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The organochlorine *o,p′*-DDD disrupts the adrenal steroidogenic signaling pathway in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The mechanisms of action of *o,p′*-DDD on adrenal steroidogenesis were investigated in vitro in rainbow trout (*Oncorhynchus mykiss*). Acute exposures to *o,p′*-DDD inhibited ACTH-stimulated cortisol secretion while cell viability decreased significantly only at the highest concentration tested (200 μM *o,p′*-DDD). Stimulation of cortisol secretion with a cAMP analogue (dibutyryl-cAMP) was inhibited at a higher concentration than that needed to inhibit ACTH-stimulated cortisol synthesis in cells exposed to *o,p′*-DDD. Forskolin-stimulated cortisol secretion and cAMP production, and NaF-stimulated cAMP production were inhibited in a concentration-dependent manner by *o,p′*-DDD. In contrast, basal cortisol secretion was stimulated while basal cAMP production was unaffected by *o,p′*-DDD. Pregnenolone-stimulated cortisol secretion was enhanced by *o,p′*-DDD at a physiologically relevant pregnenolone concentration, while *o,p′*-DDD inhibited cortisol secretion when a pharmacological concentration of pregnenolone was used. Our results suggest that the cAMP generation step is a target in *o,p′*-DDD-mediated disruption of ACTH-stimulated adrenal steroidogenesis in rainbow trout but that other downstream targets such as steroidogenic enzymes responsible for cortisol synthesis might also be affected.

Keywords: Teleost; Cortisol; Steroidogenesis; Signaling disruption; Adenylate cyclase; *o,p′*-DDD; ACTH; cAMP; Adrenal; Cytochrome P450

Introduction

Previous studies, both in vivo and in vitro, revealed that pesticides have endocrine-disrupting effects on corticosteroid synthesis in teleost fish (Benguira et al., 2002; Bisson and Hontela, 2002). Moreover, recent studies with other steroidogenic systems in mammalian models reported that a variety of pesticides interfere with components of the signaling pathway that trigger steroidogenesis (Walsh and Stocco, 2000; Walsh et al., 2000a, 2000b). The organochlorine 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (*o,p′*-DDD) is one pesticide that seems to specifically target the adenosine 3′:5′-cyclic monophosphate-(cAMP) mediated signaling pathway that triggers cortisol synthesis in fish (Ilan and Yaron, 1980; Leblond and Hontela, 1999; Benguira and Hontela, 2000).

o,p′-DDD is a metabolite of organochlorine pesticide dichlorodiphenyltrichloroethane (DDT), which has been banned for use since the early 1970s in the United States but is still present in the North American environment as detectable residues in soil, glaciers, and fauna due to its extremely high persistence and biomagnification in the food chains (Brooks, 1974; WHO, 1989; Blais et al., 2001). Moreover, it is still used in some parts of the world such as Central America and India for pest control, notably in malaria-sensitive areas (ATSDR, 1989).

DDT and its metabolites are adrenolytic compounds that have been well characterized in mammals, birds, and fish (Hart et al., 1973; Yaron and Ilan, 1974; Brandt et al., 1992; Jönsson et al., 1993, 1994; Benguira et al., 2002). Of these metabolites, *o,p′*-DDD is the most notorious for its highly deleterious effects on corticosteroid biosynthesis in a wide range of species (Hart and Straw, 1971a, 1971b; Ilan and Yaron, 1980, 1983; Brandt et al., 1992; Benguira and Hontela, 2000; Breuner et al., 2000). Its high specificity for the

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adrenal has even earned it an important chemotherapeutic role in the treatment of adrenocortical carcinoma and Cushing's syndrome (Orth, 1995), medically used under the name mitotane.

Major species differences in the proposed mechanisms of action through which *o,p′*-DDD disrupts corticosteroid synthesis have been reported. In mammalian studies, steroid hydroxylases, particularly the 11-β hydroxylase (CYP11B1) solely found in the adrenal, seem to be targeted by the organochlorine, which hinders the catalytic conversion of 11-deoxycortisol to cortisol (Hart et al., 1971; Young et al., 1973; Jönsson et al., 1993; Lund, 1994; Lindhe et al., 2002). Furthermore, covalent binding of *o,p′*-DDD and/or reactive metabolites in mitochondria and to microsomal and mitochondrial cytochrome P450 has been demonstrated in all mammalian adrenal cortex models tested, including humans (Martz and Straw 1977, 1980; Jönsson et al., 1993; Lund, 1994; Cai et al., 1997; Lindhe et al., 2002). On the other hand, studies on teleost fish seem to pinpoint a site of action of the organochlorine located between the adrenocorticotropic hormone- (ACTH) receptor and the cAMP generation step, since ACTH-stimulated cortisol secretion suppressed by *o,p′*-DDD can be restored when a cAMP analogue is used (Ilan and Yaron, 1980; Leblond and Hontela, 1999; Benguira and Hontela, 2000).

The main objective of this study was therefore to characterize the mechanisms of action of *o,p′*-DDD in adrenocortical cells of rainbow trout, a model teleost. We used NaF, a universal activator of all G proteins and forskolin, a diterpene derived from the plant *Coleus forskohlii*, as a specific adenylate cyclase activator (Seamon and Daly, 1986; Coleman et al., 1994), to test the hypothesis that disruption of fish adrenal steroidogenesis mediated by *o,p′*-DDD occurs at the cAMP generation step, therefore impairing the signaling processes essential for the conversion of cholesterol into cortisol.

