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Role of calcium channels in cadmium-induced disruption of cortisol synthesis in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The mechanisms of toxicity of cadmium (Cd^{2+}) in adrenal steroidogenesis were investigated *in vitro* in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*). Toxicity of Cd^{2+} was increased in absence of extracellular Ca^{2+}, but was prevented in Ca^{2+}-supplemented medium. Pretreatment of cells with BAY K8644 (BAY), an agonist of voltage-dependent calcium channels, increased the Cd^{2+}-mediated inhibition of ACTH-stimulated secretion but not pregnenolone (PREG)-stimulated secretion. Nicardipine, an antagonist of voltage-dependent calcium channels, also increased the inhibition of adrenocorticotropic hormone (ACTH)-stimulated secretion by Cd^{2+}. These results suggest that opening of voltage-dependent calcium channels with BAY may allow Cd^{2+} entry at the same time as calcium, thus increasing toxicity of Cd^{2+}, however voltage-dependent calcium channels may not be the only way of entry into adrenocortical cells. The influx of Cd^{2+}, measured as intracellular Cd^{2+} using Fluo-3 in PREG-stimulated adrenocortical cells, was significantly enhanced by the stimulation. These results suggest that the deleterious effect of Cd^{2+} on cortisol steroidogenesis may be enhanced when the endocrine stress response is triggered.

Keywords: Cortisol; Adrenal cells; Cadmium; Calcium channels; Ca^{2+} signalling; Teleost fish

1. Introduction

Contamination by cadmium (Cd^{2+}), a nonessential metal and endocrine disrupter (Zacharewski, 1998; Hontela and Lacroix, 2006), poses a serious environmental threat to aquatic organisms. The hypothalamo-pituitary-adrenocortical (HPA) axis and cortisol secretion of teleost fish are impaired by chronic environmental exposures to heavy metals, including Cd (Brodeur et al., 1997; Norris et al., 1999; Laflamme et al., 2000). Cortisol, important in maintenance of physiological homeostasis under stress conditions (Mommsen et al., 1999), is the main corticosteroid hormone secreted by adrenocortical cells located, along with other cell types such as immune and chromaffin cells, in the teleost head kidney tissue (Vijayan et al., 2005; Hontela, 2005). *In vitro* studies demonstrated that adrenocorticotropic hormone (ACTH)- and dibutyryladenosine cAMP (dbcAMP)-stimulated cortisol secretion was significantly compromised by exposure to Cd^{2+} in rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*) (Leblond and Hontela, 1999; Lacroix and Hontela, 2004). However, experiments using pregnenolone (PREG) as a substrate for cortisol synthesis, bypassing the cytosolic steps of signal transduction to the steroid biosynthesis in the mitochondria, demonstrated that PREG-stimulated cortisol secretion remained normal until cytotoxic concentrations of Cd^{2+} were reached (Lacroix and Hontela, 2004). These studies suggested that Cd^{2+} disrupts the signalling pathways leading to cortisol synthesis at a step prior to the synthesis of pregnenolone.

Although the mechanisms of action of Cd^{2+} on adrenal steroidogenesis of fish remain to be elucidated, potential interactions of Cd^{2+} with calcium (Ca^{2+}), a key messenger in the steroidogenic pathways, warrant careful investigations. There is substantial evidence that Ca^{2+} and Cd^{2+} compete for binding sites in tissues such as gill or intestinal cells, and calcium protects against

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cadmium toxicity (Baldisserotto et al., 2004). Species-specific differences in sensitivity to Cd toxicity may be linked to differences in these interactions (Niyogi and Wood, 2004). In mammalian Y-1 adrenocortical cell line, the effect of Cd^{2+} on ACTH-stimulated steroid secretion was completely reversed in calcium-supplemented media, suggesting that Cd^{2+} competes and/or interferes with Ca^{2+} during adrenal steroidogenesis (Mathias et al., 1998). The uptake of Cd^{2+} through calcium channels was also demonstrated *in vitro* in adrenal PC12 chromaffin cells (Hinkle et al., 1987; Hinkle and Osborne, 1994). Nimodipine (dihydropyridine), an organic calcium channel antagonist, protected the cells from the cytotoxic effects of Cd^{2+}, whereas, BAY K8644, a calcium channel agonist, increased the toxicity (Hinkle et al., 1987; Hinkle and Osborne, 1994). Moreover, data from experiments using modulators of calcium channels and fluorescent dyes to monitor intracellular free Cd^{2+}, confirmed the involvement of calcium channels in the transport of Cd^{2+} within the PC12 adrenal cells (Hinkle and Osborne, 1994). Although Cd^{2+} permeability through calcium channels is low compared to Ca^{2+}, Cd^{2+} has been used as an effective inorganic calcium channel blocker to reduce Ca^{2+} uptake in secretory or excitable cells (Enyeart et al., 1993; Hinkle and Osborne, 1994; Yamazaki et al., 1998). Recently, Raynal et al. (2005) characterized the Cd uptake in isolated adrenocortical cells of rainbow trout and yellow perch. Inhibition of cadmium uptake by calcium was observed in cells of rainbow trout but not perch. Whether uptake of Cd^{2+} through calcium channels is linked to the disruption of corticosteroidogenic signalling pathways in teleost adrenocortical cells has not been determined thus far.

