

Regulation of acute cortisol synthesis by cAMP-dependent protein kinase and protein kinase C in a teleost species, the rainbow trout (*Oncorhynchus mykiss*)

M Lacroix and A Hontela

Département des Sciences Biologiques, Université du Québec à Montréal, TOXEN Research Center, Canada

(Requests for offprints should be addressed to A Hontela, Département des Sciences Biologiques, Université du Québec à Montréal, TOXEN Research Center, C.P. 8888, succ. Centre-Ville, Montréal, Québec, Canada H3C 3P8; Email: hontela.alice@uqam.ca)

Abstract

The effects of cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) on acute ACTH-stimulated cortisol secretion were assessed using a specific PKA inhibitor (H-89) and a PKC activator (phorbol 12-myristate 13-acetate, PMA) in dispersed head kidney cells of rainbow trout (*Oncorhynchus mykiss*). To investigate the sites of action of both PKA and PKC, pregnenolone (a cortisol precursor stemmed from the rate limiting step in cortisol synthesis) and 25-OH-cholesterol (an exogenous substrate that bypasses the rate limiting step) were used as substrates, with and without ACTH stimulation. Inhibition of PKA decreased ACTH-stimulated cortisol secretion while activation of PKC had the same effect,

demonstrating that PKA stimulates and PKC inhibits cortisol synthesis. Inhibition of PKA and activation of PKC had no significant effect on pregnenolone-stimulated cortisol synthesis, indicating that both PKA and PKC act upstream from the pregnenolone step. Inhibition of PKA and activation of PKC had no significant effect on basal cortisol secretion in the presence of 25-OH-cholesterol, suggesting that PKA and PKC exert their effects on the mitochondrial cholesterol translocation step. This study provided evidence for the stimulatory role of PKA and the inhibitory role of PKC in the signalling pathways leading to cortisol synthesis in teleosts.

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Introduction

Cortisol is the main steroid hormone produced in the interrenal tissue of teleost fish, the tissue homologous to the mammalian adrenal. It plays an important role in maintaining physiological homeostasis through effects on glycaemia, growth, and osmoregulation (Hontela 1997, Wendelaar Bonga 1997). Toxicological studies have demonstrated the disruptive effects of xenobiotics on adrenal steroidogenesis in teleosts (Ilan & Yaron 1983, Brodeur *et al.* 1998, Hontela 1998) as well as in birds and mammals (Jönsson *et al.* 1993, 1994, Lund 1994, Mgbonyebi *et al.* 1994). It has also been reported that acute (60 min) non cytotoxic *in vitro* exposures of interrenal cells to *o,p'*-DDD, a DDT metabolite, inhibit cortisol secretion through effects prior to cAMP formation while heavy metals such as cadmium, zinc, and mercury seem to exert their effects downstream from cAMP production in the signalling cascade leading to acute cortisol synthesis (Leblond & Hontela 1999, Benguira & Hontela 2000). These studies have shown that xenobiotics may target different intracellular steps of adrenal steroidogenesis, some of which are relevant to the intracellular signalling pathways leading to cortisol synthesis.

The understanding of the pathways and intracellular messengers that regulate cortisol synthesis in fish is limited. Among studies on the control of cortisol synthesis by adrenocorticotrophin (ACTH), Patiño *et al.* (1986) demonstrated the importance of cAMP as an intracellular second messenger in the stimulation of cortisol production in the coho salmon (*Oncorhynchus kisutch*). Furthermore, dibutyryl-cAMP (dbcAMP) has been shown to stimulate cortisol secretion in a dose-related manner independently of ACTH in rainbow trout dispersed interrenal cells (Leblond *et al.* 2001). Mammalian studies had already reported that the main pathway leading to corticosteroid synthesis in the adrenal gland involves ACTH which stimulates a signalling cascade integrating G-proteins, adenylyl cyclase, cAMP and protein kinase A (PKA) (Miller 1988, Schimmer 1995). Other pathways involving protein kinase C (PKC) via stimulation by angiotensin II (AII) or acetylcholine, both known secretagogues of cortisol in fish (Perrott & Balment 1990, Kloas *et al.* 1994), at least share a role in the regulation of corticosteroid synthesis, both chronic and acute (Bird *et al.* 1990).

