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Ultrastructural Immunoperoxidase Demonstration of Relaxin in Corpora Lutea from a Pregnant Sow

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ABSTRACT

Corpora lutea from a pregnant sow were prepared for immunocytochemical localization of relaxin in granulosa lutein cells at the ultrastructural level. The antiserum used in the staining procedure was raised against a highly purified porcine relaxin prepared from pregnant sows' ovaries. The primary criterion for specificity was the identification of the peroxidase-antiperoxidase complex and a reduction in staining intensity upon prior incubation with porcine relaxin. Anti-relaxin serum stained a population of small granules which were commonly found in clusters within the cytoplasm or bordering the plasmalemma. The localization of relaxin in these granules extends the observations of others who have correlated changes in the number of granules with relaxin levels as determined by bioassay.

INTRODUCTION

Relaxin has recently been purified from pregnant sows' ovaries and chemical studies have shown this hormone to be a series of low molecular weight proteins consisting of two peptide chains linked by disulphide bonds (Schwabe et al., 1977; James et al., 1977). Opinions vary as to the relative location of relaxin and progesterone in the corpus luteum of the sow and other species. It has been suggested that the small granules evident in the granulosa lutein cell are the sites of storage of either relaxin (Belt et al., 1971) or progesterone (Gemell et al., 1974). Fluctuations in relaxin and progesterone concentrations in corpora lutea and plasma correlate with the abundance of these membrane bound granules. Fluorescent antibody techniques have demonstrated by light microscopy the presence of relaxin in the corpus luteum of the pregnant rabbit (Zarrow et al., 1966) and rat (Anderson et al., 1975) but neither relaxin nor progesterone has been localized at the subcellular level. The present report demonstrates that relaxin may be localized immunocytochemically in granules of the granulosa lutein cells in the pregnant sow ovary.

MATERIALS AND METHODS

The tissues were obtained from a pregnant sow (estimated by assessment of fetal size to be between Day 20 and 30 of gestation) immediately after slaughter. Eight corpora lutea were cut from the ovaries, diced into small pieces and fixed in one of the following fixatives for 12 h at room temperature; 1 percent paraformaldehyde (pH 7.35, 312 mOsm) Zamboni's (pH 7.5, 809 mOsm) (Zamboni and De-Martino, 1967) 1 percent glutaraldehyde (pH 7.35, 343 mOsm) 2.5 percent glutaraldehyde (pH 7.35, 470 mOsm). The tissues were rinsed in 0.1M phosphate buffer (pH 7.4, 240 mOsm) for 20 min (three changes), dehydrated through ascending concentrations of ethanol, infiltrated with propylene oxide and then 150 pieces were embedded in Araldite 6005. Using a diamond knife, ultrathin sections were cut from 26 blocks, 20 of which were fixed in 1 percent glutaraldehyde. The sections were collected on nickel grids, etched with 5 percent H_2O_2 for three min and stained by the immunocytochemical bridge technique of Moriarty et al. (1973).

Normal goat serum and goat antiserum to rabbit IgG (Kallestad, Minnesota) were used for the blocking and coupling serum respectively. For whole tissue identification 1 μm sections were stained with azure II:methylene blue (1:1).

The primary criterion of specificity in this study was the identification of peroxidase-antiperoxidase (PAP) molecules (Moriarty, 1973). In order to satisfy more precisely two important requirements in the interpretation of results, method specificity on the one hand and antibody specificity on the other, a number of immunocytochemical controls for the staining procedure were performed. The purpose for

