Stimulation with LH of progesterone production by rabbit corpora lutea *in vitro**

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Summary. Quartered CL from 7-day pseudopregnant rabbits were incubated at 37°C for 0–180 min in the presence of BSA, LH or adrenaline in Krebs–Ringer–bicarbonate buffer. Total progesterone at each time point was quantified in homogenates of tissue plus incubation media and expressed relative to CL protein. Progesterone increased linearly with time during the first 30 min of incubation in the presence of BSA. LH and adrenaline markedly accelerated progesterone accumulations relative to the BSA control. At 10 min, progesterone accumulation in the presence of LH and adrenaline were 2.4 and 5.9 times that in the absence of stimulators, respectively. Both hormones caused concentration-dependent increases in progesterone and the apparent ED₅₀ was 0.75 µg/ml for LH and adrenaline. The CL obtained from ovaries of 7-day pseudopregnant rabbits are therefore capable of an acute steroidogenic synthetic response to LH as well as adrenaline.

Introduction

Previous studies have clearly documented that while LH is absolutely required to initiate corpus luteum formation in all species, progesterone production by corpora lutea (CL) and CL maintenance in rabbits requires oestrogen and not LH (Bill & Keyes, 1983; Hunzicker-Dunn & Miller, 1983). Oestradiol is therefore considered to be the principal luteotrophic hormone in rabbits because it is sufficient for maintenance of serum progesterone concentrations in hypophysectomized pseudopregnant and pregnant rabbits and withdrawal causes rapid luteal regression (Keyes & Nalbandov, 1967; Rennie, 1968; Spies *et al.*, 1968; Holt *et al.*, 1975; Bender *et al.*, 1978; Bill & Keyes, 1983). The rabbit CL in fact appears to be a typical oestradiol target tissue, exhibiting specific, high-affinity and low-capacity oestrogen receptors (Yuh & Keyes, 1979; Drake & Cook, 1979; Miller & Toft, 1983) which appear with the physiological dependence of CL function on oestrogen (Mills & Osteen, 1977; Miller & Keyes, 1978) and disappear with luteal demise (Miller & Toft, 1983). However, an acute and strong steroidogenic response to oestradiol has not been demonstrated.

Steroidogenesis in virtually all other tissues is thought to be regulated by trophic hormones via surface membrane receptors and a cAMP-mediated process. Rabbit CL, like other steroidogenic tissues, also exhibit the initial responses for LH- and catecholamine-directed functions. These include LH and catecholamine surface receptors (Abramowitz *et al.*, 1982; Miller *et al.*, 1986), an adenylate cyclase responsive to both effectors (Hunzicker-Dunn & Birnbaumer, 1976; Hunzicker-Dunn, 1982) and type I and II forms of cAMP-dependent protein kinase (Hunzicker-Dunn & Jungmann, 1978). Changes in these measures appear to correlate with luteal function. LH receptor

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concentrations and LH- and adrenaline-stimulated adenylate cyclase activities increase dramatically during CL formation and decrease markedly with luteal demise (Hunzicker-Dunn & Birnbaumer, 1976; Hunzicker-Dunn, 1982; Miller *et al.*, 1986). The appearance and disappearance of the type I form of cAMP-dependent protein kinase similarly correlate with luteinization and luteolysis (Hunzicker-Dunn & Jungmann, 1978), and it is this type I form of cAMP-dependent protein kinase which is activated acutely after i.v. injection of hCG (Hunzicker-Dunn, 1981).

Since rabbit CL require oestrogen to sustain progesterone production yet also possess at least the initial response for LH- and catecholamine-directed functions, we questioned whether the rabbit CL was able to produce progesterone acutely in response to LH or adrenaline. Previous studies had not firmly established or rejected steroidogenic actions for LH or adrenaline in rabbit CL. In-vivo studies have been clouded by the robust steroidogenic response of rabbit interstitial tissue to LH (Yuh & Keyes, 1981), making it impossible to distinguish a luteal response. Ectopic CL (transplanted to the kidney capsule of ovariectomized rabbits) gave an inconsistent acute progesterone response, as measured in peripheral serum, to the i.v. injection of hCG (50 i.u.), presumably due to the low amounts of transplanted luteal tissue present (J. B. Miller, unpublished observations). In-vitro studies by Dorrington & Kilpatrick (1966, 1967) and Wilks *et al.* (1970) demonstrated only slight (30–35%) increases in progesterone accumulation by rabbit CL slices incubated with LH for 3 or 2 h, respectively.

