Responsiveness *in vitro* of porcine luteal tissue recovered at two stages of the luteal phase

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Summary. Corpora lutea (CL) were recovered from pigs at 6½ or 12½ days after induced ovulation and fragments of tissue and isolated cells were incubated for 3 h *in vitro*. Progesterone synthesis by tissue and cells from 12½-day CL was stimulated by bovine LH (1 µg/ml), dibutyryl cyclic AMP (10 mM) and hCG (10–50 i.u./ml) but 6½-day CL, although having a higher level of control synthesis, did not respond to these substances. The addition of testosterone (100 ng/ml) to the incubations was found to suppress the response to bovine LH in the 12½-day tissue and cells. Separation of the dissociated luteal cells into populations of large and small sized cells demonstrated that the large cells were more synthetically active than the small cells, but cells of both sizes in the 12½-day CL responded to bovine LH. These results support the concept that the porcine CL is autonomous for much of its lifespan.

Introduction

Although several studies have been carried out on porcine luteal tissue *in vitro* (Duncan, Bowerman, Anderson, Hearn & Melampy, 1961; Cook, Kaltenbach, Norton & Nalbandov, 1967; Cook, Niswender, Sutterlin, Norton & Nalbandov, 1968; Preumont, Cooke & Ryan 1969; Birnbaumer, Yang, Hunzicker-Dunn, Bockaert & Duran, 1976), there remains considerable controversy about stimulation of progesterone production *in vitro* by gonadotrophins of porcine or other origin. It has been shown that the porcine corpus luteum (CL) *in vivo* is virtually autonomous, in that if hypophysectomy is carried out after the ovulatory LH surge then the CL are maintained for the approximate length of one oestrous cycle (see Anderson & Melampy, 1967). Also, the gland is refractory to the luteolytic effects of anti-ovine LH and prostaglandin F-2α until at least 10–12 days after ovulation (Spies, Slyter & Quadri, 1967; Diehl & Day, 1974). The present study was to examine the progesterone production and responsiveness of luteal tissue recovered during and after this refractory period.

Materials and Methods

Tissue collection

Immature gilts (pure-bred White or White crosses) aged 6–9 months and weighing approximately 100 kg were induced to ovulate by subcutaneous injections of 1000 i.u. PMSG (Organon Laboratories, Morden, Surrey, U.K.) followed by 500 i.u. hCG (Organon) given intramuscularly 4 days later at a similar time (09:00 h); ovulation was expected to occur at 40 h

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after the hCG injection, i.e. 01:00 h (Dziuk & Gehlbach, 1966; Baker & Coggins, 1968). The animals were sent for slaughter 9 or 15 days after the hCG injection and the ovaries were obtained immediately after slaughter, transported to the laboratory and the CL dissected out. The interval between slaughter and dissection was always less than 1 h. The ovaries were therefore taken $6\frac{1}{2}$ or $12\frac{1}{2}$ days after ovulation.

Incubations

Luteal tissue fragments. The luteal tissue obtained from several pooled CL from each of 4 animals was finely minced with two scalpel blades and washed twice in 50 ml Hank's balanced salt solution without calcium and magnesium (HBSS) (Flow Laboratories, Irvine, U.K.). The tissue was then weighed into aliquots of approximately 20 mg, placed into glass scintillation vials and 2 ml Eagle's minimum essential medium (EMEM) (Flow Laboratories) were added. The substances added to the incubations were bovine LH (NIH-LH-B10), hCG (Sigma, Poole, Dorset, U.K.), dibutyryl cAMP (Sigma) and testosterone (Sigma) to give final concentrations in 1 ml medium of 1 μ g bLH, 10–50 i.u. hCG, 10 mM-dbcAMP and 100 ng testosterone. After gassing with 95% O₂/5% CO₂ for 10 sec the vessels were sealed and incubated in replicates of 4 or 6 in a shaking water bath for 3 h at 37°C. The tissue and medium were then separated by centrifugation at 100 g for 5 min and the tissue subsequently homogenized in absolute alcohol. The ethanolic extract and the medium were assayed for progesterone as described below. Progesterone production was calculated by subtracting the progesterone concentration in tissue aliquots before incubation from the total progesterone in the medium and tissue after incubation.