Materials and methods

Chemicals. *o,p′*-DDD was purchased from Aldrich Chemicals (Montréal, Quebec, Canada). Porcine $ACTH_{1–39}$, N^6,2′-*o*-dibutyryladenosine cAMP (dbcAMP), forskolin, 3β-hydroxy-5-pregne-20-one (pregnenolone), minimal essential medium (MEM), Hepes, and bovine serum albumin (BSA) were obtained from Sigma–Aldrich (Oakville, Ontario, Canada). Collagenase/dispase mixture (collagenase from *Achromobacter iophagus* and dispase from *Bacillus polymyxa*) and lactate dehydrogenase (LDH) activity kit were bought from Boehringer Mannheim (Laval, Quebec, Canada). The *m*-aminobenzoic acid ethyl ester methanesulfonate (MS-222) was bought from ICN Pharmaceuticals (Orangeburg, NY). Other chemicals used were reactive grade. Forskolin and *o,p′*-DDD were disolved in 100% dimethylsulfoxide (DMSO) while pregnenolone was dissolved in 100% ethanol. All other agents used were directly dissolved into the medium. Concentrations of DMSO and ethanol in the final cell suspension medium were 5 (v/v) and 2% (v/v), respectively. These concentrations do not have an effect on cortisol secretion, cAMP production, or cell viability (data not shown).

Experimental animals. Male and female rainbow trout (150–200 g body wt) were bought from Pisciculture Laurentienne (Labelle, Quebec, Canada). Fish were kept in freshwater flow-through basins (600 L) maintained at 15 ± 2°C, supplied with filtered and oxygen-saturated water (hardness 70 mg/L $CaCO_3$) at a rate of 3.8 L/min. They were fed daily with a commercial trout chow at the manufacturer's recommended rate (10 g/kg of fish). A 2-week acclimation period was allowed before the beginning of the experiments.

Cell suspensions. The cell suspension method has been described elsewhere (Leblond et al., 2001). Fish were anesthetized in MS-222, bled from the caudal vasculature, and perfused twice with a 30-ml syringe containing a saline solution (0.7% NaCl), to remove as much blood as possible. The head kidney was then dissected and placed in a tube containing MEM supplemented with 5 g/L BSA and 2.2 g/L $NaHCO_3$, pH 7.4 (complete medium). The tissue was washed twice with complete medium, resuspended in 2.5 ml of complete medium with 2.0 mg/ml of collagenase/dispase, and incubated in a 15-ml conical tube for 60 min at room temperature with gentle agitation, resuspending the cells every 15 min with a transfer pipette. Following enzymatic digestion, the solution was filtered with a 30-μm mesh cloth and the filtrate was centrifuged at 300*g* for 5 min. The supernatant was then removed, the pellet resuspended in 1.5 ml of complete medium, and cellular density adjusted to 75 $\times$ 10^6 cells/ml using a hemacytometer.

In vitro exposures to pesticides and stimulation of cortisol secretion. The cells were distributed in a 96-well microplate at 150 μl of 75 $\times$ 10^6 cells/ml per well and incubated under optimal conditions, as described previously (Leblond et al., 2001). In summary, a 2-h preincubation at 15°C (acclimation temperature of basins in which fish were held) with light agitation was necessary to reach basal cortisol secretion. Cells were then resuspended in complete medium and the microplate was centrifuged at 300*g* for 3 min. The supernatants were then removed and the pellets were resuspended in 150 μl of a Ringer solution (in g/L: 6.5 NaCl, 0.2 $CaCl_2$, 0.2 $NaHCO_3$, 1.0 dextrose, and 5.95 Hepes) containing 50, 75, 100, or 200 μM *o,p′*-DDD. After a 60-min incubation at 15°C, cells were washed with Ringer and then resuspended in complete medium containing the optimal concentrations (Leblond et al., 2001; Lacroix and Hontela, unpublished results) of the various stimulating agents: 1 IU/ml ACTH, 2 mM dbcAMP, 5 μM forskolin, 60 mM NaF, and 1 and 10 μM pregnenolone. For cortisol determination, the cells were incubated for 60 min at 15°C with

light agitation with the different stimulating agents with the exception of a 120-min incubation required for optimal dbcAMP stimulation. For stimulation by NaF, a Ca^{2+}-free Ringer solution (in g/L: 6.5 NaCl, 0.2 $NaHCO_3$, 1.0 dextrose, and 5.95 Hepes) was used to avoid precipitation. Control wells were always run at the same time as wells exposed to the pesticide. Cells were then centrifuged at 300*g* and the supernatants were collected for RIA determination using the ICN Cortisol RIA KIT (ICN Diagnostics, Costa Mesa, CA).

