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Original article

The effect of PCB126, 77, and 153 on the intracellular mobilization of Ca^{+2} in bovine granulosa and luteal cells after FSH and LH surge *in vitro*

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Abstract

Polychlorinated biphenyls (PCBs) are a group of persistent environmental pollutants that impair cattle reproduction. Among other effects, PCBs can disturb the intracellular mobilization of Ca^{+2} in several cell types. Hence, it is possible that they disrupt the transduction of intracellular signals generated from gonadotropin (FSH/LH) receptors. In steroidogenic ovarian cells, a defect in Ca^{+2} mobilization may have a detrimental influence on two important processes: the secretion of steroids (E2 or/and P4) and their morphological and functional differentiation.

The aim of this study was to determine the influence of PCBs: 126 (dioxin-like) 77 (ambivalent) and 153 (estrogen-like) and a mixture of PCBs (Aroclor 1248) on these processes. Bovine granulosa and luteal cells were incubated for 72 hrs with PCBs (100 ng/ml), followed by Fura 2AM dye, and the fluctuations in intracellular Ca^{+2} mobilization after FSH/LH treatment were determined using an inverted microscope coupled with a CCD camera. The intensity and area of fluorescence excited by UV light were detected in the green spectrum of visible light. Aroclor 1248 and PCBs 153 and 77 significantly decreased ($P < 0.01$-0.001) the effect of FSH on intracellular Ca^{+2} mobilization in granulosa cells. In luteal cells, the most effective PCB on this process was PCB 77. The results revealed adverse effects of PCBs on the mobilization of intracellular Ca^{+2}. Moreover, the estrogen-like congeners were found to more effectively disturb this process than the dioxin-like PCB 126. Hence, it is possible for PCBs to have a negative influence on reproductive processes by affecting calcium mobilization.

Key words: PCB, calcium mobilization, FSH, LH, cattle

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Introduction

Polychlorinated biphenyls (PCBs) belong to a class of persistent environmental pollutants. As endocrine disruptors, PCBs may impair many vital processes, including reproduction in humans and animals (Ulbrich et al. 2004, Kotwica et al. 2006, Meeker and Hauser 2010). These substances are often detected in bovine reproductive organs (Thomas et al. 1999, Glynn et al. 2000), including ovaries, where they are accumulated mainly in follicular fluid (Kamarianos et al. 2003). PCBs can disturb ovarian steroidogenesis (Roselli et al. 2000) as well as the secretion of oxytocin (OT) from bovine luteal cells (Mlynarczuk and Kotwica 2005) and prostaglandins (PGE2 and PGF2α) from the myometrium (Wrobel et al. 2009), endometrium and endothelial cells from oviduct (Wrobel et al. 2010). Moreover, certain individual congeners impair the effect of FSH-stimulation on OT secretion from bovine granulosa cells (Mlynarczuk et al. 2005) and suppress the effects of LH on progesterone (P4) and OT secretion from bovine luteal cells (Mlynarczuk and Kotwica 2006). The mechanism underlying this effect in not known, but it has been suggested that xenobiotics may disrupt the signals transmission, generated from membrane receptors (Nikula et al. 1999). Additionally, PCBs can disturb the intracellular concentration of Ca^{+2} in human granulocytes (Voie and Fonnum 1998), decrease the concentrations of cAMP and phosphoinositols concentrations in cortical cells (Inglefield et al. 2001) and decrease intracellular calcium mobilization in bovine myometrial cells (Bae et al. 1999, Wrobel and Kotwica 2005) and mouse thymocytes (Yilmaz et al. 2006). Thus, PCBs may influence the affinity of some membrane receptors for their ligands or impair intracellular signaling pathways.

