Serotonin-induced stimulation of progesterone production by cow luteal cells *in vitro*

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Summary. The addition of acetylcholine or histamine (10^{-7} to 10^{-4} M), γ -aminobutyric acid, a dopamine agonist, and melatonin (10^{-7} to 10^{-5} M) did not alter basal or LH-stimulated progesterone production ($P > 0.05$). The addition of the specific β_2 -adrenergic agonist terbutaline and salbutamol did not significantly elevate progesterone production. Treatment of luteal cells with serotonin (5-HT), 10^{-6} to 10^{-4} M, increased the production of progesterone ($P < 0.05$). This stimulated production was inhibited by the addition of mianserin (10^{-5} M, a 5-HT antagonist; $P < 0.05$). Isoproterenol (10^{-7} to 10^{-4} M) also resulted in significant increases in progesterone production ($P < 0.05$). The combined treatments of 5-HT + LH, isoproterenol + LH, or isoproterenol + 5-HT did not result in a further increase in progesterone above that observed in response to LH or isoproterenol alone ($P > 0.05$). The isoproterenol-induced progesterone production could not be blocked by butoxamine (10^{-5} M, a β_2 -antagonist), or practolol (10^{-5} M, a β_1 -antagonist), but was inhibited by propranolol (10^{-5} M, a general β -antagonist; $P < 0.05$). The response to isoproterenol was unaffected by mianserin (10^{-5} M). These results demonstrate a possible role for 5-HT in the regulation of steroidogenesis by the corpus luteum of the cow. Furthermore, these results suggest that serotonin-induced progesterone production is a receptor-mediated event.

Introduction

At the level of the ovary, the adrenergic agents adrenaline, noradrenaline and isoproterenol have been shown to stimulate cAMP and progesterone production by luteal tissue from the cow (Condon & Black, 1976; Godkin, Black & Duby, 1977; Milvae, Alila & Hansel, 1983), rat (Hartwood, Richert, Dufau & Catt, 1980; Ratner, Sanborn & Weiss, 1980a; Norjavaara, Selstam & Ahren, 1982), rabbit (Birnbaumer, Yang, Hunzicker-Dunn, Brockaert & Duran, 1976), and ewe (Jordan, Caffrey & Niswender, 1978). In contrast to the stimulatory effects of catecholamines in the aforementioned species, corpora lutea of the mare (Condon, Ganjam & Kenney, 1979) and women (Richardson & Masson, 1980; Casper & Cotterell, 1984) appear to be refractory to stimulation by exogenous adrenergic agonists.

Catecholamines act directly on luteal cells, since fibroblasts and erythrocytes separated from ewe luteal cell suspensions did not produce significant amounts of cAMP in response to adrenaline (Jordan *et al.*, 1978). Adrenergic agents are thought to induce cAMP and subsequent progesterone production by binding to specific receptors in the luteal cell membrane (Lefkowitz, 1976). Initial support for this hypothesis is suggested by the finding that propranolol can block the catecholamine-induced increase in luteal cAMP and progesterone production in the cow (Condon & Black, 1976; Godkin *et al.*, 1977), rat (Hartwood *et al.*, 1980; Norjavaara *et al.*, 1982) and ewe (Jordan *et al.*, 1978). These results not only suggest specific adrenergic receptors, but also that they are of the β -adrenergic type. In the rat the β -adrenergic stimulation of ovarian cAMP and

progesterone has been shown to be mediated by β_2 -adrenergic receptors (Coleman, Paterson & Somerville, 1979a, b; Ratner, Weiss & Sanborn, 1980b; Adashi & Hsueh, 1981; Norjavaara, Rosberg, Gafvels & Selstam, 1984). In the cow and ewe the characterization of a specific β_2 -adrenergic mediation of catecholamine response by luteal cells has not been established. The purpose of this study with cow luteal cells was to determine (a) whether the catecholamine induced increase in progesterone production is mediated by a β_2 -adrenergic receptor and (b) the role of other biogenic amines on basal and LH-stimulated progesterone production.