The chronic regulation of transcription factors and transcription of genes coding for proteins necessary for

cortisol synthesis (i.e. CytP450 hydroxylases) by cAMP, PKA or PKC has been investigated in numerous mammalian models (Schimmer 1995). However, the time course of the disruptive effects reported in recent toxicological studies in fish (Leblond & Hontela 1999, Benguira & Hontela 2000) suggests that mechanisms regulating acute cortisol synthesis may be targeted by some adreno-toxicants. Acute trophic hormone-mediated steroidogenesis calls for rapid regulation mechanisms involving intracellular messengers such as PKA and PKC and proteins that act upon the rate limiting step in cortisol synthesis in the adrenal gland: the transfer of cholesterol to the inner mitochondrial membrane (Clark *et al.* 1994, Lin *et al.* 1995, Stocco & Clark 1996).

Since the signalling pathways leading to cortisol synthesis have not been investigated in teleosts, the purposes of this study were (1) to characterize the roles of PKA and PKC in acute cortisol synthesis in the rainbow trout (*Oncorhynchus mykiss*) with the use of a specific PKA inhibitor, H-89 and a PKC activator, phorbol 12-myristate 13-acetate (PMA), and (2) to allocate specific sites of action for these intracellular messengers in their regulation of acute cortisol synthesis.

Materials and Methods

Animals

Male and female rainbow trout (mean weight 80–110 g) were bought from Labelle Piscicultures, Inc. (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 CaCO₃) 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 two-week acclimation period was allowed before the beginning of the experiments.

Chemicals

Porcine adrenocorticotrophic hormone (ACTH₁₋₃₉), pregnenolone (3 β -hydroxy-5-pregne-20-one), MEM (minimal essential medium), BSA (bovine serum albumin), PMA, and 25-OH-cholesterol were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Collagenase/dispase mixture (collagenase from *Achromobacter iophagus* and dispase from *Bacillus polymyxa*) was bought from Boehringer Mannheim (Laval, Quebec, Canada). H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline-sulphonamide·2HCl) was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). MS-222 (*m*-aminobenzoic acid ethyl ester methane-sulphonate) was bought from ICN Pharmaceuticals (Orangeburg, NY, USA). Other chemicals used were reactive grade. Pregnenolone and 25-OH-cholesterol were dissolved in 100% ethanol, PMA and H-89 were

dissolved in dimethylsulphoxide (DMSO). Concentrations of DMSO and ethanol in the final cell suspension medium were 5% (v/v) and 2% (v/v) respectively. These concentrations have no effect on cortisol secretion or cell viability (data not shown) (Leblond & Hontela 1999).

Cell suspensions

The cell suspension method has been described elsewhere (Leblond *et al.* 2001). The head kidneys of 8–10 fish were used in each treatment, each head kidney being treated individually as *n*=1 replicate. Fish were anaesthetized 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 NaHCO₃, pH 7.4 (complete medium). The tissue was washed twice with complete medium, resuspended in 2.5 ml complete medium with 2.0 mg/ml 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 μ m mesh cloth and the filtrate was centrifuged at 300 \times g (1000 r.p.m.) 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 using a haemocytometer.

In vitro effects of agonists and antagonists on cortisol secretion

The cells were distributed in a 96-well microplate at 150 μ l of 75×10^6 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 \times g for 3 min. The supernatants were then removed and the pellets were resuspended in 150 μ l complete medium containing the test agent(s). Depending on the experiment, the test agents were: the optimal dose of ACTH (1 IU/ml or 2.4×10^{-6} M) as previously investigated by Leblond *et al.* (2001) and various concentrations of 25-OH-cholesterol, pregnenolone, H-89 and PMA. Cells were then incubated at 15 °C for 60 min with the test agents with light agitation and centrifuged one more time at 300 \times g for 3 min. The supernatants were collected and cortisol determined by RIA (ICN Pharmaceuticals). Blanks of every agent used in the assays (H-89, PMA, 25-OH-cholesterol and pregnenolone) revealed no cross-reactivity with the cortisol RIA used in the study (data not shown). Cell viability was assessed using Trypan Blue exclusion method.

