BIOLOGY OF REPRODUCTION **61,** 1099–1103 (1999)

Dichlorodiphenyldichloroethylene Potentiates the Effect of Protein Kinase A Pathway Activators on Progesterone Synthesis in Cultured Porcine Granulosa Cells[1]

N.K. Crellin, M.R. Rodway, C.L. Swan, C. Gillio-Meina, and P.J. Chedrese[2]

Reproductive Biology Research Unit, Department of Obstetrics and Gynecology and Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N OW8

ABSTRACT

The insecticide dichlorodiphenyltrichloroethane (DDT) and its major metabolite *p,p′*-dichlorodiphenyldichloroethylene (DDE) have been implicated as endocrine-modulating chemicals. The DDT metabolite *p,p′*-DDE has been found contaminating human tissues and follicular fluid because of dietary exposure. We investigated the effects of DDE on progesterone synthesis in a stable porcine granulosa cell line, JC-410, and in primary cultures of porcine granulosa cells. Progesterone synthesis was not affected by 0.1–100 ng/ml DDE in the JC-410 cells. However, 10 ng/ml DDE increased 8-bromo-cAMP (8-Br-cAMP)-stimulated progesterone synthesis 0.4-fold ($P < 0.05$) over the levels observed with 1 mM 8-Br-cAMP alone. The effect of cholera toxin (CT) on progesterone synthesis was increased 0.7-fold ($P < 0.05$) by 10 ng/ml DDE over the value observed with 30 ng/ml CT alone. In primary cultures of porcine granulosa cells, 10 ng/ml DDE potentiated CT-stimulated progesterone synthesis 1.2-fold over the value observed with CT alone. In the JC-410 cells, 1 and 10 ng/ml DDE increased CT-stimulated cytochrome P450-cholesterol side-chain cleavage ($P450_{scc}$) mRNA levels 0.3- and 0.4-fold, respectively, over the values obtained with CT alone. Neither basal nor CT-stimulated cAMP levels were changed by DDE. We conclude that DDE affects granulosa cell response to protein kinase A activators by altering the expression of the $P450_{scc}$ gene.

INTRODUCTION

The use of dichlorodiphenyltrichloroethane (DDT) was banned in North America and Western Europe in the 1970s because of evidence of persistence in soils and aquatic sediments and the potential to bioconcentrate in birds and mammals. However, the use of DDT has continued in developing countries [1, 2]. The persistence and biomagnification of DDT and its metabolites ensure that residues are carried far from their point of application [1]; thus human exposure may continue even in countries where DDT has been banned.

The insecticide DDT and its major and most stable metabolite, *p,p′*-dichlorodiphenyldichloroethylene (DDE), have been described as endocrine-modulating chemicals (EMCs) [3–7] that have estrogenic effects on the female reproductive system [8–11]. Dietary exposure to *p,p′*-DDE has resulted in contamination of human blood, adipose tissue, and ovarian follicular fluid [12,13]. The presence of *p,p′*-DDE in follicular fluid is of great concern because of the intimate contact between the granulosa cells and the developing oocyte. Toxins can impair fertility by altering follicular growth and hormone biosynthesis or by changing granulosa cell-oocyte interactions [14].

Definitive research investigating the molecular mechanisms by which EMCs cause reproductive dysfunction is vital to the development of appropriate screening protocols for potential EMCs [15]. The objective of this study was to investigate the effects of DDE on mammalian ovarian steroidogenesis. Studies were conducted in JC-410, a stable steroidogenic porcine granulosa cell line [16, 17], and in primary cultures of porcine granulosa cells.