Materials and Methods

Materials and drug preparation

Serotonin (5-hydroxytryptamine oxalate salt; 5-HT), DL-isoproterenol HCl, acetylcholine chloride, histamine dihydrochloride, salbutamol, γ -amino-*n*-butyric acid (GABA), melatonin (*N*-acetyl-5-methoxytryptamine) and DL-propranolol HCl were purchased from Sigma Chemical Co., St Louis, MO. Mianserin HCl and 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide were from Research Biochemicals, Inc., Wayland, MA, and butoxamine HCl was from Burrough's Wellcome Co., Research Triangle Park, NC. Terbutaline was donated by Ciba-Geigy Corp., Summit, NJ. Practolol was donated by Imperial Chemical Industries PLC, Macclesfield, Cheshire, U.K. Luteinizing hormone (LH) was kindly supplied by the National Institutes of Health (NIAMMD-bLH-4). Ham's F12 culture medium was purchased from Grand Island Biological Co., Grand Island, NY. The collagenase was Worthington, Type 1, Freehold, NJ, and the *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (Hepes) was purchased from Calbiochem-Behring, San Diego, CA.

Salbutamol and terbutaline are specific β_2 -adrenergic receptor agonists, while isoproterenol is a nonspecific β -agonist, activating both subtypes of β -receptors. Butoxamine is a specific β_2 -adrenergic receptor antagonist and practolol a specific β_1 -antagonist; propranolol is a non-specific β -adrenergic blocking agent. Mianserin is a nonspecific serotonin receptor antagonist which blocks both 5-HT₁ and 5-HT₂ receptor subtypes and 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide is a nonspecific dopamine agonist. All hormone and drug preparations were prepared in cold 0.05 M-phosphate-buffered saline (pH 7.4) before use in each experiment and used at a final concentration of 10^{-7} to 10^{-4} M. In all cases *N* equals the number of animals tested. Duplicate determinations of all treatments were performed for each corpus luteum.

Experimental procedures

Corpora lutea (CL) were obtained *per vaginam* from regularly cycling, nonlactating dairy cows on Days 10–12 of the oestrous cycle (oestrus = Day 0). The luteal tissue was placed into Ham's F12 culture medium containing 24 mM-Hepes (pH 7.4) at 4°C for transport back to the laboratory. The luteal tissue was dissociated using collagenase (2000 U/g tissue) as described by Simmons, Caffrey, Phillips, Abel & Niswender (1976) and modified by Pate & Condon (1982). Cell viability was determined by the trypan blue exclusion method (Tennant, 1964). Incubations were conducted utilizing 2.5×10^5 viable cells/culture tube in a final volume of 1 ml Ham's F12 culture medium. Luteal cell suspensions were incubated in a Dubnoff metabolic shaker water bath at 37°C starting with a 15-min preincubation period, after which the cells were centrifuged (800 *g*, 10 min at 4°C) and the medium discarded. The cells were then resuspended in fresh medium and the appropriate treatment solutions added. After the incubation period of 2 h, cell suspensions were centrifuged and the culture medium was collected and stored (–20°C) for subsequent determination of progesterone concentration.

Progesterone present in the culture medium was quantitated by radioimmunoassay of unextracted samples using antiprogestrone-11-bovine serum albumin (No. 337, Niswender). This antiserum does not cross-react significantly with any other steroid present in the culture medium (Gibori, Antczak & Rothchild, 1977). The progesterone tracer used was [1,2-³H]progesterone and was purchased from New England Nuclear (Boston, MA). The sensitivity of the assay, as determined by the lower 95% confidence limit of the maximum binding in the absence of any unlabelled progesterone, was 0.1 ng/ml medium. The intra-assay and interassay coefficients of variability were 5.3% and 13.3% respectively. All standards were assayed in quadruplicate, and all unknowns were assayed in duplicate.

