Progesterone Synthesis by Pig Corpus Luteum Tissue during Superfusion

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A modified superfusion technique is described with which it was demonstrated that the action of gonadotrophin on progesterone secretion by pig corpus luteum tissue is twofold, in that it first stimulates the rapid release of progesterone (either preformed or partially synthesized), which is followed by prolonged synthesis of the steroid *de novo* from acetate.

The application of a continuous superfusion method to the study of ovarian steroidogenesis has been described for pig luteal tissue (Watson & Leask, 1973, 1975) and for rat ovarian tissue (Anderson *et al.*, 1973; Hashimoto, 1975). The technique for pig luteal tissue allows both the introduction of regulatory factors to the incubating tissue and sampling of the tissue, but has the disadvantage that it is unsuitable for long-term superfusion owing to its obvious susceptibility to bacterial contamination. Further, short-lived changes in steroid secretion, such as those described for adrenal cells under superfusion conditions (Schulster, 1973; Lowry & McMartin, 1974), could not be studied by the technique owing to the relatively slow complete turnover rate of the incubating medium.

The present paper describes an improved method for superfusion of ovarian tissues, more suitable for long-term studies of tissue dynamics, and confirms our previous hypothesis (Watson & Leask, 1975) that luteinizing hormone has a twofold effect on progesterone secretion from pig luteal tissue, in that it initially stimulates a rapid increase in progesterone secretion from the tissue due to either release or partial synthesis of the steroid, and this is followed by the synthesis of the steroid *de novo* from acetate.

Experimental

Materials. All chemicals were of A.R. grade, and solvents were further purified by chromatography on H₂SO₄-impregnated silica gel, followed by distillation in an all-glass system. Sodium [1,2-¹⁴C]acetate (57 mCi/mmol), 3 β -hydroxy[7(*n*)-³H]pregn-5-en-3-one (pregnenolone; 10.5 Ci/mmol) and [1,2,6,7-³H]- or [4-¹⁴C]-pregn-4-ene-3,20-dione (progesterone; 82 Ci/mmol and 58 mCi/mmol respectively) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Luteinizing hormone (NIH-LH-B8) was supplied by the National Institutes of Health, Bethesda, Md., U.S.A. Human chorionic gonadotrophin was supplied by Organon Laboratories, Newhouse, Lanarkshire, U.K. Dibutyl 3':5'-cyclic AMP was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Scintillators used consisted of PPO (2,5-diphenyl-oxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)-benzene] (2 g and 0.1 g respectively; Koch-Light Laboratories, Colnbrook, Bucks., U.K.) dissolved in (a) toluene (1000 ml) for organic materials, and (b) toluene (800 ml) and Triton X-100 (200 ml; Hopkin and Williams, Chadwell Heath, Essex, U.K.) for aqueous solutions (Watson & Leask, 1975).

Watson-Marlow MHRK/4/Delta pumps (Watson-Marlow Ltd., Falmouth, Cornwall, U.K.) were used in the superfusion system. Samples were counted for radioactivity in a Nuclear-Chicago Isocap/300 liquid-scintillation counter.

Pig corpus luteum. Slices of suitable pig corpus luteum tissue, obtained from ovaries of mature sows, were prepared as previously described (Watson & Leask, 1975).

Superfusion. The modified superfusion chambers consisted simply of readily available chromatography columns (1 cm long $\times$ 1 cm internal diam.) with fine gauze filters at each end (Boehringer Corp., Ealing, London W.5, U.K.; BM-combination column 10). These provided a totally closed and aseptic system. Sterilized medium, Krebs-Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing glucose (10 mmol/l), bovine serum albumin (0.1%; BDH Biochemicals, Poole, Dorset, U.K.) and gassed with O₂+CO₂ (95:5), or Wellcome medium 199 (Wellcome Reagents Ltd., Beckenham, Kent, U.K.) was pumped at a rate of 27 ml/h from a reservoir at 0°C via glass coils and upwards through the columns containing slices. The glass coils and columns were maintained in a water bath at 37°C. A second pump on the outlet side of the columns, operating at the same rate as the first pump, was also used to maintain an even flow rate. Superfusates were collected for 10 or 20 min intervals and stored at -15°C until analysed.

Progesterone analysis. The concentration of progesterone in the superfusates was measured by a modification of the rapid competitive protein-binding assay method of Johansson (1969). In this method, plasma from women taking combined contraceptive pills was used as binding protein, and free and

bound steroid were separated by dextran-charcoal (Watson & Leask, 1975). This was removed by filtering through glass-fibre filters packed in Pasteur pipettes, rather than by centrifugation, thereby allowing very careful timing and large numbers of samples to be analysed at one time.

