GnRH AND NEUROPEPTIDE REGULATION OF GONADOTROPIN SECRETION FROM CULTURED HUMAN PITUITARY CELLS

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) and its superactive analogues are currently being used in the treatment of a number of endocrine disorders, such as endometriosis, precocious puberty, infertility and prostatic cancer. Selection of these analogues for clinical use have been previously based on their activities in animal models. This thesis has therefore investigated the binding characteristics of the human GnRH receptor, in comparison to those of the rat receptor, as well as the activities of a number of GnRH analogues for stimulating luteinising hormone (LH) and follicle stimulating hormone (FSH) secretion from cultured human pituitary cells. The establishment of a human pituitary bioassay system has further made possible the investigation of the direct regulatory roles of GnRH and other neuropeptides in man. To date, such studies in man have been performed in vivo and are thus complicated by the simultaneous interactions of numerous modulators.

A specific, high affinity receptor for GnRH in human pituitaries obtained post-mortem is described. The human pituitary GnRH receptor bound GnRH, GnRH agonists 1, 3 and 4 ([D-Ala^{6},N$^{\alpha}$-MeLeu^{7},Pro^{9}NEt]-GnRH, [Trp^{7}]-GnRH and [His^{5}]-GnRH), and GnRH antagonist 1 ([Ac-D-Nal(2)^{1},D-α-Me-4-ClPhe^{2},D-3-Pal^{3},D-Arg^{6},D-Ala^{10}]-GnRH) with similar affinities (K_{d}s of 4.81 nM, 0.32nM, 6.60 nM, 9.89 nM and

0.32 nM respectively) to those of the rat pituitary (K_ds of 4.71 nM, 0.31 nM, 2.04 nM, 3.16 nM and 0.40 nM respectively). GnRH agonist 2 ([D-Trp^6,D-Trp^7]-GnRH), GnRH antagonists 2 and 3 ([D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-GnRH and [Ac-D-Nal(2)^1,D-α-Me-4-ClPhe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]-GnRH), and chicken GnRH II ([His^5,Trp^7,Tyr^8]-GnRH), however, were bound with much lower affinities by the human receptor (K_ds of 5.50 nM, 4.21 nM, 11.4 nM and 110.0 nM respectively) than by that of the rat (K_ds of 0.59 nM, 0.09 nM, 1.00 nM and 28.7 nM respectively).

Monovalent and divalent cations affected [^{125}I]-GnRH agonist binding to rat and human pituitary receptors differently. In the presence of Mg^{2+} or Ca^{2+}, binding to the human receptor was significantly lower than in the rat. At near physiological concentrations, Na^+ and K^+ (100 mM and 10 mM respectively) inhibited [^{125}I]-GnRH agonist binding to the receptors to a similar extent in both species. At unphysiological concentrations (10 mM Na^+ and 100 mM K^+) these ions decreased binding to the human pituitary receptor to a greater extent than to the rat receptor.

Using a ligand-immunoblotting technique, the human receptor or binding component of the receptor complex was found to be of greater molecular size (64,000 daltons) than that of the rat (60,000 daltons).

active in stimulating LH release from both human and rat pituitary cells (0.08 nM and 0.35 nM respectively). GnRH agonist 2 and chicken GnRH II also had similar LH-releasing activities in the human bioassay (EC_{50}s of 0.53 nM and 16.8 nM respectively) as in that of the rat (EC_{50}s of 0.80 nM and 30.0 nM respectively). From receptor binding data, however, it was expected that these two analogues would be less active in the human than in the rat. GnRH agonists 3 and 4, although bound by the human and rat receptors with similar affinities, were more active in releasing LH from human pituitary cells than from rat. Further apparent differences between the human and the rat are demonstrated by the LH-inhibiting activities of the GnRH antagonists. GnRH antagonists 1, 2 and 3 were less active in the human (IC_{50}s of 2.60 nM, 72.0 nM and 23.6 nM respectivity) than in the rat (IC_{50}s of 0.52 nM, 18.7 nM and 0.47 nM respectively). The lower activity of antagonists 2 and 3 in the human, compared with the rat, were predictable from receptor studies, but GnRH antagonist 1 was expected to have a similar potency in both species. The biological potencies of these analogues for affecting FSH release from the human pituitary cells were similar to those for LH. It is thus apparent that rat studies are not a valid index for GnRH analogue activity in man.

Stimulation of the human pituitary cells with 100 nM GnRH for 44 min resulted in a biphasic release of LH and

The results show that the human and rat pituitary GnRH receptors have similar binding affinities for GnRH and certain GnRH analogues but differ in their binding of at least four GnRH analogues, their sensitivity to cationic effects on GnRH agonist binding, and in the molecular size of the receptor GnRH-binding protein.

In view of these differences between the human and rat pituitary GnRH receptors, a method for culturing human pituitary cells from autopsy tissue was established in order to investigate the biological potencies of the GnRH analogues tested in the receptor binding assays, as well as the regulation of gonadotropin secretion by GnRH and other neuropeptide hormones at the level of the pituitary.

The culture of functionally active human pituitary cells was validated by virtue of their ability to respond to the hypothalamic releasing hormones; GnRH, TRH, CRH and GHRH, by releasing LH and FSH, TSH and prolactin, ACTH and GH. Immuno-peroxidase staining of the cultured human pituitary cells showed that 15.2% of the total cell population were LH-containing cells, and 12.3% were FSH-containing cells.

The EC_{50} of GnRH-stimulated LH secretion from human pituitary cells was 0.95 nM, similar to that found with rat pituitary cells (2.00 nM). GnRH agonist 1 was 10-fold more

FSH. The initial phase occurred during the first 4 min of stimulation, and the second protracted plateau phase continued for at least the ensuing 40 min. The kinetics of LH and FSH release were identical, with no evidence of differential release at any stage.

GnRH-stimulated LH and FSH secretion was Ca^{2+} dependent. The presence of 3 mM EGTA prevented the gonadotropin response to GnRH, and the Ca^{2+} ionophore, A23187, stimulated release of both LH and FSH. The phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate, also stimulated gonadotropin secretion, indicating that activation of protein kinase C is able to elicit LH release. In addition to the effects of Ca^{2+} and phorbol ester, cAMP was associated with a dose-dependent increase in LH and FSH secretion, suggesting that this cyclic nucleotide also plays a role in gonadotropin secretion in man.

GnRH desensitised the cells to subsequent stimulation with GnRH. After an initial 2 h incubation with 0.1-100 nM GnRH, LH release during a second 2 h incubation with the same doses was reduced by 50-100%. The reduction in FSH ranged from 63-92%. Depletion of gonadotropin pools may contribute to desensitisation, since total LH and FSH cell content decreased 48% and 49%, respectively, after 100 nM GnRH stimulation for 4 h. When the initial incubation was carried out under Ca^{2+}-free conditions, the cells still

became desensitised to a subsequent stimulation with GnRH, although to a lesser degree than in cells incubated in the presence of Ca^{2+}, indicating that the desensitisation phenomenon is partly due to depletion of LH and FSH pools and partly due to some other Ca^{2+}-independent mechanism. A GnRH antagonist was unable to induce desensitisation suggesting that receptor activation is required and not merely receptor occupancy.

Pulsatile administration of GnRH (3 nM for 4 min) to cultured human pituitary cells, at frequencies of 45 min, 90 min and 180 min, resulted in identical secretory profiles of LH and FSH. The cells, however, only to responded to 3 pulses of GnRH before becoming desensitised. These data suggest that the cultured human pituitary cells are unable to synthesise new stores of LH and FSH.

Having established a method for culturing human pituitary cells the effects of other neuropeptides were investigated.

The human GnRH precursor consists of the GnRH sequence followed by a 59-amino acid carboxyl-terminal extension. A 56-amino acid peptide within this extension (designated GAP) has been shown to stimulate gonadotropin release from cultured rat pituitary cells. A series of ten overlapping peptide fragments were synthesised in our laboratory and

tested for their ability to stimulate LH and FSH release in the human pituitary bioassay. Six of the peptides tested stimulated gonadotropin secretion from the cultured human pituitary cells. All the active peptides were localised to the amino-terminal region of the carboxyl-terminal extension, and incorporated the decapeptide sequence, Asn-Leu-Ile-Asp-Ser-Phe-Gln-Glu-Ile-Val, which is regarded as the minimal structural requirement delineated for gonadotropin-releasing activity. A further flanking sequence extending this active region from its carboxyl-terminus was found to enhance gonadotropin-releasing activity, although the flanking sequence itself was inactive. The gonadotropin release stimulated by these peptides was shown to be a dose-dependent, specific, and Ca^{2+}-dependent phenomenon which occurs via a mechanism involving activation of the GnRH receptor, since a GnRH antagonist inhibited the activity and the peptides were able to displace [^{125}I]-GnRH agonist from human pituitary membranes.

Substance P (SP) has been implicated as a putative hypothalamic regulatory peptide in the control of gonadotropin secretion. A specific, saturable, high affinity receptor for SP is demonstrated in human pituitaries obtained post-mortem. The human pituitary SP receptor bound SP and two analogues, [D-Tyr^{0}]-SP and [D-Tyr^{0}, NorLeu^{11}]-SP, with similar affinities (K_ds of 17.6 nM, 35.3 nM and 19.3 nM respectively). The fragments, SP(3-11), SP(7-10), SP(5-8)

and SP(1-4), had lower affinities for the receptor than SP (K_ds of 92.4 nM, 286 nM, >1.0 μM, >1.0 μM respectively). The progressive decrease in binding with the removal of amino acid residues from the amino- or carboxyl-termini of the molecule is similar to that observed in the rat. GnRH and TRH did not compete for the SP binding site. SP inhibited GnRH-stimulated LH secretion from cultured human pituitary cells in a dose-dependent manner, while FSH release was largely unaffected. These findings suggest that SP may have a physiological role in the regulation of LH secretion in man.

These data thus demonstrate that functionally active cells can be cultured from human pituitaries obtained post-mortem, and that the mechanism of GnRH action and the regulation of gonadotropin release by other neuropeptides closely parallels that in the rat. The human pituitary, however, differs from that of the rat in its response to a number of GnRH analogues.

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CHAPTER 1

LITERATURE REVIEW:

GnRH AND NEUROPEPTIDE REGULATION OF GONADOTROPIN SECRETION

INTRODUCTION

The major neuroendocrine regulator of gonadotropin secretion is gonadotropin-releasing hormone (GnRH). The decapeptide hormone, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, is synthesised in the hypothalamic neurons as a higher molecular weight prohormonal form (Millar et al., 1977; Millar et al., 1978; Millar et al., 1981; Seeburg and Adelman, 1984) following which it is processed to GnRH and other fragments in the cell bodies or during transportation along the axons, such that the processed decapeptide predominates in the nerve termini (Millar et al., 1978; Rubin et al., 1987). GnRH is secreted into the hypophyseal-portal vessels in a pulsatile manner (Filicori and Crowley, 1984; Crowley et al., 1985) and reaches the anterior pituitary gland where it binds to specific receptors on the gonadotrope cells (Clayton and Catt, 1981; Sandow, 1983a), resulting in the activation of the second messenger systems, namely phospholipid turnover and Ca^{2+} mobilisation (Conn et al., 1987), and finally in the pulsatile secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH) (Marshall and Kelch, 1986). Following gonadotropin release, the hormone-receptor complexes are internalised (Jennes et al., 1983; Hazum et al., 1985) where they may play a role in such events as receptor recycling, hormone degradation and control of LH and FSH gene expression.

Other putative neuropeptide regulators of gonadotropin secretion are substance P, an inhibitor of GnRH-stimulated gonadotropin release (Kerdelhue et al., 1983); and the endogenous opioid peptides, several of which have also been shown to inhibit gonadotropin secretion at the pituitary level (Blank et al., 1985; Blank et al., 1986).

End organ feedback via the gonadal steroids occurs both at the hypothalamic level, by altering the firing rates of the GnRH neurons (Clarke and Cummins, 1985), and at the pituitary level by increasing the responsiveness of the gonadotropes to GnRH (Kamel et al., 1987a). This area is covered extensively in the literature (see reviews: Kalra and Kalra, 1985; Marshall and Kelch, 1986; Kalra et al., 1987; Mahesh and Muldoon, 1987) and will not be reviewed here. The intention is to address only the neuropeptide regulators of gonadotropin secretion which act directly on the pituitary.

GnRH SECRETION

The mode of secretion of GnRH from the hypothalamus is an important factor in the regulation of gonadotropin secretion from the anterior pituitary. In earlier years, studies on GnRH secretion were difficult due to the fact that the peptide is diluted to such a degree in the peripheral circulation that it is unmeasurable by radioimmunoassay (RIA). Indirect methods for assessing GnRH secretion were therefore established. These were: (1) detailed studies on plasma LH levels which provide reasonable approximations of GnRH levels in the portal vessels; (2) the "ablation-replacement model" which utilises the administration of GnRH to GnRH-deficient animals; and (3) the "in vitro model" wich uses dispersed pituitary cells to investigate the direct effects of GnRH and other putative regulators on gonadotropin secretion.

