,5,5′-HeCB, which effectively inhibited Ca^{2+}-buffering by mitochondria and microsomes, but had no effect on PKC translocation in our previous studies (Kodavanti et al. 1995). Neither congener was studied by Shain et al. (1991). Although we have no clear explanation for the differences with these two congeners, they do possess some unique structural properties that may play a role. Since the 3,3′,5,5′-TeCB is *nonortho*-substituted, it is anticipated that it might be involved in some interactions involving the coplanar state; however, the lack of *para*-substitutions may weaken such interactions. This is supported by the predicted binding affinities (3,3′,5,5′-TeCB, pEC_{50} 5.19; 3,3′,4,4′-TeCB, pEC_{50} 6.04) to the dioxin receptor using the previously developed Quantitative Structure-Activity Relationship (QSAR) model (Waller and McKinney 1992). In fact, there is also experimental evidence that the 3,3′,5,5′-TeCB can act like a "coplanar" congener (Matthews et al. 1984). The 2,2′,3,4,4′,5,5′-HeCB is the *diortho*-substituted derivative of the most dioxin-like PCB known (the 3,3′,4,4′,5-PeCB). It can be predicted again, based on the previous QSAR model (Waller and McKinney 1992), that its binding to the dioxin receptor (2,2′,3,4,4′,5,5′-HeCB, pEC_{50} 4.28; 3,3′,4,4′,5-PeCB, pEC_{50} 6.33) would be reduced by *diortho*-substitution

(Waller and McKinney 1992). Thus, in both cases there may be sufficient dioxin-like character to complicate the binding picture and yield unpredictable results.

The present SAR analysis also revealed that activity of PCBs in vitro does not appear to be directly related to the hydrophobicity of PCB congeners. Table 1 illustrates that several congeners were very effective in inhibiting Ca^{2+}-buffering by microsomes and mitochondria, regardless of the number of chlorines, suggesting that hydrophobicity does not play a major role in the activity of PCBs in vitro. The activity of PCBs appears to be more directly associated with the structural configuration of PCB congeners.

Since most of the selected congeners represent the ones found in the environment, the effectiveness of these congeners may represent a potential for health risk. For example, 2,2'-DCB, 2,4,4'-TCB, 2,2',5,5'-TeCB, 2,2',3,3',4,4'- and 2,2',4,4',5,5'-HCBs have been found in soil (total PCB concentrations ranged from 1.4 to 61 mg/kg) and drinking water (total PCB concentrations ranged from 100 to 450 ng/l) samples (WHO 1993), and are active in vitro in the present preparation (Table 1). Similarly, 2,4,4'-TCB, 2,2',5,5'-TeCB, 2,3,3',4,4'- and 2,3',4,4',5-PeCBs, 2,2',3,3',4,4'-, 2,2',4,4',5,5'-, and 2,3,3',4,4',5-HCBs have been found in brain, human breast milk, fish, meat and dairy products with total PCB concentrations up to 235 mg/kg (WHO 1993), and are also active in vitro (Table 1). Of particular interest, 2,2',4,4',5,5'-HCB which accounted for 22% of the PCBs in human tissue and may be the single most important congener representing many *ortho*-substituted PCBs that constitute PCB residue in humans (Jensen and Sundstrom 1974), was also active (Table 1). Seegal and his associates reported an accumulation of several *ortho*-substituted PCB congeners in the brains of rat pups (up to 624 ng/g) when the dams were exposed to 300 ppm Aroclor 1254 in their diet from conception to weaning (Shain et al. 1986), as well as in brains (up to 53 μg/g) of adult rats exposed to 1000 ppm Aroclor 1254 in diet for 30 days (Seegal et al. 1991a). Similarly, Rosin and Martin (1981) reported that disposition of ^{14}C-Aroclor 1254 in intracellular organelles following an oral dose of 500 mg/kg, which caused neurobehavioral changes, yielded levels comparable to those used to study in vitro effects on neurotransmitter function (up to 100 μM). In the present study, effects of PCBs on Ca^{2+}-buffering were observed at much lower concentrations (Figs 1–7; Table 1). Since these *ortho*-lateral congeners exist in the environment, accumulate in brain tissue, and are active in vitro, the effects of PCBs on the nervous system could be mediated by these congeners.

Observations with PCB mixtures indicated that all the Aroclors 1016, 1254 and 1260 were active. It is interesting to find that one of the major contaminants in the PCB mixtures, 1,2,3,7,8-pentachlorodibenzofuran (Erickson 1986), was not active in vitro. This suggests that the effects of PCB mixtures on the nervous system are due to the PCB congeners present in those mixtures.