The objective of this study is to determine whether Cd^{2+} competes with extracellular Ca^{2+} during the stimulated signal transduction leading to cortisol synthesis. The interactions of Ca^{2+} and Cd^{2+} were investigated *in vitro* in rainbow trout adrenocortical cell preparations. Extracellular Ca^{2+} was either lowered using EGTA as a Ca^{2+} chelator, or supplemented using $CaCl_2$, cells were exposed to $CdCl_2$, and the adrenotoxic effect of Cd^{2+} was evaluated using cortisol secretion as an endpoint. Voltage-dependent calcium channels modulators were used to elucidate the implication of calcium channels in the mechanisms of adrenotoxcicity of Cd. It was hypothesized that BAY K8644, an agonist of voltage-dependent calcium channels, will increase Cd^{2+} toxicity, while nicardipine, a non specific antagonist for L- or T-type voltage-dependent calcium channels, will provide a protection against Cd^{2+} adrenotoxicity. To determine if the entry of Cd^{2+} in adrenocortical cells is enhanced under stimulation, Cd^{2+} influx was also measured using Fluo-3, a permeant fluorescent dye.

2. Materials and methods

2.1. Chemicals

Porcine adrenocorticotropin (ACTH 1-39), N^6,2′-o-dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP), 3α-hydroxy-5-pregnen-20-one (pregnenolone; PREG), minimum essential medium (MEM), bovine serum albumin (BSA), HEPES, propidium iodide (PI), ethylene glycol *bis*(2-aminoethylether) *N*,*N*,*N*′,*N*′ tetraacetic acid (EGTA), nicardipine (an antagonist of voltage-dependent calcium channels), BAY K8644 (an agonist of voltage-dependent calcium channels), cadmium chloride ($CdCl_2$) and calcium chloride ($CaCl_2$) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Collagenase/dispase mixture (collagenase from *Achromobacter iophagus* and dispase from *Bacillus polymyxa*) was obtained from Boehringer Mannheim (Laval, QC, Canada). RIA kits for cortisol assays and 3-aminobenzoic acid ethyl ester (MS 222) for anesthesia were purchased from ICN Pharmaceuticals (Orangeburg, NY, USA). The membrane-permeant Fluo-3/AM Ca^{2+} probe was purchased from Molecular Probes (Eugene, OR, USA).

2.2. Experimental animals

Rainbow trout, *O. mykiss*, (body mass 100–150 g) were obtained from Labelle Piscultures Inc. (Labelle, QC, Canada). Upon arrival to the aquatic facilities, fish were maintained in a 600 L freshwater tank at 15±1 °C, supplied with a constant flow rate of 3.8 L/min of filtered and oxygen-saturated water (hardness=70 mg/L $CaCO_3$). Rainbow trout were fed daily with commercial trout food 10 g/kg of fish body mass. Two weeks of acclimation were allowed before the beginning of the experiments.

2.3. Preparation of head kidney cell suspensions

The method described by Leblond et al. (2001) was used to prepare the adrenocortical cell suspensions. Fish anaesthetized with MS 222 were bled from the caudal vasculature and perfused with saline solution (0.7%), to remove as much blood as possible. Head kidney, where the adrenocortical cells are located, was then dissected out and deposited into fresh MEM supplemented with 5 g/L BSA and 2.2 g/L $NaHCO_3$ (complete medium), pH adjusted to 7.4. The tissue was washed and resuspended in 2.5 mL complete medium with 2.0 mg/mL collagenase/dispase, and incubated 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 complete medium and cellular density adjusted to 75×10^6 cells/mL. Cells were plated in a 96-well microplate at 150 μL of 75×10^6 cells/mL per well and incubated, as described by Leblond et al. (2001). A 2-h preincubation with slow agitation was required to reach basal cortisol secretion. All incubations were performed at 15 °C.