Intracellular cAMP determination. Validation experiments (data not shown) demonstrated that a 10-min incubation with the various stimulating agents induced a significant rise in cAMP production, in agreement with Leblond et al. (2001) reporting a significant increase in cortisol secretion 15 min after stimulation with ACTH. To determine cAMP production in the present study, cells were incubated with the various agents for 10 min at 15°C with light agitation; they were centrifuged at 300*g*; supernatants were discarded; and cells were lysed using 3% perchloric acid. Intracellular cAMP concentrations were determined using the DPC Liquid Phase cAMP RIA (Diagnostic Products Company). None of the stimulating agents cross-reacted with either the cortisol RIA or the cAMP RIA (data not shown).

Cell viability. After the acute 60-min exposure to *o,p′*-DDD, supernatants were collected to evaluate cell viability. Viability was assessed using the LDH extrusion method. Cell lysis provokes emptying of the intracellular content into the medium (including LDH), therefore enzyme activity can be measured as an indicator of loss of cellular membrane integrity. Viability was expressed as a percentage by subtracting maximal LDH activity obtained from cells lysed in deionized water from the values obtained from cells exposed to *o,p′*-DDD as follows:

$$\%\ \text{cell viability} = \frac{[1 - \text{LDH activity of cells exposed to pesticides}]}{\text{LDH activity at 100\% mortality (deionised water)}}.$$

Statistical analysis. In each experiment, the number of replicates represents the number of different fish used. Data relevant to a single determination are relevant to a single fish; no pooled determinations were made. Statistical significance in each experiment was determined using a one-way ANOVA followed by Dunnett's comparison with control test with a $p < 0.05$ as measure of significance. Some data were analyzed by a two-way ANOVA to test for effects of exposure and stimulatory agents or various concentrations of stimulatory agent. In all cases the interaction between exposure and stimulatory agents or concentrations was not significant. Data have been log-transformed, when necessary, in order to respect normality and homoscedasticity requirements prior to the ANOVA.

Table 1
Cortisol secretion of rainbow trout adrenocortical cells elicited by various stimulating agents

Treatment	Cortisol (ng/ml)[a]	n[b]
Basal	0.65 ± 0.14	11
ACTH (1 IU/ml)	18.36 ± 4.09*	18
dbcAMP (2 mM)	26.67 ± 5.69*	14
Forskolin (5 μM)	11.96 ± 2.36*	13
NaF (60 mM)	0.27 ± 0.18	9
Pregnenolone (1 μM)	11.97 ± 2.57*	7
Pregnenolone (10 μM)	61.75 ± 8.65*	8

[a] Means ± SE.
[b] Number of duplicate cortisol determinations.
* Significant difference from basal cortisol secretion (Dunnett's comparison with control test $p < 0.05$, following a one-way ANOVA: $F = 62.93$, $p < 0.0001$).

Results

Cortisol secretion elicited by various agents

Table 1 summarizes the cortisol responses elicited by various stimulating agents used throughout the study, with ACTH and dbcAMP producing an optimal secretory response. Stimulation with forskolin (5 μM as the optimal concentration; Lacroix and Hontela, unpublished observations) led to a marked increase in cortisol secretion without however reaching the same levels as with ACTH, while NaF did not have an effect. Pregnenolone, a product of the side chain cleavage of cholesterol, bypasses the regulated step in steroidogenesis and is therefore immediately converted to cortisol by the steroidogenic enzymes. The two concentrations of pregnenolone used in this study were nonsaturating for the steroidogenic enzymes (unpublished data).

Effects of o,p′-DDD on ACTH-, dbcAMP-, and forskolin-stimulated cortisol secretion and cell viability

Cortisol secretion of adrenocortical cells stimulated with either ACTH or dbcAMP and cell viability following an acute (60 min) exposure to *o,p′*-DDD (expressed as a percentage of non-exposed controls) are shown in Fig. 1A (see Table 1 for cortisol secretion of controls). Exposure to *o,p′*-DDD had a significant inhibitory effect on cortisol secretion (two-way ANOVA, exposure to *o,p′*-DDD, $F = 31.57$, $p < 0.0001$). Inhibition of ACTH-stimulated cortisol secretion was concentration dependent where 75, 100, and 200 μM *o,p′*-DDD induced a significant 39, 57, and 96% inhibition, respectively. Cell viability was maintained at 85% and higher over all concentrations tested, except at the highest concentration of *o,p′*-DDD (200 μM), for which cell viability dropped to 62%. Although no significant differences were obtained when comparing both ACTH and dbcAMP (two-way ANOVA, ACTH vs dbcAMP, $F = 1.95$, $p = 0.1653$), *o,p′*-DDD inhibited dbcAMP-stimulated cortisol secretion at a higher concentration than that needed to