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which each control was used has recently been concisely reviewed by Petrusz et al. (1976). In the first group of controls one of the components of the stain was substituted. Normal rabbit serum or phosphate buffer was substituted for anti-relaxin serum: normal goat serum or phosphate buffer was substituted for the heterologous coupling antiserum or phosphate buffer was used instead of the PAP complex. Omitting each reagent systematically from the regular staining sequence allowed us to identify nonspecific staining by reagents acting directly with tissue components. The second series of controls involved the use of sequentially higher dilutions of primary antiserum (1:00–1:25,000) both at 4°C for 48 h and at room temperature for 24 h. This procedure not only facilitates recognition and elimination of contaminating antibodies of undesired specificity but also allows the determination of the optimal dilution correlated to the reaction. From this series a 1:1000 dilution of anti-relaxin serum, incubated at room temperature for 24 h, was chosen for future tests. In the third series this dilution was absorbed with varying amounts of porcine relaxin for two days at 4°C (450 ul of diluted antiserum/tube) before it was used in the stain. This is the only direct way to establish the specificity of the reaction to the tissue hormone. In addition to these series of controls, sections of rat pituitary (fixed and embedded as described above) were processed in each immunocytochemical staining run. The pituitary tissue was stained for localization of adrenocorticotrophin hormone (ACTH) and was included as an internal standard following a principle used in radioimmunoassay to detect interassay errors in the method. These blocks and the anti-ACTH serum were kindly provided by Dr. G. Moriarty and the results obtained with this tissue were consistent and identical to those described by this investigator (Moriarty, 1973).

Highly purified porcine relaxin was prepared from frozen pregnant sow ovaries by the method of Sherwood and O'Byrne (1974). Of the three microheterogeneous peaks designated as CM-B, CM-a and CM-á by these authors, CM-á and antiserum to CM-á were used for this study. The preparation of CM-á was 3.97 times more potent than NIH-R-PI porcine relaxin in the mouse interpubic ligament bioassay (Steinetz et al., 1960) NIH-R-PI having 442 GPU/mg. Therefore, this preparation corresponded very closely with the biological potency of CM-á reported by Sherwood and O'Byrne (1974).

Antiserum was prepared in an adult male New Zealand White rabbit by the method of Vaitakaitis et al. (1971). The rabbit was exsanguinated 7 weeks after the initial immunization and the antiserum stored in frozen aliquots. The antiserum was titrated against ^{125}I-labeled CM-á using a modification of the acylating technique of Bolton and Hunter (Kwok et al., 1976). Maximum binding of 50pg CM-a-I^{125} was achieved with a final concentration of 1:24,000 antisera equilibrated for two days at 4°C.

RESULTS

Antigenicity was preserved with all fixatives. Tissues fixed in Zamboni's fixative showed the most intense staining reaction in a 1:1000 dilution of antirelaxin serum. One percent glutaraldehyde was best for preservation of ultrastructure. A 1:10,000 dilution of anti-relaxin serum incubated with the tissue for 24 h at room temperature gave a reaction whereas when the tissue was incubated at 4°C for 48 h a 1:25,000 dilution of antiserum was still discernable from control sections.

The granulosa lutein cell is a large polyhedral cell. The appearance of these cells was consistent with the description of Belt et al. (1971) for granulosa lutein cells at Day 28 of gestation. The mitochondria were found to be small with tubular cristae and are reminiscent of mitochondria identified in many but not all steroid secreting cells (See review by Christensen and Gillim, 1969). Large areas of cytoplasm were occupied by endoplasmic reticulum coiled in tight whorls. Short segments of granular endoplasmic reticulum were interspersed between the mitochondria throughout the rest of the cytoplasm. Lipid droplets appeared as vacant circular spaces in our unstained tissue. Two prominent populations of granules were identified as lysosomes and smaller granules.

In granulosa lutein cells anti-relaxin serum stained a population of granules which ranged from 0.2 μm to 0.6 μm in diameter and included larger heterogenous granules of up to 1.0 μm in diameter. However a proportion of this granule population did not stain. The former group of smaller granules were commonly in clusters within the cytoplasm (Fig. 1a) or bordering the plasmalemma (Fig. 1b). The larger irregular granules tended to be separated from the clusters. PAP molecules were recognized within both the clustered and irregular granules (Fig. 3) and to a slight extent within the cytoplasm. Larger granules previously recognized as lysosomes did not stain (Belt et al., 1971).

Prior absorption of the serum antibody with 5ng of relaxin reduced staining in granules (Fig. 2a) and cytoplasm and higher amounts (5μg–200μg) abolished staining in these locations. In view of the similarity in chemical structure of insulin, proinsulin and relaxin (James et al., 1977) it was significant that absorption with 10μg of porcine proinsulin did not reduce the staining density. No stain was evident in granules or cytoplasm of controls in which phosphate buffer was substituted for anti-relaxin serum or for PAP complex (Fig. 4), or in which goat anti-rabbit IgG was omitted from the stain. PAP molecules could be distinguished on occasional secretion granules

when normal rabbit serum replaced the specific antiserum. However, in all control sections, including unprocessed sections which were stained only with 4 percent OsO_4, osmiophilia was observed over areas of granular endoplasmic reticulum. This latter reaction was intensified in sections reacted with 3,3′-diaminobenzidine tetrahydrochloride and further intensified in the complete procedure.

