**Effect of polychlorinated biphenyls on the secretion of oxytocin from luteal and granulosa cells in cow: possible involvement of glucocorticoid receptors**

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**ABSTRACT:** Polychlorinated biphenyls (PCBs) stimulate oxytocin secretion from bovine granulosa and luteal cells. Since oxytocin on the one hand is released from ovarian cells by cortisol and on the other hand PCBs can be bound by glucocorticoid receptors (GCr), we have tested the hypothesis that PCBs acting via GCr can stimulate oxytocin secretion. In preliminary studies the effect of RU486 (GCr blocker) on cells viability was tested. Thereafter, a selected dose of RU486 (10^5 M), which did not affect cell viability, was used in further experiments. It appears that this dose of RU486 completely blocked GCr against cortisol-stimulated oxytocin secretion, in both types of cells. Furthermore, granulosa cells (10^5) from follicles of two sizes (>1 cm < in diameter) and luteal cells (10^5) from day 5–10 of estrous cycle were incubated for 72 h with congeners of PCB (126, 77 or 153) at doses of 1, 10 or 100 ng/ml each, separately or jointly with RU486. The effect of PCB 77 and 153 on oxytocin secretion was blocked by RU486, but it did not change the effect evoked by PCB126 in both granulosa and luteal cells. We assume that some PCB congeners can affect oxytocin secretion from granulosa and luteal cells acting via GCr.

**Keywords:** polychlorinated biphenyls; oxytocin; glucocorticoid receptors; ovary; cow

PCBs are recognised as environmental pollutants due to their slow rate of degradation (Borja et al., 2005) and ability to be accumulated in various tissues, including tissues of the reproductive tract in humans and animals (Archibeque-Engle et al., 1997; Pauwels et al., 1999; Kamarianos et al., 2003; De Saeger et al., 2005; Mlynarczuk et al., 2005). They are known to elicit diverse effects causing impairment of reproductive processes (Safe, 1994). Recently we found that PCBs essentially impair contractility of the bovine uterus (Wrobel et al., 2005) via their influence on prostaglandin synthesis in the endometrium (Wrobel and Kotwica, 2005). Moreover, a mixture of PCBs (Aroclor 1248) and individual congeners slightly affected steroid release, but significantly stimulated oxytocin secretion from bovine corpus luteum (CL) and from follicles of different sizes (Mlynarczuk et al., 2003; Mlynarczuk and Kotwica, 2005). It should be noted that ovarian oxytocin plays a crucial role in the regulation of the estrous cycle in cows (Stormshak, 2003). It plays an important role in the growth and maturation of follicles (Voss and Fortune, 1993; Jo and Fortune, 2003), it supports the function of early CL (Miyamoto and Schams, 1991) and also participates in luteolysis at the end of the luteal phase (Hansel et al., 1987; McCracken et al., 1999; Kotwica et al., 1999). PCBs are suggested to affect cell function acting via the estradiol receptor (ER) (Bonefeld-Jorgensen et al., 2001), as agonists or antagonists (Nesaretnam et al., 1996; Nesaretnam

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and Darbre, 1997) and via the arylhydrocarbon receptor (AhR; Lind et al., 2000).

Estradiol was found to be a stimulator of oxytocin secretion from bovine follicle (Voss and Fortune, 1993). Although some PCBs have a lower affinity to ER than native hormone, they can stimulate oxytocin secretion from granulosa cells more efficiently than estradiol does (Mlynarczuk and Kotwica, 2005). On the other hand cortisol was found to be a strong stimulator of oxytocin secretion from ovarian cells (Luck, 1988). PCBs were also found to be able to affect adrenal steroidogenesis via GCr (Goldman and Yawetz, 1992). Therefore it is possible that PCBs can influence on oxytocin secretion from ovarian cells via the same receptors.