Both gonadotropins, FSH and LH, play a pivotal role in the regulation of bovine ovarian functions (Shemesh and Hannsel 1976, Baird 1992, Hunter et al. 2004). FSH is an essential factor for the recruitment and growth of antral follicles (Fortune 1993) and oocyte maturation (Armstrong et al. 1994, Adriaens et al. 2004). Additionally, LH is responsible for the survival of the dominant follicle to ovulation after FSH surge (Campbell et al. 1999), the alteration of follicular steroidogenesis towards P4 synthesis and secretion (Brendtson 1995) and the initiation of luteinization in theca interna and granulosa cells (Fortune et al. 2001). The receptors for these hormones belong to a structurally unique subfamily of G-protein-coupled receptors (Ji et al. 1998) that are linked to the adenyl cyclase and phospholipase C signal transduction pathway (Zhu et al. 1994). After activation of these receptors by their ligands and signal transduction initiation, Ca^{+2} ions are released into the cytoplasm. Hence, intracellular fluctuations in Ca^{+2} concentration are an indicator of these receptors activity (Zhu et al. 1994, Ryu et al. 1998).

In this study, ovarian steroidogenic cells were incubated with different individual congeners of PCBs or a mixture of them (Aroclor 1248) and treated with FSH or LH, after which intracellular calcium mobilization was measured. The estrogen-like congener PCB 153 (Katzenellenbogen et al. 1993), the dioxin-like congener PCB 126 (Lind et al. 1999), the bifunctional congener PCB 77 (Nesaretnam et al. 1996, Pang et al. 1999) and industrial mixture of PCBs – Aroclor 1248 were chosen for the experiments.

Materials and Methods

Collection of ovaries and cell preparation

Bovine ovaries were collected from cows or mature heifers at a defined stage of estrous cycle (Ireland et al. 1980, Fields and Fields 1996) at a commercial slaughterhouse within 15-20 min after killing the animals. The ovaries were placed in ice-cold 0.9% NaCl containing penicillin (10 IU/ml), streptomycin (100 μg/ml), amphotericin (2 μg/ml) and L-glutamine (100 μg/ml) and then transported to the laboratory in an ice bath within 1 h. Atretic follicles were eliminated on the basis of the procedure described by Henderson et al. (1987). All materials used were purchased from Sigma (Poznan, Poland) unless otherwise stated. The ovaries utilized for the collection of granulosa cells were from days 16-21 of the estrous cycle. For each experiment, cell mixtures from 10 follicles > 1 cm were collected via vigorous aspiration of follicular fluid (Voss and Fortune 1991). The cells were suspended in DMEM/HAM-12 medium supplemented with 10% NCS and transferred to 4-well plates (5×10^4 cells/ml) (Nunclon NUNC, Denmark). After a 24-h preincubation, the medium was changed, and the cells were washed twice with M-199 medium containing 0.1% BSA. The DMEM/HAM-12 incubation medium (1 ml) was supplemented with 0.1% BSA, ascorbic acid (20 μg/ml), transferrin (5 μg/ml) and sodium selenite (5 ng/ml).

A suspension of luteal cells was obtained from the corpus luteum (CL) between days 5-10 of the estrous cycle ($n = 3$) by perfusion with a mixture of enzymes (1 mg/ml collagenase IA and 5 μg/ml DNase I) as described by Okuda et al. 1992. Cells showing a viability above 85% were used for further analyses. Luteal cells were suspended in DMEM/HAM-12 medium supplemented with 10% FCS and transferred to 4-well plates (5×10^4 cells/ml) (Nunclon NUNC, Denmark). After a 24 hrs preincubation, the medium was

changed, and the cells were washed twice with M-199 medium containing 0.1% BSA. The incubation medium (1 ml) was the same as was used for granulosa cells.

Both, granulosa and luteal cells were then incubated with PCB: 126, 77, 153 and Aroclor 1248 at a dose of 100 ng/ml for 72 h. The cells were cultured in an atmosphere of air containing 5% CO_2 at 100% humidity at 38°C (Heraus BB-6060, Hanau, Germany). All of the media used were phenol red-free and contained 20 µg/ml gentamycin (ICN).