MATERIALS AND METHODS

Reagents

Culture media and reagents for tissue culture were purchased from Gibco (Burlington, ON, Canada). Plastic culture plates were purchased from Falcon (Lincoln Park, NJ) and Costar (Corning, NY). The DDE used in this study was part of a chlorinated pesticide kit from PolyScience (Niles, IL) and consisted of approximately 96% *p,p′*-DDE and 4% *o,p′*-DDE. Cholera toxin (CT) and 8-bromo-cAMP (8-Br-cAMP) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{125}I]iodosodium, iodinated progesterone (11-α-hydroxyprogesterone 11-β-D-glucuronide-[^{125}I]iodotiramine), and Hybond-N nylon membranes were purchased from Amersham (Arlington Heights, IL; now Amersham Pharmacia Biotech, Piscataway, NJ). ^{32}P was purchased from New England Nuclear (Boston, MA). The Bio-Rad DC Protein Assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

Granulosa Cell Culture

Granulosa cells for primary culture were obtained from small-sized follicles (1–3 mm) in ovaries of prepubertal gilts collected at the local slaughterhouse; they were cultured as previously described [18]. The JC-410 cell line is a stable cell line originating from a spontaneously immortalized primary culture of porcine granulosa cells [16, 17]. The JC-410 cells synthesize approximately 10% of the progesterone synthesized by primary cultures of granulosa cells from small follicles. Cells were maintained in a CO_2 incubator (Forma Scientific Inc., Marietta, OH) at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. Cells were grown in phenol red-free Medium 199 supplemented with 5% newborn calf serum, 5 μg/ml insulin, 100 IU/ml penicillin, and 100 μg/ml streptomycin (culture medium). In experiments in which progesterone and cAMP were measured as end points, 24-well (1.5 × 2.0-cm wells) culture plates were used. In experiments in which RNA was extracted, cells were cultured in 10 × 2.0-cm plates. Once 70–80% confluency was attained, generally within 48 h, culture medium was replaced with serum-free culture medium containing the treatments, and incubation was per-

Accepted June 1, 1999.
Received November 9, 1998.
[1]This research was supported by the Canadian Network of Toxicology Centres, Saskatchewan Health (Government of Saskatchewan), Medical Research Council of Canada and Natural Sciences Engineering Research Council of Canada grants to P.J.C.
[2]Correspondence: P. Jorge Chedrese, Reproductive Biology Research Unit, Department of Obstetrics & Gynecology, Royal University Hospital, 103 Hospital Dr., Saskatoon, SK, Canada S7N OW8. FAX: 306 966 8040; e-mail: chedresj@duke.usask.ca

formed for 24 h. Treatments were delivered in 5-μl volume to wells containing 1 ml of medium. Control groups received vehicle, consisting of ethanol and benzene, up to a maximum of 0.01% benzene and 0.49% ethanol. This percentage is volume:volume and was not adjusted for the specific gravity of alcohol.

Quantification of Progesterone, cAMP, Cellular Protein, and DNA

Progesterone content was determined by RIA in 100 μl of culture medium, as previously described [18]. The inter- and intraassay coefficients of variation for the progesterone assay were below 10%. The minimum detectable amount of progesterone was 6.25 pg. The generation of cAMP was tested in cells cultured for 24 h with DDE and then exposed to control medium or CT in the presence of the phosphodiesterase inhibitor IBMX (125 μM) for 30 min. Medium was then discarded and cAMP was extracted with absolute alcohol. The extract was dried and resuspended in 0.01 M acetate buffer, pH 6.2, and cAMP levels were determined by RIA [19]. The inter- and intraassay coefficients of variation for the cAMP assay were below 15%. The minimum detectable amount of cAMP was 3.25 fmol. Protein was determined in cells solubilized with 0.1% SDS using the Bio-Rad DC Protein Assay Kit. Trypsin-dispersed cells were collected, and DNA was estimated by a fluorometric assay using the *bis*benzimidazole fluorescent dye [20].