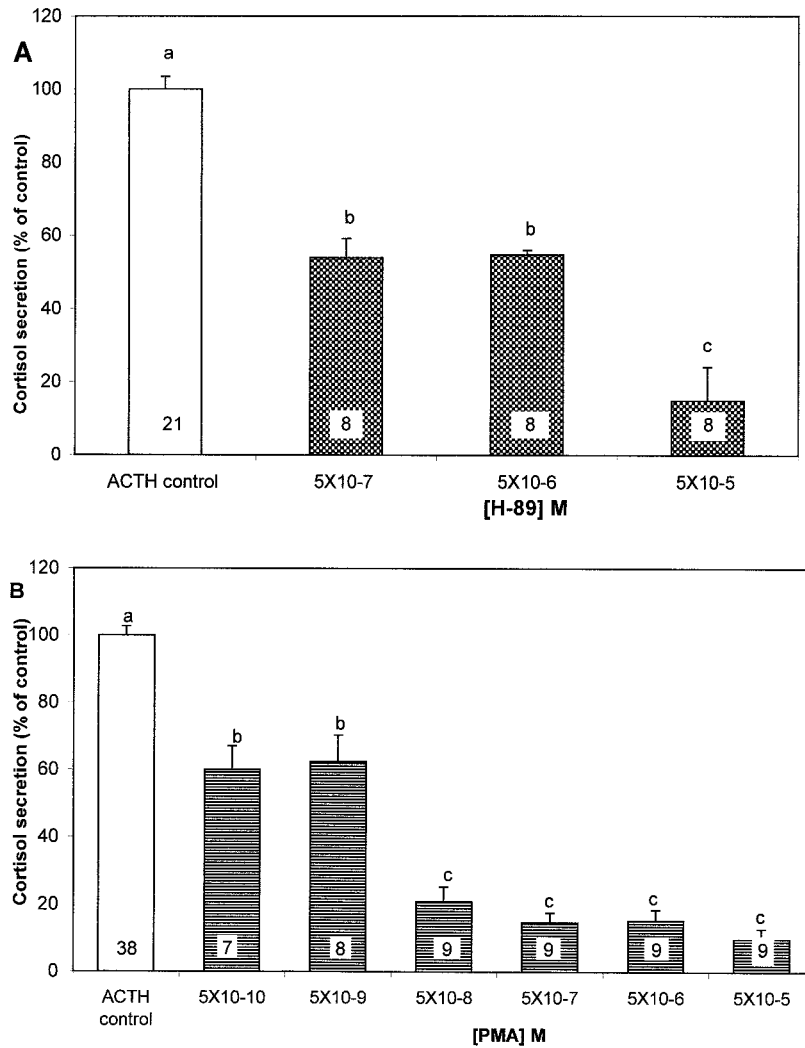


Figure 1 Effect of (A) PKA inhibitor H-89 and (B) PKC activator PMA on cortisol secretion of rainbow trout adrenocortical cells stimulated with ACTH (1 IU/ml), and incubated for 1 h at 15 °C (expressed as mean percentage of ACTH-stimulated secretion used as positive control, \pm S.E.M.). Significant differences between treatments are represented by different letters (ANOVA and Tukey-Kramer HSD test, $\alpha=0.05$). Cells obtained from the interrenal tissue of one fish correspond to $n=1$, numbers of replicates are indicated within each bar.

Statistical analysis

Statistical significance in each experiment was determined using a one-way analysis of variance (ANOVA) and a Tukey-Kramer Highly Significant Difference (HSD) test ($\alpha=0.05$). A value of $P<0.05$ was considered significant.

Results

Effect of H-89 and PMA on ACTH-stimulated acute cortisol synthesis

To investigate the roles of both PKA and PKC in acute cortisol synthesis in rainbow trout, adrenocortical cells

were incubated for 1 h with increasing concentrations (5×10^{-7} to 5×10^{-5} M) of H-89, a PKA specific inhibitor (Fig. 1A), or increasing concentrations (5×10^{-10} to 5×10^{-5} M) of PMA, a PKC activator (Fig. 1B), and they were stimulated by ACTH (1 IU/ml). A dose-related decrease in ACTH-stimulated cortisol secretion was observed when cells were exposed to H-89 or PMA. Concentrations of 5×10^{-7} M and 5×10^{-10} M H-89 and PMA respectively, significantly decreased cortisol secretion ($\approx 55\%$ to 60% of control). The ACTH-stimulated cortisol secretion of untreated controls (ACTH only) was 18.3 ± 1.8 ng/ml (mean \pm S.E.M., $n=38$, Fig. 1B).