2.4. Exposure to Cd at various concentrations of Ca

Following preincubation, cells were washed with teleost Ringer solution (269 mosM/L, 6.5 g/L NaCl, 0.2 g/L KCl, 0.2 g/L $NaHCO_3$, 1.0 g/L Dextrose, 5.95 g/L Hepes, pH 7.4) without $CaCl_2$, then exposed to Cd^{2+} (0.1 mM as $CdCl_2$) in Ringer solution with various concentrations of $CaCl_2$ (0, 2, 8 and 20 mM) for 60 min. Cells treated without $CaCl_2$ (0 mM) were incubated with and without 0.1 mM EGTA. Cortisol secretion was then stimulated for 120 min with ACTH (1 IU/mL) and PREG (0.5 μM), in

medium with concentration of $CaCl_2$ used in the previous treatment. Supernatants were frozen (−20 °C) until cortisol determination by RIA.

2.5. Exposure to Cd and modulators of calcium channels

To assess the role of voltage-dependent calcium channels in the adrenotoxicity of Cd^{2+}, BAY K8644, a calcium channel agonist, and nicardipine, a channel blocker, were used. Following preincubation, adrenocortical cells were treated with 1 μM of BAY K8644 or 25 μM of nicardipine in Ringer solution in presence of extracellular Ca^{2+} (2 mM $CaCl_2$) for 60 min. The optimal concentration of nicardipine was previously determined as the EC_{50} (the effective concentration reducing by 50% ACTH-stimulated cortisol secretion). Nicardipine and BAY K8644 were dissolved in ethanol before dilution in the Ringer solution. The final concentration of ethanol (2%) had no effect on cortisol secretion and viability of adrenocortical cells (data not shown), as reported previously (Lacroix and Hontela, 2004).

Following the 60 min treatment with BAY K8644 or nicardipine cells were exposed to various concentrations of Cd^{2+} (0–10 mM $CdCl_2$) in Ringer solution, for another 60 min. Microplates were then centrifuged (300×*g* for 3 min) and cells were washed once with 150 μL of Ringer solution, the pellets were resuspended in 150 μL of MEM (2 mM Ca) containing 1 IU/mL ACTH or 0.5 μM PREG and incubated. Supernatants were frozen (−20 °C) until cortisol determination by RIA.

2.6. Cell viability

Viability was determined by flow cytometry using the exclusion of dye propidium iodide (PI) following $CdCl_2$ exposure before the stimulation with ACTH and PREG. To assess viability, 5 μL samples from each well of the microplate were resuspended in a test tube containing 370 μL of complete medium to which the PI (1 μg/mL) was added. The cells were analyzed by flow cytometry using a FACScan (Becton Dickinson) equipped with an argon laser emitting at 488 nm. For each sample 10,000 events were analysed and the results were expressed as the percentage of live cells. Data analyses were performed with a FACSCAN software.

2.7. Measurement of intracellular Cd^{2+} influx in head kidney cells

The evolution of free intracellular Cd^{2+} ($[Cd^{2+}]_i$) was determined by flow cytometry using Fluo-3, according to the method of Vandenberghe and Ceuppens (1989). Head kidney cells (10^7 cells/mL, including adrenocortical cells) resuspended in Ringer solution with and without Ca^{2+} (2 mM $CaCl_2$), were loaded with 5 μM of acetoxymethylester of Fluo-3. This permeant probe crosses the cell membrane and is hydrolyzed in the cytoplasm to the impermeant trapped form. Cells were incubated with Fluo-3 for 20 min at 15 °C, in the dark. Whereafter, cells were diluted 5 times with respective Ringer solutions and incubated at 15 °C for additional 40 min. Cells were then washed and resuspended at a concentration of 1×10^6 cells/mL in Ringer solution, with respective Ca^{2+} concentration. Experiments were conducted with and without extracellular Ca^{2+} to assess Cd^{2+} influx into the head kidney cells since Cd^{2+} induces intracellular Fluo-3 fluorescence and the Cd^{2+}-induced fluorescence is undissociable from Ca^{2+}-induced fluorescence (Marchi et al., 2000). Forward scatter, right angle scattter and emission at 530 nm of the cells were registered before and after treatment of cells with appropriate additions on a FACScan (Becton Dickinson), with excitation at 488 nm from an argon laser. Fluorescence was measured over time (102 s), with interruptions for treatment (0.5 μM Preg, 0.1 mM Cd, 25 μM nicardipine or 1 μM Bay) additions at 20 and 40 s. Data were analyzed with FACScan software. PREG, a precursor of cortisol synthesis, was used to measure $[Cd^{2+}]_i$, in stimulated cortisol synthesis. The EC_{50} of $CdCl_2$ (0.1 mM), concentration that reduced stimulated-cortisol secretion by 50% in adrenocortical cells from rainbow trout (Leblond and Hontela, 1999; Lacroix and Hontela, 2004) was used.