DISCUSSION

With the availability of purified porcine relaxin and an antiserum to this material it has been possible to use a recently developed technique to localize relaxin at the fine structural level. The pig was the animal of choice for this study since the purified antigen was isolated from sow ovaries and the chemical structure is known. It is not known at present whether antibodies raised to porcine relaxin will cross react with relaxin in the tissues of other species to make localization possible.

Previous attempts to localize relaxin in luteal cells had limited success. Incubation of luteal cells from pregnant rats with fluorescent labelled antibody caused fluorescence in the juxtanuclear area but it was difficult to define the intracellular organelle with which the antibody was associated (Anderson et al., 1975). Techniques using frozen tissue have demonstrated that relaxin readily diffuses into the intercellular space during processing (Zarrow et al., 1966; Anderson et al., 1975).

Our results agree with those of other investigators who have correlated changes in the number of small granules in porcine luteal cells with relaxin levels as determined by bioassay (Belt et al., 1971). Although PAP complex was observed in granules in a few of the endothelial cells we saw adjacent to a granulosa lutein cell no relaxin staining granules were seen in the intercellular spaces. Belt et al. (1971) failed to observe exocytosis or other evidences of granule release from porcine luteal cells during the rise from low concentrations of granules and relaxin early in pregnancy to maximum levels reached at Day 105–110 of gestation. Their suggestion that ovarian relaxin is mostly stored over this time-course is supported by Sherwood et al. (1975) who found that the levels of relaxin in plasma remained constant. Similar studies in late pregnancy suggest that there is a secretory pattern of ovarian relaxin (Belt et al., 1971; Sherwood et al., 1975) which is in keeping with a role for relaxin in the promotion of soft tissue changes and in the regulation of myometrial activity associated with farowing (Zarrow et al., 1954; see review by Steinetz et al., 1959). Undoubtedly extension of our present report to a systematic investigation of intracellular localization of ovarian relaxin later in gestation may help to define more precisely the position of relaxin in the hierarchial control of the initiation of labour proposed for the sow by Heap et al. (1977).