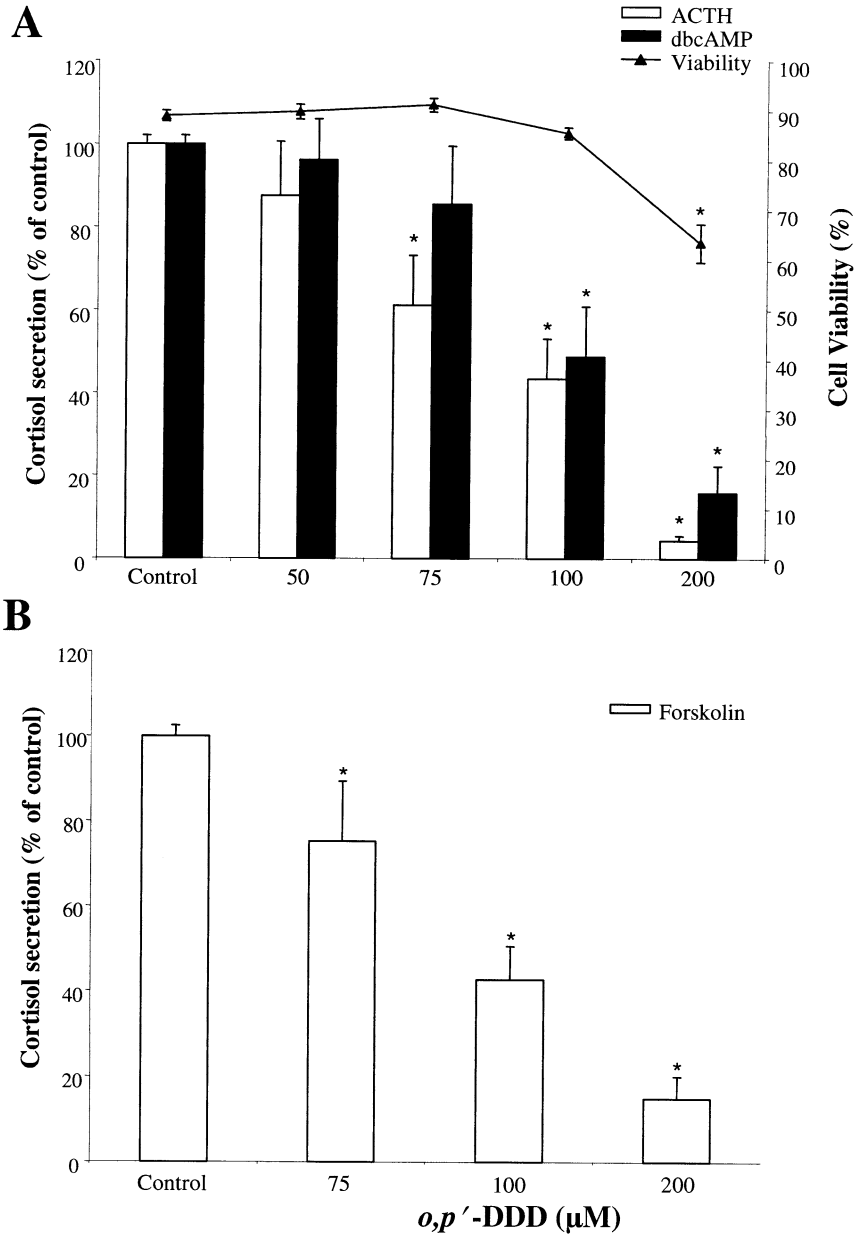

Fig. 1. Cortisol secretion and viability of rainbow trout adrenocortical cells stimulated with ACTH (1 IU/ml), dbcAMP (2 mM) (A), or forskolin (5 μM) (B) following an acute in vitro exposure to *o,p′*-DDD. Bars represent the mean ± SE of eight or nine duplicate determinations. Two-way ANOVA revealed a significant effect of exposure to *o,p′*-DDD ($F = 31.57$, $p < 0.0001$) but no difference between ACTH and dbcAMP ($F = 1.95$, $p = 0.1653$). *Significant difference from respective control (Dunnett's comparison with control test $p < 0.05$, following a one-way ANOVA for ACTH: $F = 39.69$, $p < 0.0001$; dbcAMP: $F = 7.66$, $p < 0.0001$; viability: $F = 29.62$, $p < 0.0001$; forskolin: $F = 29.99$, $p < 0.0001$).

inhibit ACTH-stimulated cortisol secretion (100 μM). At higher concentrations of *o,p′*-DDD, dbcAMP-stimulated cortisol secretion was inhibited. Cortisol secretion of adrenocortical cells stimulated with forskolin (5 μM) following an acute (60 min) exposure to *o,p′*-DDD is shown in Fig. 1B (see Table 1 for cortisol secretion of controls). Inhibition of forskolin-stimulated cortisol secretion was concentration dependent, with significant 25, 53, and 85% inhibition over the concentrations of *o,p′*-DDD tested.

Effect of o,p′-DDD on NaF- and forskolin-stimulated cAMP production

NaF- and forskolin-stimulated cAMP production following an acute (60 min) exposure to *o,p′*-DDD are shown Fig. 2. Production of cAMP in NaF- and forskolin-stimulated controls was 20.18 ± 3.52 and 35.72 ± 4.14 pmol/ml, respectively (expressed as the means of eight duplicate determinations ± SE). Exposure to *o,p′*-DDD had a signif-