2.8. Statistical analysis

All the data (cortisol secretion and cell viability) are expressed as % of a control, the group not exposed to Cd and treated with 2 mM Ca, selected as the most physiologically relevant treatment, as done in previous studies (Hontela, 2005). Statistical significance in each experiment was determined either using a one-way analysis of variance (ANOVA) and a Tukey-Kramer Highly Significant Difference (HSD) test or a *t* test. A value of $P<0.05$ was considered significant.

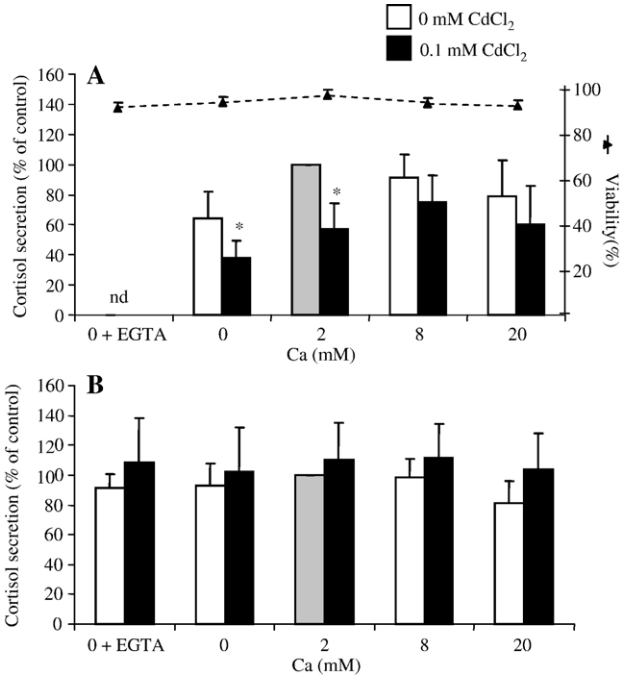

Fig. 1. Cortisol secretion of rainbow trout adrenocortical cells exposed *in vitro* to Cd^{2+} (0.1 mM $CdCl_2$ in Ringer; solid bars) or Ringer only (empty bars) in presence of various concentrations of Ca and stimulated with ACTH at 1 IU/mL (A) or PREG at 0.5 μM (B). Results are expressed as percentage of control (2 mM of Ca for ACTH and PREG, respectively, shaded bars). Each bar represents the mean of eight experiments ± SD. * indicate significant differences from the control (ANOVA and Tukey Kramer HSD test, $P<0.05$).

3. Results

To evaluate the influence of extracellular Ca^{2+} on adrenotoxicity of Cd^{2+}, ACTH-and PREG-stimulated cortisol secretion by trout adrenocortical cells exposed to 0.1 mM $CdCl_2$ (EC_{50}), was measured in incubation medium at 0, 2, 8 and 20 mM $CaCl_2$ (Fig. 1). The data are shown as % of controls, cells not exposed to Cd^{2+} and stimulated either with ACTH or PREG in Ringer solution containing 2 mM of $CaCl_2$. The ACTH-stimulated cortisol secretion was suppressed by the removal of extracellular Ca^{2+}, using EGTA as a chelator. The toxic effect of Cd^{2+} on ACTH-stimulated cortisol secretion was significantly greater at low Ca^{2+}concentrations (0 and 2 mM $CaCl_2$) in the incubation medium, compared to the control (Fig. 1A). However, at non-physiological concentrations of Ca^{2+} (8 and 20 mM of $CaCl_2$), the difference between ACTH-stimulated secretion of cells exposed to Cd^{2+} was not significantly different from the non-exposed controls, and moreover, 20 mM of extracellular Ca tended to diminish the ACTH-stimulated cortisol secretion. In contrast, the use of EGTA and the variation of extracellular Ca^{2+} concentration did not have a significant effect on PREG-stimulated cortisol secretion (Fig. 1B). There were no effects on cell viability in any of the treatments (Fig. 1A).

To evaluate the role of calcium channels in toxicity of Cd^{2+} in trout adrenocortical cells, BAY K8644 (BAY), an agonist and nicardipine, an antagonist of voltage-dependent calcium channels were used. Pretreatment of adrenocortical cells with 1 μM of BAY significantly increased the adrenotoxicity of Cd^{2+} (Fig. 2A) on ACTH-stimulated cortisol secretion. At 0.01 mM, Cd^{2+} had no significant effect on ACTH-stimulated cortisol secretion, however pretreatment with BAY produced a significant decrease of ACTH-stimulated secretion at this concentration of Cd and the secretion was completely abolished at 0.1 mM. Pretreatment of

