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MECHANISM OF ACTION OF NORADRENALINE ON SECRETION OF PROGESTERONE AND OXYTOCIN BY THE BOVINE CORPUS LUTEUM *IN VITRO*

Grażyna MISZKIEL and J. KOTWICA*

Division of Reproductive Endocrinology and Pathophysiology,
Institute of Animal Reproduction and Food Research of Polish Academy of Sciences,
10-718 Olsztyn-Kortowo, Poland

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The present studies were conducted: (1) to determine which β-adrenoceptor subtypes are involved in progesterone and oxytocin (OT) secretion, (2) to examine whether noradrenaline (NA) acts directly on the cytochrome P-450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD), and (3) to study the effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on NA-stimulated steroidogenesis in luteal cells. The effect of NA on progesterone secretion from luteal slices of heifers on days 8–12 of the oestrous cycle was blocked by both atenolol (β_1-antagonist) and ICI 118.551 hydrochloride (β_2-antagonist). OT secretion was blocked only after treatment with ICI 118.551 hydrochloride ($P < 0.05$). Dobutamine (10^{-4}–10^{-6}M), a selective β_1 agonist and salbutamol (10^{-4}–10^{-6}M), a selective β_2 agonist, both increased progesterone production ($P < 0.01$) with an efficiency comparable to that produced by NA ($P < 0.01$). The increase of OT content in luteal slices was observed only after treatment with salbutamol at the dose of 10^{-5}M ($P < 0.01$). Dobutamine had no effect on OT production at any dose. A stimulatory effect of NA on cytochrome P-450scc activity ($P < 0.05$) was demonstrated using 25-hydroxycholesterol as substrate. 3β-HSD activity also increased following NA ($P < 0.01$) or pregnenolone ($P < 0.05$) and in tissue treated with pregnenolone together with NA ($P < 0.01$). PGF decreased progesterone synthesis ($P < 0.05$) and 3β-HSD activity ($P < 0.01$) in tissue treated with NA. We conclude that NA stimulates progesterone secretion by luteal β_1- and β_2-adrenoceptors, while OT secretion is probably mediated only via the β_2-receptor. NA also increases cytochrome P-450scc and 3β-HSD activity. PGF inhibits the luteotropic effect of NA on the luteal tissue.

Key words: Cattle, corpus luteum, noradrenaline, progesterone, oxytocin

The stimulatory influence of catecholamines on secretion of progesterone and oxytocin (OT) from the corpus luteum (CL) of cattle has been found to be e xerted through β-receptors in both *in vitro* (Godkin et al., 1977) and *in vivo* studies (Kotwica et al., 1991; Skarżyński and Kotwica, 1993). β-adrenoceptors are of three

*Corresponding author; E-mail: janko@pan.olsztyn.pl; Fax: +48 (89) 524-0347

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subtypes, β_1, β_2 and β_3. The response of the female reproductive system to catecholamines is modulated by the concentration of adrenoceptor sites on target cell surfaces. In the rat ovary receptors of the β_2 subtype mediate ovarian steroidogenic response (Adashi and Hsueh, 1981). In the rabbit and sow, β_1-receptors predominate in the ovary (Abramovitz et al., 1982; Perkins et al., 1986), while in the cow both β_1- and β_2-receptors appear to be present (Battista and Condon, 1986; Pesta et al., 1994). The β_3-adrenoceptor was isolated at first in human adipocytes, however its role in reproductive tract is uncertain. Whereas beta-mimetics increase pregnenolone production and stimulate 3 β-HSD activity in rat granulosa cells (Jones et al., 1983), denervation of the ovary or chemical sympathectomy decreases 3 β-HSD activity in rats CL (Burden and Lawrence, 1977).