Measurement of gonadotropin secretion

Since the gonadotropins are released from the pituitary in a pulsatile manner, this suggests that GnRH is released in a similar pattern from the hypothalamus. Thus studies on the pulsatile release patterns of LH, which are measurable by RIA, give indirect assessments of the regulation of the hypothalamic-pituitary axis by GnRH (Santen and Bardin,

1973; Reame et al., 1984; Filicori et al., 1986). LH pulses vary in frequency during the human menstrual cycle (Filicori et al., 1986). The LH interpulse interval decreases during the follicular phase from 94 min in early follicular phase to 71 min by late follicular phase (Filicori et al., 1986), while in the luteal phase it progressively increases from 103 min in early luteal phase to 216 min by late luteal phase. The amplitude of the LH pulses also varies significantly throughout the cycle (Filicori et al., 1986). The pulse amplitude declines from early follicular phase to midfollicular and rises again by late follicular phase. It reaches a maximum in the early luteal phase and progressively decreases until the late luteal phase. These observations suggest a varied GnRH pulse frequency during the reproductive cycle and changes in GnRH pulse amplitude and/or gonadotrope sensitivity to GnRH.

This method of measuring LH pulsatility as an indirect assessment of GnRH pulsatility has two major disadvantages; the first being that the model is based on the assumption that all the major LH pulses are fully visualised. The observed LH interpulse interval has been shown to correlate well with the blood sampling interval (Crowley et al., 1985), which has resulted in a number of conflicting reports depending on the sampling times used in the studies. Much of this apparent controversy disappears when a blood sampling interval of 5 to 10 min is used. This is the optimal time

interval in order to visualise all major pulses of LH. The second disadvantage of this model is that it relies on the assumption that only GnRH is capable of eliciting a pulse of LH. This is perhaps reasonable as there is presently little evidence for the existance of another gonadotropin-releasing factor. More to the point is the converse of this assumption that every pulse of GnRH results in a measurable pulse of LH being secreted from the gonadotropes. Thus, using this approach, small pulses of GnRH which might not be sufficient to cause release of LH but could sensitise gonadotropes, would be missed.

Other indirect methods, such as the "ablation-replacement model" and the "in vitro model", have therefore been established, and may be used in conjunction with this method in an attempt to maximise physiological information.

"Ablation-replacement model"

The "ablation-replacement model" utilises the administration of exogenous GnRH to hypothalamic lesioned animals (Wildt et al., 1981; Clarke et al., 1986), or humans (Spratt et al., 1987; Gross et al., 1987) lacking endogenous hypothalamic GnRH. Pulsatile administration of the releasing hormone is able to restore full biological function in the rhesus monkey (Belchetz et al., 1978) while continuous exposure results in desensitisation or refractoriness of the

pituitary gonadotrope. This model has been used to investigate the significance of altered GnRH pulse frequency and amplitude on LH and FSH secretion in the ovariectomised, hypothalamic lesioned rhesus monkey (Wildt et al., 1981). Increasing the frequency of GnRH administration from the physiological 1 pulse/h to 2, 3 or 5 pulses/h resulted in a gradual decline of both gonadotropins, while decreasing the pulse frequency to 1 pulse in 3h led to a decline in plasma LH but an increase in FSH (Wildt et al., 1981). Reduction in GnRH pulse amplitude from 1 μg/min to 0.1 μg/min resulted in an abrupt decline in gonadotropin levels, while a 10-fold increase in amplitude caused a decline in plasma FSH but did not affect LH. Alterations in GnRH pulsatility therefore appear to be responsible for changes in the LH/FSH ratio during the cycle.

The effect of GnRH pulse frequency on LH and FSH secretion in man has been investigated by administrating different regimens of GnRH, in pulsatile fashion, to men with idiopathic hypogonadotropic hypogonadism (IHH) (Spratt et al., 1987; Gross et al., 1987). It has been shown that decreasing the frequency of GnRH pulses from 1 pulse/h to 1 pulse/2h and 1 pulse/3h, while keeping the total amount of GnRH administered constant, resulted in a progressive increase in serum FSH levels while serum LH levels were unaltered (Gross et al., 1987). Similarly, when the dose per pulse was held constant, decreasing the frequency from 1

pulse/0.5h to 1 pulse/1.5h again resulted in a selective increase in serum FSH levels (Gross et al., 1987). These results conflict with a previous report in which decreasing the frequency of low dose pulsatile GnRH administration did not cause a preferential rise in serum FSH (Finkelstein et al., 1986). However, this particular study was carried out using IHH men whose serum testosterone levels had been normalised for at least 6 months (Finkelstein et al., 1986), while the above mentioned study was carried out on IHH men at a time when serum testosterone and oestradiol levels were not normal to minimise the influence of testicular steroids (Gross et al., 1987). The importance of the steroid hormone milieu on the differential regulation of LH and FSH secretion by changes in GnRH pulse frequency have been previously demonstrated (Adams et al., 1986). In orchidectomised juvenile monkeys in which testosterone levels were raised to adult levels using testosterone-containing silastic capsules, the differential regulation of LH and FSH secretion by GnRH pulse frequency was abolished.

The mechanism for such frequency modulation is at present unknown. It has, however, recently been shown that pituitary GnRH receptor number is dependent on GnRH pulse frequency (Khalid et al., 1987), and thus the differential control of LH and FSH secretion may be mediated at the GnRH receptor level. Alternatively postreceptor mechanisms may be involved.

Similar studies, using ovariectomised, hypothalamic lesioned sheep, have indicated that this effect of GnRH pulse frequency on the relative amounts of LH and FSH secreted is not as pronounced in this species as in the primate (Clarke et al., 1984). Hourly pulses of GnRH restored gonadotropin secretion in these sheep, as has been reported in the rhesus monkey (Belchetz et al., 1978) and in man (Crowley et al., 1985). Increasing the amplitude of the GnRH pulses from 250 ng to 500 ng in sheep caused an increase in plasma LH baseline, peak values and pulse amplitudes, while there was no significant change in plasma FSH over 10 pulses. Decreases in GnRH pulse frequency from 1 pulse/h to 1 pulse/2h and 1 pulse/4h led to a decrease in plasma LH baseline but an increase in LH pulse amplitude. This was reversed immediately on reverting pulse frequency back to 1 pulse/h. Mean plasma FSH levels rose significantly to 111% of values on hourly pulses following the change from hourly to 2-hourly pulses. The mean increase in FSH was greater after 4 days of 2-hourly pulses but this change was rendered non-significant by the variability between individual animals. There was no change in FSH levels, within 7 days, on reversion back to hourly pulses of GnRH. In contrast, in the rhesus monkey plasma FSH levels were shown to be inversely related to GnRH pulse frequency (Wildt et al., 1981), where on reversion of pulse frequency back to 1 pulse/h, FSH levels immediately decreased reaching hourly

pulse levels within 5 days. It may be necessary to conduct these experiments in the sheep for longer durations as is suggested by previous studies using adult Soay rams with low circulating gonadotropin levels as a result of 12 weeks of exposure to long day lengths (Lincoln, 1979a; Lincoln, 1979b). In these animals, a GnRH pulse frequency of approximately 1 pulse/3h caused a progressive rise in plasma FSH which reached a maximum after 8 to 10 days (Lincoln, 1979a). There was no change in mean plasma LH release during this period. At the end of the study, when administration of GnRH had ceased, the concentration of FSH remained high for at least 24 h, decreasing slowly over the ensuing 8 to 14 days (Lincoln, 1979a; Lincoln, 1979b), while LH pulsatility immediately ceased. Thus, while the trends in FSH secretion in the sheep are similar to those in the monkey, it is apparent that the kinetics are different in that the preferential rise in FSH secretion caused by a less than 1 pulse/h GnRH frequency was more gradual in the sheep than in the primate, and the effect was longer lasting on removal of the GnRH stimulus.

The physiological relevance of alterations in GnRH pulsatility, and thus the LH/FSH ratios, during the reproductive cycle, however, is not clear since it has been shown that a constant GnRH pulse frequency administered to hypothalamic lesioned rhesus monkeys (Belchetz et al., 1978) and sheep (Clarke et al., 1984), and GnRH-deficient women

(Crowley et al., 1985) is capable of restoring a fully functional reproductive cycle. This latter finding draws attention to the major shortfall of this model which is that it makes use of the assumption that if an experiment is successful in restoring a normal pituitary-gonadal axis then this goal must have been achieved by physiological means. Studies on LH pulse frequency during the reproductive cycle, however, clearly indicate that a constant frequency of GnRH pulses is not the physiological norm. Ablation-replacement studies of this nature are therefore misleading in that the data are easy to misinterpret if not evaluated in the light of what is known from other studies using different approaches.

"In vitro model"

This model involves the use of two cell culture techniques: namely the perifusion system and the static culture system. In the perifusion system, anterior pituitary cells are dispersed, cultured on biocarrier beads, and placed in columns where they may be perifused with repeated pulses of GnRH, and other secretagogues, at different doses, durations and frequencies (Liu and Jackson, 1984; McIntosh and McIntosh, 1986; Kamel et al., 1987b). Using perifused rat anterior pituitary cells (Liu and Jackson, 1984) it has been shown that pulsing with low doses of GnRH (1 and 5 x 10^{-10} M) results in the stabilisation of the pituitary cells

and the release of similar amounts of LH in response to each sequential GnRH pulse for up to 24 h. In contrast, at high doses (10^{-8} and 10^{-6} M) of GnRH, the pituitary cells gradually released less LH with each pulse, suggesting that, similar to constant infusion, pulsatile delivery of GnRH at high doses can result in desensitisation of the pituitary cells to GnRH. This desensitisation may be as a result of the uncoupling of post-receptor mediating events, receptor down-regulation or depletion of the releasable LH pool.

A recent study, using perifused sheep pituitary cells, was carried out in order to determine whether the release of LH and FSH can be controlled differentially by varying the patterns of GnRH stimulation alone, free of the effects of steroid feedback or other influences from the whole animal (McIntosh and McIntosh, 1986). Varying the dose, interval and duration of GnRH pulses did not alter the proportions of LH and FSH released, in the short-term, from freshly dissociated cells. However, as stated previously, in the whole animal the alterations in the LH/FSH ratio induced by varying the GnRH pulse frequency were only observed after a period of 1 to 2 weeks and not within 8 h, which was the duration of the in vitro study. There are also other factors which may be responsible for the alterations in LH/FSH ratio seen in vivo, such as differential clearance from the circulation, differential synthesis in intact tissue (no

gonadotropin synthesis occurred under the conditions used in vitro) or other hormones influencing FSH secretion.

A disadvantage of the in vitro model is, therefore, that only relatively short-term studies can be performed and thus studies on the long-term effects of GnRH and other hormones must be carried out in the whole animal, excluding as many variables as possible by performing the necessary surgical manipulations.

Static cultures have been used to determine the LH-releasing potencies of GnRH and other synthetic analogues which have potential clinical applications. Investigations such as these have shown that the concentration of GnRH required to stimulate the release of 50% of maximum releasable LH from cultured rat (Loumaye et al., 1982; Leiser et al., 1986) and sheep (Millar et al.,1986a; Sower et al., 1987) pituitary cells is in the nanomolar range, while a number of GnRH agonists have been shown to be between 4 and 60 times more potent (Loumaye et al., 1982).

This technique has also been used to investigate the the mechanism of GnRH action (Conn et al., 1987), as well as the direct regulatory effects of other hormones, such as substance P (Kerdelhue et al., 1983) and the endogenous opioids (Blank et al., 1985; Blank et al., 1986), on

gonadotropin secretion. These areas will, however, be covered in more detail in later sections of this chapter.

Direct measurement of GnRH

Because of the limitations of the indirect methods for assessing GnRH secretion from the hypothalamus, a number of techniques have been established for directly measuring GnRH in the portal blood of monkeys (Carmel et al., 1976; Carmel et al., 1979), man (Carmel et al., 1979; Antunes et al., 1978) and rats (Sarkar and Fink, 1980). These early studies confirmed the hypothesis that GnRH is secreted into the portal vessels in a pulsatile fashion. A disadvantage of these experiments, however, was that they were performed on anaesthetised animals.

This led to the development of the push-pull perfusion system in rats (Levine and Ramirez, 1980; Levine and Ramirez, 1982) and sheep (Levine et al., 1982), thus allowing the measurement of GnRH in the portal vessels of conscious, freely moving animals. The technique comprises the implantation of an outer permanent cannula, through which an inner cannula assembly is inserted such that the tip protrudes past the outer cannula into the mediobasal hypothalamus (Levine and Ramirez, 1980). Artificial cerebrospinal fluid is "pushed" through the inner cannula

and "pulled" or drawn up between the inner and outer cannulae for measurement of GnRH immunoreactivity.