**MATERIAL AND METHODS**

**Collection of ovaries.** Bovine ovaries from cows or mature heifers at a defined stage of the estrous cycle (as described by Ireland et al. (1980) and Fields and Fields (1996)), were collected from a commercial slaughterhouse within 15–20 min of killing the animals. Ovaries were placed in an ice-cold 0.9% NaCl solution 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 on ice within 1 hour. Atretic follicles were eliminated on the basis of examinations described by Henderson et al. (1987). All materials used were purchased from Sigma (Poznan, Poland), unless otherwise stated.

**Preparation of granulosa cells.** Ovaries for the collection of granulosa cells were from days 6–16 for the follicles <1 cm, and from days 16–21 for follicles >1 cm. The cells were obtained by vigorous aspiration of follicular fluid (Voss and Fortune, 1993) and separated from the follicles of above and below 1 cm in diameter. Suspension of cells were collected into conical tubes and stored on ice. Cells collected for one 48-well plate, 8–10 follicles >1 cm or 12–14 follicles <1 cm were used. Cells were washed twice with M-199 containing Earle's salt and 0.1% BSA and suspended in DMEM/F-12 HAM enriched with 10% FCS. Viability of the cells, estimated by exclusion of trypan blue dye (0.04%), from follicles <1 cm of diameter, were 65 to 75% and about 75% for cells from follicles >1 cm of diameter. Cell suspensions (10^5/0.5 ml) were transferred into 48-well plates (Nunc, NUNC). After 24 h incubation, they were washed twice with M-199 containing 0.1% BSA, to remove cells which did not attach to well bottoms, and covered with 0.5 ml of DMEM/F-12 HAM supplemented with 0.1% BSA, ascorbic acid (20 µg/ml), transferin (5 µg/ml) and sodium selenite (5 ng/ml, ICN). Cells were then cultured in air containing 5% CO₂ with 100% humidity, and at 38°C (Heraus BB-6060, Hanau, Germany). All media were enriched with gentamycin (20 µg/ml; ICN Pharmaceuticals, Inc., Costa Mesa, USA).

**Preparation of luteal cells.** Suspension of luteal cells were obtained from CL (n = 8) on day 5–10 of the estrous cycle by perfusion with a mixture of enzymes (Collagenase IA 1mg/ml and DNAse I, 5 µg/ml), as described by Okuda et al., 1992. The cells with viability above 85% were used for further studies. Luteal cells were suspended in DMEM/HAM-12 medium supplemented with 10% NCS and placed (10^5/0.5 ml) into 48-well plates (Nunc NUNC, Denmark). After 24 h

![Figure 1](image_url)  
Figure 1. Influence different doses of RU486 (1 × 10⁻⁴M, 1 × 10⁻⁵M and 1 × 10⁻⁶M) on the viability of granulosa cells obtained from follicles <1 cm (a), >1cm (b) and luteal cells from day 5–10 of oestrus cycle (c), after 72h incubation, before statistical calculations row data were log-transformed (P < 0.05).
incubation, medium was changed and the cells were washed twice with medium M-199 containing 0.1% BSA. The incubation medium (0.5 ml) was DMEM/HAM-12 supplemented with 0.1% BSA, ascorbic acid (20 µg/ml), transferrin (5 µg/ml), and sodium selenite (5 µg/ml). All used media were without phenol red and contained gentamycin 20 µg/ml (ICN). The conditions of culture were the same as for granulosa cells.

**Preliminary studies.** Doses of RU486 used to block of GCr are higher than those applied for the blockade of P4 receptors (Mulholland et al., 2005). Therefore we measured the effect of different RU486 doses (10⁻⁴ M, 10⁻⁵ M or 10⁻⁶ M; n = 8 each) for 72 h on the viability of cells, as determined by TOX1 test (in vitro toxicity assay kit, MTT based). The procedure was performed according to manufacturers’ instructions. Actinomycin D (500 ng/ml) was used as a negative control. Absorbance of enzymatic reaction product was determined by Multiscan EX (Labsystems, Finland) at the 570 nm of wavelength. It appeared that neither 10⁻⁵ M nor 10⁻⁶ M of RU486 affected cell viability compared to the control (Figure 1).