Although the primary function of PGF is induction of luteal regression (McCracken et al., 1970; Hansel et al., 1973), several studies in the cow and other species have demonstrated that PGF increases progesterone production in *in vitro* experiments. While there is agreement on the luteolytic role of PGF $_{2\alpha}$ *in vivo*, studies examining its direct effect on luteal tissue gave inconclusive results. Steroidogenic cells occupied about 68% of the luteal tissue (O'Shea et al., 1989). Response of bovine small and large luteal cells is different after PGF $_{2\alpha}$ treatment. It was demonstrated that small luteal cells do respond to PGF $_{2\alpha}$ with an increase of inositol phosphates (Davis et al., 1988) and progesterone synthesis (Alila et al., 1988; Meidan et al., 1992), whereas in large luteal cells, PGF $_{2\alpha}$ slightly elevates (Meidan et al., 1992) or inhibits progesterone secretion in cells stimulated by LH or forskolin (Alila et al., 1988). This may be caused, in part, by variable homogeneity in enriched small and large luteal cell preparations, which contain other nonsteroidogenic cell types. For this reason we have used slices of CL rather then dispersed luteal cells to examine the role of PGF $_{2\alpha}$ in steroidogenesis. Bovine luteal cells and those of other species produce PGF (Milvae and Hansel, 1983; Pate, 1988), and therefore it is possible that PGF produced locally within the CL may affect luteal cells by paracrine/autocrine mechanisms thereby interfering effects of NA.

The aim of this study was to examine the effect of adrenergic agonists and antagonists on progesterone and OT production to determine which receptor subtypes are involved in the response of bovine luteal slices to NA. Furthermore, we wished to determine the influence of NA on cytochrome P-450scc and 3 β-HSD activity and to investigate the possibility that PGF can affect NA-stimulated steroidogenesis in luteal cells.

Materials and methods

Corpora lutea collection and preparation of slices

Bovine ovaries (n = 35) were collected from a commercial slaughterhouse within 15–20 min of death, immediately placed in flask with ice-cold medium (M-199; Sigma) put in crashed ice. Within 2 hours from ovaries collection corpora lutea (CL) were dissected from ovaries and cut with a tissue slicer (Natsume Seisakusho Co., Ltd; 250–400 μm) in sterile conditions and washed 3 times before being incubated. The medium for collection of CL and for culture of slices was supplemented with 10% of calf serum (Sigma), penicillin (10 IU ml^{-1}; Sigma), streptomycin (100 μg ml^{-1}; Sigma), amphotericin (2 μg ml^{-1}; Sigma) and L-glutamine (100 μg ml^{-1}; each suspended in 2 ml of enriched M-199 were pre-incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. All experiments were performed on CL from days 8–12 of the cycle, as estimated on the basis of morphological observations published by Miyamoto et al. (2000). Data are presented as a rate of progesterone secretion into the medium (ng/ml medium) and as a cumulative progesterone production in luteal slices (μg/g protein). Oxytocin content in luteal tissue was expressed as ng/g tissue.

Experiment 1

The aim of experiment was to define the receptor subtype involved in the response of luteal cells to NA, by their secretion of OT and progesterone. Slices from bovine CL (n = 9) each in 4 replicates were pre-incubated for 24 h in medium. The medium was then replaced and the CL slices were incubated for 2 h with LH (100 ng/ml; gift from Dr A. F. Parlow – Pituitary Hormones & Antisera Center, Torrance, CA) as a positive control, NA (10^{-5}M; Polfa, Poland) and atenolol (β_1-antagonist; Research Biochemical International, U.S.A.) or ICI-118,551 hydrochloride (β_2-antagonist; Research Biochemical International, U.S.A.) added 20 min prior to NA. In parallel experiment CL slices were treated for 2 h with dobutamine (β_1-agonist; Sigma) or salbutamol (β_2-agonist; Sigma). After the time of incubation the medium M-199 and slices were collected for progesterone and OT determination. Concentrations of added LH and NA were defined previously (Bogacki and Kotwica, 1999).

Experiment 2

To determine whether the NA influence on cytochrome P-450scc and 3 β-HSD activity in CL (n = 9 and n = 8, respectively), luteal slices after 24 h of pre-incubation were treated for 2 h with LH (100 ng/ml), NA (10^{-5}M) and with pregnenolone (10 μg/ml; Sigma) alone or jointly with NA. In parallel experiment CL slices were treated for 3 h with 25-hydroxycholesterol (20 μg/ml; Sigma) alone or together with NA. The 25-hydroxycholesterol utilisation by luteal tissue is an

indirect measure of cytochrome P-450scc activity, hence its activity was est i-mated by quantifying progesterone secretion into the medium. The 3 β-HSD activity was measured in the slices.