The present studies were therefore designed to determine whether rabbit CL are able to produce progesterone *in vitro* in response to an acute stimulus by adrenaline or LH. A preliminary account of the findings has been published in abstract form (Damle *et al.*, 1984).

Materials and Methods

Materials. LH (NIH-LH-S18) was obtained from the Pituitary Hormone Distribution Program, NIAMMD, Bethesda, MD. Adrenaline HCl was from Parke-Davis and Co., Detroit, MI, bovine serum albumin (BSA, fraction V, charcoal purified) from Sigma Chemical Co., St Louis, MO, [1,2,6,7-³H]progesterone (sp. act. 112 Ci/mmol) from New England Nuclear, Boston, MA, scintillation fluid 3a70B from Research Products International, Mount Prospect, IL, gelatin from Difco Laboratories, Detroit, MI, Dextran T70 from Pharmacia Fine Chemicals, Piscataway, NJ, and petroleum ether (reagent grade) was from American Scientific, McGaw Park, IL. Charcoal (carbon, decolorizing Norit A) was purchased from Eastman Kodak Co., Rochester, NY, and was washed before use. All other biochemical reagents were obtained from Sigma Chemical Co.

Preparation and incubation of corpora lutea. New Zealand White rabbits (3–3.5 kg) which had littered at least once were obtained from Lesser Rabbitry, Union Grove, WI. Animals were individually caged in a temperature-controlled room with 14 h of light each day. Purina rabbit chow and water were freely available. Pseudopregnancy was induced on Day 0 with an i.v. injection of 100 i.u. hCG (Ayerst Laboratories Inc, NY) dissolved in 0.9% (w/v) NaCl. The rabbits (30) were killed by cervical dislocation on Day 7 of pseudopregnancy. Ovaries were removed and CL were immediately dissected, quartered with razor blades, and placed into capped 20-ml glass vials (1 CL equivalent per vial) containing 2.5 ml Krebs–Ringer–bicarbonate buffer (KRB), prepared with half the recommended amount of CaCl₂ and equilibrated with 95% O₂/5% CO₂ (Cohen, 1951). Incubations for each datum point in a single experiment were in duplicate and tissues for each duplicate incubation were obtained from different animals. Two rabbits were used for each experiment. BSA (diluted from 1 mg/ml stock), LH (NIH-LH-S18, diluted from 1 mg/ml stock prepared in 0.1 M-NaCl) or adrenaline (diluted from 1 mg/ml isotonic NaCl stock) were diluted into KRB just before the addition of tissue. CL were incubated at 37°C for up to 180 min in a shaking water bath. For incubations longer than 1 h, incubation vessels were re-gassed every hour. Incubations were terminated by rapidly freezing incubation vessels in an acetone–solid CO₂ bath.

For studies with rat CL, 28-day-old female rats (Charles River, Boston, MA; CrI:CD⁸(SD)BR Sprague–Dawley outbreds) were injected s.c. with 50 i.u. PMSG followed 54 h later with 50 i.u. hCG. Rats were killed 7 days after the hCG injection by cervical dislocation. Ovaries were sliced with a razor blade, and incubated as described above (1/8 equivalent ovary per vial).

Progesterone radioimmunoassay. The total progesterone concentration (tissue plus medium) was quantified by the radioimmunoassay described by Day & Birnbaumer (1980). The antiserum (GDN No. 337, kindly provided by Dr G. D. Niswender, Colorado State University) was raised in sheep. Gibori *et al.* (1977) have reported that GDN No. 337 showed only minimal cross-reactivity to 20 α -hydroxypregn-4-en-3-one (1.7%) compared with progesterone (100%). For progesterone determinations, the contents of each incubation vessel were thawed and homogenized with 15–20 strokes of a 5-ml Potter–Elvehjem tissue grinder (Kontes, Vineland, NJ). Progesterone was extracted from triplicate

10- μ l aliquants of homogenates with 8 ml petroleum ether by shaking at room temperature for 30 min in an automatic shaker. Tritiated progesterone (3000 c.p.m.) was added to each tube before extraction to correct for procedural losses, which averaged about 30%. The ether extracts were then evaporated to dryness and reconstituted with 0.5 ml PBS-gel buffer (0.15 M-NaCl 0.5 M-potassium phosphate buffer, pH 7.2, 0.1% gelatin). Aliquants of samples (0.1 ml) or standards were incubated (4°C, 18 h) in triplicate with tritiated progesterone (14 000 c.p.m.) and antiserum (final dilution 1:3000) in a total volume of 0.3 ml. Unbound progesterone was separated from the antigen-antibody complex by dextran-coated charcoal (3.12% charcoal, 0.62% dextran, T70) adsorption according to Hotchkiss *et al.* (1971). Antibody-bound [3 H]progesterone was quantified by scintillation spectrometry. Assay sensitivity was 5–15 pg, the midpoint of the standard curve was 90–150 pg, and the interassay and intra-assay coefficients of variation were <10%. Results were calculated with the aid of a CDC Cyber 170 computer using the 4 parameter model logistic curve-fitting program of Rodbard (DeLean *et al.*, 1978) and are expressed as units of total progesterone accumulated per μ g luteal protein. Tissue content of progesterone at the start of the incubation was subtracted from values obtained at each incubation time.