The development of this technique allowed, for the first time, the measurement of GnRH release within the mediobasal hypothalamus of conscious rats during the four stages of the oestrus cycle and after ovariectomy (Levine and Ramirez, 1982). GnRH release rates were measured throughout the afternoon and early evening hours of each day of the oestrus cycle. This study showed that a biphasic GnRH surge occurs on the afternoon of proestrus which may act to prime and then stimulate the pituitary gonadotropes thus resulting in the LH surge which occurs prior to ovulation. During oestrus, GnRH output was low and mostly undetectable, while during dioestrus I and dioestrus II small but significant rises occurred for a brief period during the late afternoon. In long-term ovariectomised rats serum LH levels, but not GnRH levels, were increased, suggesting that the negative feedback effects of ovarian steroids operate primarily at the pituitary level (Levine and Ramirez, 1982). In contrast, a recent study which used this technique to measure GnRH output at the anterior pituitary of intact and castrated male rats (Dluzen and Ramirez, 1987), rather than within the hypothalamus (Levine and Ramirez, 1982), has demonstrated that GnRH output, following castration, significantly increased due to larger and more frequent pulses arriving at the pituitary. This is supported by

earlier studies in which GnRH levels in rat portal blood were shown to increase after castration (Sherwood and Fink, 1980). The current hypothesis is, therefore, that following castration there is an increased frequency and decreased amplitude of individual GnRH signals from discrete loci within the mediobasal hypothalamus, but an increased synchrony of GnRH signals throughout the hypothalamus resulting overall in an increased frequency and amplitude of GnRH pulses in the portal blood.

The temporal relationship between GnRH and LH secretion was demonstrated in the ovariectomised sheep, by Levine et al., using this push-pull perfusion technique (1982) and, at the same time by Clarke and Cummins (1982) using a novel approach which involved the insertion of a needle into an artificial sinus anterior to the pituitary gland through which blood is removed for sampling. The GnRH pulses are measured on arrival at the pituitary and therefore reflect the sum of all the individual episodes that are secreted from the hypothalamus. The results confirmed earlier hypotheses in that each LH pulse was synchronous with a GnRH pulse.

More recently, however, it has been shown that apart from the "large" amplitude pulses of GnRH that elicit the release of LH from the pituitary, there are a number of smaller GnRH pulses that are not necessarily associated with

pulses of LH into the peripheral blood (Clarke et al., 1985; Clarke and Cummins, 1987). These "small" GnRH pulses can sustain ongoing LH synthesis without release, leading to an accumulation of releasable LH, suggesting that insertion of these "small" GnRH pulses may modify the pattern of pituitary responsiveness to "large" GnRH pulses.

GnRH RECEPTORS

The first step of GnRH action is the binding of the neuropeptide to specific, high affinity receptors located on the plasma membrane of the gonadotropes in the anterior pituitary (Clayton and Catt, 1981). The characterisation of pituitary GnRH receptor binding requirements was made possible by the development of superactive GnRH analogues (agonists) (Schally et al., 1976; Vale et al., 1976) with high affinity for the receptor (Perrin et al., 1980a) and low susceptibility to proteolytic degradation (Koch et al., 1977). The radioreceptor assay utilises the technique of displacing radiolabelled ligand from the receptor with increasing concentrations of unlabelled peptide. In early studies the labelled ligand used was native GnRH which was easily degraded and thus gave artifactual results of multiple binding sites with varying affinities for GnRH (Spona et al., 1973; Grant et al., 1973). Later studies using a GnRH agonist as the radiolabelled ligand, showed that the pituitary possessed a single class of saturable, high affinity receptors that were specific for GnRH (Clayton et al., 1979; Clayton and Catt, 1980).

Homologous regulation of GnRH receptors

The pituitary GnRH receptors are subject to homologous regulation by GnRH (Loumaye and Catt, 1982; Clayton 1982). This phenomenon is responsible for maintaining gonadotrope sensitivity during variations in hormone concentration. Fluctuations in GnRH receptor number and cell responsiveness have been observed during the rat oestrus cycle, pregnancy and lactation (Clayton et al., 1980; Clayton and Catt 1981). As mentioned previously, frequency and amplitude of GnRH pulsatility also vary during these reproductive stages suggesting a direct modulatory role of GnRH pulse frequency on receptor number. A recent study using seasonally anoestrus ewes has demonstrated that pulsatile GnRH therapy results in an up-regulation of GnRH receptors (Khalid et al., 1987). In addition, earlier studies, using castrated male rats have also shown a direct relationship between GnRH pulse frequency and receptor number, which was modulated by gonadal steroids (Garcia et al., 1984; Katt et al., 1985).

In vitro studies, in which the direct effect of GnRH was investigated, have demonstrated that following the administration of a physiological dose of GnRH there is an initial rapid loss in GnRH binding sites that results from internalisation and processing of the receptors and is independent of protein synthesis (Loumaye and Catt, 1983).

Following this is a protein-synthesis-dependent increase in GnRH receptor number (Loumaye and Catt, 1983). This increase in receptor number can also be induced by 50mM KCl (i.e. not dependent on receptor occupancy), but not by a GnRH antagonist (Loumaye and Catt, 1982; Loumaye and Catt, 1983), and must therefore be linked to activation of postreceptor events. High concentrations of GnRH (greater than 100nM) causes down-regulation of GnRH receptors, with no subsequent rise in receptor number, possibly due to massive internalisation and processing of the hormone-receptor complexes (Loumaye and Catt, 1982; Zilberstein et al., 1983).

The development of biologically active fluorescent-labelled analogues of GnRH have allowed direct visualisation of the hormone-receptor complex by image-intensified fluorescent microscopy (Hazum et al., 1980; Naor et al., 1981). During the first 10min the labelled peptide is distributed homogeneously on the surface of the gonadotropes, following which time patching, capping and internalisation occurs. More detailed studies using a radiolabelled GnRH analogue and the technique of electron microscopic autoradiography, have shown that the label is initially associated with the plasma membrane of the gonadotropes, and later with smooth membrane vesicles, lysosome-like structures and secretory granules (Hazum et al., 1985). The presence of GnRH receptor complexes in

secretory granules suggests that a portion of the receptor molecules escape degradation and are recycled.

MODE OF ACTION OF GnRH

Microaggregation of GnRH receptors

The crucial step in GnRH action on gonadotropin release, following binding to the receptor, is microaggregation of the GnRH receptors (Conn et al., 1982a; Conn et al., 1982b). Internalisation of the hormone receptor complex does not appear to be required for initial gonadotropin release since LH release occurs within seconds of incubation with GnRH while internalisation occurs only 20 to 30 min later (Hazum et al., 1980; Naor et al., 1981; Hazum et al., 1985). Further evidence comes from studies in which a GnRH agonist, although immobilised on agarose beads, was still able to stimulate LH release from pituitary cells with full efficacy (Conn and Hazum, 1981). The presence of GnRH binding sites in rat nuclear membrane preparations (Millar et al., 1983) suggests that internalisation does play some role perhaps involving other intracellular events such as receptor recycling, hormone degradation and control of LH and FSH gene expression.

Role of ionic calcium

Ionic calcium (Ca^{2+}) has been shown to fulfill all the requirements of a second messenger for GnRH action on

gonadotropin secretion. It is well established that GnRH-stimulated LH release is inhibited substantially by the removal of extracellular Ca^{2+} (Marian and Conn, 1979), while when Ca^{2+} is replaced the cells again respond to the releasing hormone (Conn and Rogers, 1979). The addition of Ca^{2+} channel blockers, such as D600, also inhibit the gonadotropin response to GnRH (Borges et al., 1983; Bates and Conn, 1984) implicating voltage-sensitive Ca^{2+} channels in the mechanism of Ca^{2+} entry. The converse also holds true, whereby addition of a Ca^{2+}-ionophore, which elevates intracellular Ca^{2+} levels by smuggling extracellular Ca^{2+} across the plasma membrane, stimulates the secretion of LH and FSH from cultured rat pituitary cells (Conn et al., 1979a).

The Ca^{2+} indicator, Quin 2, has been used to directly measure intracellular Ca^{2+} levels before and after stimulation with GnRH. Such studies have shown that GnRH does cause an increase in the cytosolic Ca^{2+} levels of gonadotroph enriched cell populations (Clapper and Conn, 1985). More recent investigations have indicated that the initial increase in intracellular Ca^{2+} caused by GnRH is due to the release of Ca^{2+} from intracellular stores and is independent of extracellular Ca^{2+} (Limor et al., 1987). Other studies, using cultured chicken pituitary cells, have gone so far as to demonstrate that the initial phase of GnRH-induced gonadotropin secretion requires not only the

mobilisation of intracellular Ca^{2+} but also the influx of extracellular Ca^{2+} via a mechanism other than a voltage-sensitive Ca^{2+} channel; possibly a receptor-operated Ca^{2+} channel (Davidson et al., 1988). The second phase of the biphasic gonadotropin response to GnRH involves the participation of both a voltage-sensitive Ca^{2+} channel and Ca^{2+} entry via another route (Davidson et al., 1988).

The evidence thus suggests that activation of the GnRH receptor by binding the ligand results in the mobilisation of cytosolic Ca^{2+} and in the opening of a receptor operated Ca^{2+} channel, followed by a voltage-sensitive Ca^{2+} channel, thus resulting in an increase in cytosolic Ca^{2+} levels and finally in gonadotropin release.

Calmodulin

Calmodulin is a multifunctional receptor for Ca^{2+}, which regulates the activity of many Ca^{2+}-dependent enzymes, including several protein kinases. GnRH has been shown to induce a redistribution or translocation of calmodulin from the cytosolic fraction to the plasma membrane (Conn et al., 1981). Subsequent studies have confirmed these findings by demonstrating the association of immunoreactive calmodulin with GnRH receptor patches (Jennes et al., 1985). This GnRH-stimulated redistribution of calmodulin appears to be of functional significance since several calmodulin inhibitors

have been shown to inhibit the effect of GnRH on gonadotropin secretion (Hart et al., 1983; Davidson et al., 1987). It therefore seems likely that the GnRH-induced elevation of intracellular Ca^{2+} enables the formation of a Ca^{2+}-calmodulin complex, which in turn regulates the activity of a number of enzymes involved in the gonadotropin synthesis and secretory processes.

Role of phospholipid turnover

Turnover of membrane phospholipids has also been implicated in the mechanism of GnRH action (Conn et al., 1987). It has been shown that phosphatidylinositol (PI) turnover is an early step in the mechanism of GnRH action (Raymond et al., 1982). PI turnover is believed to precede the opening of Ca^{2+} channels (Michell, 1975) and results in the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), both of which have second messenger functions (Berridge, 1984). It appears that IP_3 is responsible for the mobilisation of Ca^{2+} since it has been shown to stimulate release of Ca^{2+} from the endoplasmic reticulum of permeabilised cells (Berridge, 1984). It has also been suggested that phosphatidic acid, another biproduct of PI turnover, may function as an endogenous Ca^{2+}-ionophore (Putney et al., 1980). The resulting increased levels of intracellular Ca^{2+} may interact with calmodulin (Conn et al., 1981) and activate phospholipase A_2

resulting in phospholipid metabolism and the formation of free arachidonic acid (Naor et al., 1975). Some arachidonic acid metabolites may serve as activators of guanylate cyclase resulting in cGMP formation, while others are thought to initiate the processes leading to granule fusion and gonadotropin secretion (Naor et al., 1984). DAG is an activator of the ubiquitous enzyme, protein kinase C (PKC). Under resting conditions PKC is found in an inactive form in the cell cytosol or loosely associated with membranes. Activation by DAG results in tight association of the enzyme with the membrane, and a resultant increase in affinity of PKC for Ca^{2+} and phospholipid (Naor et al., 1985). PKC has a wide substrate specificity and may play a role in phosphorylating a number of intracellular proteins that are involved in the secretion and/or synthesis of the gonadotropins.

There appears to be a synergistic effect between the PKC and Ca^{2+} pathways when they are activated simultaneously (Harris et al., 1985; Naor and Eli, 1985; Davidson et al., 1987). This effect is blocked in the presence of calmodulin antagonists (Davidson et al., 1987) suggesting that it is a calmodulin-dependent process and not a direct effect of Ca^{2+} on PKC. It is hypothesised that a protein phosphorylated by PKC interacts synergistically with a calmodulin-dependent enzyme to stimulate LH secretion.

It is therefore apparent that the two major second messenger systems, namely PI turnover and Ca^{2+} mobilisation, are closely coupled and that for a full biological response of the gonadotropes to GnRH both systems are required.

Role of cyclic nucleotides

The role of cyclic nucleotides in GnRH-stimulated gonadotropin release is controversial. Several early reports have supported the concept of cyclic nucleotides as mediators of GnRH actions (Borgeat et al., 1972; Adams et al., 1979) while others have not (Conn et al., 1979b; Benoist et al., 1981). Recent studies have shown that GnRH-stimulated LH release could be facilitated by cAMP (Sundberg et al., 1976; Turgeon and Waring, 1986), although cAMP did not directly stimulate LH secretion (Sundberg et al., 1976) or did so only after a considerable time lag (Magness et al., 1981; Turgeon and Waring, 1986). More recently it has been shown, that although cAMP was not involved in the acute release of LH and FSH, it appeared to play a pivotal but indirect role in the protein synthesis dependent phase of release by stimulating de novo protein synthesis of the gonadotropins and/or the synthesis of proteins that are integrally related to the secretion of these hormones (Bourne and Baldwin, 1987a; Bourne and Baldwin, 1987b).

In view of the initial conflicting reports regarding the role of cAMP, it was suggested that cGMP may be the intracellular second messenger since GnRH also stimulated an increase in intracellular cGMP levels (Snyder et al., 1980). The use of an inhibitor of GTP formation, however, demonstrated that GnRH-stimulated LH release was not blocked in the absence of measurable amounts of cGMP (Naor and Catt, 1980). The role of this cyclic nucleotide is thus not yet clear.