Preparation of cells for measuring Ca^{+2} mobilization

After incubation, the cells were washed three times in DMEM/HAM-12 with 0.1% BSA, and calcium-free PBS was added to each well, supplemented with 0.1% BSA (500 µl) and Fura 2AM dye (5 µM/ml), as described by Grynkiewicz et al. (1985). The cells were incubated for 60 min in a cells culture incubator and then washed three times again in DMEM/HAM-12 with 0.1% BSA. The wells were subsequently replenished with calcium-free PBS at half of the previous volume of medium (250 µl). The cells were cultured for 45 min in heater (ZALMED 32/250, Poland) at 30°C with a controlled atmosphere (95% air + 5% CO_2).

Determination of Ca^{+2} mobilization

The fluctuations in intracellular Ca^{+2} mobilization were determined using an inverted microscope (Olympus IX70) with a fluorescence objective (Olympus Fl, 40x) and a UV filter for Fura 2AM (340 nm wavelength). The total magnification was 400x. The Olympus VLS 100HG UV burner was used as the source of UV light. Images were collected (frame by frame) for 70 sec at 5 sec intervals using an Ikegami FL3 CCD color camera (at a VGA resolution of 640 × 480). The files were saved in the uncompressed TIFF format. The smallest scale of measurement was 3 × 3 pixel squares. The images were analyzed using graphics software (Olympus MicroImage 4.0, Japan). The intensity and area of fluorescence excited by the UV Fura 2AM dye were detected in the green spectrum of visible light (510-520 nm).

Preliminary studies

The spontaneous mobilization of intracellular Ca^{+2} was visualized either with the shutter of the microscope open during the entire time of the measurements (SPO) or with the shutter was open only during a specific time required to the acquire images by the camera (STO, 0.5-1 sec/picture). Luteal cells (from 3-4 CL and 5-10 days of the cycle) and granulosa cells from 10 follicles (> 1 cm in diameter) were used for imaging. The cells were prepared as previously described. Two luteal cells or 4 granulosa cells were placed in the photo frame of the microscope. The mean ± SEM of the first four points in each measurement was used as the control value. The means ± SEM of further points were compared with the control using ANOVA and Tukey's post-test. The obtained data (Fig. 1) showed that the constant exposure of cells treated with Fura to UV light (SPO) provoked a progressive, but statistically significant ($P < 0.05$) decline in dye luminescence after either 40 sec (luteal cells) or 45 sec (granulosa cells). However, when the shutter was opened only at the exposure time (STO), the luminescence intensity was not significantly changed ($P > 0.05$) during the time of measurement (70 sec); hence, this method was chosen for further investigations.

Detection of intracellular Ca^{+2} mobilization

Intracellular Ca^{+2} mobilization was induced by treating the cells with LH (luteal cells) or FSH (granulosa cells). The gonadotropins were microinjected (1 ng/5 µ l of solvent) near the observed cells after the fourth image. The first four pictures were used to determine the control value of excitation. The obtained images were analyzed as described above.

Statistical analyses

For this study, luteal cells from 3 different CL were used, and detection was repeated twice for each of the groups of CL cells ($n = 6$). For granulosa cells, 3 cultures were used (each contained cells from 10 follicles > 1 cm in diameter), and detection was also repeated twice for each culture ($n = 6$). The means ± SEM of the green area and green intensity of the products in the next measurement points were obtained. Analogical points in the control and PCB treatment curves were compared with each other. The mean ± SEM areas under the curves were then analyzed. For these calculations, ANOVA and the Tukey post-test were used. The statistical procedures were performed with the software program PRISM 4.0 (GraphPad Software Inc., CA, USA).