Extraction and isolation of radioactive progesterone. To 7 ml of each 20 min superfusate sample, [^3H]- or [^{14}C]progesterone (1000 d.p.m.) was added to assess recovery. The samples were extracted three times with ethyl acetate (7 ml, 5 ml and 2 ml respectively) and the combined extracts back-washed with water (1 ml). The samples were evaporated to dryness for subsequent purification by t.l.c. Kieselgel G-Kieselgel HF-254 (1:1, w/w) plates (0.5 mm thick) were used, and the extracts were first chromatographed in the solvent system chloroform-ethanol (9:1, v/v). Progesterone was located by viewing the plate under short-wave u.v. light, and was eluted with acetone. The steroid was further purified by re-chromatography in the solvent system hexane-ethyl acetate (1:1, v/v). Radioactive progesterone was again eluted with acetone, the extract evaporated to dryness, and finally added to counting vials with ethanol (0.6 ml). A quench correction was used to compensate for this volume of ethanol. Samples were then counted for radioactivity in the scintillation counter linked to an on-line computing system (Seaton, 1974). Several representative radioactive progesterone samples were diluted with unlabelled progesterone and recrystallized to constant radioactivity. No loss of radioactivity was noted, thus establishing that the radioactivity measured was entirely due to progesterone.

Results

A representative pattern of progesterone secretion from pig corpus luteum tissue slices under control conditions is shown in Fig. 1. Concentrations of progesterone during the first 2 h of superfusion are not shown, as these were always high, owing to flushing out of endogenous steroid, but after this preincubation values remained fairly constant. Superfusate was collected for 12 h, followed by a break overnight during which medium was still pumped through the chambers. Collection was restarted after 11 h for a further 3 h. Even after 24 h the tissue continued to secrete steroid, but at a rate approximately half that of the initial steady-state value.

The effect of infusions of luteinizing hormone and dibutyryl cyclic AMP over a 3 h interval on progesterone secretion from the tissue is also shown in Fig. 1. The gonadotrophin caused a fairly rapid rise in progesterone secretion, which increased to a maximum by 3–4 h and thereafter fell only slightly during the 10 h of sample collection. By 24 h, however, the steroid concentrations were similar to the initial

Fig. 1. Progesterone secretion by control (●) and luteinizing hormone (▲) or dibutyryl cyclic AMP (○)-treated pig corpus luteum tissue during superfusion

Each point represents the mean of four determinations, and s.e.m. values did not exceed $0.2 \mu\text{g/g}$ at any point. The bar indicates the period of addition of luteinizing hormone ($500 \mu\text{g}$) or dibutyryl cyclic AMP (1 mg). The differences between control and treated groups first become statistically significant 30 min after addition of luteinizing hormone or dibutyryl cyclic AMP.

steady-state values, unlike the control chambers, where the value had fallen to half the initial steady-state value by this time, indicating that some stimulation was possibly still occurring. This type of pattern in response to gonadotrophin and dibutyryl cyclic AMP has been consistently obtained in separate experiments (two each with luteinizing hormone and dibutyryl cyclic AMP, three with human chorionic gonadotrophin).

Fig. 2(a) shows the incorporation of [^{14}C]acetate into progesterone under control and stimulated conditions similar to those described above. [^{14}C]Acetate was added to both chambers 30 min before any addition of stimulant, and little conversion into progesterone occurred in either control or stimulated tissue during the first 2–3 h of superfusion with radioactive acetate. After this, however, the specific radioactivity of progesterone in the superfusates rose to considerably higher values with stimulated tissue than in the control situation. This difference was maintained over the total period of superfusion, although infusion of [^{14}C]acetate was for 3 h only, indicating that radioactive acetate was probably being stored at some intermediate stage. Even after 24 h, radioactive progesterone could be isolated, and there was a significant difference between control and

Fig. 2. Incorporation of radioactive precursors into progesterone during superfusion of pig corpus luteum tissue

(a) Incorporation of $[^{14}\text{C}]$ acetate during control conditions (●) and stimulation with luteinizing hormone (▲) or dibutyryl cyclic AMP (○). Differences between control and treated groups first become statistically significant 160 min after addition of luteinizing hormone (LH) or dibutyryl cyclic AMP (cAMP). (b) Incorporation of $[^3\text{H}]$ pregnenolone during control conditions (●) or dibutyryl cyclic AMP stimulation (○). Differences between control and treated groups first become statistically significant 90 min after addition of dibutyryl cyclic AMP.

stimulated tissue. This pattern has been consistently obtained with luteinizing hormone, dibutyryl cyclic AMP and human chorionic gonadotrophin (two experiments each).