Northern Blot Analyses

Total RNA was isolated by acid phenol-chloroform extraction according to the method described by Chomczynski and Sacchi [21]. Samples of total RNA were denatured, size-fractionated by electrophoresis on a 1% agarose-formaldehyde gel, and transferred onto a nylon membrane by diffusion blotting. Human type II 3β-hydroxysteroid dehydrogenase (3β-HSD) [22], porcine P450-cholesterol side-chain cleavage ($P450_{scc}$) [23], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [24] cDNAs were used as probes. Probes were labeled by primer extension [25] with [α-^{32}P]dCTP (> 3000 Ci/mmol; New England Nuclear) to a specific activity of 1.5–3.0 × 10^9 dpm/mg DNA. Membranes were hybridized and autoradiographed as previously described [19]. A Kodak Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, Rochester, NY) was used for gel photography and densitometric analysis of autoradiographs.

Statistical Analysis

Data are presented as the mean ± SEM of four independent experiments. Data were examined using a two-way ANOVA between two treatment groups. Statistical differences were determined based on difference from control or difference from protein kinase A (PKA) activator (CT or 8-Br-cAMP) treatment groups. Significant differences were established as $P < 0.05$.

RESULTS

The effect of DDE on 8-Br-cAMP-stimulated progesterone accumulation in the JC-410 cells is depicted in Figure 1. No changes in progesterone accumulation were observed with 10 ng/ml DDE (Fig. 1). The PKA activator, 1 mM 8-Br-cAMP, induced a 0.7-fold increase in progesterone accumulation. Addition of 10 ng/ml DDE potentiated 8-Br-cAMP-stimulated progesterone accumulation 0.4-fold over

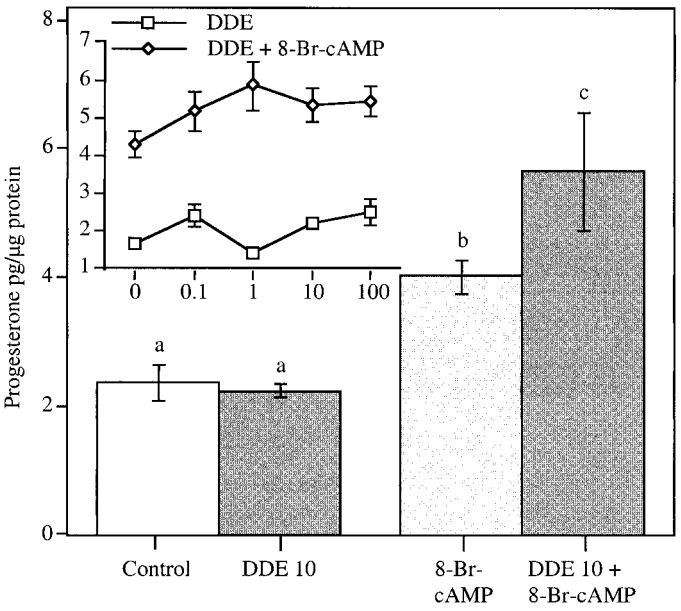

FIG. 1. Effect of DDE on 8-Br-cAMP-stimulated progesterone accumulation in JC-410 cells. Cells were cultured with 10 ng/ml DDE in the presence or absence of 1 mM 8-Br-cAMP for 24 h. Each bar represents the mean ± SEM of four independent replications. Different letters indicate significant differences. Inset is a dose-response curve from a single experiment, comparing the effect of increasing concentrations of DDE to that of increasing concentrations of DDE with 1 mM 8-Br-cAMP.

the levels observed with 8-Br-cAMP alone. This effect appeared to be dose dependent, since 0.1, 1, 10, and 100 ng/ml DDE potentiated 8-Br-cAMP-stimulated progesterone accumulation 0.2-, 0.4-, 0.2-, and 0.3-fold, respectively, over the levels observed with 8-Br-cAMP alone (Fig. 1, inset). Although an elevation was observed, there was no significant stimulation of progesterone accumulation with 30 ng/ml CT (Fig. 2). Addition of 10 ng/ml DDE potentiated the effect of CT on progesterone accumulation 0.7-fold over the levels observed with CT alone (Fig. 2). This effect also appeared to be dose dependent, since 1, 10, and 100 ng/ml DDE potentiated the effect of CT on progesterone accumulation 0.1-, 0.5-, and 0.6-fold, respectively, over the levels observed with CT alone (Fig. 2, inset).