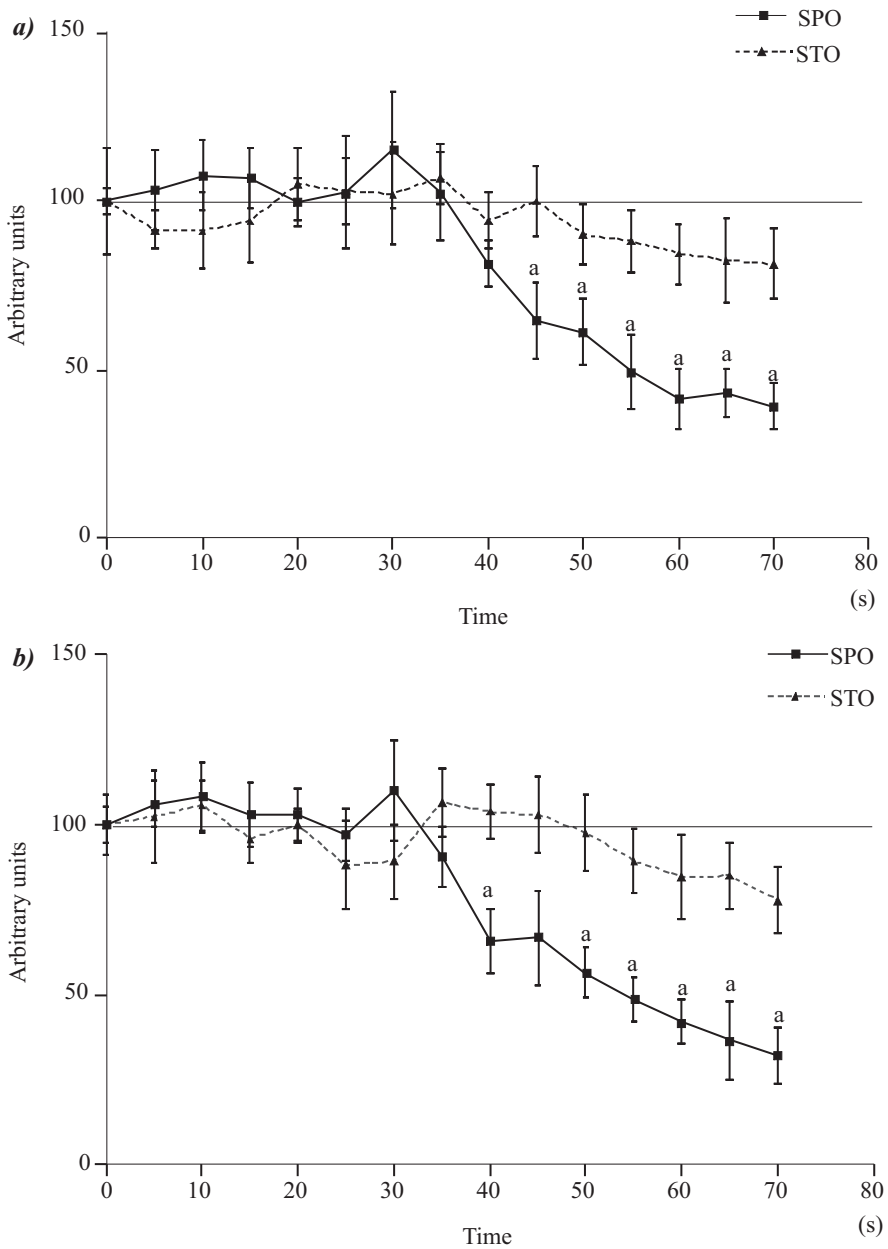

Fig. 1. The effect of still and intermitted of UV stream light on the spontaneous luminescence of Fura 2AM in granulosa (*a*) and luteal (*b*) cells ($P < 0.05$). SPO – Shutter permanently open, STO – Shutter temporary open.