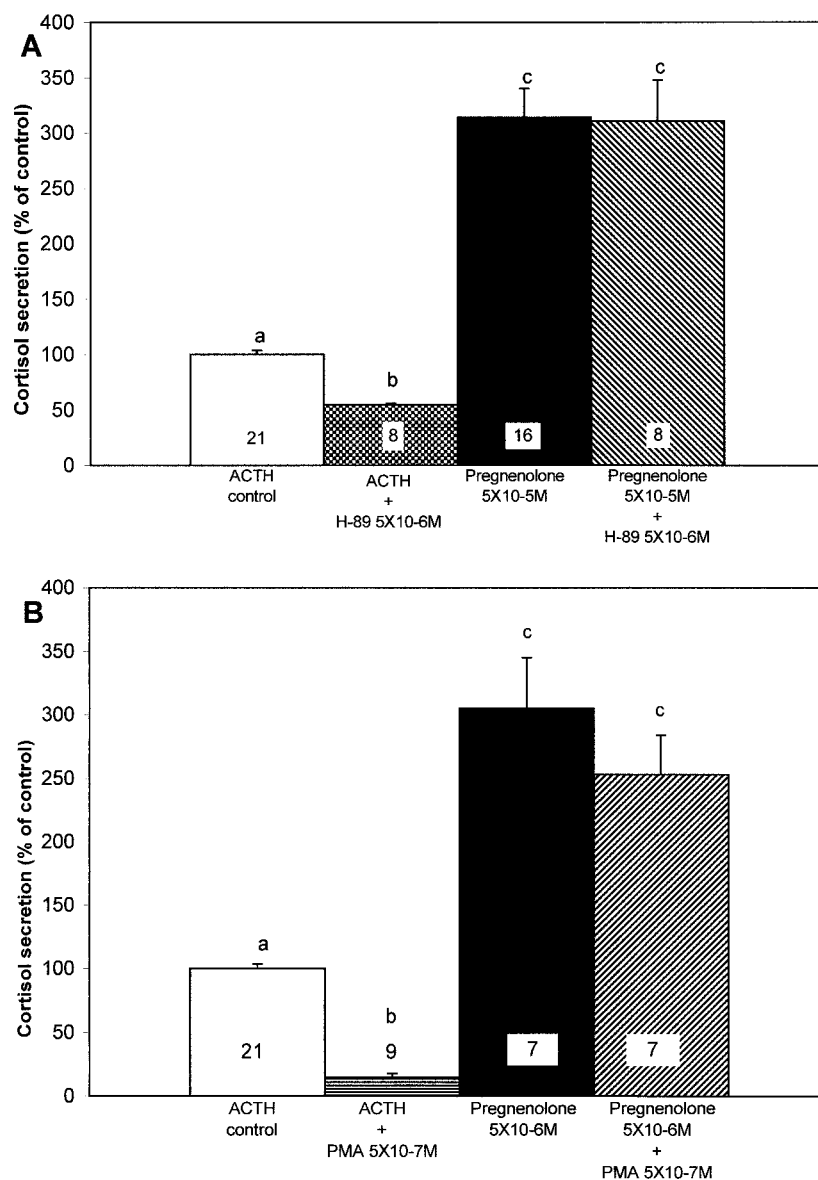


Figure 2 Effect of (A) PKA inhibitor H-89 and (B) PKC activator PMA on cortisol secretion of rainbow trout adrenocortical cells stimulated with ACTH (1 IU/ml) or pregnenolone (5 and 50 μ M), and incubated for 1 h at 15 °C (expressed as mean percentage of control, see Fig. 1 for details). Significant differences between treatments are represented by different letters (ANOVA and Tukey-Kramer HSD test, $\alpha=0.05$).

Effect of H-89 and PMA downstream from the rate limiting step in acute cortisol synthesis

To identify the sites of action of both PKA and PKC in the signalling pathways leading to cortisol synthesis, pregnenolone (a cortisol precursor stemmed from the cholesterol side chain cleavage reaction immediately following the rate limiting step in cortisol synthesis) was used as a

substrate. Pregnenolone produced a dose-related increase in cortisol secretion, concentrations of 5×10^{-7} M, 5×10^{-6} M and 5×10^{-5} M pregnenolone produced respectively 100%, 305% and 315% of the optimal ACTH-induced cortisol secretion. Furthermore, there were no significant effects of either H-89 or PMA on cortisol secretion stimulated with pregnenolone, compared with ACTH-stimulated cortisol secretion (Fig. 2).