From the present SAR data, it is evident that *ortho-/meta*-substitution or *ortho*-substitution in the presence of some degree of lateral (*meta*, *para*) substitution is required for the activity in vitro. Only *para*-substitution or high lateral content in the absence of *ortho* substitutions, a characteristic of good dioxin receptor ligands, were devoid of activity. Thus, low lateral substitution (especially without *para*-substitution that can favor coplanarity) or high lateral content in the presence of *ortho* substitution (to hinder coplanarity) may be the most critical structural requirement underlying the in vitro activity of most PCB congeners. The results further indicate the interplay of multiple structural requirements in the expression of PCB neuroactivity that may reflect multiple binding sites and/or different binding modes to the same site. Further studies are needed to clarify these issues.

Acknowledgements The present work is supported by EPA award CR 818550-01-0 to PRSK. The authors thank Ms. Theresa Freudenrich for technical assistance and Drs. Kevin M. Crofton and Chris J. Waller for critical comments on an earlier version of the manuscript.

References

Borle AB (1981) Control, modulation, and regulation of cell calcium. Rev Physiol Biochem Pharmacol 90: 13–153

Carafoli E (1987) Intracellular calcium homeostasis. Annu Rev Biochem 56: 395–433

Choi DW (1985) Glutamate neurotoxicity in cortical cell culture is calcium dependent. Neurosci Lett 58: 293–297

Cottman CW, Matthews DA (1971) Synaptic plasma membranes from rat brain synaptosomes: Isolation and partial characterization. Biochem Biophys Acta 249: 380–394

Erickson MD (1986) Analytical chemistry of PCBs. Butterworth, Boston

Eboli ML, Ciotti MT, Mercanti D, Calissano P (1993) Differential involvement of protein kinase C in transmitter release and response to excitatory amino acids in cultured cerebellar neurons. Neurochem Res 18: 133–138

Fabiato A, Fabiato F (1978) Effect of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. J Physiol 276: 233–255

Farber JL (1990) The role of calcium in lethal cell injury. Chem Res Toxicol 3: 503–508

Jensen S, Sundstrom G (1974) Structures and levels of most chlorobiphenyls in two technical PCB products and in human adipose tissue. Ambio 3: 70–76

Johnson JD, Meisenheimer TL, Isom GE (1986) Cyanide-induced neurotoxicity: role of neuronal calcium. Toxicol Appl Pharmacol 84: 464–469

Johnson JD, Conroy WG, Isom GE (1987) Alteration of cytosolic calcium levels in PC12 cells by potassium cyanide. Toxicol Appl Pharmacol 88: 217–224

Kiedrowski L, Costa E, Wroblewski JT (1992) Glutamate receptor agonists stimulate nitric oxide synthase in primary cultures of cerebellar granule cells. J Neurochem 58: 335–341

Kodavanti PRS, Shin D, Tilson HA, Harry GJ (1993) Comparative effects of two polychlorinated biphenyl congeners on Ca^{2+}-homeostasis in rat cerebellar granule cells. Toxicol Appl Pharmacol 123: 97–106

Kodavanti PRS, Shafer TJ, Ward TR, Mundy WR, Freudenrich T, Harry GJ, Tilson HA (1994) Differential effects of polychlorinated biphenyl congeners on phosphoinositide hydrolysis and protein kinase C translocation in rat cerebellar granule cells. Brain Res 662: 75–82

Kodavanti PRS, Ward TR, McKinney JD, Tilson HA (1995) Increased [^{3}H]-phorbol ester binding in rat cerebellar granule cells by polychlorinated biphenyl mixtures and congeners: structure-activity relationships. Toxicol Appl Pharmacol 130: 140–148

Kohli KK, Gupta BN, Albro PW, Mukhtar H, McKinney JD (1979) Biochemical effects of pure isomers of hexachlorobiphenyl: fatty livers and cell structure. Chem Biol Interact 25: 139–156

Kuratsune M, Youshimara T, Matsuzaka J, Yamaguchi A (1972) Epidemiologic study on Yusho: a poisoning caused by ingestion of rice oil contaminated with a commercial brand of polychlorinated biphenyls. Environ Health Perspect 1: 119–128

Kwan CY, Takemura H, Obie JF, Thastrup O, Putney JW Jr (1990) Effects of MeCh, thapsigargin, and La^{3+} on plasmalemmal and intracellular Ca^{2+} transport in lacrimal acinar cells. Am J Physiol 258: C1006–C1015

