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Evidence for the Existence of a Luteal Cell Type That Is Steroidogenic and Releases Relaxin[1] (42571)

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Abstract. Secretion of relaxin from cultured luteal cells derived from pregnant sows was detected by a reverse hemolytic plaque assay. In this method, luteal cells are cultured in monolayers together with protein-A-conjugated ovine red blood cells. In the presence of porcine relaxin antiserum and complement, relaxin-releasing cells become surrounded by an area of hemolysis—a plaque—which can be microscopically visualized. After fixation, these same luteal cells in monolayers were stained for the presence of 3β-hydroxysteroid dehydrogenase, an enzyme marker for steroidogenic cells. Cells could then be classified by their ability to form plaques (relaxin-releasing cells) and/or steroidogenic capability (positive staining). Dual-secretors (large luteal cells that were steroidogenic and released relaxin) could be identified in dispersed luteal cells derived from pigs at all stages of pregnancy examined (Day 22–112 of gestation, $n = 9$; term is Day 114 ± 2 days). In addition, luteal cells were detected that were either steroidogenic only or released relaxin, and finally, cells that appeared to possess neither endocrine capability. Frequency analysis of functional subtypes indicated approximately equal representation of each in the first half of pregnancy, but an apparent fall in relaxin-releasing cells in the preparturient period. It is suggested that dual-secretors may represent one mechanism that allows the corpus luteum to express multiple endocrine function during pregnancy without the requirement for increased cell numbers.

The simultaneous presence of two separate and distinct hormones within porcine corpora lutea during pregnancy—the steroid, progesterone (1) and the polypeptide hormone, relaxin (2–4)—raises the obvious and important question as to whether these hormones are secreted by the same luteal cell (a dual-secretor), or whether functional subtypes of luteal cells exist that are dedicated to the release of either progesterone or relaxin. The existence of dual-secretors may be inferred from two indirect lines of evidence.

First, large luteal cells of other species appear to possess the ultrastructural features characteristic of steroid-secreting cells (abundant mitochondria, smooth endoplasmic reticulum, lipid droplets), *and* those of protein hormone-secreting cells (abundant rough endoplasmic reticulum, electron-dense granules) (1, 5, 6). Second, large porcine luteal cells derived from pregnant pigs release high amounts of progesterone in culture (1) and relaxin has been immunocytochemically detected within the secretory granules of this cell type (2, 4).

However, there are major limitations to such approaches that preclude positive identification of dual-secretors. Analysis of hormone release from cell populations provides information only on the average amount of hormones released, and provides no data on the contribution of individual cells. Likewise, morphological and immunocytochemical approaches may provide inconclusive information, since many cells that contain a hormone may not release it at any given time (7). Therefore, there is a requirement to use direct approaches in order to demonstrate unambiguously that a single endocrine cell can release both a steroid and polypeptide hormone. In this study, we attempted to meet this requirement by using two assay procedures in sequence, with the goal of defining endocrine function of individual luteal cells. First, a reverse hemolytic plaque assay was employed to detect the release of relaxin by individual luteal cells in culture. Second, enzyme histochem-

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istry was immediately carried out on the same cells to monitor the presence of 3β-hydroxysteroid dehydrogenase (3β-HSD), an essential enzyme marker of progesterone-secreting cells (5, 6, 8).

Materials and Methods. *Luteal cell culture.* An ovary was obtained about 10 min after slaughter of pregnant pigs (weight approximately 100–160 kg). A total of nine pigs were used with gestational ages ranging from 22 to 112 days (term is 114 ± 2 days). The stage of pregnancy was determined by the day of mating to an intact boar, and confirmed by fetal crown–rump length (9). The ovary was immediately placed in about 30 ml Dulbecco's modified Eagle's medium [DMEM, obtained from Grand Island Biological Co. (GIBCO), Grand Island, NY] containing 0.1% bovine serum albumin (BSA; Fraction V, Sigma, St. Louis, MO), 100 units penicillin G/ml and 100 μg streptomycin sulfate/ml (GIBCO); this medium will be subsequently referred to as DMEM-0.1% BSA.