Experiment 3

To examine the effect of PGF on NA-stimulated progesterone production, the luteal slices (from 9 CL) after 24 h of pre-incubation were incubated for 4 h with LH (100 ng/ml), NA (10^{-5}M), $PGF_{2\alpha}$ (0.5 μg/ml; Sigma) or NA together with PGF. Progesterone concentration was measured in the medium and in two of four luteal slices, while 3β-HSD activity was measured in two other luteal slices of each treatment. The concentrations and the time of incubation with $PGF_{2\alpha}$ were established in preliminary studies.

Luteal slices homogenisation

Immediately following incubation luteal slices were frozen in liquid nitr o-gen. Before assays frozen tissue was homogenised with a Vibratory Mill (Retsch MM-2). OT and progesterone were then from powdered tissue extracted as d e-scribed previously by Tsang et al. (1990). Recovery of OT and progesterone a v-eraged 90% and 85% respectively. Data were co rrected for procedural losses.

Activity of 3β-HSD determination

The 3β-HSD enzyme assay was based on the procedure described by Murono and Payne (1979) in which the conversion of radioactive pregnenolone to progesterone was measured. Luteal slices were homogenised by Ultra-Turrax (T25-IKA) in 3 ml of potassium phosphate buffer (0.05 M) containing 1 mM EDTA (pH 7.4). The assay was performed in glass tubes, which contained 50 μl of [^{3}H]pregnenolone (~2.86×10^5 dpm; Amersham, sp. act. 21.1 Ci/mmol) and 7.5 μl of unlabeled pregnenolone (5 nmol), 20 μl of assay buffer containing 10 nmol NAD in 15% dimethyl sulphoxide and 80 μl tissue homogenate. All as-says were incubated for 20 min. The reaction was stopped by placing the tubes in an ice bath. The samples were then extracted with 1 ml of diethyl ether. St eroids were separated as described by Jones and Hsueh (1982).

Hormone determinations

Progesterone content in tissue and in medium was determined by RIA (Kotwica et al., 1990). The sensitivity was 15 pg/tube and intra- and interassay variations were 8.4 and 16.2%, respectively. The relationship between real (x) and determined (y) amounts of four different concentrations of progesterone added to the plasma samples is expressed by the linear regression equation ($y = 0.99x - 0.14$).

OT concentration in luteal slices was determined by the method of Tsang et al. (1990). The hormone (2 μg) was iodinated with 5 μCi of ^{125}I using chloramine T (Greenwood et al., 1963). The procedure of OT assay in medium was described previously (Kotwica et al., 1996). The sensitivity was 3 pg/ml. Intra- and interassay variations were 7.5 and 14.6%, respectively. The accuracy of the procedure is expressed by the linear regression equation ($y = 0.99x + 0.14$).

Protein determination

Protein assay was done by the burette Weichselbaum (1946) method using bovine albumin as standard. The sensitivity of the method was 0.5 mg ml^{-1}.

Statistical analysis

The secretion of progesterone and OT after antagonists treatment in Experiment 1 was measured by comparison values with NA by means of ANOVA and Newman-Keuls as a post test. In all other experiments differences between mean (± SEM) values were determined by one-way analysis of variance (GraphPad). At least $P < 0.05$ was accepted as significant.

Results

Experiment 1

The viability of cells in luteal slices after 29 h of incubation was over 75% (trypan blue exclusion). The β_1-adrenoceptor antagonist atenolol (0.5, 2 and 5 μg/ml), significantly ($P < 0.05$) decreased progesterone production after 2 h of incubation with NA (Fig. 1a) but it did not influence OT secretion at any dose (Fig. 1b). The β_2-adrenoceptor antagonist ICI 118.551 hydrochloride decreased ($P < 0.05$) progesterone and OT secretion at all doses tested (Fig. 1a, b). Dobutamine (1, 10, 100 μM), a selective β_1 agonist and salbutamol (1, 10, 100 μM), a selective β_2 agonist, both increased progesterone production ($P < 0.01$) relative to NA ($P < 0.01$; Fig. 2a). OT concentrations in medium was not changed. Therefore the OT content in luteal slices shown; it was significantly higher compared to control only after treatment with salbutamol at 10 μM ($P < 0.01$; Fig. 2b). Dobutamine had no effect on OT production at any dose (Fig. 2b).