OTHER NEUROPEPTIDE REGULATORS OF GONADOTROPIN SECRETION

As is the case in other hormonal systems, gonadotropin secretion is under the control of more than one modulator. While GnRH is the major regulator, there are a number of other neuropeptides such as substance P (Kerdelhue et al., 1983) and the endogenous opioids (Blank et al., 1985; Blank et al., 1986) which also have a direct effect on gonadotropin secretion from the pituitary.

Substance P

Substance P (SP) is an undecapeptide which, although first described in the gastrointestinal tract (Von Euler and Gaddum, 1931), has since been shown to be widely distributed within the peripheral and central nervous systems of mammals (Nicholl et al., 1980; Aronin et al., 1983; Pernow, 1983). Fluctuations in hypothalamic SP occur during the rat oestrus cycle (Kerdelhue et al., 1982), and it has been shown to affect the release of gonadotropins (Kerdelhue et al., 1978; Vijayan and McCann, 1979) and prolactin (Vijayan and McCann, 1979; Eckstein et al., 1980) in vivo, thus suggesting that the peptide plays a role in control of reproductive function. More recently, SP has been found to inhibit GnRH-stimulated LH and FSH release from cultured rat anterior pituitary cells (Kerdelhue et al., 1979; Kerdelhue et al.,

1982) and to bind to specific, high affinity binding sites in rat anterior pituitary membranes (Kerdelhue et al., 1982; Kerdelhue et al., 1985), implicating a direct modulatory role for SP on gonadotropin secretion. Further evidence for a physiological role of SP comes from in vivo studies which have demonstrated that after administration of SP antisera to rats, there was an increase in LH and FSH (Kerdelhue et al., 1979; Kerdelhue et al., 1982). The administration of SP to female rhesus monkeys, however, had no effect on gonadotropins, while it induced a significant increase in prolactin secretion (Eckstein et al., 1980). This suggests that the peptide may not have a role in the control of reproduction in primates. It would therefore be of interest to determine whether SP exerts an effect on gonadotropin secretion in man.

Endogenous opioid peptides

Opioid agonists when administered acutely inhibit gonadotropin secretion in man (Azizi et al., 1973; Santen et al., 1975) and other species (Cicero et al., 1979). These acute effects of opioids on LH and FSH release are mediated mainly at the hypothalamic level (Wilkes and Yen, 1981). However, in view of the fact that the anterior pituitary has a 250-fold greater concentration of B-endorphin than the hypothalamus (Bloom et al., 1978), a number of studies have been carried out to investigate the possible direct effects

of opioid peptides on gonadotropin secretion from pituitary cells. The results of these investigations have shown that gamma-endorphin (Matteri and Moberg, 1985), human B-endorphin (Matteri and Moberg, 1985; Blank et al., 1986; Snchez-Franco and Cacicedo, 1986) and morphine (Blank et al., 1985; Blank et al., 1986) are capable of directly inhibiting GnRH-stimulated gonadotropin secretion at the pituitary level. Specific, saturable opiate binding sites have also been described in pituitary tissue (Blank et al., 1985; Blank et al., 1986), suggesting that the opiates exert their effects by binding to specific receptors on the gonadotropes. These direct inhibitory actions of endogenous opioids at the pituitary level may be of particular importance in alterations of reproductive function during times of stress, strenuous athletic exercise and other conditions accompanied by secretion of large amounts of pituitary opioids.

OBJECTIVES OF THIS STUDY

GnRH and its superactive analogues are being used clinically in the treatment of various endocrine disorders, such as endometriosis, precocious puberty, infertility and prostatic cancer, as well as being investigated as possible contraceptives (Sandow 1983b). To date, these superactive analogues have been selected for clinical use on the basis of their relative activities in rats. The first objective of the present study was therefore to determine whether the human and rat pituitary receptors have similar properties, using the radioreceptor assay technique, thus vindicating the use of rat receptor binding studies as a reasonable index of GnRH analogue activity in man.

The second objective of this study was to establish a system for culturing human pituitary cells from post-mortem tissue in order to investigate the neuropeptide regulation of gonadotropin secretion, at the pituitary level, in man. In particular, the biological potencies of a number of GnRH analogues and the mechanism of GnRH-stimulated gonadotropin secretion was investigated, as well as the direct regulatory effects of substance P and a range of novel peptides, which are potentially processed from the GnRH precursor. To date, such studies have been restricted to laboratory and domestic animals. In vivo studies on hypothalamic peptide regulation of pituitary hormone secretion are complicated by the simultaneous interaction of various modulators, such as

neurotransmitters, steroids and other hormones. In vitro studies using cultured pituitary cells are, therefore, advantageous in that the direct effects of individual hormones may be investigated.

CHAPTER 2

CHARACTERISATION OF THE HUMAN GnRH RECEPTOR

INTRODUCTION

The initial step in GnRH action is the binding of the peptide to specific receptors on the plasma membrane of pituitary gonadotropes. The binding affinity of GnRH analogues for rat receptors is a good index of gonadotropin-releasing activity of these analogues (Clayton and Catt, 1980; Clayton and Catt, 1981; Loumaye et al., 1982) and receptor binding characteristics are, therefore, predictors of biological activity. The specificity and binding affinity of the human GnRH receptor, however, are unknown. The characterisation of the human receptor may have important implications since GnRH and its superactive analogues are being used clinically in the treatment of endometriosis, precocious puberty, infertility, and prostatic cancer, as well as being investigated as possible contraceptives (Sandow, 1983b). For this reason it is of value to determine whether the human and rat GnRH receptors have similar properties, thus vindicating the use of rat receptor binding assays as a reasonable index of GnRH analogue activity in the selection of superactive analogues for use in human studies. I have therefore developed methodology for investigating the GnRH receptor in membranes isolated from human pituitaries obtained at autopsy and compared the characteristics with those of the rat pituitary receptor.

MATERIALS AND METHODS

Hormones

Synthetic GnRH was obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland); GnRH agonist 1, (D-Ala^{6},N$^{\alpha}$-MeLeu^{7},Pro^{9}NEt)-GnRH, from Dr J E Rivier, The Salk Institute (San Diego, CA); GnRH agonist 2, (D-Trp^{6},Trp^{7})-GnRH, from Dr R deL Milton, University of Cape Town Medical School (Cape Town, South Africa); GnRH agonist 3, (Trp^{7})-GnRH, from Dr R deL Milton; GnRH agonist 4, (His^{5})-GnRH, from Dr R deL Milton; GnRH antagonist 1, (Ac-D-Nal(2)^{1},D-α-Me-4-ClPhe^{2},D-3-Pal^{3},D-Arg^{6},D-Ala^{10}NH$_2$)-GnRH, from Dr R Roeske, Indiana University School of Medicine (Indianapolis, IN); GnRH antagonist 2, (D-pGlu^{1},D-Phe^{2},D-Trp^{3,6})-GnRH, from Dr J E Rivier; GnRH antagonist 3, (Ac-D-Nal(2)^{1},D-α-Me-4-ClPhe^{2},D-Trp^{3},D-Arg^{6},D-Ala^{10}NH$_2$)-GnRH, from Dr R Roeske; chicken GnRH II , (His^{5},Trp^{7},Tyr^{8})-GnRH, from Dr R deL Milton; GnRH 1-5 from Dr R deL Milton; (D-Ala^{1})-somatostatin-14 from Dr D H Coy, Tulane University (New Orleans, LA); and synthetic porcine TRH from Bechman Instruments Inc. (Palo Alto, CA).

Binding studies

Human anterior pituitaries were obtained from nine healthy women (25-35 yr of age) between 12 and 48h after accidental death. They were immediately snap frozen in

liquid nitrogen and stored at -70°C until use. The membranes were prepared by homogenising the tissue in buffer A (10 mM Tris HCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% BSA, pH7.4), centrifuging at 1000 x g for 15 min, and centrifuging the resulting supernatant for 30 min at 10.000 x g (Hazum, 1981a). The membrane pellet was resuspended in the same buffer. Rat anterior pituitaries were obtained from adult male Long-Evans rats (350-450 g) and were processed as described for the human.

All binding studies were performed in triplicate in buffer A. Unlabelled peptides (50 µl) and 50 µl labelled peptide (60.000-70.000 cpm) were added to 400 µl membrane suspension (0.1 human or 0.4 rat pituitary equivalents/ml), mixed, and incubated for 150 min at 4°C. The contents of these tubes were transferred with 3 ml buffer B (0.04 M sodium phosphate, 0.15 M NaCl, 1.0% BSA, pH 7.4) onto Whatman GF/C filters (presoaked for 24 h in this buffer) and the filters washed three times with 3 ml buffer B. Bound radioactivity was determined in a γ-spectrometer. Total binding (6-10%) was determined in the absence of unlabelled peptide and nonspecific binding (2.5-3.5%) by the addition of 10^{-6} M or 10^{-7} M GnRH, agonist or antagonist.

GnRH agonist 1 was labelled with $Na^{125}I$ (Kerdelhue et al., 1973) and purified by QAE Sephadex A-25 chromatography (Nett and Adams, 1977). The specific activity, as determined

by self-displacement in the rat pituitary receptor assay, was 959-1066 μCi/μg. The proportion of [^{125}I]GnRH agonist 1 bound by excess pituitary membranes (35-40%) was taken into account in the Scatchard analysis of data. ED_{50} (effective dose required to displace 50% of the labelled agonist) values were determined using an Allfit program of DeLean et al. (1978). K_ds (dissociation constants) were estimated from Scatchard analysis and from ED_{50}s using the formular of Cheng and Prusoff (1973). The binding studies were performed with pools of membranes from two to three pituitaries, and each study included a displacement curve of the GnRH agonist 1 as a reference point for determining relative potencies. These findings were then confirmed by examining binding of all the analogs in a single study.

The validity of the GnRH binding assay using partially purified membranes was previously established by comparison of the relative potencies of a range of GnRH analogues in binding to membranes and dispersed pituitary cells in the rat (Loumaye et al., 1982). Similarly, dispersed human pituitary cells specifically bound [^{125}I]GnRH agonist (data not shown). High performance liquid chromatography analysis of GnRH, GnRH analogues and [^{125}I]GnRH agonist revealed no measurable breakdown of the peptides when incubated with the pituitary membranes under the receptor assay conditions (150 min at 4°C). GnRH was partially degraded (about 20%) by membranes when incubated at 37°C.

Studies on the effects of cations on [^{125}I]GnRH agonist 1 binding were carried out as described above, with the exception that EDTA was omitted from the assay buffer and various concentrations of $CaCl_2$, $MgCl_2$, NaCl and KCl were included. Ionic concentrations were confirmed by atomic absorption spectrometry. The amount of Ca^{2+} bound by BSA in the assay buffer was calculated to be less than 3%. Specific binding in the presence of the monovalent and divalent cations was compared with control tubes lacking these ions.

The effect of pH on [^{125}I]GnRH agonist 1 binding to human and rat pituitary membranes was also investigated using 10 mM HEPES containing 1 mM EDTA, 1 mM DTT and 0.1% BSA as the assay buffer for pH 6.0 to 7.5 and buffer A (described above) for pH 7.5 to 8.5.

Ligand-immunoblotting technique for receptor visualisation

The procedure was as previously described (Eidne et al., 1985). Membranes were prepared from human and rat pituitaries in 20 mM Tris HCl, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM phenylmethane-sulfonyl fluoride, pH 8.0 and solubilised in 125 mM Tris maleate, 160 mM NaCl, 40 mM β-D-octylglucopyranoside, pH 6.0, at 4°C for 10 min. The

solubilised membranes were immediately electrophoresed at 4°C on a 0.1% sodium dodecyl sulphate, polyacrylamide 6-12% linear gradient gel (Laemmli, 1970) at 25 mA for 45 min and then at 35 mA for 60 min. The electrophoresed proteins were Western-blotted onto nitrocellulose (Bio-Rad Laboratories, Richmond, CA) in 50 mM Tris, 200 mM glycine, and 20% (vol/vol) ethanol at 195 mA for 2 h at 23-25°C. Protein molecular weight markers (Pharmacia Fine Chemicals, Uppsala, Sweden) transferred onto the nitrocellulose were stained with Amido black. Nitrocellulose strips with separated membrane proteins were incubated with buffer C (50 mM Tris HCl, 90 mM NaCl, 50 mg/ml BSA, fatty acid free (Miles Laboratories, Inc., Elkhart, IN) pH 8.0) at 23-25°C for 1 h. They were then incubated with buffer C containing 10^{-6} M GnRH for 45 min and washed several times with 50 mM Tris HCl, 90 mM NaCl, 5 mg/ml BSA, pH 8.0. The strips were incubated for a further 30 min in 10 ml buffer C containing 200 µl antiserum 1076, which is directed towards the middle region of GnRH (King and Millar, 1982), or non-immune serum followed by a series of washes and then a 30 min incubation with horseradish peroxidase-conjugated sheep antirabbit immunoglobulin G (N.L.Cappel Laboratories, Cochranville, PA) at a concentration of 50 µl in 25 ml buffer C. The strips were washed and the GnRH receptors visualised by incubating with 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO) (0.5 mg/ml in 50 mM Tris HCl, 150 mM NaCl, pH 8.0 and

0.0003% H_2O_2) for 5 min. The reaction was terminated by several washes in distilled water.