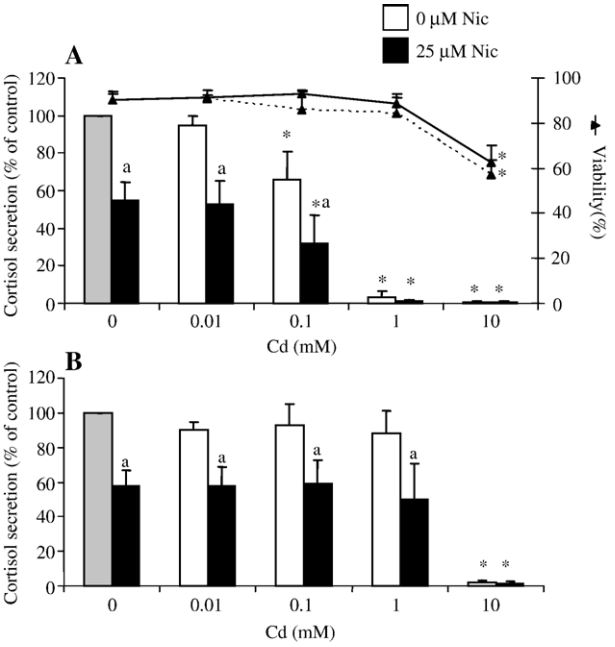

Fig. 3. Effects of Cd^{2+} ($CdCl_2$) on ACTH (A)- and PREG (B)-stimulated cortisol secretion and viability of rainbow trout adrenocortical cells pretreated with nicardipine (25 μM). Results are expressed as the mean percentage of respective controls (shaded bars) for ACTH and PREG without nicardipine or Cd. Each bar represents the mean of eight experiments±SD (ANOVA and Tukey-Kramer HSD test, $P<0.05$). * indicate significant differences from the control, letter *a* indicates significant differences between treatments without nicardipine (0 μM, solid line for viability) and with nicardipine (1 μM, dotted line for viability) for the same concentration of Cd.

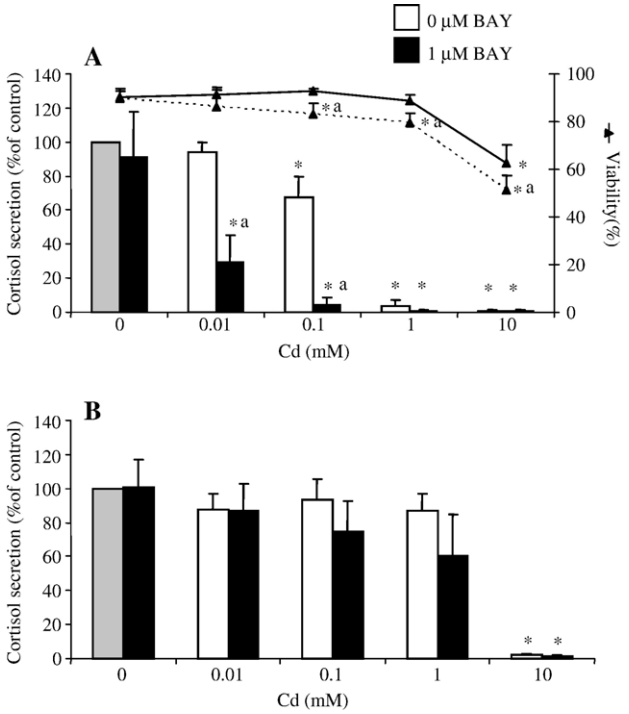

Fig. 2. Effects of Cd^{2+} ($CdCl_2$) on ACTH (A)- and PREG (B)-stimulated cortisol secretion and viability of rainbow trout adrenocortical cells pretreated with BAY K8644 (1 μM). Results are expressed as the mean percentage of respective controls (shaded bars) for ACTH and PREG without BAY K8644 or Cd. Each bar represents the mean of eight experiments±SD (ANOVA and Tukey-Kramer HSD test $P<0.05$). * indicate significant differences from the control (shaded bar), letter *a* indicates significant differences between treatments without BAY (0 μM, solid line for viability) and with BAY (1 μM, dotted line for viability) for the same concentration of Cd.

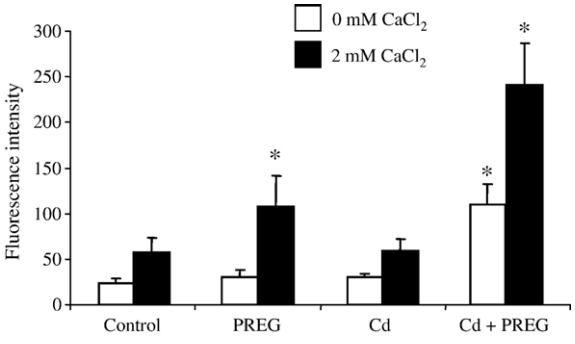

Fig. 4. Variations in fluorescence in rainbow trout adrenocortical cells loaded with Fluo-3 in Ringer solution supplemented with $CaCl_2$ (solid bars) or without (empty bars) treated with pregnenolone (PREG 0.5 μM), Cd (0.1 mM), and Cd+PREG. Maximal values of mean fluorescence were noted at fixed time points after treatment. Each bar represents the mean of six experiments±SD. * indicates significant difference from their respective control (basal fluorescence at 0 or 2 mM $CaCl_2$ before treatment with PREG, Cd, or Cd+PREG), (ANOVA and Tukey-Kramer HSD test, $P<0.05$). Differences between $CaCl_2$ treatments (0 and 2 mM $CaCl_2$) were significant for all experiments (*t* test, $P<0.05$).

