Pulsatile GnRH administration stimulates the number of pituitary GnRH receptors in seasonally anoestrous ewes

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Summary. Twenty seasonally anoestrous ewes were pretreated with progesterone for 4 days and divided into four equal groups. Ewes in Group 1 received no GnRH treatment and were slaughtered immediately after progesterone removal. Ewes in Groups 2, 3 and 4 received i.v. injections of 250 ng GnRH every 2 h for 36 h starting at the time of progesterone removal. Ewes in Group 2 were slaughtered immediately after the 36 h GnRH pulsing, while ewes in Groups 3 and 4 were given a bolus injection of 125 µg GnRH at this time and were slaughtered 2 and 10 h after the bolus injection, respectively. Blood samples were collected every 30 min from ewes in Group 4 only, from 4 h before the start of GnRH treatment until 10 h after the bolus injection. Pulsing with GnRH resulted in episodic release of LH, and the bolus injection of GnRH was immediately followed by a preovulatory type LH surge in those ewes in which an endogenous surge had not already begun. The pituitary GnRH receptor numbers were significantly higher for the ewes in Group 2 than for any of the other treatment groups, while there was no significant difference in the receptor numbers between Groups 1, 3 and 4. The results suggest an up-regulation of GnRH receptors resulting from pulsatile GnRH therapy.

Introduction

In cyclic and anoestrous ewes, luteinizing hormone (LH) is secreted in an intermittent manner (Yuthasastrakosol *et al.*, 1977), suggesting that gonadotrophin releasing hormone (GnRH) is also secreted in a pulsatile fashion, a view supported by the demonstration of simultaneous pulses of GnRH in hypothalamic portal blood and LH in jugular vein blood (Clarke & Cummins, 1982). It has been suggested that intermittent delivery of GnRH to the pituitary gland is essential for gonadotrophin secretion to be maintained. Exogenous GnRH given in a pulsatile manner every 1–2 h to GnRH-deficient humans or monkeys can mimic the hormonal changes of puberty and the menstrual cycle (Knobil *et al.*, 1980; Valk *et al.*, 1980; Wildt *et al.*, 1980). McLeod *et al.* (1982a) have also reported that injections of low doses of GnRH every 2 h result in a sustained increase in pulsatile LH secretion in seasonally anoestrous ewes until the onset of a preovulatory LH surge, and that such animals, if primed with progesterone, will eventually ovulate and exhibit normal luteal function. Although short-term continuous infusion of low doses of GnRH is equally effective in this respect (McLeod *et al.*, 1983), only pulsatile administration of GnRH appears to be able to maintain cyclic activity for long periods in anoestrous ewes (McNatty *et al.*, 1982; M. Khalid & W. Haresign, unpublished data). These results have, therefore, been interpreted to indicate that the pulsatile manner of GnRH stimulation is of great importance in determining the normal patterns of gonadotrophin secretion.

The importance of the manner of GnRH delivery to the pituitary is well-established (Knobil, 1980) but few data are available on the cellular mechanisms that mediate the different gonadotrophin responses under different physiological conditions. There is evidence that GnRH acts to

increase gonadotrophin secretion by binding to a membrane receptor (Clayton *et al.*, 1979). The involvement of hypothalamic GnRH in the regulation of pituitary receptors for GnRH has also been reported (Fraser *et al.*, 1982; Pieper *et al.*, 1982). This study was therefore undertaken to determine whether pulsatile GnRH treatment of anoestrous ewes has any effect on the number of pituitary receptors for GnRH.

Materials and Methods

Animals and management

The 20 seasonally anoestrous Romney Marsh ewes were treated in late June, 1985. Throughout the experiment they were housed under conditions of natural daylight and temperature and fed a diet of 'indoor' ewe concentrates and hay, with water always available.

Treatment and blood sampling

All the ewes were treated with progesterone; 2 implants (Sil-Estrus: Ceva Chemicals, Hornsby, Australia), each containing 375 mg progesterone in a silicone-elastomer matrix, were inserted subcutaneously in the axilla region and remained *in situ* for 4 days.

The ewes were randomly divided into 4 equal groups. Group 1 (Ewes 1–5) received no GnRH treatment and progesterone implants were removed immediately before slaughter. All the ewes in Groups 2, 3 and 4 were given injections of 250 ng GnRH (Lutal: Fabwerke Hoechst AG, Frankfurt, West Germany) in 2 ml saline (9 g NaCl/l) at 2-h intervals for 36 h via an indwelling jugular vein catheter. The ewes in Group 2 (Ewes 6–10) were slaughtered immediately after the last pulsatile GnRH injection. A single bolus injection of 125 µg GnRH to synchronize the timing of the preovulatory LH surge was administered via the catheter to all ewes in Groups 3 (Ewes 11–15) and 4 (Ewes 16–20) 36 h after the start of pulsatile GnRH therapy, and these animals were slaughtered 2 and 10 h after the bolus injection, respectively.