RESULTS

Affinity and specificity of the human GnRH receptor

Membranes prepared from human anterior pituitaries removed at autopsy bound [^{125}I]GnRH agonist 1 with high affinity and specificity (Fig. 2.1A). Scatchard analysis indicated that the binding was saturable and involved a single class of high affinity sites. The K_d of the human

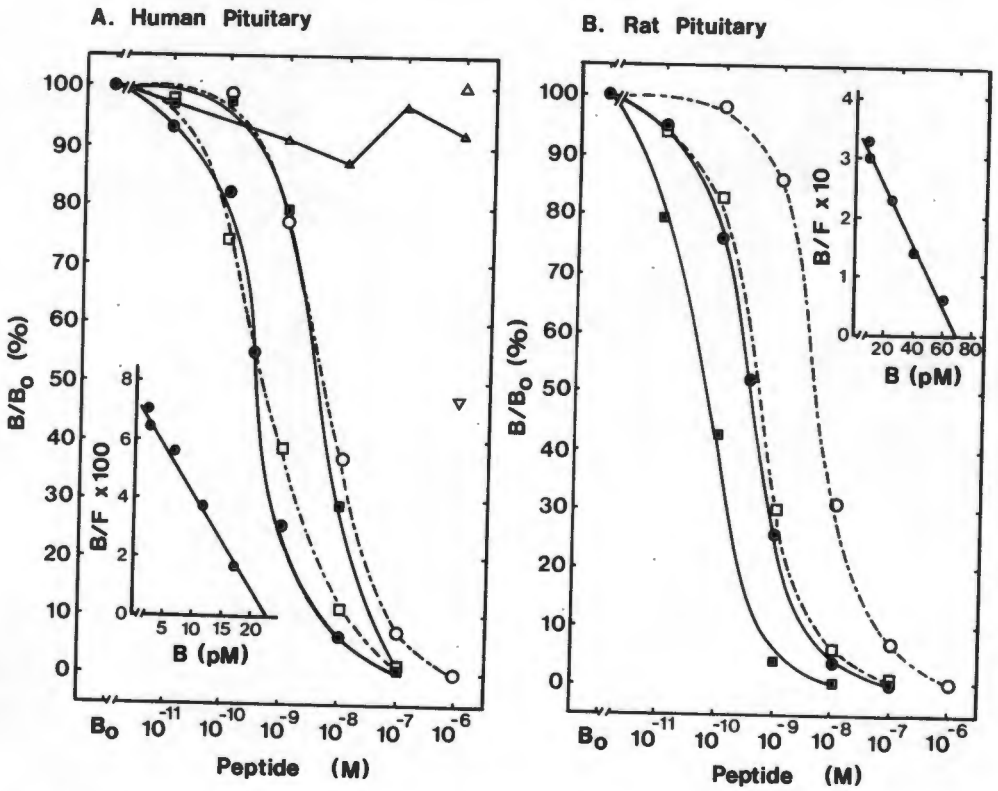

Fig. 2.1 Displacement of [^{125}I]-GnRH agonist binding to human (A) and rat (B) pituitary membranes by unlabelled GnRH (O), GnRH agonist (●), GnRH antagonist 1 (□), GnRH antagonist 2 (■), GnRH 1-5 (▲), (D-Ala^1)-somatostatin (▽), and TRH (△). Inserts show Scatchard analysis of the binding of GnRH agonist.

TABLE 2.1. Apparent dissociation constants of GnRH, agonist 1 and antagonists 1 and 2 binding to human and rat pituitary membranes

	Human pituitary membranes (12–48h post-mortem) (nM)	Rat pituitary membranes (0h post-mortem) (nM)	Rat pituitary membranes (48h post-mortem) (nM)
GnRH	4.81±1.53	4.71±1.14	2.04±0.85
GnRH agonist 1	0.32±0.14	0.31±0.03	0.20±0.10
GnRH antagonist 1	0.32±0.26	0.40±0.04	0.66±0.46
GnRH antagonist 2	4.21±0.79*	0.09±0.02	0.06±0.01

All values represent mean ± SEM of binding studies carried out in triplicate using pooled membranes from 3 cadavers or 12 rats. K_ds were calculated by Scatchard analysis for GnRH agonist 1 and by the method of Cheng and Prusoff (1973) for GnRH and GnRH antagonists 1 and 2. Binding studies were carried out on three different pools of pituitary homogenates. The mean K_d values for the human GnRH receptor in the three pituitary homogenate pools ranged from 2.8–9.0nM for GnRH, 0.11–0.40nM for GnRH agonist 1, 0.14–0.32nM for GnRH antagonist 1, and 2.8–4.6nM for GnRH antagonist 2.

*Significantly different from K_d for rat pituitary ($p<0.005$) as determined by the unpaired Student's t test.

pituitary receptor, calculated from the ED_{50} (Cheng and Prusoff, 1973), was 4.81 nM, which was similar to that of the rat receptor (4.71 nM) as determined in parallel studies (Fig. 2.1; Table 2.1). GnRH agonist 1 and GnRH antagonist 1 had higher affinities for the human receptor (K_ds of 0.32 nM) than GnRH, and these values were similar to those of the rat (Fig. 2.1; Table 2.1). GnRH antagonist 2, however, bound the human GnRH receptor with a much lower affinity than the rat receptor (K_ds of 4.21 nM and 0.09 nM respectively).

There was no difference in the binding of GnRH, agonist 1 and both antagonists 1 and 2 to freshly prepared rat pituitary membranes and membranes prepared 48 h post-mortem (Table 2.1), which was the maximum period that human material was obtained post-mortem. Rat pituitary GnRH receptor number was unchanged 24 h post-mortem (Table 2.2). At 48 h post-mortem, receptor number was significantly diminished and by 96 h it was 25% that of the fresh material.

A further five GnRH analogues were tested in the human and rat GnRH receptor binding assays, of which three were shown to have significantly lower affinities for the human receptor than the rat receptor (Table 2.3).

TABLE 2.2. GnRH receptor number of rat pituitary membranes prepared at various times post-mortem

Time post-mortem (h)	GnRH agonist bound (fmol/pituitary)
0	217 ± 5
24	247 ± 17
48	148 ± 2*
96	53 ± 9*

All values represent mean ± SEM of studies performed in triplicate using pooled membranes prepared from 3 rats.
* Significantly different from receptor number of freshly prepared rat pituitary membranes ($p<0.001$) as determined by the unpaired Student's t test.

TABLE 2.3. Apparent dissociation constants of GnRH, agonists 2, 3 and 4, antagonist 3, and chicken II GnRH binding to human and rat pituitary membranes

	Human pituitary membranes (nM)	Rat pituitary membranes (nM)
GnRH	4.90 ± 0.81	4.14 ± 0.22
Chick GnRH II	95.3 ± 4.7*	28.7 ± 2.4
GnRH agonist 2	5.50 ± 0.60*	0.59 ± 0.16
GnRH agonist 3	6.60 ± 2.0	2.04 ± 0.22
GnRH agonist 4	9.89 ± 5.91	3.16 ± 0.35
GnRH antagonist 3	11.4 ± 2.3**	1.00 ± 0.20

All values represent mean ± SEM of binding studies carried out in duplicate or triplicate using pooled membranes from 3 cadavers or 16 rats.
* Significantly different from K_d for rat pituitary ($p<0.01$) as determined by the unpaired Student's t test.
** Significantly different from K_d for rat pituitary ($p<0.05$) as determined by the unpaired Student's t test.

The effect of cations on human and rat GnRH receptor binding

In the presence of 0.6 mM Mg^{2+} [^{125}I]GnRH agonist 1 binding to the human receptor was reduced to a greater degree than occurred with the rat receptor (Fig. 2.2A). This differential effect of Mg^{2+} was even more pronounced at 6.0 mM. A greater degree of inhibition of binding to the human receptor also occurred in the presence of 1.0 and 4.0 mM Ca^{2+}. The presence of 10 mM Na^{+} or 100 mM K^{+} also resulted in inhibition of [^{125}I]GnRH agonist 1 binding, which was significantly greater in the human than in the rat (Fig. 2.2B). At more physiological concentrations (100 mM Na^{2+} and 10 mM K^{+}), however, the inhibition of binding due to the presence of these monovalent cations was similar for both species.

The effect of pH on human and rat GnRH receptor binding

The concentration of H^{+} had a similar effect on receptor binding in the human to that in the rat (Fig. 2.3); the optimum pH for binding being between 7.0 and 7.5 for both species.

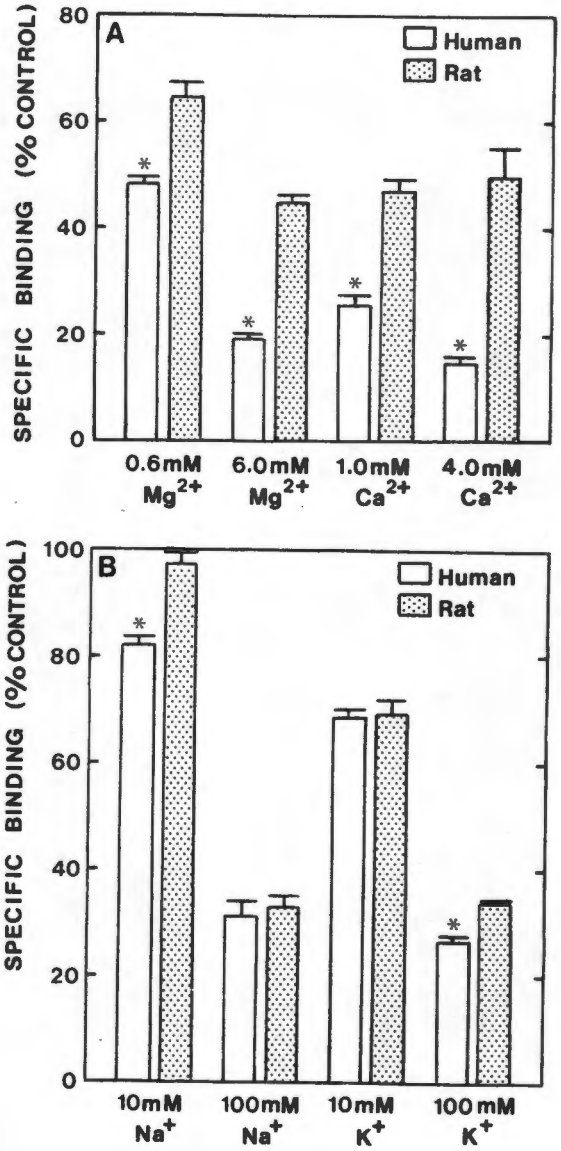

Fig. 2.2 The effect of divalent (A) and monovalent (B) cations on [^{125}I]-GnRH agonist binding to human and rat pituitary membranes, expressed as per cent of specific binding in the absence of ions.
* Significantly different from specific binding to rat GnRH receptor ($p<0.01$) as determined by the unpaired Student's t test.

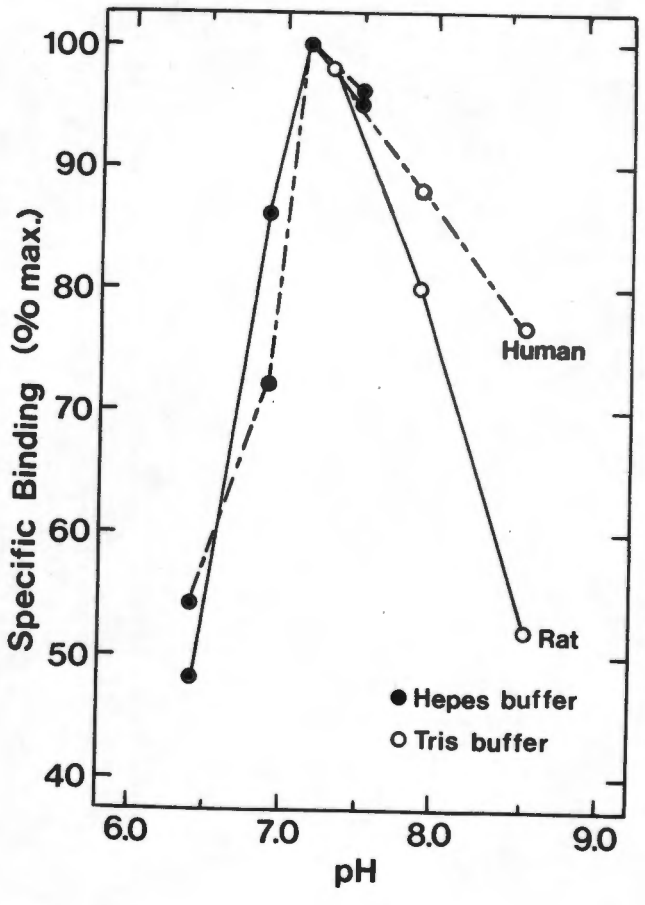

Fig. 2.3 The effect of pH on [^{125}I]-GnRH agonist binding to human (broken line) and rat (solid line) pituitary membranes. HEPES buffer was used for pH 6.0 to 7.5 and Tris buffer for pH 7.5 to 8.5.