Fig. 2(b) shows the incorporation of $[^3\text{H}]$ pregnenolone into progesterone under control and dibutyryl cyclic AMP-stimulated conditions. Additions of these materials were made as described above, and more rapid incorporation of radioactivity into progesterone was obtained than when acetate was used as precursor. Increased incorporation caused by dibutyryl cyclic AMP stimulation, however, did not occur until some 90 min after the addition of dibutyryl cyclic AMP, whereas Fig. 1 shows that the increased progesterone secretion occurred within 30 min. Further, there was little prolonged secretion of radioactive progesterone when the addition of $[^3\text{H}]$ pregnenolone to the tissue ceased, indicating little binding or build-up of $[^3\text{H}]$ pregnenolone or intermediates in the tissue.

Discussion

It has previously been demonstrated (Watson & Leask, 1975) that during superfusion the normal steroid secretory products of pig luteal tissue appear to be maintained, and that secretion of both progesterone and oestrogen by the tissue can be stimu-

lated by gonadotrophin. If the gonadotrophin is added as a pulse, however, considerable variation in the response of steroid secretion is obtained, owing to the variable uptake of the gonadotrophin by luteal tissue, whereas if the gonadotrophin is infused over a short time-period it is more consistently taken up by the tissue (Leask *et al.*, 1974) and, as the present results demonstrate, gives a consistent pattern of response. In either case, however, the effect of gonadotrophin appears to be to cause an immediate increase in steroid secretion followed by a prolonged response. A transient increase is more obvious (but variable) where the gonadotrophin is added as a pulse.

The present studies were carried out in an attempt to resolve whether the increased secretion in response to gonadotrophin was due to release of progesterone followed by synthesis *de novo* as postulated previously (Watson & Leask, 1975), or whether the ovary had the capacity rapidly to synthesize and secrete steroid. From the results obtained it appears that little synthesis of progesterone occurs in response to the gonadotrophin for some considerable time after infusion. Further, as the $[^{14}\text{C}]$ acetate was added 30 min before any gonadotrophin, in order to allow some build-up of intermediates, it appears unlikely that any of the early stages of biosynthesis are being affected by the gonadotrophin.

When [^3H]pregnenolone was used as precursor, a more rapid stimulation of incorporation of radioactivity into progesterone due to dibutyryl cyclic AMP was noted, but even this took some 90 min to become significant relative to control tissue. Increased secretion of progesterone, on the other hand, was stimulated within 30 min by gonadotrophin or dibutyryl cyclic AMP. These results support the hypothesis that the initial stimulation of progesterone secretion in response to gonadotrophin or dibutyryl cyclic AMP is due to the release of pre-formed steroid.

The phenomenon that gonadotrophins cause a transitory rise in steroid secretion by the ovary is one for which considerable evidence is now accumulating, and has probably not been detected in the past owing to the experimental and sampling procedures previously used. McCracken *et al.* (1971) have demonstrated that in the sheep *in vivo* a transitory rise in oestrogen secretion occurs 20–30 min after an infusion of luteinizing hormone, but the steroid concentrations decline again within a further 30 min. Moor (1974) has demonstrated that, although the overall effect of luteinizing hormone is to inhibit oestrogen production from sheep follicles cultured *in vitro*, the concentrations of steroid secreted during the first few hours of culture are considerably enhanced. These latter data are supported by preliminary studies (Watson & Dodson, 1975) in which oestrogen secretion from superfused isolated theca cells was inhibited by luteinizing hormone, although an initial rise in oestrogen secretion during the first 2 h of luteinizing hormone infusion was observed.

These results again demonstrate the difference between ovarian and adrenal steroid secretion and their response to trophic hormones. The latter gland

gives very rapid and short-lived increases in steroid secretion in response to corticotrophin stimulation during superfusion (Schulster, 1973; Lowry & McMartin, 1974), which is probably a characteristic evolved by adrenal steroid-secreting cells. The ovary, however, does not require such rapid changes in steroid output and, as has been demonstrated, gives a relatively slow, but much more prolonged, overall response after a transient increase in steroid output.

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