Next, we investigated the ability of RU486 (10⁻⁵ M) to block the cortisol (10⁻⁵ M) effect, which stimulates oxytocin secretion from both granulosa and luteal cells. RU486 at the dose of 10⁻⁵ M completely blocked the stimulatory (P < 0.05) effect of cortisol on oxytocin secretion from luteal cells and granulosa cells from follicles of both >1 cm < in diameter (Figure 2). Therefore, this dose of RU486 was used in further experiments.

To avoid the criticism that RU486 blocks the stimulatory effect of P4 on oxytocin secretion from granulosa cells, as suggested by Voss and Fortune (1993), we have treated these cells with P4 (10⁻⁵ M) and jointly with P4 and RU486. Oxytocin medium concentrations did not differ between these two sets of treated cells (data not shown). Thus we assumed that RU486 would block the oxytocin-stimulated effect of PCBs via GCr in our experimental model. It should be mentioned that Voss and Fortune (1993) used luteinised granulosa cells pre-treated with gonadotropins, so this could explain differences in the results.

**Experimental design.** In this study, the influence of RU486 (10⁻⁵ M) on three different PCB congeners to stimulate oxytocin secretion from granulosa (n = 8 repetitions) and luteal cells (n = 8 repetitions), which were incubated for 72 h, was investigated. We used dioxin-like PCB-126 (Lind et al., 2000), estrogen-like PCB-153 (Bonefeld-Jorgensen et al., 2001) and PCB-77, which is able to mimic both the effect of dioxins and estrogens (Nesaretnam et al. 1996; Nesaretnam and Darbre, 1997). Each PCB (AccuStandard, USA) was used at the dose of 1, 10, or 100 ng/ml. Cortisol (10⁻⁵ M, Serva) was used as a positive control. Days 5–10 of the estrous cycle were selected for luteal cell collection, since in these days the synthesis of oxytocin precursor in CL is complete (Fehr et al., 1984).

**Oxytocin analysis.** The concentration of oxytocin in the culture medium were determined by EIA, as reported earlier by Skarzynski and Okuda (1999). Anti-oxytocin serum (R-1), which has been previously characterised by Kotwica and Skarzynski (1993), was used at a final dilution of 1:30 000. The range of the curve was 3.9–1 000 pg/ml and the sensitivity of the procedure was 16–18 pg/ml. Intra- and inter-assay coefficients of variation were on average 8.2% and 10.4%, respectively. The relationship between the added and measured amounts of hormone (n = 8) was significant (r = 0.92).

**Statistical analysis.** The mean (± SEM) oxytocin concentrations were analysed by one-way ANOVA
and by Tukey for all pairs of columns, as a post test (GraphPad PRISM software, USA).

RESULTS

All doses of PCB 77 and 153 significantly increased ($P < 0.05$–0.001) oxytocin secretion from granulosa cells obtained from follicles of both sizes, whilst only the highest dose of PCB 126 increased ($P < 0.05$) oxytocin secretion. RU486 significantly reduced ($P < 0.05$) the stimulatory effect of PCB 153 and even more efficiently the effect of PCB 77 on oxytocin secretion from granulosa cells obtained from follicles of both $>1$ cm in diameter (Figure 3). RU486 had a modest effect on the secretion of oxytocin from granulosa cells culture recruited from follicles $<1$ cm in diameter caused by dioxin-like PCB-126. The effects of PCB congeners on oxytocin secretion from luteal cells was very similar.
to those observed from granulosa cells (Figure 4). And similarly, RU486 clearly reduced oxytocin-secretion from luteal cells evoked by all doses of PCB 77 ($P < 0.01$), while it reduced ($P < 0.05–0.01$) the effect by PCB 153 (10 and 100 ng/ml), and it inhibited ($P < 0.05$) the effect for 100 ng/ml of PCB 126-stimulated oxytocin secretion (Figure 4).