Blood samples (2 ml) were collected at 30-min intervals from all ewes in Group 4 from 4 h before the start of GnRH pulses until 10 h after the bolus injection to monitor plasma LH concentrations.

Radioimmunoassay of LH

Plasma LH concentrations were determined by the specific double-antibody radioimmunoassay of Foster & Crighton (1974) as modified by McLeod *et al.* (1982b). Within this study limit of sensitivity of the LH assay was 0.14 ng NIH-LH-S24 equiv./ml and the inter-assay and intra-assay coefficients of variation were 13.4% and 11.7%, respectively.

Measurement of pituitary GnRH receptors

Pituitary membrane preparation. The pituitary glands were collected immediately after slaughter and dissected free of connective tissue, intermediate and posterior lobes. The anterior lobe was weighed and then stored at -196°C in liquid nitrogen for no more than 2 weeks until analysed for GnRH receptor content.

The numbers of GnRH receptors were determined in a partly purified membrane fraction. Each anterior pituitary gland was thawed, sliced and homogenized on ice with a ground-glass homogenizer using 4 ml of ice-cold assay buffer (10 mM-Tris-HCl, pH 7.4, containing 0.1% BSA (Sigma Chemical Company, Dorset, U.K.) and 0.1% sodium azide). The homogenate was centrifuged at 300 g for 5 min, after which the supernatant was decanted and centrifuged at 10 000 g for a further 10 min. Binding experiments were initially carried out in the 300 g pellet, the supernatant after 300 g centrifugation, the 10 000 g pellet and the supernatant after 10 000 g centrifugation. The binding was negligible in all the fractions except the 10 000 g pellet. After this second centrifugation stage, the supernatant was carefully decanted and discarded and the pellet was resuspended in 1 ml assay buffer and used for the binding assay. All the steps were performed at 4°C .

General binding procedure. All GnRH binding studies were undertaken using an ^{125}I -labelled preparation of the D-Ser(TBu)⁶des Gly¹⁰-GnRH-ethylamide analogue of GnRH as radioligand. This was supplied by Fabwerke Hoechst A.G. (Frankfurt, West Germany), and was stated to have a specific activity of 932 µCi/µg. All receptor assays were performed in polycarbonate tubes precoated with 3% BSA to reduce non-specific adsorption of labelled peptide. Since there was insufficient material to permit Scatchard analysis of individual pituitaries, concentrations of GnRH receptors in the partly purified membrane fraction were determined in triplicate as follows. Total binding was measured by incubating 100 µl aliquants of the partly purified membrane fraction (101–192 mg wet weight) with 50 pg labelled GnRH analogue (100 µl) and made up with assay buffer to a total volume of 300 µl. Non-specific binding was

assessed by incubating the membrane fraction and label in triplicate in the presence of an excess (1 $\mu\text{g}/\text{tube}$) of non-radioactive GnRH analogue. After an incubation period of 120 min at 4°C, 2 ml ice-cold assay buffer were added to stop the reaction, and the tubes were centrifuged at 10 000 g and 4°C for 10 min. The supernatant was carefully aspirated and discarded, and the pellet was washed twice in this manner with ice-cold assay buffer. The amount of labelled hormone bound to the membrane fraction was determined by gamma-spectrometry. Each tube was counted for 1 min and the efficiency of the counter was 75%. Specific binding was then calculated by subtracting the non-specific binding from the total binding for each pituitary, and ranged from 4 to 23% of the total counts added. The results for binding of the GnRH-analogue have been expressed as c.p.m. bound per g wet weight of pituitary tissue, and taken to indicate GnRH receptor numbers. The intra-assay coefficient of variation was 7.17%.

Fig. 1. Scatchard plot analysis of ^{125}I -labelled D-Ser(TBu)⁶-des-Gly¹⁰-GnRH ethylamide binding to a crude membrane fraction prepared from wether anterior pituitary glands.

Fig. 2. Displacement curves of ^{125}I -labelled D-Ser(TBu)⁶-des-Gly¹⁰-GnRH ethylamide by unlabelled GnRH (▲) and the D-Ser⁶GnRH-analogue (●).