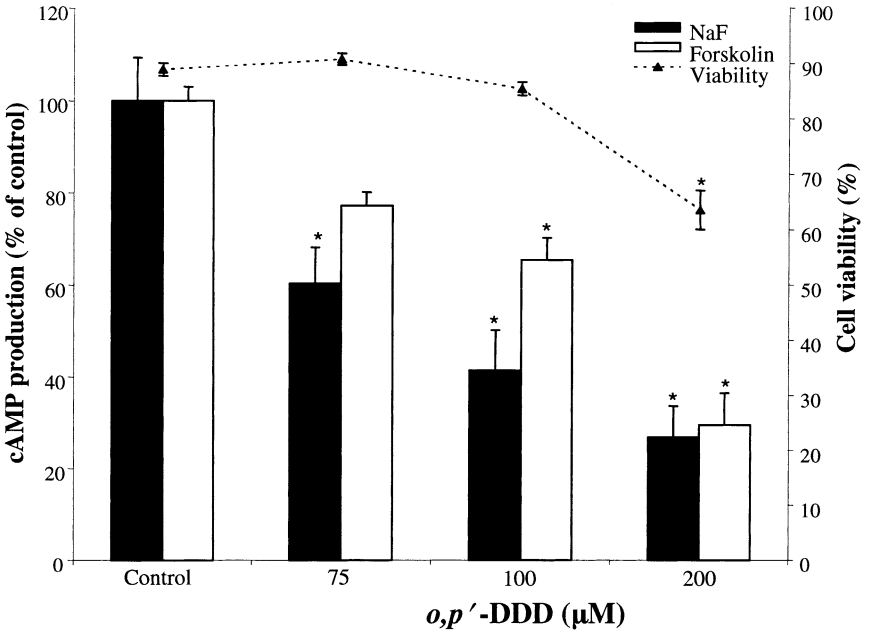

Fig. 2. cAMP production of rainbow trout adrenocortical cells stimulated with NaF (60 mM) and forskolin (5 μM) following an acute in vitro exposure to *o,p′*-DDD. Bars represent the mean ± SE of seven to nine duplicate determinations. Viability data are transposed from Fig. 1. Two-way ANOVA revealed a significant effect of exposure to *o,p′*-DDD ($F = 39.66$, $p < 0.0001$) but no difference between NaF and forskolin ($F = 2.01$, $p = 0.1608$). *Significant difference from respective control (Dunnett's comparison with control test $p < 0.05$, following a one-way ANOVA for NaF: $F = 15.85$, $p < 0.0001$; forskolin: $F = 50.08$, $p < 0.0001$).

icant inhibitory effect on cortisol secretion (two-way ANOVA, exposure to *o,p′*-DDD, $F = 39.66$, $p < 0.0001$). Both NaF- and forskolin-stimulated cAMP production of cells exposed to *o,p′*-DDD were similarly inhibited in a concentration-dependent manner (two-way ANOVA, NaF vs forskolin, $F = 2.01$, $p = 0.1608$). NaF-stimulated cAMP production was significantly inhibited by 40, 59, and 74%, similar to forskolin-stimulated cAMP production (23, 35, and 70%), over the concentrations of *o,p′*-DDD tested.

Effect of o,p′-DDD on basal cortisol secretion and cAMP production

Basal (unstimulated) cortisol secretion of cells exposed to *o,p′*-DDD is shown in Fig. 3A (see Table 1 for cortisol secretion of basal control). Basal cortisol secretion increased in a concentration-related manner following exposure to the pesticide, reaching a peak (1660% compared to basal control) at the highest noncytotoxic concentration of *o,p′*-DDD (100 μM). Basal cAMP production (Fig. 3B) did not seem to be affected by exposure to *o,p′*-DDD, except for a decrease at the cytotoxic concentration of 200 μM *o,p′*-DDD. Basal cAMP production was 4.95 ± 0.67 pmol/ml (expressed as the mean of eight duplicate determinations ± SE).

Effect of o,p′-DDD on pregnenolone-stimulated cortisol secretion

Pregnenolone-stimulated cortisol secretion following an acute (60 min) exposure to *o,p′*-DDD is shown in Fig. 4 (see Table 1 for cortisol secretion of controls). Exposure to *o,p′*-DDD had a significant effect on cortisol secretion (two-way ANOVA, exposure to *o,p′*-DDD, $F = 8.31$, $p < 0.0001$), yet the effect varied depending on the concentration of pregnenolone used to stimulate cortisol secretion (two-way ANOVA, 1 vs 10 μM of pregnenolone, $F = 11.36$, $p = 0.0012$). Cortisol secretion was slightly modulated by *o,p′*-DDD, with a statistically significant increase at 100 μM when a physiological concentration of pregnenolone (1 μM) was used as substrate to stimulate cortisol secretion. However, *o,p′*-DDD inhibited cortisol secretion in a concentration-dependent fashion when a 10-fold higher concentration of pregnenolone was used.

Discussion

Earlier studies of the effects of *o,p′*-DDD on cortisol secretion in teleost fish have revealed that the site of action of *o,p′*-DDD may be located between the ACTH receptor and cAMP production within the signaling cascade that leads to cortisol synthesis (Ilan and Yaron, 1980; Leblond and Hontela, 1999; Benguira and Hontela, 2000). It is generally understood that this signaling cascade is composed of the ACTH receptor, which activates a G_s protein once its ligand ACTH is bound, which in turn stimulates adenylate cyclases to produce cAMP (Patiño et al., 1986; Miller, 1988; Lacroix and Hontela, unpublished results). cAMP acts acutely to stimulate steroidogenesis by activating multiple targets, such as the cAMP-dependent protein kinase