Dissociated cells. Washed and minced luteal tissue obtained from the same 4 animals as above was placed in 0.1% collagenase/dispase (Boehringer Ltd, Lewes, East Sussex, U.K.) in HBSS at a volume of 10 ml/g tissue and incubated at 37°C for 1 h while being agitated by a magnetic stirrer. Several different enzyme preparations were tested for cell yield and viability and collagenase/dispase produced the optimum yield, viability and responsiveness to gonadotrophins (Hunter, 1980). The dissociation process was aided by drawing the tissue gently up and down a syringe every 20 min. The cell suspension was then filtered through nylon mesh (100 μ m) and washed twice in 50 ml HBSS. The cells were collected by centrifugation at 100 g for 10 min and counted twice with a haemocytometer; viability was estimated by the trypan blue exclusion test (Tennant, 1964). After dissociation, the average cell yield was 20×10^6 viable cells/g tissue, and viability values were >80% viable before incubation and >75% viable after incubation. Incubations were set up of 100 000 viable cells in 1 ml EMEM in vials, and 4 aliquots were immediately frozen for subsequent estimation of the steroid in the medium before incubation. The incubations were then carried out in quadruplicate or triplicate as described above for the tissue fragments.

Some of the dissociated luteal cells were separated into populations of large and small cells by a modification of the method of Lemon & Loir (1977), which involved sedimenting the cells on a BSA gradient at unit gravity. The pellets of separated cells obtained were examined under the light microscope and fractions containing the same cell type were pooled. The incubations involved 30 000–50 000 cells of each size in triplicate and were carried out as described above.

Radioimmunoassay

The progesterone assay was based on the method of Scaramuzzi, Corker, Young & Baird (1975) using an antiserum raised in a sheep to a progesterone 11 α -hemisuccinate-bovine serum albumin conjugate. The antiserum was used at a dilution of 1:25 000 and showed negligible cross-reactivity with androgens and oestrogens (<0.1%), pregnenolone (0.22%), 17 α -hydroxyprogesterone (<0.01%) and 20 α - and 20 β -dihydroprogesterone (<1%). The limit of sensitivity of the assay was 25 pg/tube and the intra- and inter-assay co-efficients of variation were 7.9% ($n = 24$) and 14.8% ($n = 40$) respectively.

Statistical analysis

All data were analysed by Student's *t* test (unpaired).

Results

CL tissue fragments and dissociated cells

The results in Table 1 show that bLH and cAMP significantly enhanced progesterone production by the 12½-day tissue and cells, but if testosterone was also present then the response to bLH was no longer significant. Progesterone production by the tissue and cells recovered 6½ days after ovulation was not significantly increased by any of the treatments, but the control level of synthesis was greater than that of the tissue and cells recovered later in the luteal phase.

Table 1. Progesterone production by minced tissue and dissociated cells of CL recovered at 6½ and 12½ days after ovulation induced in gilts

Age of tissue (days)	Minced tissue (µg progesterone/g tissue/3 h)					Isolated cells (pg progesterone/1000 cells/3 h)				
	Control	bLH (1 µg/ml)	dcAMP (10 mM)	Testosterone (100 ng/ml)	bLH (1 µg/ml) + testosterone (100 ng/ml)	Control	bLH (1 µg/ml)	dcAMP (10 mM)	Testosterone (100 ng/ml)	bLH (1 µg/ml) + testosterone (100 ng/ml)
6½	59.8 ± 6.9	68.6 ± 5.4	58.5 ± 1.8	67.3 ± 3.7	NI	130.0 ± 26.3	150.0 ± 12.5	125.0 ± 5.0	111.3 ± 10.0	86.3 ± 8.8
6½	55.8 ± 7.0	54.9 ± 7.9	60.8 ± 5.2	57.6 ± 12.3	NI	75.0 ± 5.0	77.5 ± 6.3	81.3 ± 6.3	72.5 ± 7.5	70.0 ± 6.3
12½	4.1 ± 1.2	11.7 ± 2.7*	26.6 ± 3.2**	2.7 ± 0.9	4.9 ± 3.5	19.0 ± 6.3	50.5 ± 3.8**	66.0 ± 16.3*	31.3 ± 5.0	31.3 ± 2.5
12½	4.2 ± 1.0	9.0 ± 1.0**	17.8 ± 1.7**	4.9 ± 1.6	5.7 ± 1.3	53.8 ± 3.8	77.5 ± 5.0**	78.8 ± 5.0**	48.8 ± 1.2	53.8 ± 3.8

Values are mean ± s.e.m. for 6 (minced tissue) or 4 (isolated cells) samples from several pooled CL from 1 animal. Each row represents 1 animal. NI = not incubated.