Figure 3 depicts the effect of DDE in the presence or absence of 30 ng/ml CT in cultures of primary porcine granulosa cells. Progesterone levels were not altered by DDE alone at any of the concentrations used in this study. Progesterone accumulation was elevated 0.4-fold when CT was added to the cultures. Addition of 0.1, 1, 10, and 100 ng/ml DDE potentiated CT-stimulated progesterone accumulation 0.4-, 1.9-, 1.2-, and 1.7-fold, respectively, over the levels observed with CT alone.

Figure 4 depicts the effect of DDE on CT-stimulated cAMP production in the JC-410 cells. Addition of 10 ng/ml DDE had no effect on cAMP production. Intracellular levels of cAMP were stimulated 6-fold by the addition of 30 ng/ml CT to the cultures. No difference in the CT-stimulated cAMP levels was observed with the addition of DDE.

The effect of DDE on the expression of the $P450_{scc}$ and 3β-HSD genes in the JC-410 cells is depicted in Figure 5. Expression of the $P450_{scc}$ and 3β-HSD genes was not altered by DDE alone. The presence of 30 ng/ml CT increased mRNA levels of $P450_{scc}$ and 3β-HSD 26- and 4-fold, respectively. Addition of 1 and 10 ng/ml DDE poten-

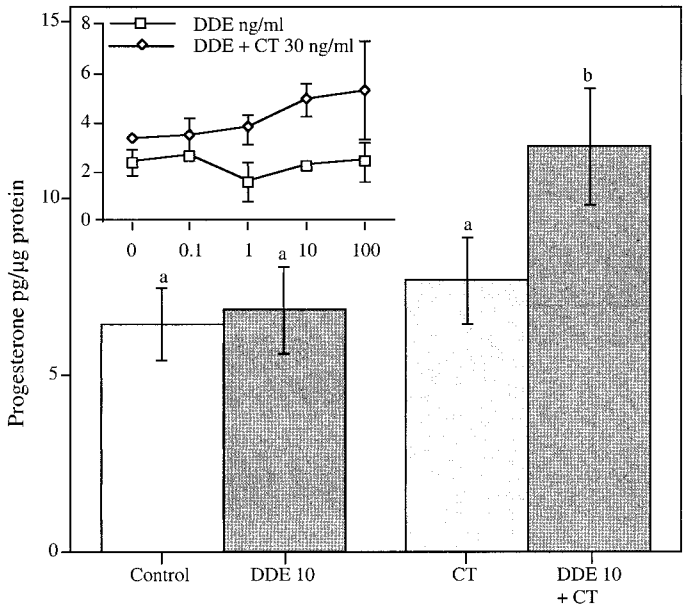

FIG. 2. Effect of DDE on CT-stimulated progesterone accumulation in JC-410 cells. Cells were cultured with 10 ng/ml DDE in the presence or absence of 30 ng/ml CT for 24 h. Each bar represents the mean ± SEM of four independent replications. Different letters indicate significant differences. Inset is a dose-response curve from a single experiment, comparing the effect of increasing concentrations of DDE to that of increasing concentrations of DDE with 30 ng/ml CT.