Experiment 2

Treatment of slices with 25-hydroxycholesterol stimulated progesterone production ($P < 0.01$; Fig. 3) and NA amplified this effect of 25-hydroxycholesterol ($P < 0.05$). Addition of pregnenolone increased progesterone production nearly twofold above the control level ($P < 0.01$; Fig. 4). Activity of 3β-HSD

was also increased in luteal slices treated with NA ($P < 0.01$) and pregnenolone ($P < 0.05$). Incubation of slices with NA together with pregnenolone significantly elevated progesterone concentration ($P < 0.05$) and 3β-HSD activity ($P < 0.01$) when compared to the effect of NA and pregnenolone separately.

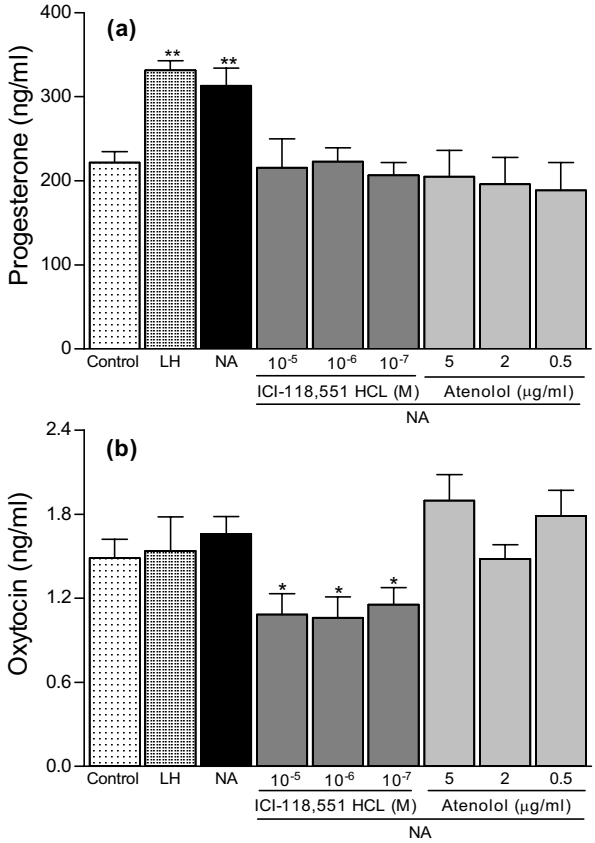

Fig. 1. Progesterone (a) and oxytocin (b) concentrations in medium after 2 h incubation of bovine corpus luteum slices from days 8–12 of the cycle with luteinising hormone (LH; 100 ng/ml), noradrenaline (NA; 10^{-5}M) and NA together with atenolol (β_1-adrenoceptor antagonist) or ICI-118.551 hydrochloride (β_2-adrenoceptor antagonist). Results are means (± SEM) of 9 CL. Different from NA ($^{*}P < 0.05$) by ANOVA

Experiment 3

The concentration of progesterone in the medium was increased after 4 h of incubation of luteal slices with $PGF_{2\alpha}$ ($P < 0.05$; Fig. 5). $PGF_{2\alpha}$ did not alter the stimulatory effect on NA on progesterone secretion into the medium. However, $PGF_{2\alpha}$ suppressed the stimulatory effect of NA on tissue progesterone content ($P < 0.05$) and 3β-HSD activity ($P < 0.01$; Fig. 6).