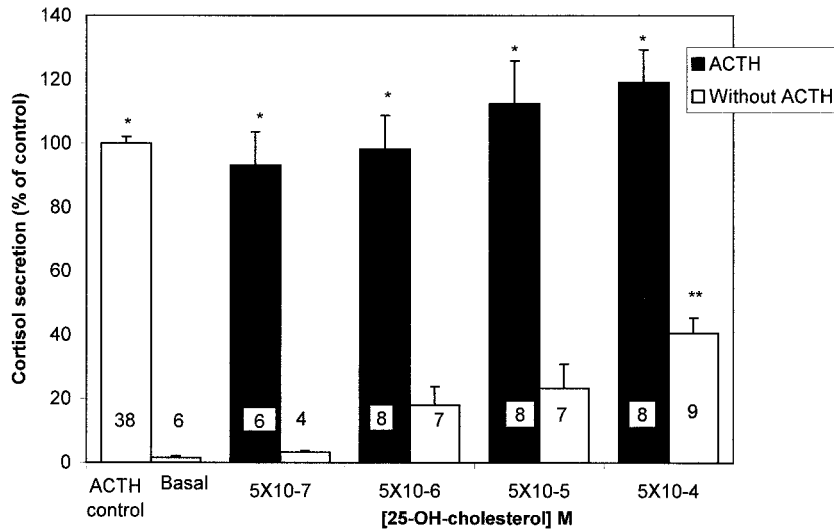


Figure 3 Effect of exogenous substrate 25-OH-cholesterol on cortisol secretion in ACTH-stimulated and non-stimulated rainbow trout adrenocortical cells incubated for 1 h at 15 °C (expressed as mean percentage of control, see Fig. 1 for details). *Significant difference from corresponding treatment without ACTH stimulation, and **significant difference from basal secretion without 25-OH-cholesterol (ANOVA and Tukey-Kramer HSD test, $\alpha=0.05$).

Effect of H-89 and PMA on the rate limiting step in acute cortisol synthesis

To investigate the role of both PKA and PKC on the rate limiting step in cortisol synthesis, 25-OH-cholesterol

(an exogenous substrate that bypasses the regulated endogenous cholesterol transport and translocation steps) was first tested on both ACTH-stimulated and basal cortisol secretion (without ACTH stimulation) (Fig. 3), as well as with both agents (H-89 and PMA) (Fig. 4). Figure 3

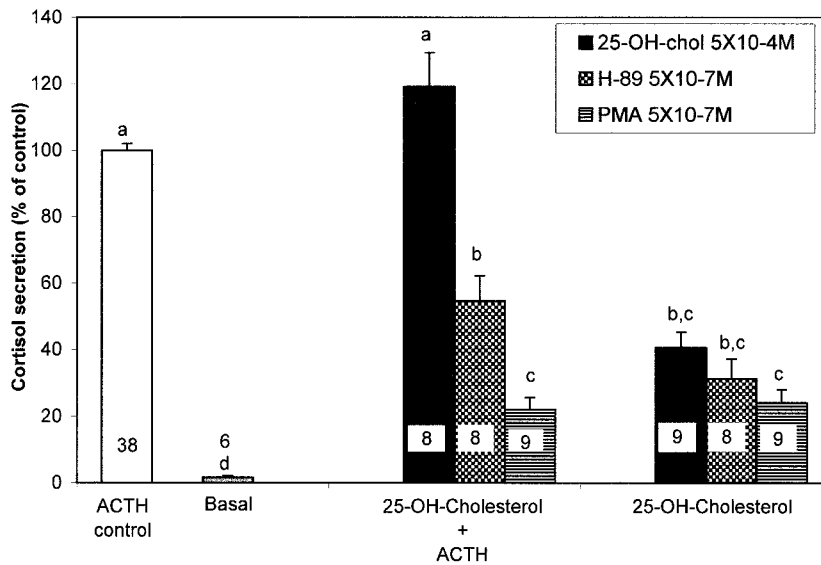


Figure 4 Effect of PKA inhibitor H-89 and PKC activator PMA on cortisol secretion with and without ACTH (1 IU/ml) stimulation in the presence of exogenous substrate 25-OH-cholesterol in rainbow trout adrenocortical cells incubated for 1 h at 15 °C (expressed in mean percentage of control, see Fig. 1 for details). Significant differences between treatments are represented by different letters (ANOVA and Tukey-Kramer HSD test, $\alpha=0.05$).