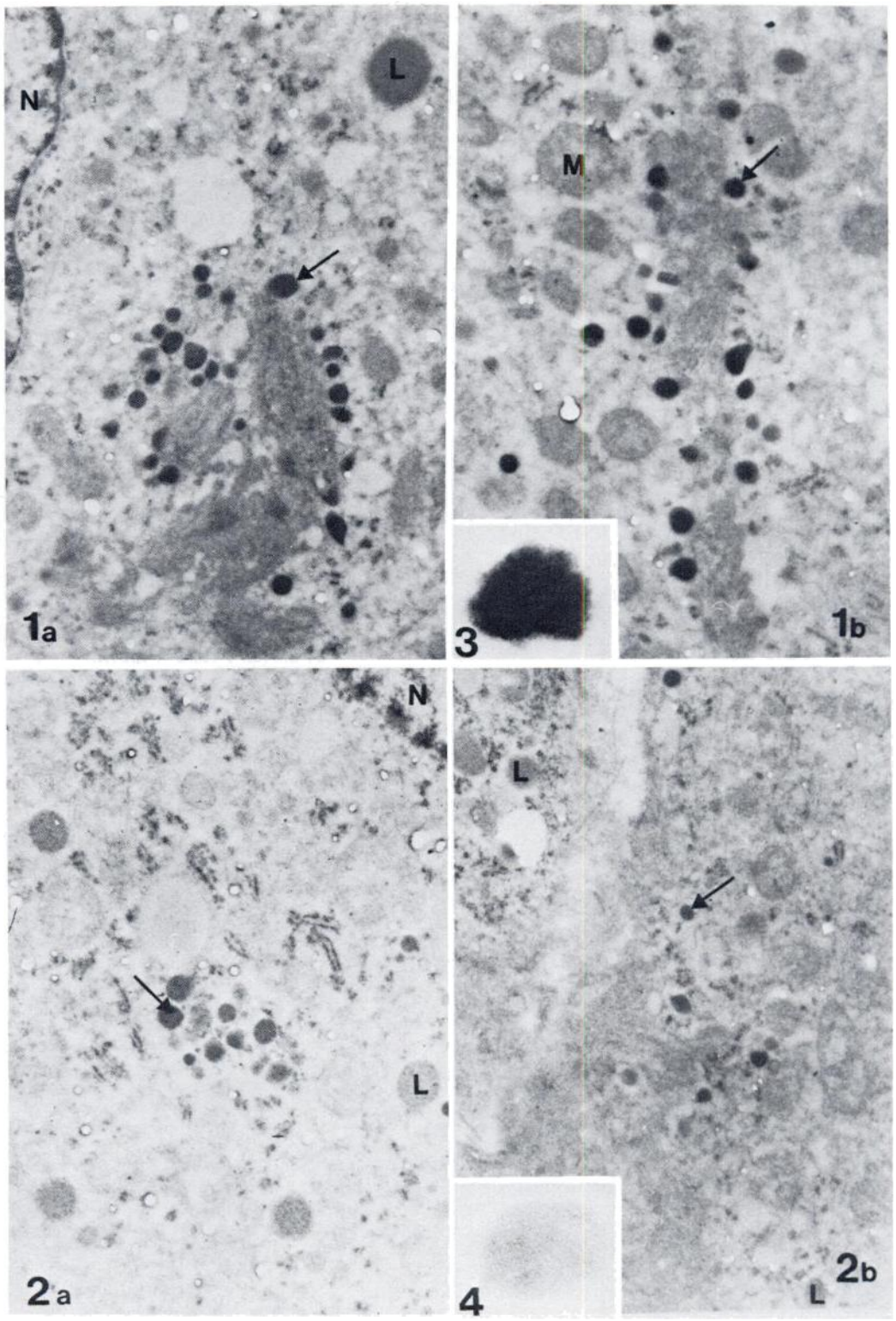
Small granules similar in appearance to those in the pig have also been described in granulosa lutein cells of a number of species including rats (Long, 1973), human (Crisp et al., 1970), sheep (Gemell et al., 1974), and cow (Kramers et al., 1975). It has been suggested that the small granules present in ovine and bovine tissue have as their function the storage and secretion of progesterone (Gemell et al., 1974; Kramers et al., 1975). It is possible that the population of small granules contain both progesterone and relaxin. Alternatively as suggested by Deane et al. (1966) there may be two cell types, and one may secrete relaxin, the other progesterone. Our observations are not entirely consistent with this proposal in that the large polyhedral

FIGS. 1a and 1b. Sections of granulosa lutein cells from a pig at 20 to 30 days of pregnancy. Stained with a 1:1000 dilution of antiserum to porcine relaxin and the PAP complex unlabelled antibody technique. Fixation 1 percent glutaraldehyde, embedding Araldite. After immunochemical staining the sections were floated on 4 percent OsO_4 for 15 min. No counterstain. Stain is on a population of granules (arrowed) distributed in clumps with the cytoplasm (a) or bordering the plasmalemma as can be observed in the two adjacent cells in (b). N = nucleus, L = lysosome, M = mitochondria. ×12,500.

FIGS. 2a and 2b. Staining intensity in secretory granules (arrowed) incubated with anti-relaxin serum is reduced by absorption with 5ng of porcine relaxin (a) Control section (b) to that in Fig. 1b. Normal rabbit serum (1:1000 dilution) was substituted for the antiserum. This section was run through the same immunochemical staining sequence as the grid in Fig. 1b. N = nucleus, L = lysosome. ×12,500.

FIG. 3. The appearance of a secretory granule which has accumulated a large number of PAP molecules. ×56,000.

FIG. 4. The appearance of secretory granule stained as for Fig. 3 but phosphate buffer was substituted for the PAP complex. ×56,000.



cell these authors designated as progesterone-secreting cell was found to contain relaxin. Confirmation is required using immunocytochemical techniques that these polyhedral cells contain progesterone.

The localization of relaxin in luteal cells now opens the way for future work on the quantitation of this hormone in different physiological states and the study of the mechanism of secretion thereby clarifying the role for relaxin in ovarian function.

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