Determination of the molecular size of human and rat GnRH receptors

The molecular sizes of the human and rat GnRH receptors were studied using a ligand-immunoblotting technique (Eidne et al., 1985). A single GnRH binding protein was present in human pituitary membranes which was consistently (as determined on 11 separate occasions) of higher molecular weight than that of the rat (Fig. 2.4A). The molecular weights, determined from a plot of log-molecular weight of molecular weight markers vs. relative migration, were 64,000 daltons for the human receptor and 60,000 daltons for the rat receptor. The receptor bands were not visible when the nitrocellulose strips were incubated with non-immune rabbit serum (Fig. 2.4B) or with GnRH antisera requiring those regions of GnRH which bind the receptor (data not shown).

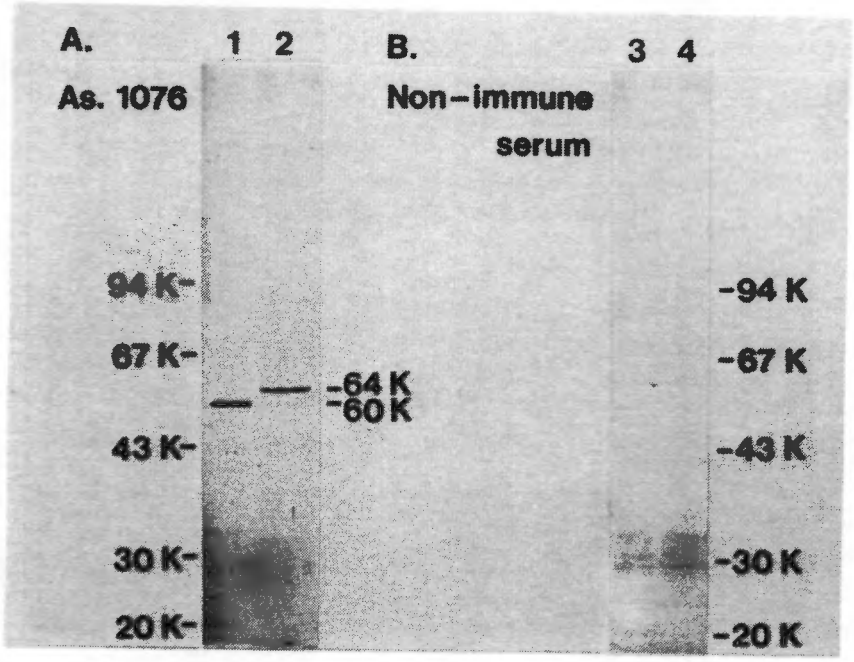

Fig. 2.4 Electrophoretic separation and ligand-immunoblotting visualisation of human (lanes 2 and 4) and rat (lanes 1 and 3) pituitary GnRH receptors using (A), antiserum 1076, which is directed towards the middle region of GnRH (King and Millar, 1982); and (B), non-immune serum.

DISCUSSION

The human GnRH receptor has been shown to bind GnRH with a high affinity and specificity which was similar to what has been observed in the rat in this and previous studies (Clayton and Catt, 1980; Clayton and Catt, 1981; Millar et al., 1982a; Perrin et al., 1980b). While the human receptor also bound GnRH agonist 1 and GnRH antagonist 1 with similar affinities to the rat receptor, it differed from the rat in its binding of GnRH antagonist 2, indicating a possible species difference. Human and rat ovarian GnRH receptors have also been found to differ in their affinity for GnRH analogues (Pieper et al., 1981; Popkin et al., 1983).

To ensure that this difference in the binding affinities of the human and rat GnRH receptors for GnRH antagonist 2 was not due to post-mortem changes in the human receptor, pituitary membranes prepared from groups of three rats kept under similar conditions to those in the mortuary were compared with fresh material. There was no difference in the binding of any of the GnRH analogues to freshly prepared rat pituitary membranes and membranes prepared 48 h post-mortem.

In the light of these findings, a further five GnRH analogues were selected, by virtue of their relatively poor

binding affinities for the sheep GnRH receptor, and tested in the human receptor binding assay. Three of the analogues tested, namely GnRH agonist 2, GnRH antagonist 3 and chicken GnRH II, bound the human GnRH receptor with significantly lower affinities than they bound the rat receptor, thus further substantiating the contention that there is a species difference between the two receptors. In order to fully characterise the ligand binding requirements of the human GnRH receptor, the binding affinities of several more analogues, with different hydropathic and steric properties, need to be investigated.

The human GnRH receptor also exhibited differences from the rat receptor in the inhibitory effects of cations on [^{125}I]GnRH agonist 1 binding. The divalent cations caused a significantly greater inhibition of binding to the GnRH receptor in the human than in the rat. Differences in the effects of monovalent cations on binding in the human and the rat were not as marked as the binding differences in the presence of the divalent cations. Diminished binding to the rat GnRH receptor in the presence of various cations has been previously described (Marian and Conn, 1980), and reported to be due to a decrease in receptor affinity rather than receptor number. In view of the scarcity of human material it was not possible to determine whether the decreased binding to human pituitary membranes was due to changes in receptor number or affinity.

In spite of these cationic differences, the concentration of H^+ had a similar effect on receptor binding in the human to that in the rat; the optimum pH for binding being between 7.0 and 7.5 for both species.

The apparent difference in rat and human pituitary receptor binding characteristics may be due to molecular differences in the receptors which affect charge, conformation and hydrophobicity. The molecular sizes of the rat and human receptors were therefore studied using a ligand-immunoblotting technique (Eidne et al., 1985). The molecular weights were 64,000 daltons for the human receptor and 60,000 daltons for the rat receptor. The latter estimation is in agreement with a previous report (Hazum, 1981b). These receptor bands may represent only the binding component of a receptor protein complex, as a recent study using target size analysis of the rat pituitary GnRH receptor gave a molecular size of 136,346 daltons (Conn and Venter, 1985). This is approximately twice that in our studies, which were carried out under denaturing conditions. The GnRH binding protein bands were specific by a number of criteria. They were not visible when the nitrocellulose strips were incubated with non-immune rabbit serum or with GnRH antisera requiring those regions of GnRH which bind the receptor.The differences in apparent molecular size between the human and rat GnRH binding proteins may be due to

differences in glycosylation or amino acid composition of the processed receptor proteins. It is unlikely that these changes are due to the presence of different degradative enzymes in the respective tissues since the membranes were prepared in the presence of phenylmethane-sulfonyl fluoride, a potent serine protease inhibitor. A more detailed study would be required to determine the relevance of this molecular size difference with regard to the binding characteristics of the human and rat GnRH receptors.

These studies have established that the human pituitary contains a single class of high affinity specific binding sites for GnRH. A previous study reported low binding of [^{125}I]GnRH analogue to human pituitary membranes (Clayton and Huhtaniemi, 1982), but the nature of the binding sites was not established. In this study, the pituitaries obtained at autopsy from premenopausal women had high specific binding (3.1-6.7% of total [^{125}I]GnRH analogue) comparable to that found in the rat, whereas the male pituitaries had lower binding (0.7-1.0% of total [^{125}I]GnRH analogue) (data not shown). No other differences in binding related to cause, or time after death were found.

The present investigation has demonstrated the feasibility of using human pituitaries obtained post-mortem for characterising GnRH binding sites. It is clear that although the human pituitary receptor is similar to the rat

receptor in its affinity for GnRH and certain analogues, it differs from the rat receptor in its interaction with at least four GnRH analogues, its ionic requirements for monovalent and divalent cations, and the molecular size of the GnRH binding protein.

Detailed characterisation of the specificity of the human pituitary GnRH receptor may provide data of value in predicting which GnRH analogues are appropriate for clinical application, thereby possibly reducing the number of clinical investigations employed in the selection of potentially useful GnRH analogues.

CHAPTER 3

CULTURE OF FUNCTIONALLY ACTIVE HUMAN PITUITARY CELLS FOR THE INVESTIGATION OF GONADOTROPIN REGULATION

INTRODUCTION

In vivo studies on hypothalamic peptide regulation of pituitary hormone secretion in man are complicated by the simultaneous interaction of various modulators such as neurotransmitters, steroids and other hormones (Pohl and Knobil, 1982; Sandow, 1983; Chappel, 1985; Kalra, 1985; Kalra and Kalra, 1985; Plant, 1986). In vitro studies using cultured pituitary cells are therefore advantageous in that the direct effect of individual hormones, hormone analogs and neurotransmitters may be investigated. To date, such studies have been restricted to laboratory and domestic animals. The lack of culture systems for normal human pituitary cells and the resultant dearth of information on the mechanisms of pituitary hormone regulation in vitro encouraged us to explore the possibility of culturing functionally viable human pituitary cells from post mortem tissue. This chapter describes methodology for the culture of human pituitary cells which respond to all of the hypothalamic releasing hormones, and the results of studies of the mechanisms and kinetics of gonadotropin secretion and the relative potencies of GnRH analogs.

MATERIALS AND METHODS

Hormones

GnRH, chicken GnRH II, (His^5,Trp^7,Tyr^8)-GnRH, GnRH agonist 1, (D-Ala^6, N$^{\alpha}$MeLeu^7, Pro^9 NEt)-GnRH, GnRH agonist 2, (D-Trp^6,Trp^7)-GnRH, GnRH agonist 3, (Trp^7)-GnRH, and GnRH agonist 4, (His^5)-GnRH, were synthesized by Dr R C deL Milton, University of Cape Town Medical School (Cape Town, South Africa); GnRH antagonist 1, (Ac-D-Nal(2)^1,D-α-Me-4-ClPhe^2,D-3-Pal^3,D-Arg^6,D-Ala^{10}NH$_2$)-GnRH, was supplied by Dr R Roeske, Indiana University School of Medicine (Indianapolis, IN); GnRH antagonist 2, (D-pGlu^1,D-Phe^2,D-Trp^{3,6})-GnRH, was supplied by Dr J E Rivier, The Salk Institute (San Diego, CA); and GnRH antagonist 3, (Ac-D-Nal(2)^1, D-α-Me-4-ClPhe^2,D-Trp^3,D-Arg^6,D-Ala^{10}NH$_2$)-GnRH, by Dr R Roeske. Synthetic TRH was obtained from Beckman Instruments Inc.(Palo Alto, CA); GHRH(1-29)NH$_2$ from Dr D H Coy, Tulane University (New Orleans, LA); and ovine CRH was from Dr N Ling, The Salk Institute (San Diego, CA).

Preparation of cells

Human anterior pituitary glands were obtained at autopsy from 33 men, 20 to 40 years old, within 24 h after accidental death. The glands were placed into ice cold sterile HEPES buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4,

10 mM glucose, 360 μM $CaCl_2$, 1% (w/v) BSA, 25 mM n-2-hydroxyethyl piperazine ethane sulphonic acid (HEPES) pH 7.2). After removal of the capsule, the tissue was diced and incubated in HEPES buffer containing 0.9% collagenase (142 U/mg, CLS II Worthington Biochemicals, Freehold, NJ) and 1.8 mg% DNAase (Miles Biochemicals, Eppindust, South Africa) at 37°C for 1 h (Vale et al., 1972). The cells were harvested by centrifugation at 450 x g for 7 min and washed twice with HEPES buffer before final suspension in minimal essential medium (MEM with Hank's salts, Gibco, Paisley, Scotland) containing 10% foetal calf serum, 0.1 mM glutamine and 100 U/ml penicillin (Glaxo (Pty) Ltd., Wadeville, R S A), 100 mg/ml streptomycin (Novo Industries, Johannesburg, R S A) and 100 mg/l neomycin (Upjohn (Pty) Ltd., Isando, R S A). Cells obtained from between 1 and 6 pituitary glands were pooled and distributed into tissue culture dishes (Falcon # 3046 6-well multidish) such that the dishes contained approximately 10^5 dispersed cells per well (4 ml medium). The dishes were incubated at 37°C, 95% humidity and 5% CO_2. Cells sufficient for 6 wells were usually obtained from one pituitary.

Rat anterior pituitary glands were obtained from adult male Long-Evans rats (350-450g) within minutes of death by decapitation and were processed as described above. Cells sufficient for 3 wells usually were obtained from one rat pituitary.

Incubation of cells

After 3 to 4 days in culture, the cells were washed 5 times with MEM, and test substances added to duplicate or triplicate wells. After 3 hours of incubation the medium was removed and added to tubes containing BSA, to prevent absorption of gonadotropins, such that the final concentration of BSA in the samples was 0.1%. They were then centrifuged at 400 x g and the supernatants removed and stored at -20°C until assay for LH and FSH, and in the TRH, GHRH and CRH studies for TSH, prolactin, GH and ACTH.

To study the kinetics of gonadotropin secretion during a 1 h continuous incubation with 100 nM GnRH, the cells were incubated with GnRH for 5 min following which the medium was removed and replaced with fresh medium containing GnRH. This step was repeated at 5 min intervals for the first 20 min, and at 10 min intervals for the following 40 min. At the end of 60 min the cells were stimulated with 1 μM A23187 for 5 min.