shows a dose-related increase in basal cortisol production for cells incubated with 25-OH-cholesterol with significant stimulation at a dose of 5×10^{-4} M 25-OH-cholesterol. No significant effect was observed for the ACTH-stimulated cells incubated with the exogenous substrate compared with the control. When H-89 and PMA were added (Fig. 4) to the ACTH-stimulated cells, the results were as expected from the previous experiments (Figs 1 and 2). The inhibition of PKA by H-89 produced a significant (50%) decrease in cortisol secretion while activation of PKC by PMA also decreased cortisol secretion to 20% of the control. However, H-89 and PMA had no significant effect on basal cortisol secretion in the presence of 25-OH-cholesterol, although their related cortisol secretion was significantly higher than basal values without the exogenous cholesterol substrate.

Discussion

Recent studies on fish adrenal function emphasized the need for a better understanding of the intracellular pathways responsible for the control of corticosteroidogenesis in fish in order to elucidate the mechanisms and identify the intracellular processes where endocrine modulators such as adrenotoxics affect cortisol synthesis.

The first part of this study was designed to characterize the roles of PKA and PKC, implicated in the intracellular signalling pathways, in acute ACTH-mediated cortisol synthesis in a teleost fish, the rainbow trout. The use of the specific PKA inhibitor H-89 (Chijiwa *et al.* 1990) produced a dose-related decrease in cortisol production, indicating a stimulatory role of PKA in ACTH-mediated acute cortisol synthesis. Previous studies have demonstrated the important role of the ACTH-mediated signalling pathway in the stimulation of corticosteroidogenesis in mice (Schimmer 1995), bovine (Yoshida *et al.* 1993), and fish (Gupta *et al.* 1985) species. Our study implicates, for the first time, PKA as a crucial stimulatory component in the ACTH-mediated signalling pathway in fish adrenal steroidogenesis. Other studies using the same inhibitor (H-89) on fish ovarian follicular steroidogenesis have involved PKA in a similar cAMP-mediated pathway (Srivastava & Van der Kraak 1994a,b).

The first part of our study also investigated the role of PKC, a constituent of a distinct signalling pathway which involves phosphoinositide turnover and diacylglycerol production via phospholipase C (PLC) activation (Berridge 1987, Berridge & Irvine 1989). PLC activation by hormones such as AII, vasopressin and acetylcholine, and its effect on steroidogenesis have been extensively studied in mammals and the interactions between this signalling pathway and the cAMP-mediated pathway have proved to be highly species and cell-type specific (see review by Bird *et al.* 1990). The presence of AII and acetylcholine in fish (Wendelaar Bonga 1993) could imply

similar regulation mechanisms of steroidogenesis. Our results show that PKC has an inhibitory role in the acute cortisol response in fish adrenocortical cells. Activation of PKC by a phorbol ester (PMA) caused a dose-dependent decrease in ACTH-stimulated acute cortisol synthesis. Studies using phorbol esters in human and bovine adrenal cells have shown that these PKC activators suppress cAMP-stimulated steroidogenesis (Mason *et al.* 1986, Naseeruddin & Hornsby 1990). However, other studies demonstrated the opposite effects in bovine and mouse Y1 cells (Culty *et al.* 1984, Widmaier & Hall 1985). Studies on fish ovarian follicular steroidogenesis revealed that activation of PKC by PMA has an inhibitory effect on trophically-stimulated steroidogenesis (Van der Kraak 1990, 1992). Our primary findings using H-89 and PMA indicate that acute steroidogenesis in the fish adrenal (interrenal) is under the control of at least two signalling pathways involving PKA and PKC.

The second objective of this study was to identify a specific site of action for both kinases (PKA and PKC) implicated in the control of ACTH-stimulated acute steroidogenesis in rainbow trout. Pregnenolone (product of the side chain cleavage reaction immediately following the rate limiting step of mitochondrial cholesterol translocation) was used as a substrate to determine whether PKA or PKC have effects downstream from the rate limiting step in cortisol synthesis. Our results show that neither inhibition of PKA by H-89 nor activation of PKC by PMA have any effect on pregnenolone-stimulated cortisol secretion. These results call for regulation mechanisms that act prior to pregnenolone formation for both PKA and PKC. Furthermore, higher doses of pregnenolone (data not shown) produced massive production of cortisol revealing the high capacity of the seemingly unregulated steroidogenic enzymes located downstream of pregnenolone formation. These results provide further evidence for a rate limiting step upstream of pregnenolone formation that is regulated by both PKA and PKC.