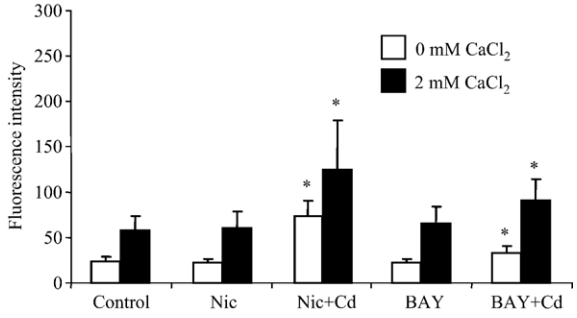

Fig. 5. Variations in fluorescence in rainbow trout adrenocortical cells loaded with Fluo-3 in Ringer solution supplemented with $CaCl_2$ (solid bars) or without (empty bars) and treated with nicardipine (NIC, 25 μM), or BAY K8644 (BAY, 1 μM) in presence or absence of Cd (0.1 mM). Maximal values of mean fluorescence were noted at fixed time points after treatment. Each bar represents the mean of six experiments ± SD. * indicates significant difference from their respective control (basal fluorescence at 0 or 2 mM $CaCl_2$ before treatment with Nic, Nic+Cd, Bay, or Bay+Cd) (ANOVA and Tukey-Kramer HSD test, $P<0.05$). Difference between $CaCl_2$ treatments (0 and 2 mM $CaCl_2$) were significant for each experiment (t test, $P<0.05$).

adrenocortical cells with BAY and exposure to Cd^{2+} had no significant effect on PREG-stimulated cortisol secretion (Fig. 2B), until cell viability was decreased by approximately 50% at 10 mM of Cd (Fig. 2A). Viability of cells pretreated with BAY was also significantly lower at the concentration of 0.1 mM of Cd^{2+} and higher, compared to cells that have not been pretreated with BAY.

An antagonist of voltage-dependent calcium channels, nicardipine had a significant effect on both ACTH- and PREG-stimulated cortisol secretion (Fig. 3A and B). Pretreatment of cells with 25 μM of nicardipine decreased by 50% the ACTH-stimulated cortisol secretion (EC_{50}) without Cd (0 mMCd). Exposure to Cd^{2+} further decreased cortisol secretion, with the effect becoming significant at 0.1 mM Cd and greater (Fig. 3A). The PREG-stimulated secretion was also compromised (~50% of the control) by nicardipine but the increasing concentrations of Cd^{2+} did not exert additional adrenotoxicity on cortisol synthesis stimulated with PREG. Cell viability was significantly decreased at 10 mM Cd^{2+} but there were no significant differences in viability of cells pretreated with nicardipine or not (Fig. 3A).

To further elucidate the interactions between Cd^{2+} and Ca^{2+} in adrenocortical cells, changes in intracellular Cd^{2+} levels were evaluated using Fluo-3 as a probe, in presence or absence of extracellular Ca^{2+}. Since the signal from ACTH-induced Cd^{2+} and/or Ca^{2+} influx in the head kidney cells was not detectable in our system (data not shown), the Ca^{2+}/Cd^{2+} influx was measured in cells stimulated with PREG (0.5 μM), a precursor for cortisol synthesis. When Cd^{2+} (0.1 mM) was added to the cell preparation, with and without extracellular Ca^{2+}, the PREG-induced fluorescence was 5 times higher than the control which represent the basal state of fluorescence (Fig. 4). Since Fluo-3 fluorescence is produced by both Cd^{2+} and Ca^{2+} (Marchi et al., 2000), the Cd^{2+} influx within cells is representative only in Ringer solution without supplemented $CaCl_2$. Our results therefore suggest that under stimulation with PREG, Cd^{2+} influx is induced in head kidney cells (Fig. 4).

Changes in fluorescence produced by Cd^{2+} were also assessed with nicardipine and BAY in Ringer solution, with and without Ca^{2+} (Fig. 5). As indicated by the increasing fluorescence, a significant influx of intracellular Cd^{2+} occurred within the head kidney cells, when Cd^{2+} was added following the addition of BAY or nicardipine, compared to the control (basal fluorescence before treatment additions). However, the results also showed that the agonist or the antagonist of calcium channels, used alone without Cd^{2+}, had no significant effect on the Ca^{2+} influx in both Ringer solution with and without $CaCl_2$ (Fig. 5).