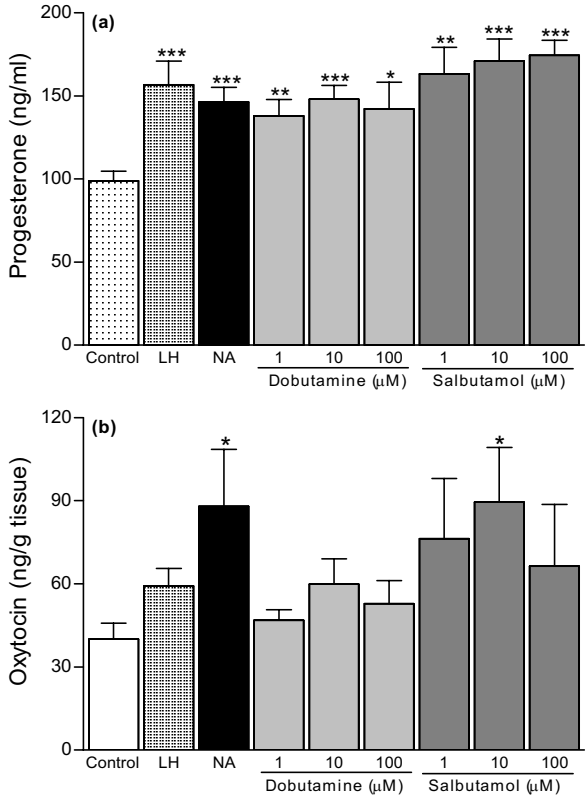

Fig. 2. Progesterone concentrations in medium (a) and oxytocin concentrations in tissue (b) after 2 h of incubation of bovine corpus luteum slices from days 8–12 of the cycle with luteinising hormone (LH; 100 ng/ml), noradrenaline (NA; 10^{-5}M) or dobutamine (β_1-agonist) and salbutamol (β_2-antagonist). Results are means (± SEM) of 8 CL. Different from control ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) by ANOVA

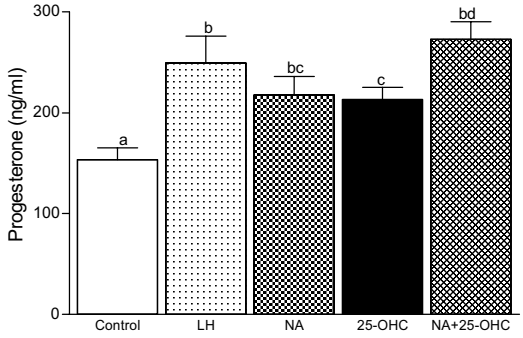

Fig. 3. Progesterone concentrations in medium after 3 h of incubation of bovine corpus luteum slices from days 8–12 of the cycle with luteinising hormone (LH; 100 ng/ml), noradrenaline (NA; 10^{-5}M), 25-hydroxycholesterol (25-OHC; 20 μg/ml) or NA together with 25-OHC. Results are means (± SEM) of 9 CL. Bars with variable superscripts are different ($P < 0.05$)

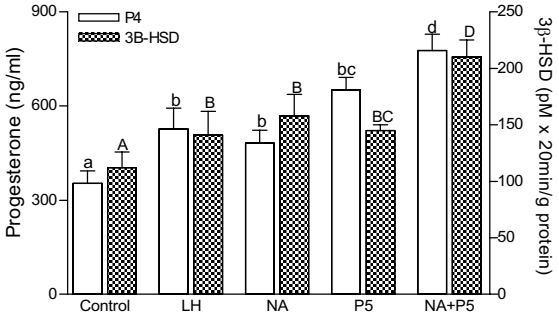

Fig. 4. Progesterone concentrations in medium and 3β-hydroxysteroid dehydrogenase (3β-HSD) activity after 2 h incubation of bovine corpus luteum slices from days 8–12 of the cycle with luteinising hormone (LH; 100 ng/ml), noradrenaline (NA; 10^{-5}M), pregnenolone (P5; 10 µg/ml) and NA together with pregnenolone. Results are means (± SEM) of 8 CL. Bars with variable superscripts are different ($P < 0.05$)

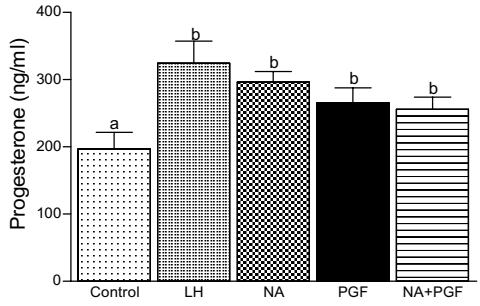

Fig. 5. Progesterone concentrations in medium after 4 h of incubation of bovine corpus luteum slices from days 8–12 of the cycle with luteinising hormone (LH; 100 ng/ml), noradrenaline (NA; 10^{-5}M), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 0.5 µg/ml) and NA together with $PGF_{2\alpha}$. Results are means (± SEM) of 9 CL. Bars with variable superscripts are different ($P < 0.05$)