Following the finding of the implication of both kinases upstream of pregnenolone formation, it was important to assess whether PKA and PKC exert their regulatory roles on the mitochondrial cholesterol translocation, believed to be the rate limiting step in the acute response (Privalle *et al.* 1983, Stocco & Clark 1996, Kallen *et al.* 1998). An exogenous cholesterol substrate, 25-OH-cholesterol, which has the capacity to diffuse across the mitochondrial membranes (Toaff *et al.* 1982) was therefore used to stimulate acute cortisol synthesis while bypassing the translocation step. Our results show that 25-OH-cholesterol did not affect ACTH-stimulated acute cortisol synthesis, providing evidence that ACTH stimulates intracellular responses that facilitate the conversion of endogenous cholesterol usually not available unless the signalling pathway is activated. However, in the absence of ACTH, 25-OH-cholesterol produced a dose-related

increase in cortisol synthesis, indicating that 25-OH-cholesterol effectively bypasses the rate limiting step and is therefore available for enzymatic catalysis. The lower values for basal secretion in the presence of 25-OH-cholesterol could be attributed to lower conversion rates for the exogenous cholesterol substrate. Although 25-OH-cholesterol is converted at a lower velocity than endogenous cholesterol in mitochondria of the human placenta and porcine ovaries (Tuckey & Holland 1989, Tuckey 1992), other studies on mitochondria from the bovine adrenal, porcine testes, rat adrenal and rat ovaries have shown that 25-OH-cholesterol has a higher conversion rate than endogenous cholesterol (Mason & Robidoux 1978, Tuckey & Atkinson 1989). Conversion rates thus seem to be species and cell-type specific. The use of other hydroxylated forms of cholesterol could provide better substrates for side chain cleavage, which in turn might be of better use since doses used for the stimulation of steroidogenesis would be decreased. The PKA inhibitor H-89 and the PKC activator PMA had no significant effect on the cells incubated with 25-OH-cholesterol only (without ACTH). These results could imply that both kinases may regulate the rate limiting step in ACTH-stimulated acute cortisol synthesis in rainbow trout interrenal cells. However, Van der Kraak (1992) has shown that PMA exerts its inhibitory effects both prior to and distal from cAMP formation in goldfish preovulatory ovarian follicles. Therefore, PKC might also exert its inhibitory effects by acting simultaneously on different steps in the steroidogenic pathway in fish adrenal steroidogenesis.

The proximity of the site of action of both PKA and PKC in the regulation of acute ACTH-mediated cortisol synthesis calls for further studies to evaluate their mechanism of action. The steroidogenic acute regulatory protein (StAR) characterized by Clark *et al.* (1994) is now recognized as the principal mediator of acute trophic hormone-stimulated steroidogenesis in mammals (Lin *et al.* 1995, Kallen *et al.* 1998). StAR protein can be phosphorylated by PKA and PKC (Hartigan *et al.* 1995, Arkane *et al.* 1997). Phosphorylation of StAR provides increased StAR activity (Arkane *et al.* 1997) and stimulation by the cAMP-PKA-mediated pathway causes rapid accumulation of StAR mRNA (Lehoux *et al.* 1998). Also, PKC has been shown to inhibit StAR mRNA production in porcine granulosa cells (Pescador *et al.* 1997). Investigations into the characterization of StAR protein in teleosts might provide valuable answers as to the mechanisms by which cortisol synthesis is regulated in fish.

In conclusion, this study provided evidence that both PKA and PKC have a role in the regulation of ACTH-stimulated acute cortisol synthesis in rainbow trout. Furthermore, our results show that PKA has a role in transducing the stimulation message initiated when ACTH binds to its receptor. On the other hand, PKC has a negative control on stimulated cortisol synthesis and effectively inhibits its production when activated.

Characterization of the specific sites of action of PKA and PKC has led us to believe that both kinases carry out their effects between the binding of the signalling molecule and the cholesterol side chain cleavage reaction, more precisely on the rate limiting mitochondrial cholesterol translocation step.

Further investigations will examine the roles of PKA and PKC in teleost adrenal steroidogenesis in relation to different xenobiotics known to impair cortisol synthesis. Characterization of the mechanisms of action of both PKA and PKC in these lower vertebrate species is also an important aspect to develop if we are to understand the complex processes that regulate acute adrenal steroidogenesis in fish.

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