After transport back to the laboratory (about 15 min), luteal tissue (about 10 g obtained from three to four corpora lutea) was stripped from the fibrous capsules, minced finely (1 mm^3 fragments) with sterile scalpel blades, and washed twice in fresh 5 ml DMEM-0.1% BSA. These procedures were carried out at 22°C. All of the luteal fragments were then placed in a Spinner's suspension flask (25-ml volume; Bellco Glass, Inc., Vineland, NY) containing 15 ml DMEM-0.1% BSA, 0.15% collagenase (139 units/mg; Type III, Cooper Biochemicals, Freehold, NJ) and incubated for 60 min at 37°C. Following gentle trituration of the flask contents, cells were incubated for an additional 10–20 min. Dispersed cells were centrifuged for 10 min at 1500*g* at 22°C, resuspended in 10 ml Spinner's minimum essential medium (GIBCO), 0.1% BSA, and washed twice more in this medium.

The cells were then passed through a nylon mesh (75 μm; TETCO, Inc., Elmsford, NY) to remove tissue clumps, and an aliquot of cells removed for cell counts (hemacytometer) and cell viability (trypan blue exclusion). For the sake of simplicity, only the easily distinguishable large luteal cells (i.e., >25 μm) in size were counted. Cell yields were $1–2 \times 10^6$ large cells/dispersion; cell viability (small and large) was >90%.

Plaque assays. This assay employs the principle of antibody-directed, complement-mediated red blood cell lysis around a relaxin-releasing cell. Microincubation chambers were constructed by placing a glass coverslip onto two strips of Scotch double stick tape attached about 20 mm apart on a poly-l-lysine-coated glass microscope slide. Luteal cells were diluted to 5×10^5 large cells per ml DMEM-0.1% BSA, mixed 1:1 with 12% protein-A conjugated sheep red blood cells (10) in the same medium, and this mixture infused into the microincubation chambers by capillary action. After a 45-min incubation (at 37°C in a humidified atmosphere of 5% CO_2:95% air) in which a monolayer of luteal cells and sheep red blood cells formed on the chamber floor, the unattached cells were washed away by drawing fresh DMEM-0.1% BSA through the chamber using a sterile gauze sponge placed on one side of the chamber. The monolayers were then incubated overnight (about 18 hr) and washed again the following day with DMEM-0.1% BSA to remove accumulated cell products. This overnight incubation was included after preliminary studies showed that the formation of plaques by freshly dispersed luteal cells was inconsistent, as compared with the rate of plaque formation by cells cultured overnight which was highly reproducible. A solution of the same medium containing rabbit anti-porcine relaxin antiserum (1:80) was then infused to each chamber and incubated for 4–8 hr to achieve maximum plaque formation. A solution of guinea pig complement (1:40, GIBCO) was finally infused into all chambers to develop the plaques, and the monolayers were fixed in a 1.8% solution of glutaraldehyde (in 0.9% NaCl, w/v) after 50 min.

Specificity of porcine relaxin antibody has been characterized previously (11). Plaque formation was abolished by preabsorption of relaxin antibody with purified porcine relaxin (10 μg relaxin/ml combined with 1:80 relaxin antibody) and by omission of relaxin antibody or complement.

Histochemical identification of 3β-hydroxysteroid dehydrogenase. Luteal cells in incubation chambers were stained for the presence of 3β-HSD immediately after plaque assay by a minor modification of the method of Payne and colleagues (8); this technique has

been successfully used to monitor the presence of 3β-HSD in ovine luteal cells (5). Briefly, monolayers were fixed for 1 hr in 1.8% glutaraldehyde in saline as previously described after completion of the plaque assay, then washed twice in normal saline (0.9% NaCl, w/v). A mixture of nitroblue tetrazolium (0.2 m*M*), β-NAD (1.5 m*M*) and 5β-androstan-3β-ol-17-one (0.2 m*M*) in phosphate-buffered saline [(pH 7.2, 0.1% bovine serum albumin, Fraction V); all reagents from Sigma] was then introduced into each chamber, the monolayer exposed to these agents overnight at 37°C, fixed again in 1.8% glutaraldehyde in saline, and stored at 4°C before microscopic examination.