Statistical analysis

Analysis of differences between treatment means was conducted using one-way analysis of variance followed by the Student–Newman–Keuls mean separation procedure.

Results

The effects of some of the biogenic amines on basal progesterone production are presented in Table 1. Progesterone production was not affected significantly by acetylcholine, histamine, GABA, dopamine or melatonin over the treatment concentrations tested. Similarly, none of these compounds had any effect on LH-stimulated progesterone production ($P > 0.05$, Table 2). Terbutaline and salbutamol did not affect the basal or LH-stimulated production of progesterone. LH (100 ng) alone resulted in a large increase in progesterone production ($P < 0.01$).

The addition of serotonin at concentrations from 10^{-6} to 10^{-4} M resulted in a dose-dependent stimulation of progesterone production by the luteal cells ($P < 0.05$; Fig. 1). Additionally, the 5-HT-induced progesterone response could be blocked by the addition of mianserin (10^{-5} M, $P < 0.05$). The 5-HT stimulation of progesterone production was significantly less than that observed for LH alone and there was no additive effect of 5-HT combined with LH. Isoproterenol at concentrations from 10^{-7} to 10^{-4} M also resulted in stimulation of progesterone production ($P < 0.05$; Fig. 2). The combination of isoproterenol plus LH or isoproterenol plus 5-HT (10^{-5} M) did not result in a significant elevation of progesterone above that observed with LH or

Table 1. Effect of biogenic amines on progesterone production (ng/ml culture medium) by dissociated luteal cells in a 2-h incubation

Treatment	N	Treatment concentration				
		0	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
Control	14	42.6 ± 3.22				
LH	14	188.3 ± 14.94*				
Acetylcholine	(N)		57.3 ± 6.26(5)	51.5 ± 5.04(8)	59.1 ± 7.28(8)	57.1 ± 4.73(7)
Histamine	4		61.7 ± 13.67	46.9 ± 8.98	44.1 ± 6.80	44.2 ± 5.90
γ-Aminobutyric acid	4			45.5 ± 4.90	53.0 ± 13.03	47.8 ± 10.86
Dopamine agonist	4			47.6 ± 3.65	43.4 ± 6.62	44.8 ± 8.07
Melatonin	4			54.9 ± 7.92	54.5 ± 4.76	43.6 ± 4.19
Terbutaline	3		65.3 ± 8.20	58.8 ± 16.49	53.6 ± 7.61	41.5 ± 9.38
Salbutamol	6		69.4 ± 5.66	61.7 ± 5.53	63.8 ± 4.41	49.7 ± 3.28

Values are mean ± s.e.m.

* $P < 0.01$ compared with control value.

Table 2. Effect of biogenic amines on LH-stimulated progesterone production (ng/ml culture medium) by dissociated luteal cells in a 2-h incubation

Treatment	N	Treatment concentration				
		0	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
Control	14	42.6 ± 3.22				
LH	14	$188.3 \pm 14.94^*$				
Acetylcholine	(N)		$191.1 \pm 36.96(4)$	$211.1 \pm 27.64(6)$	$221.1 \pm 29.99(6)$	$197.1 \pm 26.08(6)$
Histamine	3		156.0 ± 24.29	149.2 ± 21.24	153.4 ± 22.59	149.6 ± 25.19
γ -Aminobutyric acid	3			168.4 ± 11.85	176.6 ± 13.96	214.8 ± 20.03
Dopamine agonist	3			198.6 ± 45.88	188.8 ± 46.01	179.9 ± 42.02
Melatonin	3			212.7 ± 10.25	216.1 ± 9.00	212.2 ± 17.16
Terbutaline	3		186.6 ± 18.76	162.8 ± 27.04	148.1 ± 31.05	140.3 ± 24.11
Salbutamol	3		200.6 ± 51.96	203.8 ± 36.28	202.5 ± 48.06	197.7 ± 26.06

Values are mean $\pm$ s.e.m.

* $P < 0.01$ compared with control value.