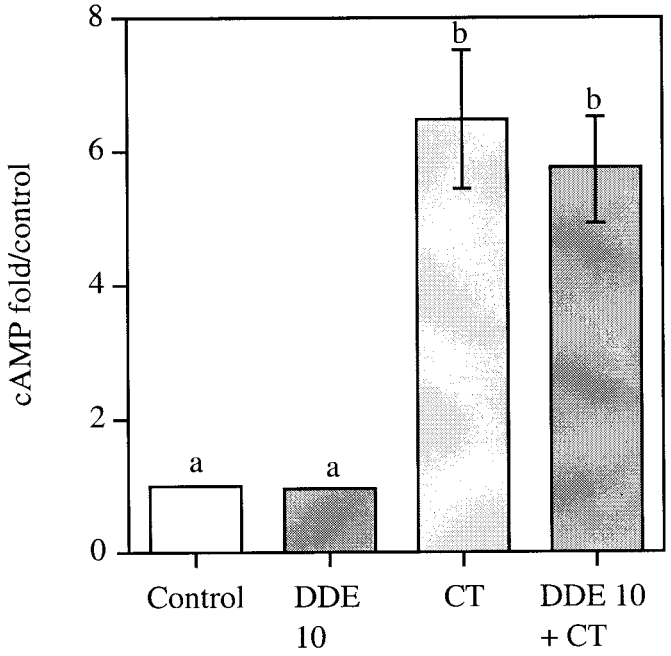

FIG. 4. Effect of DDE on CT-stimulated cAMP production in the JC-410 cells. Cells were cultured with 10 ng/ml DDE for 24 h; then 30 ng/ml CT was added for 30 min. Each bar represents the mean ± SEM of three independent replications. Different letters indicate significant differences.

tiated the CT-stimulated increase in $P450_{scc}$ mRNA levels 0.3- and 0.4-fold, respectively, over the levels observed with CT alone. The CT-stimulated 3β-HSD mRNA levels were not changed by DDE. Similar results were observed in three repetitions of the experiment.

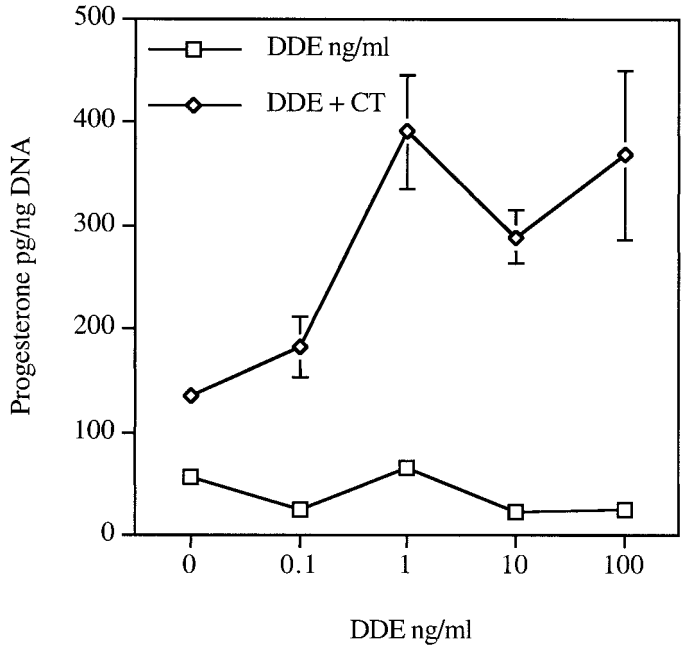

FIG. 3. Effect of DDE and CT on progesterone accumulation in primary porcine granulosa cells. Cells were cultured with increasing concentrations of DDE in the presence or absence of 30 ng/ml CT for 24 h. Figure shows a single representative experiment. Each point represents the mean ± SEM of four wells.