Other methods. Protein was determined according to the technique of Lowry *et al.* (1951), using crystalline BSA as a standard in 40 μ l aliquants of homogenate. Data comparisons were performed using the Student's *t* test.

Results and Discussion

Rabbit CL produced progesterone *in vitro*. In control (BSA) incubations total luteal progesterone increased in a linear manner, but only during the first 30 min of incubation (Fig. 1a). The addition of LH or adrenaline at 10 μ g/ml clearly accelerated progesterone accumulation. At 10 min, LH- and adrenaline-stimulated progesterone accumulation exceeded control values by 2.4-fold

Fig. 1. Effect of LH (10 μ g/ml) and adrenaline (10 μ g/ml) on progesterone accumulation by (a) rabbit CL quarters obtained from ovaries of 7-day pseudopregnant rabbits and (b) ovarian slices from PMSG-hCG-primed immature female rats. Control incubations contained BSA (10 μ g/ml). Tissue content of progesterone at the start of incubation (time zero) was subtracted from values obtained at each incubation time. Results represent means of triplicate determinations of duplicate incubations of luteal tissue from separate animals and are representative of (a) 3 and (b) 2 separate experiments. $P < 0.025$ for BSA vs LH at 10 min; $P < 0.01$ for BSA vs adrenaline at 10 min and BSA vs LH at 3 h; $P < 0.005$ for BSA vs LH at 1 h, and for BSA vs adrenaline at 1 and 3 h.

Fig. 2. Effect of LH and adrenaline at different concentrations on the accumulation of progesterone by rabbit CL quarters. Incubations were for 30 min and progesterone accumulation by CL incubated with BSA was subtracted from values obtained in the presence of hormones. Duplicates of incubations in a single experiment are presented and results are representative of 2 separate experiments.

($P < 0.025$) and 5.9-fold ($P < 0.01$), respectively. Progesterone accumulation in the presence of BSA, LH or adrenaline plateaued within 3 h. Analysis of dose-response relationships obtained using 30 min incubations showed that both LH and adrenaline caused concentration-dependent increases in progesterone accumulation (Fig. 2). Half-maximal activation for LH and adrenaline was achieved with 0.75 $\mu\text{g/ml}$.

In contrast to rabbit CL, the steroidogenic role for LH in CL of PMSG-hCG primed immature female rats has been firmly established (Rothchild, 1981). We therefore measured the progesterone synthetic response of ovarian slices from PMSG-hCG-primed immature rats to LH and adrenaline, and compared these results with those of the rabbit luteal system. A dose-response relationship between LH and adrenaline and progesterone accumulation equivalent to that shown in Fig. 2 with rabbit CL was observed with the rat system (not shown). Progesterone accumulation by the luteal-enriched rat ovarian preparation also exhibited a similar time dependency to that shown by rabbit CL quarters and a similar progesterone synthetic capacity (with our conditions) in the absence of added hormone (BSA) and in the presence of adrenaline (Fig. 1b). However, LH was slightly (15%) more effective (at 3 h) in elevating progesterone production in the rat ovarian system compared with that of the rabbit system. Clearly, rabbit luteal tissue responds as well as does the CL-enriched rat ovarian preparation to adrenaline and nearly as well to LH in terms of progesterone production.

These results unequivocally demonstrate that rabbit CL are capable of an acute steroidogenic response to LH as well as to adrenaline. The entire steroidogenic response pathway for LH and adrenaline is therefore present and functional in rabbit CL. Although neither of these hormones appears to be essential to maintain steroidogenesis (Bill & Keyes, 1983; Gadsby *et al.*, 1985), the presence of this intact cAMP-steroidogenic response pathway suggests that LH and/or adrenaline may modulate luteal steroidogenesis in rabbits. Further studies are required to clarify whether LH or adrenaline plays a physiological role in the steroidogenic response of CL in the pituitary-intact rabbit.

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