The presence of the dark formazan reaction product was considered to identify a steroidogenic cell. Control procedures were as follows: (1) omission of any one of the three reaction compounds abolished staining; (2) changing the period of fixation and/or incubation of monolayers did not significantly alter the proportion of steroidogenic cells; (3) the execution of plaque assay before histochemical procedures did not significantly influence steroidogenic cell numbers since similar results were obtained when the sequence was reversed, i.e., plaque assay carried out after histochemical staining; and (4) the percentage of 3β-HSD-positive cells was similar in freshly dispersed luteal cells and cells cultured for 24 hr.

Expression of results. To determine cell function, monolayers were viewed through an inverted microscope (Olympus IMT2) and a minimum of 200 large luteal cells were scanned per chamber. Each cell was then categorized according to (a) the presence or absence of the formazan stain in the cell cytoplasm and (b) the presence or absence of a plaque around the cell, thus giving rise to four potential classifications. We made no attempt to grade the degree of histochemical coloration since positively reacting cells usually stained intensely. A plaque was defined as a minimum of a single, concentric ring of lysed red blood cells around a luteal cell. The number of cells in each category was expressed as percentage of the total, and a mean value derived from the four to six chambers was used in each experiment.

Results are stated as means ± SEM unless otherwise defined. Differences between group means were determined by analysis of variance, followed by an appropriate multiple range test (13).

Results. Microscopic examination of monolayers revealed four detectable types of large luteal cells: (a) cells which both formed a plaque and stained, suggesting this cell type released relaxin and possessed steroidogenic capacity (dual-secretors), (b) cells which formed a plaque, but did not stain (relaxin-only releasing cell), (c) cells which stained, but did not form a plaque (steroidogenic-only cell), and, (d) cells which neither formed a plaque nor stained, suggesting that this cell did not release relaxin or possess steroidogenic capacity (non-secretor).

Quantitative analysis of cell types in dispersed luteal cells derived from early pregnant, midpregnant and preparturient pigs is shown in Table I. No significant difference was observed among the frequencies of these types in dispersions derived from early and midpregnant pigs; relaxin-only releasing cells comprised about 17% of the total, with the other three types represented approximately equally in the balance (i.e., about 28%). Taken

TABLE I. PERCENTAGES OF LUTEAL CELL SUBTYPES IDENTIFIED BY A COMBINATION OF PLAQUE ASSAY AND ENZYME HISTOCHEMISTRY IN DISPERSED LUTEAL CELL PREPARATIONS DERIVED FROM EARLY PREGNANT (DAY 22–29; $n = 3$) MIDPREGNANT (DAY 45–79; $n = 3$) AND PREPARTURIENT PIGS (DAY 105–112; $n = 3$)

	Nonsecretor	Steroidogenic only	Relaxin only	Dual secretor
Early pregnant	26.9 ± 2.0	28.2 ± 5.0	17.2 ± 1.5	28.0 ± 5.1
Mid pregnant	20.6 ± 3.9	35.6 ± 4.5	17.5 ± 2.0	26.8 ± 3.5
Preparturient	37.0 ± 5.1	42.8 ± 5.7	3.8 ± 0.4	15.6 ± 1.7

Note. Values represent the percentage of each cell type (identified as explained in the text), and determined at three different stages in pregnancy. At each stage, three independent experiments were carried out and the values shown here are the mean ± SEM.

overall, the combined numbers of all steroidogenic and relaxin-releasing cells represented about 60 and 45% of the total, respectively.

However, the total proportion of relaxin-releasing cells decreased markedly (by about 24%; $P < 0.05$) in cell dispersions derived from preparturient pigs as compared with combined values derived from first and second trimester pregnant pigs. This fall could be attributed to reductions both in relaxin-only secretors and dual-secretors. A smaller concomitant increase was observed in the categories of steroidogenic-only releasing cells (increase of about 10%) and nonsecretors (increase of about 14%) in cell dispersions derived from preparturient pigs, but these shifts were not significant.

Discussion. To analyze the functional capability of individual large luteal cells, in this study we used a reverse hemolytic plaque assay followed by histochemical staining for the steroidogenic enzyme, 3β-HSD. By employing these methods in sequence on the same luteal cells, we reasoned that the observation of cells that both formed a plaque and stained positively for the presence of 3β-HSD would provide direct confirmation of cells that both secrete relaxin and are steroidogenic—dual-secretors.