Values significantly different from the control value: **P* < 0.05, ***P* < 0.005.

Response to hCG

Tissue and cells from 6½-day CL were unaffected even by high concentrations of hCG, but the tissue and cells from 12½-day CL showed enhanced progesterone production with increasing hCG dose although the control production was lower (Text-fig. 1).

Steroid production by large and small luteal cells

After separation, a virtually pure preparation of small cells (10–20 µm diameter) was obtained (>95%). The large cell preparation (30–45 µm diam., >60% purity) did contain some contaminating small cells but the proportion of large cells was considerably enhanced above that in the original mixed cell suspension (15–20% large cells). Some very small cells <10 µm in diameter were discarded with the red blood cells after separation.

Small and large cells from 6½-day CL did not show increased progesterone production in response to LH, although progesterone production by the large cells was considerable. However, both cell types from the 12½-day tissue responded significantly.

Text-fig. 1. Progesterone production in response to increasing concentrations of hCG by luteal tissue and dissociated cells recovered at 6½ and 12½ days after ovulation. Each value is the mean $\pm$ s.e.m., $n = 4$ for minced tissue and $n = 3$ for isolated cells. Progesterone production was calculated as described in the 'Methods'. Values significantly different from the control value: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Discussion

The above results indicate that the porcine corpus luteum is autonomous for much of its lifespan. When CL are recovered 6½ days after ovulation, and the minced tissue studied *in vitro*, progesterone production is high and cannot be further stimulated by gonadotrophins or cAMP (Table 1). The fact that dbcAMP, which bypasses the gonadotrophin-receptor interaction step cannot enhance steroidogenesis suggests that the tissue is already synthesizing at maximum capacity, and this is supported by the production rates detected *in vivo* (Masuda, Anderson, Henricks & Melampy, 1967). The incubations of the dissociated cells (Table 1) confirm the results from the minced luteal tissue in that the cells also appear to be producing progesterone at maximum rate.

By contrast, for tissue recovered 12½ days after ovulation, control levels of synthesis were lower than for tissue recovered earlier in the luteal phase. However, progesterone production by the minced tissue and dissociated cells was significantly stimulated by gonadotrophins and dibutyl cAMP to levels similar to those produced by the 6½-day CL. By 12 days after ovulation, the progesterone levels *in vivo* are beginning to fall from maximal (Masuda *et al.*, 1967) and this is reflected by the production *in vitro*. The treatment doses used were based on data from previous *in-vitro* studies of luteal tissue (Savard, Marsh & Rice, 1965; Cook *et al.*, 1967). Tests with the two populations of luteal cells (Table 2) showed that it is the large luteal

cells which are the more active in terms of progesterone production, but both cell types removed 12½ days after ovulation were responsive to stimulation.

Table 2. Progesterone production by separated cells from pig corpora lutea recovered 6½ and 12½ days after ovulation

Age tissue (days)	Cell population	% Large cells	Progesterone produced (pg/1000 cells/3 h)	
			Control	bLH (1 µg/ml)
6½	Small	<5	12.5 ± 0.1	11.4 ± 1.1
6½	Large	62	446.3 ± 30.3	308.8 ± 65.0
12½	Small	<5	7.0 ± 6.6	24.1 ± 2.0**
12½	Large	73	262.3 ± 32.4	402.2 ± 41.3*

Values are mean ± s.e.m. for 3 incubations of cells recovered from several CL from 1 animal.

Values significantly different from control: * $P < 0.05$, ** $P < 0.01$.

Although the small cells were individually less active in terms of progesterone production, they are still likely to play an important role *in vivo* because they are present as a large percentage of the total luteal cells.

The major role played by androgens and oestrogens in controlling the response of the ovary to gonadotrophins has been well documented (Nimrod & Lindner, 1976; Haney & Schomberg, 1978; Thanki & Channing, 1978; Williams, Roth, Marsh & LeMaire, 1979), and in the above experiments testosterone was found to inhibit the response to bLH. The porcine CL can aromatize testosterone to oestradiol (Hunter, 1980) and so testosterone may be exerting its inhibitory effect after being aromatized to oestradiol, as oestradiol has been demonstrated to inhibit the hydroxysteroid dehydrogenase isomerase system of human and ovine CL (Depp, Cox, Pion, Conrad & Henricks, 1973; Caffrey, Nett, Abel & Niswender, 1979).

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