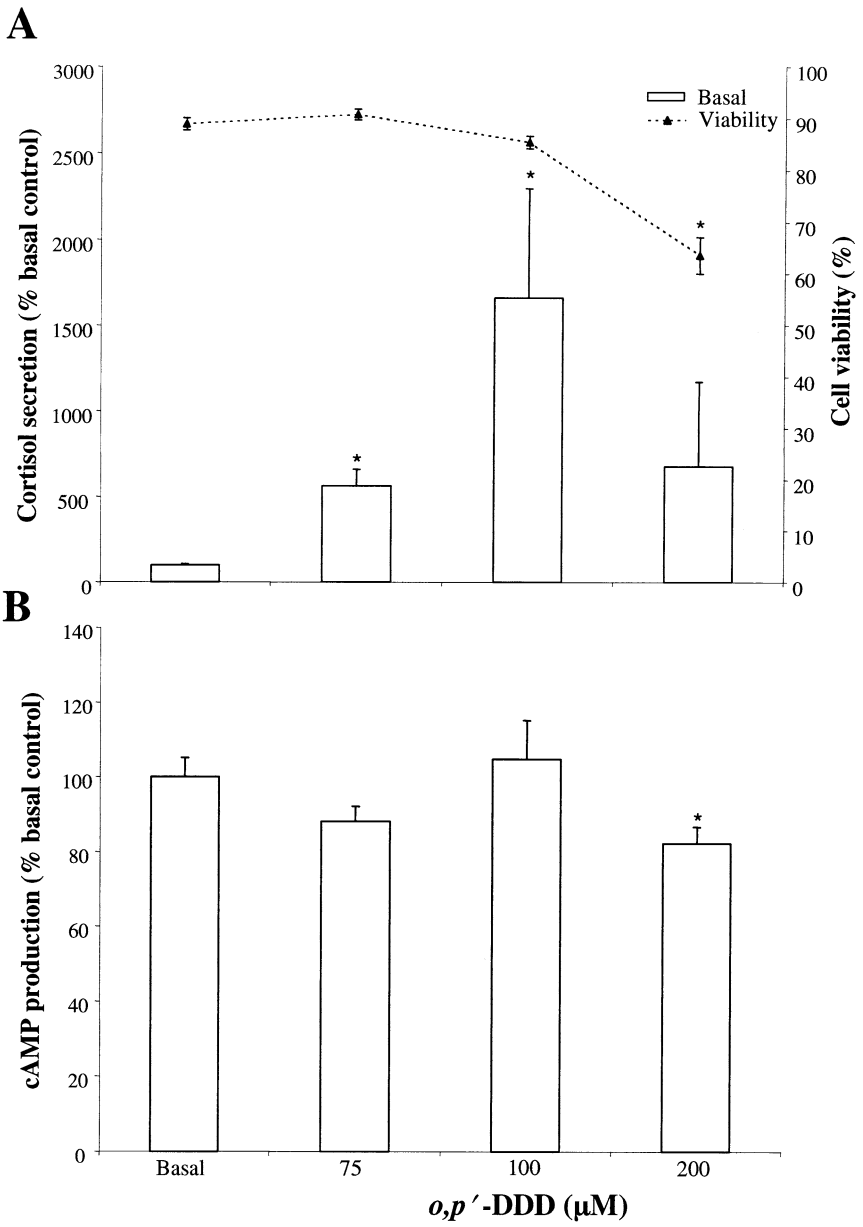

Fig. 3. Comparison of (A) basal cortisol secretion and (B) basal cAMP production in rainbow trout adrenocortical cells following an acute in vitro exposure to *o,p′*-DDD. Bars represent the mean ± SE of seven to nine duplicate determinations. Viability data are transposed from Fig. 1. *Significant difference from respective control (Dunnett's comparison with control test $p < 0.05$, following a one-way ANOVA for basal cortisol: $F = 17.31$, $p < 0.0001$; basal cAMP: $F = 4.29$, $p = 0.0101$).

(PKA) and StAR protein that regulate the rate-limiting mitochondrial cholesterol translocation step in cortisol synthesis (Lacroix and Hontela, 2001; Stocco, 2001). Steroidogenesis is also continually regulated by cAMP, which activates gene transcription of the steroidogenic enzymes responsible for the conversion of cholesterol into the appropriate steroid according to the tissue and species (Kagawa et al., 1999). All of these components may be targeted by *o,p′*-DDD to cause the loss of corticosteroid synthesis. The present study was designed to evaluate the mechanisms of action of *o,p′*-DDD on cortisol secretion in rainbow trout adrenocortical cells in vitro, with particular interest to the acute regulation of steroidogenesis mediated by the cAMP signaling pathway.

Few studies have evaluated the potential of pesticides to interact with the components of the cAMP-mediated signaling pathway, even though it is well known that many pesticides are highly lipophilic and may therefore interact with plasma membrane-bound proteins such as adenylate cyclases, phosphodiesterases, and/or receptors. Recently, Song et al. (1997) and Garcia et al. (2001) have revealed that the organophosphate chlorpyrifos impairs adenylate cyclase activity in the brain of neonatal rats and in glial cells. Earlier studies on the effects of DDT on receptor-adenylate cyclase