4. Discussion

Recent studies that demonstrated the vulnerability of the adrenal function of teleost fish to environmental pollutants (Norris et al., 1999; Leblond et al., 2001; Dorval et al., 2002; Quabius et al., 2002; Love et al., 2003) emphasized the need for a better understanding of the intracellular mechanisms of action of toxicants known as endocrine disrupters. The goal of this present study was to elucidate the role of calcium channels in Cd^{2+}-induced adrenotoxicity.

The interactions between Cd^{2+} and extracellular Ca^{2+} were investigated in adrenocortical cells from rainbow trout. Our results demonstrated that Cd^{2+} competes with Ca^{2+} since the adrenotoxic effect of Cd^{2+} was increased in absence of extracellular Ca^{2+}, but was prevented in media supplemented with Ca^{2+}. Indeed, ACTH-stimulated cortisol secretion of cells exposed to Cd^{2+} was significantly lower when extracellular Ca^{2+} was not available or was at low concentrations. A study of the competitive interactions between Cd^{2+} and extracellular Ca^{2+} in ACTH-stimulated steroid secretion in mammalian Y-1 adrenocortical cells also demonstrated that Cd^{2+} failed to inhibit ACTH-stimulated steroid secretion in $CaCl_2$ supplemented medium (Mathias et al., 1998). Moreover, recently Raynal et al. (2005) characterized cadmium uptake in adrenocortical cells of rainbow trout and yellow perch and reported that calcium inhibited cadmium uptake in rainbow trout cells. In the present study, Cd^{2+} did not have a detectable toxic effect at high (>8 mM) concentrations of calcium; in fact cortisol secretion tended to decrease even in control cells (not exposed to cadmium) at the highest (20 mM) concentration of Ca^{2+}. Since intracellular Ca^{2+} concentration is precisely regulated (Berridge, 1997; Kass and Orrenius, 1999), the high extracellular Ca^{2+} may disrupt intracellular signalling leading to cortisol steroidogenesis. Inhibition of Ca^{2+}-ATPase by Cd^{2+}, compromising intracellular Ca^{2+} homeostasis, has been shown in other cell types (Verbost et al., 1989; Zhang et al., 1990; Viarengo et al., 1993). In pharmacological studies on mammalian adrenal steroidogenesis, Cd^{2+} has been used as an inhibitor of the Ca^{2+} current associated with store depletion of Ca^{2+} (Ebisawa et al., 2000; Kawamura et al., 2003). The effects of Cd^{2+} on Ca^{2+}-ATPase in teleost adrenocortical cells and the role of this enzyme in intracellular Ca^{2+} homeostasis and adrenal steroidogenesis have not yet been investigated.

Although exposure to $CdCl_2$ disrupted ACTH-stimulated cortisol secretion at physiological calcium concentrations, PREG-stimulated secretion was maintained at a control level despite the presence of Cd^{2+} and independently of Ca^{2+} concentration (Fig. 1B). These results suggest that the competition between Cd^{2+} and Ca^{2+} occurs at steps prior to where

pregnenolone is used as a substrate in the cytoplasm, as has been reported by a previous study with teleost adrenocortical cells (Lacroix and Hontela, 2004). Laskey and Phelps (1991), using rat Leydig cells, also identified the toxic site of Cd^{2+} prior to pregnenolone synthesis in the signalling pathway leading to testosterone synthesis.