Results

Aroclor 1248 and PCBs 153 and 77 strongly decreased ($P < 0.01$-0.001) the effect of FSH on intracellular Ca^{+2} mobilization in granulosa cells (Fig. 2a). This effect was detected at every point where measurements were taken ($P < 0.01$-0.001), and it is visible as the decrease ($P < 0.01$) in the area under the curves compared with the control (Fig. 2b). The effect of PCB 126 was not as dramatic as that of the other congeners but was still statistically significant ($P < 0.05$).

In luteal cells, PCB 77 significantly decreased Ca^{+2} mobilization ($P < 0.05$) after LH surge. PCBs 126 and 153 showed a similar effect, however it was not significant ($P > 0.05$) at all points measured (for example at 45 sec of measurement, Fig. 3a). However, the area under the curve for all congeners was significantly smaller than area under the curve for the control (Fig. 3b). Although Aroclor 1248 decreased Ca^{+2} mobilization at individual points of measurement ($P < 0.05$-0.001), it did not significantly ($P > 0.05$) decrease the value of the area under the curve (Fig. 3b).

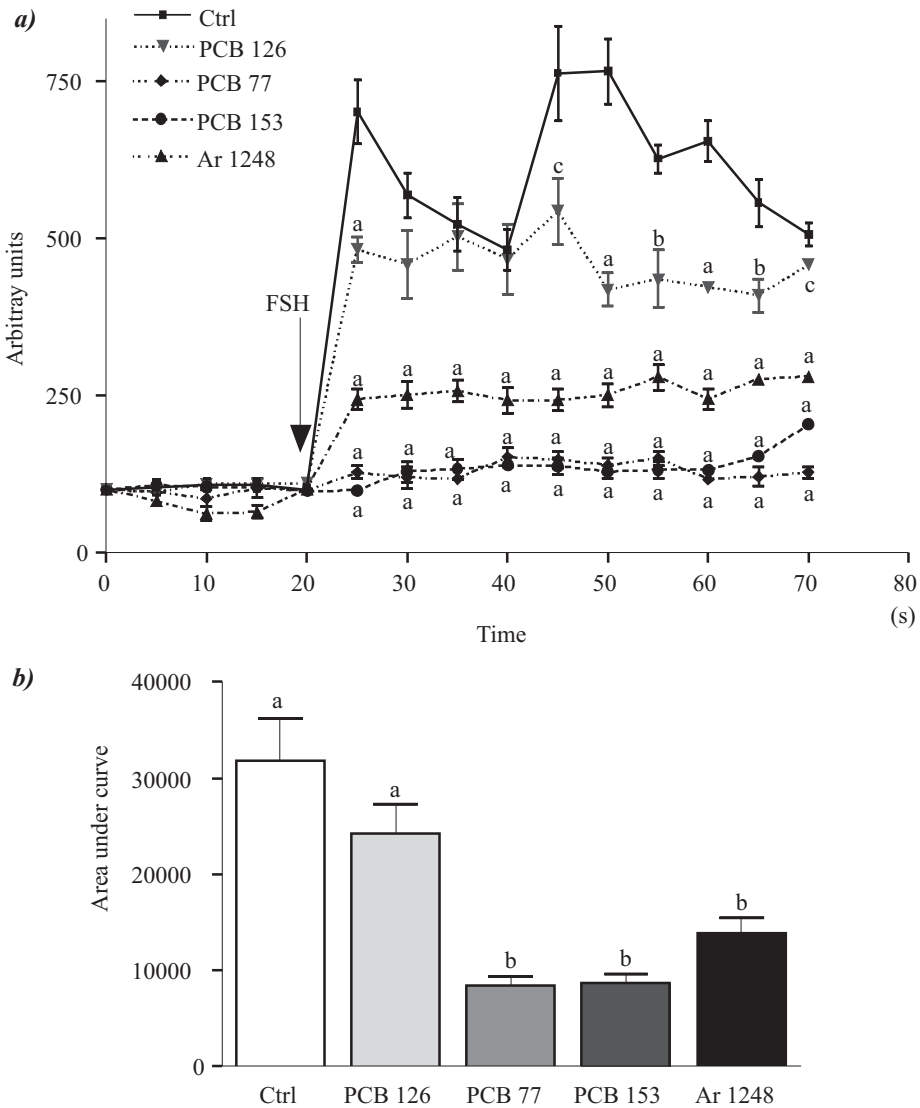

Fig. 2. The fluctuations of Ca^{+2} mobilization (*a*; a – $P < 0.001$, b – $P < 0.01$, c – $P < 0.05$) and changes in its total concentrations (b; as area under curve, $P < 0.05$) in granulosa cells treated by PCBs (100 ng/ml) after FSH surge.

Discussion