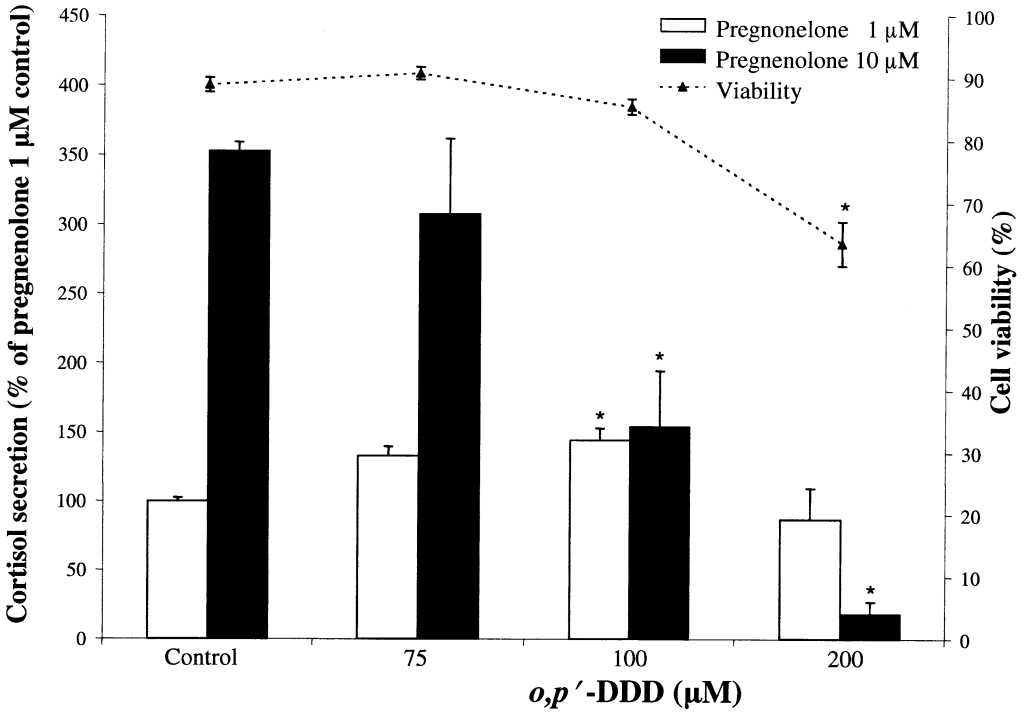

Fig. 4. Cortisol secretion of rainbow trout adrenocortical cells stimulated with pregnenolone (1 and 10 μM) following an acute in vitro exposure to *o,p′*-DDD. Bars represent the mean ± SE of eight duplicate determinations. Viability data are transposed from Fig. 1. Two-way ANOVA revealed a significant effect of exposure to *o,p′*-DDD ($F = 8.31$, $p < 0.0001$) and a difference between concentrations of 1 and 10 μM pregnenolone ($F = 11.36$, $p = 0.0012$). *Significant difference from respective control (Dunnett's comparison with control test $p < 0.05$, following a one-way ANOVA for 1 μM pregnenolone: $F = 6.21$, $p = 0.0016$; 10 μM pregnenolone: $F = 46.68$, $p < 0.0001$).

systems in various models have resulted in conflicting results, with studies reporting a decrease in cAMP (Dudeja et al., 1980), an increase, in cAMP (Kacew and Singhal, 1974), and no detectable effect (Aldridge et al., 1978; Pöschl et al., 1982; Buff et al., 1983). It is important to note, however, that none of these studies evaluated the effects of DDT on the adrenal tissue and that some employed membrane preparations that did not allow the bioactivation of DDT into a more potent metabolite such as *o,p′*-DDD. The results of the present study with teleost adrenocortical cells provided the first clear evidence that the organochlorine *o,p′*-DDD disrupts cAMP production in adrenal steroidogenesis.

Indeed, disruption of cortisol secretion occurred at concentrations of *o,p′*-DDD that did not affect cell viability, ruling out cell death as a simple cause for loss of cortisol secretion. Moreover, the use of a cAMP analogue (db-cAMP) diminished the inhibitory effect of *o,p′*-DDD on cortisol secretion at a concentration at which ACTH-stimulation was inhibited (75 μM *o,p′*-DDD), confirming previously reported work (Ilan and Yaron, 1980; Leblond and Hontela, 1999; Benguira and Hontela, 2000). This capacity of dbcAMP to sustain cortisol production pinpointed a site of action for *o,p′*-DDD directly on or upstream of cAMP production. To confirm this finding, the integrity of the signaling cascade was therefore assessed in adrenocortical cells exposed to *o,p′*-DDD.

NaF and forskolin were both used to evaluate the site of action of *o,p′*-DDD, NaF being a universal activator of all G proteins, while forskolin, a diterpene derived from the plant *C. forskohlii*, is a specific adenylate cyclase activator (Seamon and Daly, 1986; Coleman et al., 1994). Stimulation of cortisol secretion through the activation of cAMP by forskolin was inhibited by *o,p′*-DDD at all concentrations tested. Furthermore, both NaF and forskolin-stimulated cAMP production were significantly decreased after exposure to *o,p′*-DDD, providing direct evidence of impairment of the cAMP generation step, which is an integral part of signal transduction leading to cortisol synthesis. Direct measurement of cAMP production following *o,p′*-DDD exposure has not been made in any adrenal model thus far, including mammalian and avian systems in which the efforts focused on the interaction of *o,p′*-DDD with the steroidogenic enzymes that are targeted by the pesticide (Kupfer, 1975, Brandt et al., 1992). Although inhibition by *o,p′*-DDD of forskolin-stimulated cAMP production indicates that *o,p′*-DDD disrupts adenylate cyclases, other targets such as G proteins, the ACTH receptor, and phosphodiesterases that degrade cAMP may also be impaired. Inhibition of NaF-stimulated cAMP production constitutes further evidence for disruption of the signaling pathway by *o,p′*-DDD. Whether *o,p′*-DDD targets G proteins as well as adenylate cyclases is not presently known.