Fig. 1. Effect of serotonin on progesterone production by dissociated luteal cells. Data are expressed as mean $\pm$ s.e.m. with $N=6$ (10^{-4} M) and 8 (10^{-7} , 10^{-6} and 10^{-5} M) for 5-HT, $N=3$ for 5-HT+LH, $N=6$ for 5-HT+mianserin and $N=14$ for control (C) and LH. Bars with different superscript letters are significantly different ($P < 0.01$, LH and 5-HT+LH; $P < 0.05$, 5-HT and 5-HT+mianserin).

isoproterenol, respectively ($P > 0.05$). The effect of various β -adrenergic receptor antagonists and mianserin upon isoproterenol-stimulated progesterone is presented in Fig. 3. The isoproterenol-induced response could not be inhibited by butoxamine (10^{-5} M) or practolol (10^{-5} M) at any of the isoproterenol concentrations tested ($P > 0.05$). Similarly, mianserin (10^{-5} M) did not block progesterone production by isoproterenol ($P > 0.05$). In contrast, propranolol (10^{-5} M) did inhibit the isoproterenol-induced response at an isoproterenol concentration of 10^{-7} to 10^{-5} M ($P < 0.05$). None of the antagonists used in these studies significantly affected basal or LH-stimulated progesterone production (data not shown).

Fig. 2. Effect of isoproterenol on luteal cell progesterone production. Data are expressed as mean $\pm$ s.e.m. with N=5 (10^{-4} M) and 6 (10^{-7} , 10^{-6} and 10^{-5} M) for isoproterenol, N=3 for isoproterenol+LH and isoproterenol+5-HT, and N=14 for control (C) and LH. Bars with different superscript letters are significantly different ($P < 0.01$, LH and isoproterenol + LH; $P < 0.05$ for isoproterenol and isoproterenol + 5-HT).

Fig. 3. Effect of β -adrenergic antagonists and mianserin on isoproterenol-stimulated progesterone production. Data are expressed as mean $\pm$ s.e.m. with N=4 for isoproterenol (Iso.) + butoxamine and isoproterenol + mianserin, N=3 for isoproterenol + practolol and isoproterenol + propranolol and N=14 for control (C) and LH. Bars with different superscript letters are significantly different from isoproterenol alone (—) ($P < 0.05$, for isoproterenol + propranolol).

Discussion

These results demonstrate a possible role for serotonin in the regulation of steroidogenesis by the CL of the cow. That the serotonin-induced stimulation of progesterone production could be inhibited by a serotonin receptor antagonist suggests its action to be a receptor-mediated event. A preliminary experiment with C. E. Rexroad, Jr (USDA, Beltsville) has shown that serotonin is present within the CL of the cow at a relatively high concentration (152.7 ± 40.19 ng/g). In the pineal gland of the cow, serotonin concentrations range from 1000 to 2000 ng/g depending upon the time of year (Philo & Reiter, 1980). In the rat, Clausell & Soliman (1978) have reported 5-HT to be present in ovarian tissue: the concentration of 5-HT was about 900 ng/g during dioestrus and metoestrus with a significant increase to 1300 ng/g occurring at oestrus, and a role for ovarian 5-HT in the process of ovulation was proposed.

Possible sources of luteal 5-HT include mast cells, blood platelets and de-novo synthesis. In this laboratory, histological determination of mast cells using Giemsa, toluidene blue and methylene blue staining techniques has not conclusively demonstrated their presence in luteal tissue of cows. It is possible that serotonin may be working indirectly to stimulate progesterone production by acting on a secondary cell type such as endothelial cells to produce a luteotrophin. Studies are presently underway to evaluate this possibility and to define further the role of serotonin and its metabolites in the regulation of steroidogenesis by the bovine CL.