Validation of binding assay. For validation of the binding assay, pituitary glands from wethers were processed as described above. The time course of the binding reaction was estimated by incubating the partly purified membrane fraction with 80 000 c.p.m. (50 pg) of ^{125}I -labelled GnRH analogue for variable periods ranging from 45 min to 24 h at 4°C . By 120 min maximum binding was achieved and equilibrium was maintained for up to 8 h. Therefore, all the subsequent assays were incubated for 120 min at 4°C . During separation of the bound and free hormone no displacement in specific binding was observed with successive washes. In a separate experiment the specific binding for the same tissue was 10.59%, 11.97% and 11.56% after the 1st, 2nd and 3rd washing, respectively. The addition of increasing amounts ($7\text{--}1700 \times 10^3$ c.p.m.) of ^{125}I -labelled GnRH analogue to a constant amount of membrane fraction initially increased specific counts bound, and then levelled off, indicating that binding of the radioligand to the tissue was a saturable phenomenon. A Scatchard plot of the results obtained (Fig. 1) when 100 μl aliquants of pooled anterior pituitary membrane preparation were incubated with increasing concentrations of labelled hormone indicated the presence of only a single class of binding sites for the radioligand. The affinity constant (K_a) was $1.48 \times 10^9 \text{ M}^{-1}$ and the number of GnRH binding sites was 120 fmol/0.1 pituitary.

Fig. 3. Mean ($\pm$ s.e.m.) plasma LH concentrations in seasonally anoestrous ewes (a) before and after the start of i.v. injections ($\downarrow$) of 250 ng GnRH at 2-h intervals for 30 h, and (b) in relation to the start of the preovulatory LH surge. The LH surge occurred spontaneously ($\blacktriangle$ — $\blacktriangle$) in 2/5 ewes, and in response to a bolus injection ($\downarrow$) of 125 μg GnRH ($\bullet$ — $\bullet$) in 3/5 ewes (see text).

Displacement curves were performed on pooled anterior pituitary membrane preparations by incubating 45 pg ^{125}I -labelled GnRH analogue with increasing concentrations of GnRH and the GnRH analogue. Both materials inhibited the binding of the radioligand to the pituitary preparation in a similar manner, although much lower concentrations (about 60 times less) of GnRH analogue were required to produce a 50% displacement of labelled peptide (Fig. 2). When incubations were carried out with the same amount of ^{125}I -labelled GnRH analogue, increasing the amount of standard wether pituitary membrane preparation (from 0.1 to 0.2 pituitary/tube) resulted in a 1.84-fold increase in specific binding.

Fig. 4. Mean anterior pituitary GnRH receptor content (expressed as c.p.m. ^{125}I -labelled D-Ser⁶-GnRH analogue bound/g pituitary tissue) of seasonally anoestrous ewes before and at various times after the start of GnRH treatment. I indicates the s.e.d. for comparing any two treatment means.

Statistical analysis

A rise in LH concentrations was defined as an episode if (i) there was an increase of at least 50% above the preceding baseline value, (ii) there were at least two points between the peak value and the succeeding trough or baseline, and (iii) the rate of decline in concentration after the peak was no greater than that allowed by the half-life of the hormone.

The data for pituitary GnRH receptor content were analysed by analysis of variance.

Results

The pattern of change in plasma LH concentrations for ewes in Group 4, from 4 h before the start of GnRH pulses until 10 h after the bolus injection of GnRH, is presented in Fig. 3. Before the start of GnRH pulses, plasma LH concentrations were basal, and characteristic of the seasonally anoestrous ewe. Each 2-h injection of 250 ng GnRH produced an LH episode. In 2 of the ewes, the preovulatory LH peak started 4 h and 6 h respectively before the bolus injection of 125 μg GnRH, whereas in the remaining 3 ewes it occurred in response to the bolus injection of GnRH. Although the preovulatory LH peak height was not reduced in the ewes in which the surge was induced by the bolus injection of GnRH (mean 117.0 ng/ml) compared to those ewes in which it occurred spontaneously (mean 79.0 ng/ml), its mean duration was less when induced by the bolus injection of GnRH (6.3 h and 11.0 h, respectively).

The numbers of pituitary GnRH binding sites for the four different treatment groups are shown in Fig. 4. The GnRH receptor number was significantly higher ($P < 0.025$) for the ewes in Group 2

slaughtered immediately at the end of GnRH pulses and before the bolus injection than for any of the other treatment groups, while there was no significant differences in receptor numbers between ewes slaughtered 2 h (Group 3) or 10 h (Group 4) after the bolus injection of GnRH and control ewes slaughtered before the start of GnRH therapy. The number of pituitary GnRH receptors in the 2/5 animals in Group 4 in which the LH peak occurred spontaneously was only 40% of that recorded in the other 3 ewes in which the peak was induced by the bolus injection of GnRH.