To investigate GnRH desensitization, the cells were incubated with increasing doses of GnRH for 2 hours and the medium then was removed. Fresh medium containing the same doses of GnRH was added and the cells incubated for a further 2 hours. The medium was again removed. At the end of

the 4 h incubation period the cells in each well were lysed with 1 ml 1% Triton X-100 in water for 1 hour at room temperature and the lysate analysed for LH and FSH immunoreactivity.

Each experiment was repeated with similar results on at least 2 occasions. Each table and figure shows the results of a single experiment performed on a single batch of cultured cells, unless stated otherwise.

Radioimmunoassays

All samples from an individual experiment were analysed at the same time in each assay and all assays were performed in duplicate. Human LH and FSH were measured by RIA using reagents supplied by the National Hormone and Pituitary Program, Baltimore MD. The intraassay CVs for these assays were 2.9 and 4.9 % respectively. Rat LH also was measured by RIA using NHP reagents (intraassay CV 5.9 %). TSH was measured by RIA using kits obtained from Amersham, Radiochemical Centre, United Kingdom; PRL and GH by RIA using kits obtained from Serono, Woking, United Kingdom; and ACTH by RIA using kits obtained from CIS, Gif-sur-Yvette, France. The intraassay CVs for TSH, PRL, GH and ACTH were 4.5, 6.2, 13.5 and 6.2 % respectively.

Immunocytochemistry

The cultured human pituitary cells were fixed in methanol for 5 min, washed twice with PBS (0.01 M Na2HPO4, 0.15 M NaCl, pH 7.6) and drained. The cells then were covered with porcine serum diluted 1:20 in PBS for 10 min and drained. Test cells were incubated with rabbit anti-LH serum or rabbit anti-FSH serum (Dako Corp, Denmark) diluted 1:200 in PBS for 30 min at room temperature, control cells were incubated with porcine serum alone. The cells then were washed with PBS for 30 min, drained and incubated with porcine antirabbit IgG (Dako Corp) diluted 1:20 in PBS for 30 min at room temperature. The cells were drained, washed with PBS for a further 30 min and incubated with horseradish peroxidase/rabbit-antihorseradish peroxidase (Dako) diluted 1:20 in PBS for 30 min. After washing with PBS for a further 30 min, the gonadotropes were visualised by incubation for approximately 5 min with 3,31-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St Louis, MO) (0.5 g/l in PBS, and 1% H_2O_2). The reaction was terminated by several PBS washes. The cells were counterstained with hematoxylin for 5 min after which they were washed in water, dehydrated in graded ethanol (70 to 100%), cleared in xylol (Merck, Darmstadt, W. Germany) and mounted in Entellan (Merck).

Statistical analysis

The data points shown in the figures and text are the mean ± SEM of results from triplicate wells of a single experiment assayed in duplicate. Intra-experiment statistical significances were evaluated by analysis of variance or unpaired Student's t test. EC_{50} (concentration of agonist required to stimulate 50% of maximum gonadotropin release) and IC_{50} (concentration of antagonist required to inhibit 50% of GnRH-stimulated gonadotropin release) values were determined using an Allfit dose-response analysis program modified from the Allfit program of De Lean et al. (1978).

RESULTS

Sixty four percent of the human pituitary cultures were successful, in that some cell attachment occurred within 2 days after initiation of the culture. Cell viability prior to culture, as assessed by trypan blue exclusion, ranged from 40-50%, while after 3-4 days in culture the viability of the attached cells was in excess of 95% as non-viable cells did not attach. Immuno-peroxidase staining of the

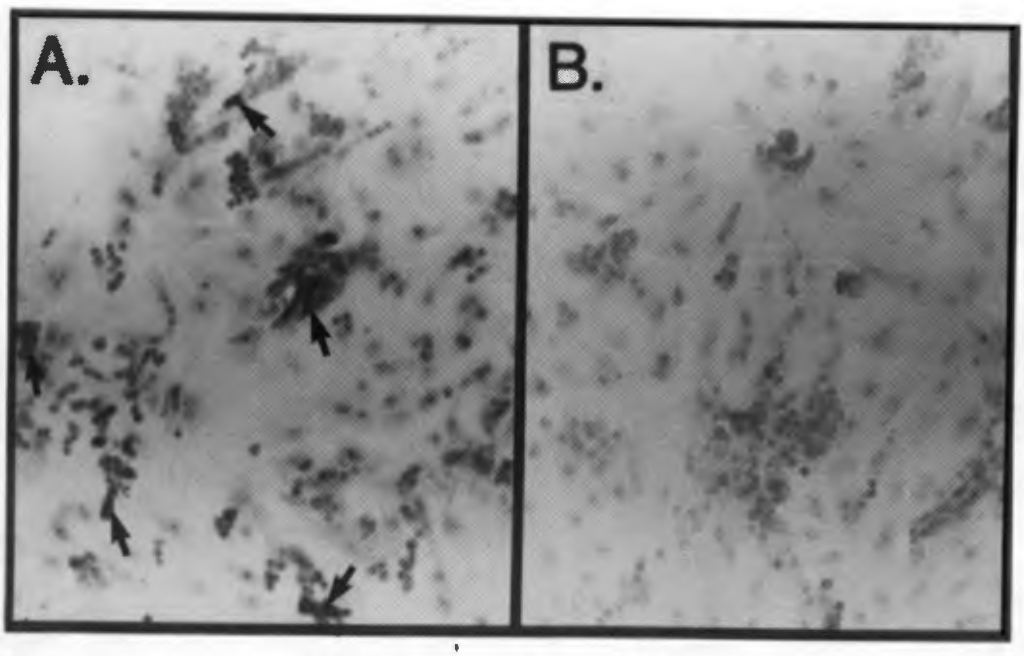

Fig. 3.1 Immunocytochemical staining of the cultured human pituitary cells. Experimental cells were immuno-peroxidase stained using a hLH antiserum and counterstained with hemotoxylin (A). Positively stained cells or cell clusters are indicated by the arrows. Of the total pituitary cell population 15.2 $\pm$ 2.4 % contained LH. Control cells (B) were incubated with non-immune serum and showed no positive staining.

cultured pituitary cells revealed that, as in the rat (Childs et al., 1983), 15.2 ± 2.4% (mean ± SEM, n=5) of the cells contained LH (Fig. 3.1A) and 12.3 ± 1.1% (n=5) of the cells contained FSH. Double staining to determine the percentage of bihormonal cells was not done. Control cells, which were incubated with non-immune serum, showed no positive staining (Fig. 3.1B).

Effect of the hypothalamic releasing hormones on hormone secretion from cultured human pituitary cells

GnRH, TRH, GHRH and CRH significantly stimulated release of LH and FSH, TSH and PRL, GH and ACTH, respectively (Table 3.1). There was an additional stimulation of TSH when the releasing hormones were added simultaneously.

TABLE 3.1. Effects of hypothalamic releasing hormones on pituitary hormone release from cultured human pituitary cells

Releasing hormone	LH (IU/l)	FSH (IU/l)	TSH (mU/l)	PRL (μg/l)	ACTH (pM)	GH (μg/l)
Basal	264±28.8	14.9±0.8	8.4±1.1	4.8±0.2	152±53.9	84.4±4.4
GnRH 100nM	718±47.6*	70.5±3.7*	6.8±0.5	5.0±0.2	217±12.7	88.1±9.1
TRH 100nM	297±19.9	17.2±0.7	14.3±0.8*	7.4±0.4*	212±6.2	94.8±0.4
CRH 100nM	267±11.6	17.2±1.3	8.9±0.1	5.6±0.2	274±15.3*	99.2±2.5
GHRH 100nM	373±31.0	23.0±1.0	8.6±0.3	6.4±0.2	211±4.0	146.9±3.5*
GnRH+TRH+ CRH+GHRH	787±28.8*	58.6±3.4*	23.5±1.6*+	8.4±0.6*	278±12.8*	156.3±12.1*

All values represent mean ± SEM of triplicate 3 h incubations using pooled cells from 3 cadavers.

* Significantly different from basal release ($p<0.01$), as determined by analysis of variance.

+ Significantly different from TRH-stimulated TSH release ($p<0.01$), as determined by analysis of variance.

The biological potencies of GnRH and GnRH analogues in cultured human and rat pituitary cells

GnRH stimulated LH release from cultured human pituitary cells in a dose-dependent manner, the EC_{50} being 0.95 nM (Fig. 3.2A; Table 3.2). Its potency in rat pituitary cells was similar (EC_{50} 2.0 nM) (Fig. 3.2B; Table 3.2). GnRH agonists 1 and 2, and chicken GnRH II were as active in stimulating GnRH release from cultured human pituitary cells (EC_{50}s of 0.08 nM, 0.53 nM and 16.8 nM respectively) (Fig. 3.3A; Table 3.2) as from rat pituitary cells (EC_{50}s of 0.35 nM, 0.80 nM and 30.0 nM respectively) (Table 3.2). GnRH agonist 3 was marginally more active in the human (EC_{50} of 0.22 nM) (Fig. 3.3 A; Table 3.2) than in the rat (EC_{50} of 1.30 nM) (Table 3.2), while GnRH agonist 4 was more than 10-fold more active in the human (EC_{50}(human) of 0.57 nM; EC_{50}(rat) of 8.20 nM) (Fig. 3.3 A; Table 3.2). GnRH antagonists 1, 2 and 3 were less active in inhibiting GnRH-stimulated gonadotropin release from human pituitary cells (IC_{50}s of 2.60 nM, 72.0 nM and 23.6 nM respectively) (Fig. 3.3 B; Table 3.2) than from rat pituitaries (IC_{50}s of 0.52 nM, 18.7 nM and 0.47 nM respectively) (Table 3.2), with the most pronounced difference being exhibited by GnRH antagonist 3 which was 50-fold less active in the human than in the rat. The biological potencies of all the analogues

tested for stimulating or inhibiting FSH release from the cultured human pituitary cells were similar to those for LH release (Table 3.2).

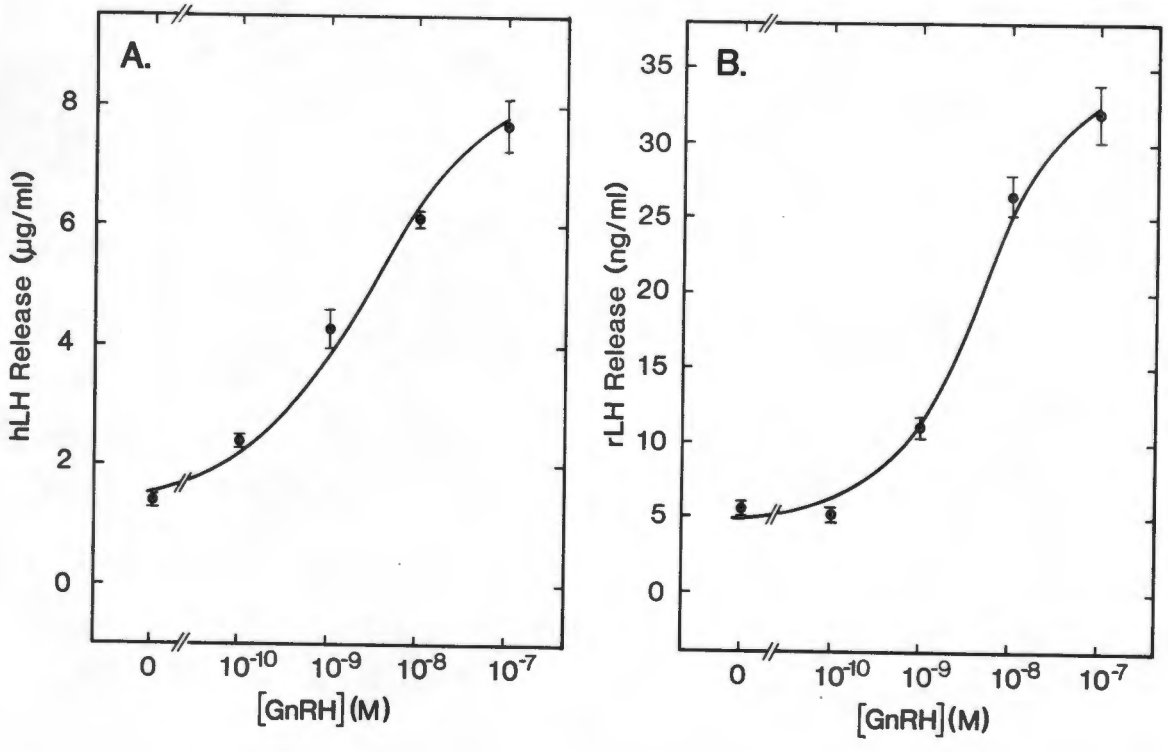

Fig. 3.2 Effect of increasing doses of GnRH on LH secretion from human (A) and rat (B) pituitary cells. All values represent mean ± SEM of triplicate 3 h incubations using pooled pituitary tissue from 3 cadavers or 10 rats. The EC_{50} for GnRH-stimulated LH release was 1.25 nM for human and 2.45 nM for rat cells.
1 µg/ml hLH is equivalent to 277 IU/l and 1 ng/ml rLH is equivalent to 1 µg/l.