In-vitro studies with human CL slices showed that melatonin stimulated progesterone production in a dose-dependent manner while serotonin and *N*-acetyl serotonin had no effect on progesterone production (MacPhee, Cole & Rice, 1975). In cultured rat granulosa cells, melatonin has been reported to increase basal and LH-stimulated progesterone production (Fiske, Parker, Ulmer, Ow & Aziz, 1984). [3 H]Melatonin has been reported to be selectively taken up by the ovaries in the rat and cat in vivo (Wurtman, Axelrod & Potter, 1964). In male rats melatonin and serotonin have been demonstrated to inhibit the in-vitro biosynthesis of androgens (Peat & Kinson, 1971; Ellis, 1972). In our studies utilizing dissociated bovine luteal cells, melatonin was unable to alter either basal or LH-stimulated progesterone production.

A possible role for GABA in the regulation of ovarian function has been suggested due to the relatively high concentration of this compound in rat ovarian tissue (Del Rio & Caballero, 1980). Shaeffer & Hsueh (1982) have reported GABA and glutamate decarboxylase (GAD), the enzyme responsible for GABA synthesis, to be present in whole ovarian homogenates but not in enriched ovarian granulosa cells. Additionally, these authors have demonstrated specific binding sites for GABA in the rat ovary, with the majority of these sites being associated with granulosa cells. The testing of GABA in our short-term incubation system does not support a role for GABA in the regulation of steroidogenesis by the CL of the cow.

To our knowledge, there is only a single report in the literature concerning the effects of acetylcholine on luteal function. In that study, acetylcholine and cabachol (a synthetic cholinergic agent) at concentrations up to 10^{-4} M did not affect basal or LH-stimulated production of progesterone by human luteal tissue in a short-term (4 h) or long-term (10 day) culture system (Casper & Cotterell, 1984). There are no data concerning the effects of histamine and dopamine on luteal steroidogenesis. However, histamine has been reported to induce the production of cortisol and corticosterone by dissociated adrenal cells of the dog (Hirose, Matsumoto & Aikawa, 1978). In our studies we have been unable to demonstrate any significant regulatory actions of acetylcholine, histamine or dopamine in controlling luteal steroidogenesis.

The isoproterenol stimulation of progesterone reported in this study is consistent with previous results reported for bovine luteal slices (Condon & Black, 1976; Godkin *et al.*, 1977) and dissociated bovine luteal cells (Milvae *et al.*, 1983). Neither terbutaline nor salbutamol, the two β_2 -adrenergic agonists used in the present study, significantly affected progesterone production by bovine luteal cells. These results differ from those reported for rat luteal tissue (Ratner *et al.*, 1980b; Norjavaara *et al.*, 1984) and FSH-primed rat granulosa cells (Adashi & Hsueh, 1981).

Additionally, isoproterenol was reported to stimulate cAMP by rat luteal tissue, and this response could not be blocked by a β_1 - or a β_2 -adrenergic receptor antagonist, although it could be blocked by propranolol (Ratner *et al.*, 1980b). Similar results have been found in this study for progesterone production by bovine luteal cells. The isoproterenol stimulation of progesterone production could not be blocked by butoxamine or practolol, but was inhibited by propranolol. The effects of isoproterenol, either alone or in combination with these antagonists, on progesterone production by rat luteal tissue was not reported by Ratner *et al.* (1980b). It may be that the β -adrenergic agonists and antagonists used in these studies lack the specificity and affinity for β -adrenergic receptors on bovine luteal cell membranes. In the rat, salbutamol and terbutaline have been shown to be partial agonists and their affinity varies between tissue types (Coleman *et al.*, 1979a, b; Adashi & Hsueh, 1981; Laszlovszky & Erdo, 1983; Norjavaara *et al.*, 1984). As suggested by Coleman *et al.* (1979b) it is also possible that these β -adrenergic agonists and antagonists are metabolized more readily than isoproterenol or propranolol, thus reducing their biological activity. Since neither β_1 - nor β_2 -adrenergic antagonists blocked the isoproterenol stimulation of progesterone but propranolol, an adrenergic antagonist which blocks both receptor subtypes, was able to, these results may suggest a mixed population of β -adrenergic receptors on luteal cells of the cow.

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