Using this approach, this study provides clear evidence for the existence of dual-secretors. Our results indicate that about 25–30% of luteal cells that are greater than 25 μm in size (which we have assumed to be large luteal cells [1, 5]) form a plaque *and* stain positively. This proportion changed little over the first half of pregnancy.

The significance of this finding may lie in at least two areas. First, we suggest that dual-secretors could represent an important cellular mechanism whereby the overall hormonal output of the corpus luteum can be increased during pregnancy without the requirement for increased cell numbers. Second, and perhaps of greater significance, the existence of individual luteal cells that have the capacity to secrete both relaxin and progesterone raises fundamental questions concerning the control of dual-hormone release from an individual cell. It is well established that the patterns of ovarian release of progesterone and relaxin in pregnancy are wholly dissimilar; progesterone secretion increases little during most of pregnancy and then falls sharply before delivery, but in contrast, relaxin secretion rises steadily throughout gestation, culminating in a marked prepartum surge (3, 14). From such information, it is plausible to infer that differential regulatory mechanisms exist to subserve these patterns of hormone release at the level of the individual dual-secretor.

We also found that at least three additional large luteal cell functional subtypes could be identified with different endocrine capabilities. Apart from dual-secretors, we also detected cells that were either steroidogenic or released relaxin, and finally, cells that appeared to possess neither hormonal capability. One objection to this interpretation could be that, in fact, all large luteal cells are dual-secretors, and that our failure to detect this phenomenon in every large luteal cell may be ascribed to insufficiently sensitive assay systems. While we cannot entirely exclude such an idea, two lines of evidence argue against this view.

First, we found in preliminary studies that peak plaque formation by relaxin releasing cells (about 50% as in the present study; see Table 1) was achieved at 3–4 hr of incubation at these gestational ages. Further prolonged incubation (up to 24 hr) did not increase this value. In addition, we found that although both prostaglandin $F_{2\alpha}$ and E_2 rapidly accelerated the rate of plaque formation, these stimulatory agents did not increase the proportion of cells releasing relaxin (Taylor, Clark and Frawley, unpublished results). We conclude that cells that do not form a plaque (despite stimulation or extended incubation) must therefore be releasing relaxin at an extremely slow rate, one, moreover, which is qualitatively different from plaque-forming cells. Second, the lengthy reaction time used in the histochemical identification of 3β-HSD (18–24 hr at 37°C) resulted in intense staining in positively responding cells. Given that these methods reliably identify endocrine function in individual cells, we suggest there are major functional differences between cells that form a plaque and/or stain positively and those that do not.

The physiological significance and ontogeny of the functionally differing subtypes remains unclear. Their existence, and the observation that for about the first half of pregnancy (i.e., up to Day 79 of gestation) these four luteal subtypes were equally distributed on an approximate basis, is not inconsistent with a luteal cell-cycle, as suggested by Niswender and

colleagues (5). But it is equally credible that a single endocrine cell can lose or acquire endocrine capabilities at different times, perhaps spontaneously.

We detected a redistribution of these subtypes several days before term. The proportion of relaxin-only secretors and dual-secretors dropped sharply, with a concomitant increase in nonsecretors and steroidogenic-only secretors. This was a puzzling result since a number of studies have clearly shown that *in vivo* there is a surge of relaxin release immediately before delivery (3, 13), and this seemingly contradicts our observation that the proportion of relaxin-releasing cells declines at this time. The most likely explanation is that luteal tissue collected in the preparturient period may already have undergone luteolytic changes that resulted in selective cell loss during dispersion. Such a possibility precludes further comment on the significance of the redistribution of functional cell types observed at this time (Table 1). On the other hand, it is also possible that the surge of relaxin release is related less to the number of relaxin releasing cells present than to the stimulatory effect of prostaglandin $F_{2\alpha}$. This compound exerts a strong stimulatory effect on relaxin release *in vivo* and is released in considerable amounts by the gravid uterus before delivery (13, 14).

In summary, we have used reverse hemolytic plaque assay combined with enzyme histochemistry to investigate the release of relaxin and steroidogenic capacity by large luteal cells derived from pigs at different stages in pregnancy. Our results unambiguously demonstrate the existence of a cell type that is both steroidogenic and secretes relaxin and indicate further functional heterogeneity exists within the large luteal cell population.

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