Although our data suggest that cAMP production is di-

rectly altered by acute (60 min) exposures to *o,p′*-DDD, targets that are located downstream of cAMP production may also play a role in the overall disruption of cortisol synthesis. Studies in both mammalian and avian systems indicate that the adrenolytic action of *o,p′*-DDD and other DDT metabolites is mediated by covalent binding to macromolecules such as cytochrome P450 and conversion to reactive intermediates that may also modulate the enzyme activities and possibly generate reactive oxygen species (Young et al., 1973; Hornsby, 1989; Jönsson et al., 1993). Our results show that, at noncytotoxic concentrations of *o,p′*-DDD, dbcAMP cannot fully restore cortisol secretion, suggesting another site of *o,p′*-DDD action distal from cAMP production. Furthermore, while basal cAMP production is unaffected by *o,p′*-DDD, basal cortisol secretion was significantly stimulated by exposure to *o,p′*-DDD at noncytotoxic concentrations, as previously noted by Ilan and Yaron (1983). This slight stimulation of basal cortisol secretion may implicate interaction of *o,p′*-DDD with steroidogenic enzymes that convert cholesterol into cortisol.

To further evaluate whether steroidogenic enzymes are inhibited by *o,p′*-DDD, pregnenolone, the product of the side chain cleavage of cholesterol by the cytochrome $P450_{scc}$ enzyme, was used to stimulate cortisol synthesis at a step downstream of cAMP production and distal from the rate-limiting step that is regulated by the StAR protein (Stocco, 2001). Cells exposed to *o,p′*-DDD mounted a modulated cortisol response with a physiological concentration of pregnenolone (a concentration that yielded 65% of the cortisol secretion elicited by optimal ACTH stimulation; Table 1). However, with a 10-fold higher concentration of pregnenolone, an *o,p′*-DDD-induced inhibition of cortisol secretion was observed. These results may imply that steroidogenic enzymes are targets of *o,p′*-DDD, as in most of the higher vertebrates tested thus far, but that their dysfunction only becomes apparent when high concentrations of the pregnenolone substrate are available and when higher enzymatic activities are solicited (Miller, 1988). Although the effects of *o,p′*-DDD on steroidogenic enzymes and/or other regulatory proteins are important, disruption of the cAMP generation step ultimately leads to suppression of the steroidogenic function of the cell, whether the enzymes located downstream are affected by the xenobiotic or not.

It is important to address the fact that this study examines the acute cortisol response of rainbow trout adrenocortical cells and that many of the effects depicted here are bound to have profound long-lasting effects. The impairment of cAMP production by *o,p′*-DDD leads to disruption of cortisol synthesis in the short term, as shown in the present study. However, gene transcription of numerous proteins, such as the steroidogenic enzymes StAR and PKA, are under strict regulation by cAMP (Miller, 1988; Kagawa et al., 1999; Stocco, 2001), and disruption of cAMP production with the resulting downregulation of gene expression may impair particular cell functions. It is known that at the higher concentrations tested or with longer exposures, *o,p′*-DDD eventually leads to cell necrosis. The mechanisms that mediate *o,p′*-DDD-induced cell death are not yet clearly established. The inhibition of specific cytochrome P450 seems improbable as the sole cause for mortality. Inhibition of 11-β hydroxylase by dexamethasone efficiently inhibits cortisol secretion without causing cell death (Morishita et al., 2001). Oxidative stress induced by the pesticide is a probable mechanism of toxicity. Interference in the cytochrome P450's catalytic cycle can generate reactive oxygen species when organochlorines interact with cytochrome P450s (Hornsby, 1989). There is evidence (Dorval et al., 2003) that endosulfan, another organochlorine pesticide, generates oxidative stress in rainbow trout adrenocortical cells. Further investigations are required to elucidate this specific mechanism of adrenal toxicity of pesticides.

In conclusion, this study provides the first evidence for the disruption by *o,p′*-DDD of the signaling pathway leading to cortisol secretion in adrenocortical cells of rainbow trout. Acute exposure to *o,p′*-DDD inhibited ACTH-stimulated cortisol secretion while inhibition was attenuated with the use of dbcAMP, a cAMP analogue. Forskolin-stimulated cortisol secretion and cAMP production, as well as NaF-stimulated cAMP production, were also inhibited in a concentration-dependent manner. Basal cortisol secretion was increased with exposure to *o,p′*-DDD while basal cAMP production was unaffected by exposure to *o,p′*-DDD. Pregnenolone-stimulated cortisol secretion provided insight into a different site of action of *o,p′*-DDD, located downstream of the cAMP generation step. The mechanisms of action identified in the present study highlight profound impacts that may develop in a more chronic scenario.

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