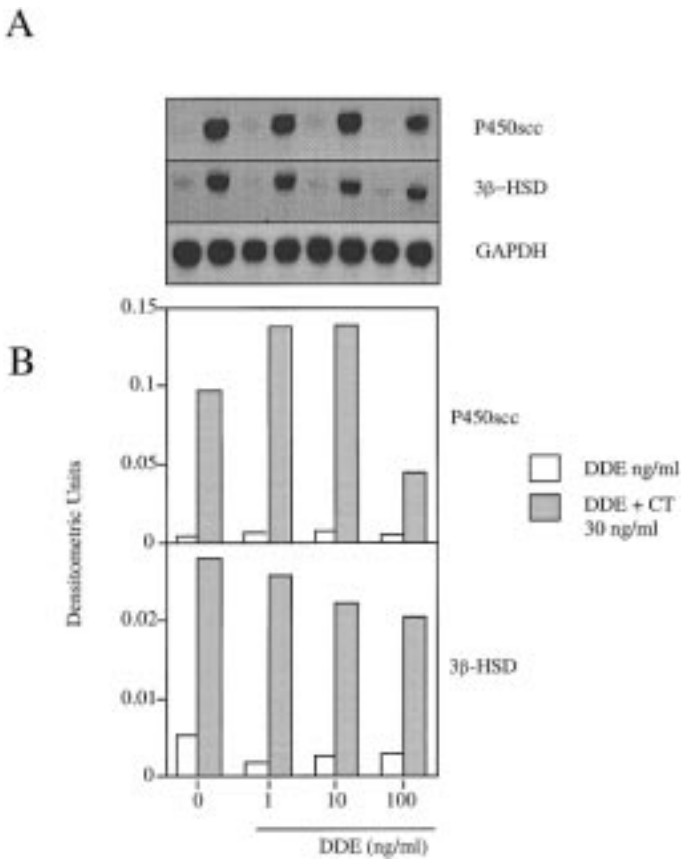

FIG. 5. The effect of DDE on the expression of $P450_{scc}$ and 3β-HSD genes in JC-410 cells. Cells were cultured with 0.1–100 ng/ml DDE in the presence or absence of 30 ng/ml CT for 24 h. Total RNA was extracted and analyzed by Northern blot. **A**) An autoradiograph of a representative experiment. **B**) A graph of densitometric analysis of the autoradiographs from the Northern hybridization represented in **A.** Values graphed are the ratio of $P450_{scc}$ or 3β-HSD to GAPDH.

DISCUSSION

Primary porcine granulosa and human luteinized granulosa cells in culture have been used to test the toxic potential of xenobiotics on reproduction [26, 27]. In the present study we used JC-410 cells and primary cultures of porcine granulosa cells to investigate the effect of DDE on progesterone synthesis. The JC-410 cell line is a stable porcine steroidogenic granulosa cell line that responds to the PKA activators, 8-Br-cAMP and CT, with an elevation in progesterone synthesis similar to that seen in primary cultures of granulosa cells [16, 17, 19]. Addition of 10 ng/ml DDE potentiated 8-Br-cAMP-stimulated progesterone synthesis (Figs. 1 and 2) in the JC-410 cells. The presence of 30 ng/ml CT elevated progesterone synthesis, although this elevation was not statistically significant at 24 h. However, 10 ng/ml DDE potentiated the effect of CT on progesterone synthesis. In primary cultures of porcine granulosa cells, DDE also potentiated the effect of PKA activators. Moreover, primary cultures appeared to be more sensitive to the potentiating effect of DDE on PKA-induced progesterone synthesis than the JC-410s. Potentiation of PKA activator-stimulated progesterone synthesis in primary cultures was observed at 0.1 ng/ml DDE, while in the JC-410 cells, potentiation was observed at concentrations greater than 1 ng/ml DDE. The effect of DDE on PKA activator-stimulated progesterone synthesis was not a result of an augmentation of cAMP synthesis. Addition of 10 ng/ml DDE, a dose that maximally potentiated the effect of CT on progesterone synthesis, did not change CT-stimulated cAMP levels. The observed changes in progesterone synthesis corresponded to changes in the levels of $P450_{scc}$ mRNA, while no effects were observed on 3β-HSD mRNA levels. These observations suggest that DDE alters $P450_{scc}$ gene expression, thus affecting an obligatory and regulated step of steroid synthesis in the ovary [28]. The addition of 10 ng/ml DDE had no effect on CT-stimulated cAMP synthesis, indicating that DDE is not exerting its effect at the level of the G protein. Therefore, it is possible that DDE mediates its effects on progesterone synthesis by modulating expression of the $P450_{scc}$ gene and not by affecting the generation of cAMP.