It was previously shown that individual congeners and Aroclor 1248 do not affect the viability of granulosa and luteal cells at doses between 1-100 ng/ml under 24-72 h of incubation (Mlynarczuk and Kotwica 2005). Hence, the results obtained here are not a result of decreasing cell viability.

Our results has shown that estrogen-like PCBs were more effective in disrupting the mobilization of intracellular Ca^{+2} than the dioxin-like PCB 126. However, it is difficult to identify which phase of signal transduction was impaired. It is well established that the interaction of LH with its receptor leads to the activation of adenylate cyclase through stimulatory guanine nucleotide regulatory protein (Gs protein), leading to an increase in cyclic AMP levels. This reaction is related to intracellular Ca^{+2} mobilization (Mennon and Mennon 2012). A similar effect has been observed on E2 secretion in the mTLC-1 cell line when treated with the estrogen-like substance bisphenol A after an FSH surge (Nikula et al. 1999). Moreover, bisphenol A and another estrogen-like chloroorganic insecticide, DDT, have been shown to decrease the cAMP concentration in cells (Nikula et al. 1999, Pohland and Tiemann 2003, Wójtowicz et al. 2007). Hence, the influence of PCBs on intracellular Ca^{+2} mobilization may occur through a similar mechanism, which is dependent on the biological properties and chemical structure of the PCBs, although PCBs can affect different steps of the intracellular signal transduction pathway (Inglefield et al. 2001,