La Rocca PT, Carlson GP (1979) The effect of polychlorinated biphenyls on adenosine triphosphatase activity. Toxicol Appl Pharmacol 48: 185–192

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin-phenol reagent. J Biol Chem 193: 265–275

Maier WE, Kodavanti PRS, Harry GJ, Tilson HA (1994) Sensitivity of adenosine triphosphatases in different brain regions to polychlorinated biphenyl congeners. J Appl Toxicol 14: 225–229

Matthews HB, Surles JR, Carver JG, Anderson MW (1984) Halogenated biphenyl transport by blood components. Fundam Appl Toxicol 4: 420–428

McKinney JD, Waller CL (1994) Polychlorinated biphenyls as hormonally active structure analogs. Environ Health Perspect 102: 290–297

McKinney JD, Chae K, Gupta BN, Moore JA, Goldstein JA (1976) Toxicological assessment of hexachlorobiphenyl isomers and 2,3,7,8-tetrachlorodibenzofuran in chicks. 1. Relationship of chemical parameters. Toxicol Appl Pharmacol 36: 65–80

Miller RJ (1991) The control of neuronal Ca^{2+}-homeostasis. Prog Neurobiol 37: 255–285

Moore L, Chen T, Knapp HR Jr, Landon EL (1975) Energy dependent calcium sequestration activity in rat liver microsomes. J Biol Chem 250: 4562–4568

Nicotera P, Bellomo G, Orrenius S (1992) Calcium-mediated mechanisms in chemically induced cell death. Annu Rev Pharmacol Toxicol 32: 449–470

Nishihara Y, Utsumi K (1986) 2,5,2′,5′-Tetrachlorobiphenyl impairs the bioenergetic functions of isolated rat liver mitochondria. Biochem Pharmacol 35: 3335–3339

Nishihara Y, Robertson LW, Oesch F, Utsumi K (1985) Interaction of tetrachlorobiphenyls with isolated rat liver mitochondria. J Pharmacobio-Dyn 8: 726–732

Rosin DL, Martin BR (1981) Neurochemical and behavioral effects of polychlorinated biphenyls in mice. NeuroToxicology 2: 749–764

Safe S (1984) Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology, and mechanism of action. CRC Crit Rev Toxicol 12: 319–395

Safe S, Safe L, Mullin M (1987) Polychlorinated biphenyls: environmental occurrence and analysis, In: Safe S, Hutzinger O (eds) Polychlorinated biphenyls (PCBs): mammalian and environmental toxicology. Springer, Berlin, pp 1–13

SAS (1989) SAS/STAT users guide, vol. 2, GLM-VARCOMP. Version 6, 4th edn. SAS Institute, Cary, N.C.

Seegal RF, Brosch KO, Bush B (1986) Polychlorinated biphenyls produce regional alterations of dopamine metabolism in rat brain. Toxicol Lett 30: 197–202

Seegal RF, Bush B, Brosch KO (1991a) Sub-chronic exposure of the rat to Aroclor 1254 selectively alters central dopaminergic function. NeuroToxicology 12: 55–66

Seegal RF, Bush B, Brosch KO (1991b) Comparison of effects of Aroclors 1016 and 1260 on non-human primate catecholamine function. Toxicology 66: 145–163

Shain W, Overmann SR, Wilson LR, Kostas J, Bush B (1986) A congener analysis of polychlorinated biphenyls accumulating in rat pups after perinatal exposure. Arch Environ Contam Toxicol 15: 687–707

Shain W, Bush B, Seegal R (1991) Neurotoxicity of polychlorinated biphenyls: structure-activity relationship of individual congeners. Toxicol Appl Pharmacol 111: 33–42

Siesjo BK (1990) Calcium in the brain under physiological and pathological conditions. Eur Neurol 30[suppl 2]: 3–9

Waller CL, McKinney JD (1992) Comparative molecular field analysis of the polyhalogenated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls. J Med Chem 35: 3660–3666

World Health Organization (1993) Environmental Health Criteria 140: polychlorinated biphenyls and terphenyls (2nd edn), prepared by Drs. S. Dobson and G.J. van Esch. International Programme on Chemical Safety, Geneva, Switzerland

Zimmerman U-J P, Schlaepfer WW (1982) Characterization of a brain calcium-activated protease that degrades neurofilament proteins. Biochemistry 21: 3977–3983