Discussion

The plasma LH response to pulsatile GnRH administration observed in this trial was similar to that reported previously (McLeod *et al.*, 1982a). Although the bolus injection of GnRH (125 µg) was designed to synchronize the preovulatory LH surge just before the time when it would have occurred naturally, it appears that it was not given early enough, since in 2/5 animals from which blood samples were collected the LH peak occurred spontaneously 4 or 6 h before the bolus injection. Although the preovulatory LH peak height was not affected, its duration was less (almost half) when it was induced compared to when it occurred spontaneously. The longer duration of the spontaneously occurring LH peak may have been attributable to either to a longer duration of exposure of the pituitary to GnRH compared to that achieved following the bolus injection, or it could reflect differences in the oestrogen-induced increase in pituitary responsiveness to GnRH stimulation.

Pituitary receptors for GnRH differed significantly amongst treatment groups. An initial significant increase (up-regulation) in the receptor numbers was observed in animals exposed to pulsatile administration of GnRH (Group 2), indicating that a high number of pituitary receptors may lead to an augmented pituitary response, which in turn may be a pre-requisite for the maximal LH release in the form of the preovulatory LH surge. An increase in the number of pituitary GnRH receptors just before the endogenous LH surge in cyclic females has previously been reported by other groups working with rats (Clayton *et al.*, 1980; Marian *et al.*, 1981), hamsters (Adams & Spies, 1981) and sheep (Crowder & Nett, 1984). The possibility that some animals in Group 2 were slaughtered after the start of a natural preovulatory LH surge cannot be ruled out because blood samples were not collected from these ewes. Indeed, there was evidence that one ewe in this group had a much lower pituitary GnRH receptor content than all of the others, and the results from ewes in Groups 3 and 4 suggest that this may have been because it produced an endogenous LH surge before the end of the pulsatile GnRH treatment.

On the basis of the plasma LH results obtained from ewes in Group 4, it would appear that animals in Group 3 were slaughtered during the LH surge. In spite of these higher gonadotrophin concentrations, there was a reduction in pituitary GnRH receptors compared to those recorded for ewes in Group 2, although not to a level lower than that observed in the control group. Whether this represents a reduction in total receptor number or a reduction in unoccupied receptor number only is not clear from the present study. However, during the preparation of the pituitary membrane fraction the tissue was incubated in assay buffer for at least 60 min before the final centrifugation, and this time period has been shown to be sufficient to facilitate the dissociation of any hormone-receptor complexes in the pituitary tissue of rats (Clayton, 1982). It is probable therefore that the reduction in measured receptor content of ewes in Groups 3 and 4 does reflect a reduction in total GnRH receptor content. Such a decrease in the receptor numbers has also been reported to occur in rats (Clayton *et al.*, 1980; Savoy-Moore *et al.*, 1980; Marian *et al.*, 1981) and sheep (Crowder & Nett, 1984) after the start of the natural preovulatory LH peak at oestrus. Although there was no further significant reduction in pituitary GnRH receptor content in animals slaughtered 10 h after the bolus injection (Group 4), the two animals with spontaneously occurring LH peaks had less than half the number of receptors compared to those animals in which a smaller surge was induced by the GnRH bolus injection. With the limited data available it is not possible to determine whether this was a causal relationship or not.

Different factors have been suggested to be involved in down-regulation of pituitary GnRH receptors such as the occupancy of the receptors by GnRH (Nett *et al.*, 1981), the internalization of hormone, and probably receptors, subsequent to occupancy (Duello & Nett, 1980; Duello *et al.*, 1983) or the decreasing concentrations of oestradiol after the LH surge (Savoy-Moore *et al.*, 1981). Since in this particular study the GnRH injections were given to intact animals and are likely to have produced changes not only in gonadotrophins but also in gonadal steroids, it is not possible to establish which specific factors were responsible for the observed changes in pituitary GnRH receptor content. Although Nett *et al.* (1981) reported an increase in GnRH receptors in ovariectomized ewes infused with high doses of GnRH, Duncan *et al.* (1986) have shown that, in rats, both pro-oestrous levels of oestradiol and a low serum prolactin level are necessary for GnRH to be able to increase the number of its own receptors, suggesting that it is the interaction of these factors which is responsible for receptor-regulation during the oestrous cycle. The possibility that GnRH may interact with pro-oestrous levels of oestradiol to regulate pituitary GnRH receptor content in the ewe therefore requires further study. Nevertheless, it seems likely that an increase in pituitary GnRH receptors, resulting from pulsatile GnRH therapy, is necessary for the pre-ovulatory LH surge to occur in the anoestrous ewe, whereas a decrease in their numbers may lead not only to the termination of the LH surge but also to a reduced release of LH thereafter.

We thank the National Institute of Health for purified LH; Prof. L. E. Reichert for purified ovine LH for the production of the radioligand; and Hoechst Pharmaceuticals for the GnRH analogue and ^{125}I -labelled GnRH analogue. This work was conducted while M.K. was in receipt of a postgraduate scholarship from the Ministry of Education of Pakistan.

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Received 17 April 1986