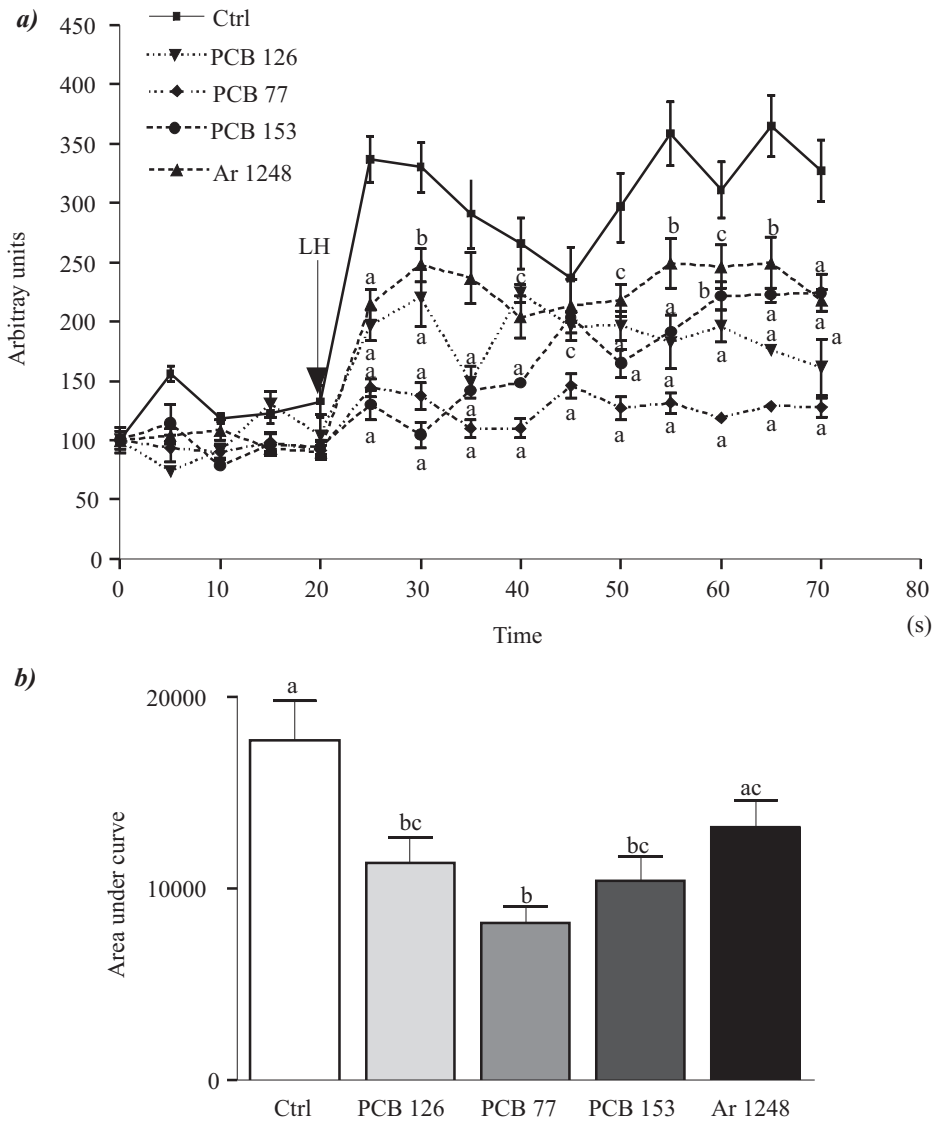

Fig. 3. The fluctuations of Ca^{+2} mobilization (*a*; a — $P < 0.001$, b — $P < 0.01$, c — $P < 0.05$, $n = 6$) and changes in its total concentrations (*b*; as area under curve, $P < 0.05$) in luteal cells treated by PCBs (100 ng/ml) after LH surge.

Llansola et al. 2009). The intermediate activity of the industrial PCBs mixture, Aroclor 1248, may have been the result of its composition. It contains both estrogen- and dioxin-like PCB congeners (Frame et al. 1996) which may partially antagonize each other effects (Suh et al. 2003).

This peculiar "desensitization" of steroidogenic ovarian cells to gonadotropins caused by PCBs can entail considerable consequences. It has been reported (Kawate 2004) that disturbances in the signals received and transduced from FSH and LH receptors may be one of the reasons for the formation of follicular cysts, which can further disturb or even disrupt the ovarian cycle. Estrogen-like chloroorganic substances, such as DDT and some PCB congeners, are also known to predispose both rats and humans to ovarian cyst formation (Heirichs et al. 1971, Jonsson et al. 1975, Gotz et al. 2001). Cystic ovarian disease is a significant problem for the reproduction of cattle and other domestic animals. This syndrome has been identified in 10-13% of dairy cows (Bartlett et al. 1986), and in some herds, ovarian cysts have been detected in 30-40% of cows (Archbald and Thatcher 1992). Disruption of the normal functioning of the hypothalamic-hypophyseal-ovarian axis is the basic cause of this illness, but its exact etiology is still poorly understood. Many factors may lead to a predisposition to this disease, including chloroorganic substances (Vanholder et al. 1997). Hence, a decrease in the reactivity of granulosa and luteal cells to gonadotropins caused by PCBs may constitute part of the mechanism responsible for the onset of cystic ovarian syndrome in cattle.

In conclusion, the PCB congeners tested here, de-

creased intracellular Ca^{+2} mobilization in granulosa and luteal cells after an FSH or LH surge, and these PCBs may disturb reproductive processes by this mechanism. The estrogen-like congeners PCBs 77 and 153 were the most effective in this process.

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