The effect of DDT and its major metabolite DDE on female reproduction is of concern because these organochlorine pesticides have been found in the follicular fluid of women with lower rates of conception [29]. Levels between 0.61 ± 0.47 and 1.07 ± 0.84 ppb (0.61 ± 0.470 and 1.07 ± 0.84 ng/ml) of DDE were found in the follicular fluid of women undergoing in vitro fertilization [12]. Although these levels of DDE appeared to have no effect on oocyte cleavage rate, or on the time of the first cleavage, the causes of infertility in the women from whom the follicular fluid was collected were not described [12]. On the basis of the observations reported here, it is possible that DDE was a contributing factor to their infertility. In the present study, 10 ng/ml (10 ppb) DDE was required to potentiate PKA activator-stimulated progesterone synthesis in JC-410 cells. This concentration is in the range of the levels reported in human follicular fluid [12]. Therefore, it is reasonable to speculate that concentrations of DDE that appear to have no effect on oocyte viability may affect fertility by altering granulosa cell steroidogenesis and the endocrine milieu that surrounds the oocyte.

Alligators hatched from eggs laid in an environment highly polluted with DDT and its metabolites, including DDE, had increased plasma estradiol levels and ovaries with prominent polyovulatory follicles and multinucleate oocytes [30]. In vitro cultures of ovaries from these exposed alligators produced lower basal estradiol than ovaries from control animals. However, LH stimulated estradiol production to a much greater magnitude than observed in ovaries from control alligators [30]. The effect of LH on steroid synthesis is mediated by an elevation in the levels of cAMP and stimulation of the PKA pathway. We have observed that DDE potentiates the effect of PKA activators on progesterone synthesis. Therefore, it is possible that a common stimulatory mechanism may mediate the effects of DDE on PKA activator-induced steroid synthesis in the ovary.

The molecular mechanism by which DDE exerts its effect on progesterone synthesis in granulosa cells is unknown. The DDT metabolite *p,p′*-DDE has been reported to act as an estrogen [3], an anti-estrogen [31], an anti-androgen [4], an anti-progestin [32, 33], and an anti-glucocorticoid [34], depending upon the system studied. We previously reported that the JC-410 cells responded to estrogens, androgens, and progestins with an increase in progesterone synthesis [17, 35]. Thus, it is likely that estrogen, androgen, and progesterone receptors are present in these cells. The effect of DDE may therefore be mediated via these receptors. However, since there is no endogenous androgen or estrogen production in the JC-410 cells [17], it is unlikely that DDE acts as an anti-estrogen or anti-androgen.

In summary, we have observed that DDE potentiates PKA activator-stimulated progesterone synthesis but does not affect CT-stimulated cAMP production. As well, DDE potentiates the CT-stimulated increase in $P450_{scc}$ mRNA levels. We conclude that the JC-410 cell line may be an appropriate model for studying the molecular mechanisms by which EMCs affect steroidogenesis in granulosa cells. Overall, these observations suggest that DDE, at concentrations found in follicular fluid of infertile women, affects PKA-stimulated progesterone synthesis and gene expression in cultured granulosa cells.

ACKNOWLEDGMENTS

We express our gratitude to Dr. Van Luu The, from Laval University, Quebec, Canada, and Dr. T. Wise, from Clay Center Nebraska, USA, for providing us with cDNA probes for 3β-HSD and $P450_{scc}$, respectively. We thank Dr. Karsten Liber for his support and the Toxicology Centre, University of Saskatchewan, for providing us with the chlorinated pesticide kit. We thank Dr. G. Irastorza for his encouragement during the initial stages of this project. We thank Christine Meaden for editorial assistance.

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