**DISCUSSION**

Since the effect of PCB 153 and 77 on oxytocin secretion, from both types of cells was significantly reduced by RU486, it is suggested that PCBs can affect oxytocin secretion from ovarian steroidogenic cells via stimulation of GCr. However, dependent on the PCB congeners used, the response of cells was different. This suggests that individual congeners may have a different affinity to these receptors. It is well-known that PCB 153, and to some extend PCB 77, have estrogen-like properties (Nesaretnam et al., 1996; Nesaretnam and Darbre, 1997) and estradiol can stimulate oxytocin secretion from bovine granulosa cells (Voss and Fortune, 1993). However, the effect of these two congeners on oxytocin stimulation was evidently reduced by RU486. Thus, it can be assumed that the observed influence of PCB on oxytocin secretion did not involve stimulation of estrogen receptors. Moreover, this suggestion can be supported by a low concentration of estradiol receptor in CL at this stage of the estrous cycle in ruminants (Zieba et al., 2000).

Although RU486 used doses that completely blocked glucocorticoid receptors in preliminary studies, it did not block oxytocin secretion evoked by PCB 126 from both types of cells. It also did not completely inhibit oxytocin secretion from granulosa cells elicited by PCB 77 and 153. However, RU486 evidently inhibited oxytocin secretion from granulosa cells elicited by PCB 77 and 153. This indicates that the effect of these two PCB congeners, in contrast to PCB 126, on oxytocin secretion from ovarian steroidogenic cells, can be partly evoked through activation of glucocorticoid receptors.

Therefore it is also possible, that PCB effect on oxytocin secretion includes stimulation of other receptors. Orphan receptors of Steroidogenic Factor 1 (SF-1) could be a candidate, since expression of its gene is correlated with gene expression for neurophysin/oxytocin (Wehrenberg et al., 1994). This suggestion is supported by the data that glucocorticoids are also bound by this orphan receptor (Li et al., 2004).

Doses of PCBs we used were within the range of those determined in different tissues in humans and animals, including bovine (Roselli et al., 2000; Kamarianos et al., 2003). Thus it is possible that secretion of oxytocin from ovarian cells evoked by PCBs can impair the course of the estrous cycle in females. It is also possible that ovarian oxytocin plays an important role in the regulation of CL life.
spans (Stormshak, 2003) and in the growth and maturation of bovine follicles (Voss and Fortune, 1993; Jo and Fortune, 2003). We could not find a direct effect of PCBs on the secretion of either estradiol from granulosa cells (Mlynarczuk et al., 2005), or progesterone from luteal cells (Mlynarczuk et al., 2003). Hence we assume that PCBs can indirectly affect the ovarian function and influence the function of the uterus. Indeed, epidemiological studies reveal that women who have aborted have higher PCB concentrations in their blood (Leoni et al., 1989), while their progesterone concentration was still in the physiological range. These observations can be supported by recent studies (Wrobel and Kotwica, 2005), which showed the same PCB congeners stimulate secretion of PGF2α from bovine endometrium and increase the ratio of PGF2α:PGE2, which is partly responsible for the rise of myometrial force contractions (Wrobel et al., 2005). Since glucocorticoids are important regulators of parturition (Liggins, 1974), it looks possible that PCBs are able to mimic their effect. Perhaps this could partly explain the mechanism of early mortality of fetuses or premature parturitions evoked by PCBs.

In conclusion, PCB 77 and 153, but not dioxin-like PCB 126, can stimulate oxytocin secretion from bovine granulosa and luteal cells acting through glucocorticoid receptors. This can markedly impair CL function and follicle growth and maturation. Chlorinated hydrocarbons, including PCBs, reduced the binding of progesterone to its receptor in the mucosa of the shell gland from egg-laying ducks, domestic fowls and from the endometrium of the rabbit uterus. Thus, although RU486 is bound by a progesterone receptor, it is assumed that this compound mainly blocked oxytocin-stimulated effect of PCBs via GC receptors.

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