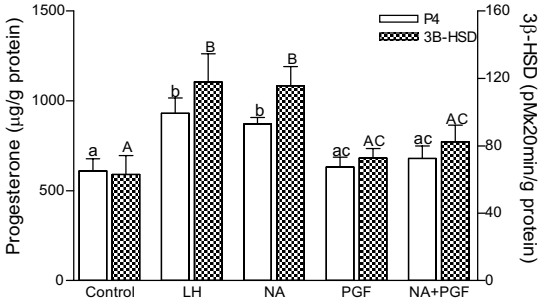

Fig. 6. Progesterone concentrations and 3β-hydroxysteroid dehydrogenase (3β-HSD) activity in slices of bovine corpus luteum (n = 9) from days 8–12 of the cycle after 4 h of incubation with luteinising hormone (LH; 100 ng/ml), noradrenaline (NA; 10^{-5}M), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 0.5 µg/ml) and NA together with $PGF_{2\alpha}$. Results are means (± SEM) of 9 CL. Bars with variable superscripts are different ($P < 0.05$)

Discussion

The present study showed that dobutamine (β_1-receptor agonist) increased only progesterone secretion from bovine luteal slices, whereas salbutamol (β_2-receptor agonist) stimulated both progesterone and oxytocin release. In agre ement with this, atenolol (β_1-receptor antagonist) and ICI 118,551 hydrochloride (β_2-receptor antagonist) significantly decreased progesterone production in luteal tissue. This suggests that both β_1- and β_2-receptor subtypes are present in mid-luteal tissue, contributing to the overall CL response. These data correspond with those of Re et al. (1995) who showed that both β_1- and β_2-adrenoceptor subtypes coexist in the ovary of adult cows. On the other hand ICI 118,551 hydrochloride diminished the stimulatory effect of NA on OT secretion. The results are consi stent with those of Luck and Münker (1991) who observed a pr edominance of β_2-receptor activity in OT secretion from cultured bovine granulosa cells. Although the presence of β-adrenoceptors in CL have been investigated in several species, the location of particular subtypes of β-receptors on large or small luteal cells has not been studied. More over, the data obtained are inconclusive. Isoprenaline, a β-agonist, increased progesterone production in large but not small ovine luteal cells in one study (Rodgers et al., 1985), but in another an opposite effect was observed in small and large cells (Niswender et al., 1985).

Since NA acting on CL has stimulated OT secretion which is only in large luteal cells (Fields et al., 1992) we assume that β_2-receptors are on large luteal cells. Differences between small and large luteal cells with respect to the amount of secreted progesterone (Weber et al., 1987) support this possibility. However, the presence of β_2-receptors on small cells cannot be excluded. Rapid commun i-cation between small and large cells cannot be ruled out, and hence β_2-receptors activation on large cells may activates the response of small cells, but this is a hypothesis which needs to be verified. Our results correspond with those by Payne and Cooke (1994) who showed that in the mid-luteal CL of goat β_2-receptors predominate in mediating the stimulatory effect of catecholamines. It is worthy noting that the proportion of β_1- and β_2-receptors varies during the oe s-trous cycle in sheep (Payne and Cooke, 1994) and cattle (Battista and Condon, 1989). In contrast, studies in the sow have shown that β_1-receptors are more prevalent in early luteal tissue (Perkins et al., 1986) and the CL of rabbit also contains predominantly the β_1-receptor subtype (Abramowitz et al., 1982).