In mammalian adrenocortical cells, Ca^{2+} is recognized as a second messenger for ACTH in the signalling cascade leading to corticosteroid synthesis and there is evidence that cytosolic Ca^{2+} concentration is raised by ACTH stimulation (Yamazaki et al., 1998). Dihydropyridines, voltage-dependent calcium channels antagonists, inhibit cortisol secretion in mammalian adrenocortical cells, indicating that these types of calcium channels are involved in the steroidogenesis of cortisol (Yamazaki et al., 1998). Nicardipine also produced a concentration-dependent inhibition of ACTH- and PREG-stimulated cortisol synthesis in rainbow trout (Lacroix, unpublished data). Thus even though removal or addition of calcium to the media did not influence PREG-stimulated cortisol secretion in the present study (Fig. 1B), treatment with the calcium channel antagonist nicardipine did decrease PREG-stimulated cortisol secretion (Fig. 3B). The specific mechanism for this effect is not yet understood. In the present study, the role of voltage-dependent calcium channels in the endocrine-disrupting action of Cd^{2+} in teleost adrenal steroidogenesis was investigated using calcium channel modulators. The ACTH-stimulated cortisol secretion was abruptly diminished by Cd^{2+}, following the treatment with BAY K8644, an agonist of voltage-dependent calcium channels. Moreover, cytotoxicity of Cd^{2+}, estimated by cell mortality, was enhanced by BAY K8644 pretreatment. These results suggest that Cd^{2+} enters the adrenocortical cells through the voltage-dependent channels. Opening them with BAY K8644 may have provoked a pronounced entry of Cd^{2+}, increasing its adrenotoxicity. However, the increased entry of Cd^{2+} into the cytosol had no effect on PREG-stimulated cortisol secretion, again suggesting that Cd^{2+} interfered at a cytosolic site of action, prior to the pregnenolone synthesis in the mitochondria. Hinkle and Osborne (1994) also concluded that the uptake of Cd^{2+} into the adrenal chromaffin cells occured via voltage-dependent calcium channels, since the use of BAY K8644 shifted the lethality response curve to the left. However, in their system, the use of a voltage-dependent calcium channel antagonist, nimodipine (dihydropyridine family), protected against Cd^{2+} toxicity by preventing Cd^{2+} entry. Thus, in the present experiment with the adrenocortical cells of rainbow trout, treatment with a dihydropyridine antagonist blocking voltage-dependent calcium channels was expected to prevent Cd^{2+} adrenotoxicity. To the contrary, use of nicardipine as an antagonist of voltage-dependent calcium channels increased adrenotoxicity of Cd^{2+} on ACTH-stimulated cortisol secretion, although there were no additional adrenotoxic effects of Cd^{2+} on PREG-stimulated cortisol secretion, except when cell viability was reduced (10 nM Cd). Voltage-dependent calcium channels may not be the only way of entry into the adrenocortical cells, since the adrenotoxic effect of Cd^{2+} was increased and not prevented by treatment with a calcium channel antagonist. Even though nicardipine compromised both ACTH- and PREG-stimulated cortisol synthesis, cortisol secretion should have been maintained at the same level without any additional adrenotoxic effect of Cd^{2+}, if voltage-dependent calcium channels were the only way of entry into adrenocortical cells.

The experiments using Fluo-3 demonstrated that Ca^{2+} influx as well as Cd^{2+} influx occurs in stimulated cortisol synthesis during exposure to Cd^{2+}. Enhanced fluorescence intensity of the head kidney cells loaded with the fluorescent probe was detected following PREG stimulation but not ACTH stimulation. Whether PREG is less specific than ACTH to enhance Cd^{2+} and/or Ca^{2+} influx, and may trigger other cell types, such as immune cells (lymphocytes, monocytes), or whether the PREG-induced influx into adrenocortical cells is simply more intense, remains to be determined in future studies. Any investigation using teleost head kidney cells *in vitro* faces the challenge of working with a mixed cell population made up of adrenocortical cells (<1% of total, unpublished data) intermingled with chromaffin and immune cells (Hontela, 2005). Despite the limitations of our approach, the use of cortisol secretion as an endpoint and the assessment of Cd^{2+} influx using the fluorescent probe provided evidence that dihydropyridine sensitive calcium channels are involved in stimulated cortisol synthesis and Cd^{2+} influx in teleosts. This is a first report on the use of PREG, as a steroid, to enhance Cd^{2+} influx in head kidney cell suspension of a teleost fish. Despite the fact that the influx of Ca^{2+} was indistinguishable from Cd^{2+} influx in presence of extracellular Ca^{2+}, as reported by Marchi et al. (2000), results without extracellular Ca^{2+} confirmed that Cd^{2+} entry into the adrenocortical cells is greater when steroidogenesis is triggered. Treatment with BAY K8644, a calcium channel agonist, significantly induced Cd^{2+} influx, confirming the implication of voltage-dependent calcium channels in the mechanism of action in Cd^{2+} adrenotoxicity. Similar to the results with ACTH- and PREG-stimulated cortisol secretion, nicardipine, the calcium channel antagonist, significantly induced Cd^{2+} influx into the head kidney cells. These results provided an explanation for the non-protective effect of nicardipine on steroidogenesis of adrenocortical cells exposed to Cd^{2+}.

The present study provided evidence that Cd^{2+} interferes in the signalling pathway leading to cortisol synthesis in teleost fish, at a step prior to pregnenolone formation in the mitochondria. The results reported here suggest that competition with Ca^{2+} mediates, at least in part, the Cd^{2+}-induced adrenotoxicity and that voltage-dependent calcium channels represent one way of entry of Cd^{2+}. Further studies are required to fully understand the complexity of Ca^{2+} homeostasis in fish adrenocortical cells and the interactions of Ca^{2+} and Cd^{2+} in adrenotoxicity.

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