The first and rate-limiting step in progesterone biosynthesis is conversion of cholesterol to pregnenolone, which is catalysed in mitochondria by the cyt o-chrome P-450 superfamily member, cholesterol side-chain cleavage enzyme. Tropic agents, such as LH and cAMP-mimetics, as well as protein synthesis i n-hibitors, markedly affect steroidogenesis by cells utilising endogenous chole s-terol as a substrate. Cholesterol side-chain cleavage enzyme is primarily co n-trolled by the availability of substrate. Thus steroidogenesis in the presence of

25-hydroxycholesterol, which readily diffuses into the mitochondria, provides a measure of the maximal rate of hormone production (Toaff et al., 1982). The increase in progesterone production in response to 25-hydroxycholesterol in the present study is consistent with results obtained in rat (Toaff et al., 1982), hamster (Silavin and Strauss, 1983) and rabbit luteal cells (McLean et al., 1987). Moreover, the results of Experiment II indicate that the response of luteal tissue to 25-hydroxycholesterol is greater after NA treatment than in the presence of 25-hydroxycholesterol alone. Thus, it can be concluded that NA directly stimulates the side-chain cleavage enzyme complex, although it may increase the availability of substrate to the enzyme rather than directly stimulates side-chain cleavage. Further investigation is necessary to elucidate the site of NA action. The second step in progesterone biosynthesis, conversion of pregnenolone to progesterone, is catalysed by the 3β-HSD. In Experiment II we showed that NA increased the activity of 3β-HSD. Thus, NA stimulates β-receptors in CL and affects progesterone synthesis by activating steroidogenic enzymes.

$PGF_{2\alpha}$ is a luteolysin in numerous species (Luck and Münker, 1991; Gadsby and Earnest, 1994). However, there are reports (Weston and Hixon, 1980; Speroff and Ramwell, 1970; Gadsby and Earnest, 1994) demonstrating a stimulatory action of PGF on progesterone production from luteal cells. The results of Experiment III showed that exposure of luteal slices to $PGF_{2\alpha}$ increased progesterone secretion into the incubation medium, whereas it did not influence progesterone synthesis as measured by 3β-HSD activity and progesterone content of the tissue. This luteotropic action of $PGF_{2\alpha}$ appears to be time- and dose-dependent, because its effect on CL slices in the present study was observed only after 4 hours of incubation. This is in contrast to the data of Girsh et al. (1994) who showed that $PGF_{2\alpha}$ increased progesterone production only in 2–4 day-old bovine CL slices, however, in CL slices obtained on days 6–12 of the oestrous cycle $PGF_{2\alpha}$ did not alter cumulative progesterone production. It is not clear whether the difference is due to dose of $PGF_{2\alpha}$ used, incubation of luteal slices or length of time between slices preparation and treatment. Studies examining a direct effect of $PGF_{2\alpha}$ on luteal tissue showed inconclusive results. $PGF_{2\alpha}$ and cloprostenol increase or decrease progesterone secretion by dispersed luteal cells in ruminants (Speroff and Ramwell, 1970; Weston and Hixon, 1980; Fitz et al., 1984).

We have also demonstrated that PGF inhibits the stimulatory effect of NA on 3β-HSD and progesterone production in CL from days 8–12 of the cycle. These findings are in agreement with those of Ahren et al. (1983) who showed that $PGF_{2\alpha}$ can inhibit the luteotropic effect of catecholamines in the rat CL. However, an opposite results were obtained in *in vivo* study, which showed that ovarian prostaglandins and NA independently regulate basal progesterone secretion (Kotwica et al., 1994). There are reports that CL of several species, e.g. the pig, rat, sheep, rabbit, human and cattle (Milvae and Hansel, 1983; Pate, 1988) locally produces $PGF_{2\alpha}$. It was suggested that the ratio of luteotropic (PGI_2) to

luteolytic ($PGF_{2\alpha}$) prostaglandins (PGs) produced by CL declines throughout the luteal phase in cattle (Milvae and Hansel, 1983). These authors also suggest that $PGF_{2\alpha}$ may antagonise the actions of PGI_2 with respect to blood flow and progesterone synthesis. Therefore, an increasing production of luteolytic $PGF_{2\alpha}$ vs. luteotropic PGs in older CL might explain the decreased response of CL to catecholamines. The observation made here that $PGF_{2\alpha}$ inhibits the luteotropic effect of NA on mid-cycle CL allows one to speculate that an inhibition of catechol amine influence on CL is part of the luteolytic mechanism. Whether this effect of $PGF_{2\alpha}$ can directly or indirectly disrupt the stimulatory effect of catecholamines on CL is not known yet.

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