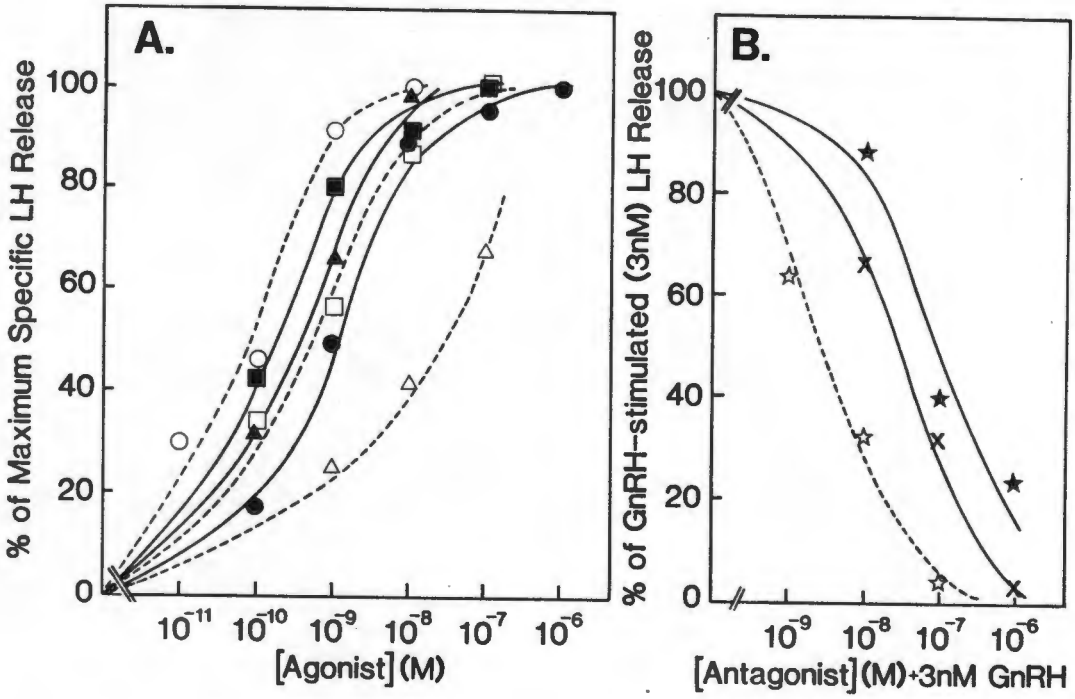

Fig. 3.3 A. Effect of increasing doses of GnRH (●), chicken II GnRH (△), GnRH agonist 1 (○), GnRH agonist 2 (▲), GnRH agonist 3 (■) and GnRH agonist 4 (□) on LH secretion from cultured human pituitary cells. B. Effect of increasing doses of GnRH antagonist 1 (☆), GnRH antagonist 2 (★) and GnRH antagonist 3 (×) on 3nM GnRH-stimulated LH secretion from cultured human pituitary cells.
All values represent the mean of triplicate 3 h incubations using pooled pituitary tissue from 2 to 5 cadavers, and are expressed as percent of maximum release in the case of the agonists, and as percent of 3nM GnRH-stimulated LH release in the case of the antagonists. Basal release was subtracted from stimulated values in each case. Each dose response curve is the result of an individual experiment.

TABLE 3.2. Gonadotropin releasing or release-inhibiting activity of GnRH and several GnRH analogues in the human and the rat.

	LH release EC_{50} human (nM)	LH release EC_{50} rat (nM)	FSH release EC_{50} human (nM)
GnRH	0.95 ± 0.25	2.00 ± 0.28	0.72 ± 0.16
Chick II GnRH	16.8 ± 8.2	30.0 ± 0.50	17.5 ± 3.50
Agonist 1	0.08 ± 0.02	0.35 ± 0.04	0.07 ± 0.03
Agonist 2	0.53 ± 0.10	0.80 ± 0.06	0.92 ± 0.38
Agonist 3	0.22 ± 0.03	1.30 ± 0.30*	0.36 ± 0.11
Agonist 4	0.57 ± 0.17	8.20 ± 0.19**	0.40 ± 0.15

	LH inhibition IC_{50} human (nM)	LH inhibition IC_{50} rat (nM)	FSH inhibition IC_{50} human (nM)
Antagonist 1	2.60 ± 0.10	0.52 ± 0.12**	3.10 ± 1.50
Antagonist 2	72.0 ± 8.0	18.7 ± 11.3*	82.0 ± 18.0
Antagonist 3	23.6 ± 1.6	0.47 ± 0.07**	25.3 ± 3.8

All values represent mean ± SEM of EC_{50}s or IC_{50}s obtained from 2 separate 3 h experiments which were carried out in triplicate using cells from 13 cadavers or 20 rats.

* Significantly different from ED_{50} for human pituitary cells ($p<0.1$) as determined by the unpaired Student's t test.

** Significantly different from ED_{50} for human pituitary cells ($p<0.01$) as determined by the unpaired Student's t test.

THe kinetics of LH and FSH secretion in response to stimulation with 100 nM GnRH

Stimulation of the cells with 100 nM GnRH for 60 minutes resulted in a biphasic release of LH (Fig. 3.4A) and FSH (Fig. 3.4B). The initial release phase was rapid, occurring during the first 3 to 4 minutes, while the plateau phase was more protracted; continuing for the ensuing 40 minutes. Stimulation with the Ca^{2+}-ionophore, A23187, at the end of the 60 min of GnRH stimulation elicited additional LH and FSH release but the response to the ionophore was significantly less than that in those cells which had not been stimulated with GnRH. Thus, GnRH stimulation leads to partial desensitization of the gonadotropes. The kinetics of both LH and FSH release were similar, so that the ratio of LH to FSH release was constant during both secretory phases (Fig. 3.4C).

Ca^{2+} and protein kinase C in gonadotropin secretion

GnRH-stimulated LH and FSH secretion from cultured human pituitary cells was Ca^{2+}-dependent. In the presence of 3 mM EGTA the gonadotropin response to GnRH was abolished (Fig. 3.5A). EGTA had no effect on LH and FSH release in the

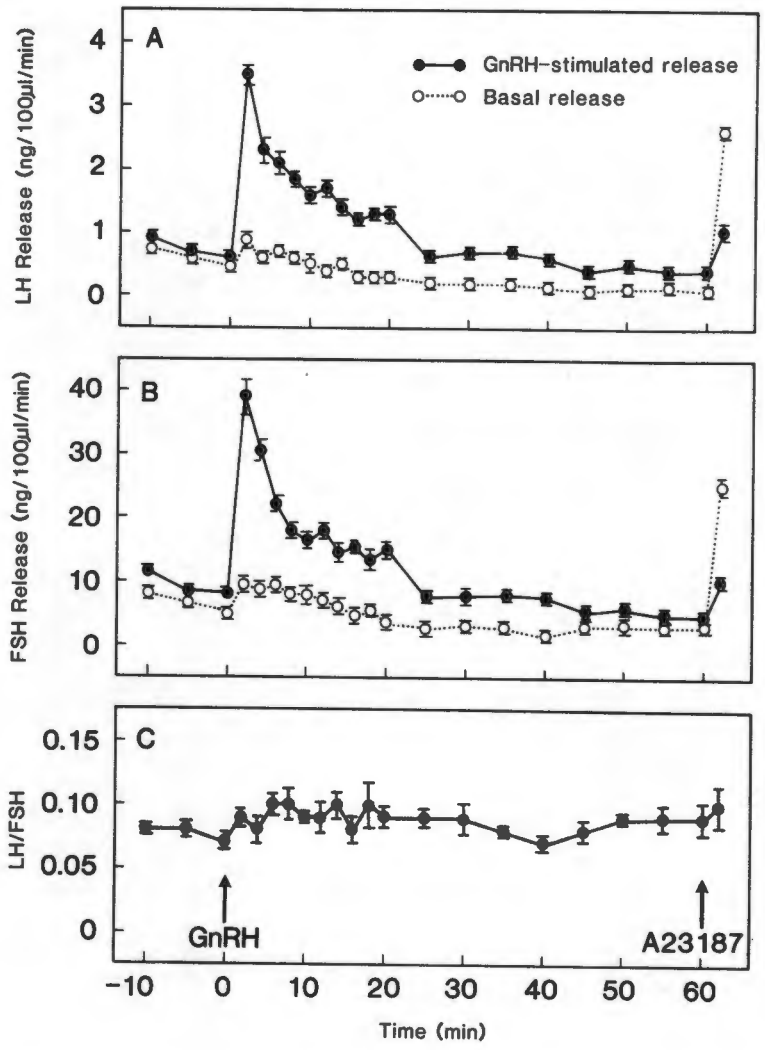

Fig. 3.4 Effect of GnRH (100 nM) stimulation on LH (A) and FSH (B) secretion from cultured human pituitary cells as a function of time. The cells were incubated with 100 nM GnRH from 0-60 min and then with 1 μM A23187. GnRH-stimulated gonadotropin secretion is represented by the solid circles and line and basal secretion by the open circles and broken line.
All values are mean $\pm$ SEM of triplicate incubations using cells from 2 cadavers.
All GnRH-stimulated values were significantly higher than the unstimulated ($p < 0.01$). The ratio of LH/FSH released (C) remained constant. The A23187-stimulated value was significantly less ($p<0.01$) in GnRH-stimulated cells than in unstimulated cells.
1 ng/100 μl LH is equivalent to 2.77 IU/l and 1 ng/100 μl FSH is equivalent to 0.53 IU/l.

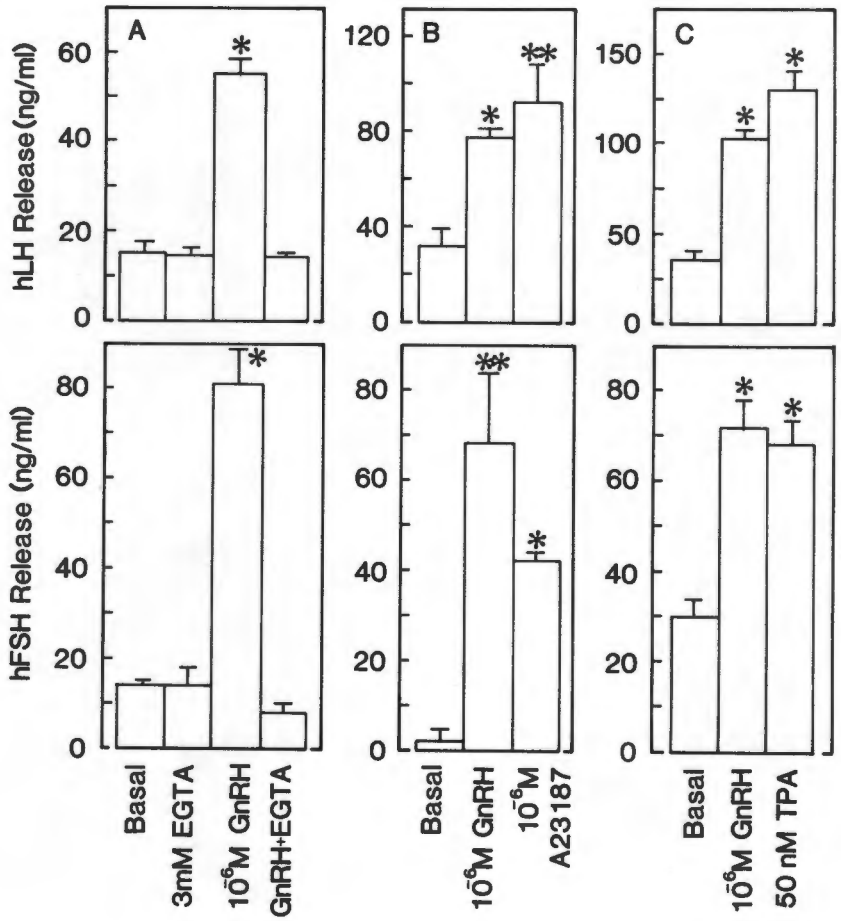

Fig. 3.5 Effect of 3 mM EGTA (A), 1 μM A23187 (B) and 50 nM TPA (C) on GnRH-stimulated LH and FSH secretion from cultured human pituitary cells.
All values represent mean $\pm$ SEM of triplicate 3 h incubations using cells from 9 cadavers.
Significantly different from basal * $p<0.01$, ** $p<0.05$ as determined by analysis of variance.
1 ng/ml LH is equivalent to 0.277 IU/l and 1 ng/ml FSH is equivalent to 0.053 IU/l.

TABLE 3.3 Effect of TPA and dbcAMP on [^{3}H]-leucine incorporation in cultured human pituitary cells

Treatment	[^{3}H]-leucine (% basal)
Basal	100.0 ± 0.24
50nM TPA	92.5 ± 0.19
1mM dbcAMP	101.0 ± 0.17

All values represent mean ± SEM of duplicate 3 h incubations using pooled cells from 6 cadavers.

absence of GnRH. The Ca^{2+}-ionophore, A23187, caused significant LH and FSH release at a dose of 1 μM (Fig. 3.5B). The phorbol ester, TPA, at a dose of 50 nM also stimulated LH and FSH release suggesting that phospholipid turnover and protein kinase C may play a role in GnRH-stimulated gonadotropin release (Fig. 3.5C). This effect of TPA was unlikely to be due to a non-specific toxic effect of the phorbol ester on the cells since incorporation of [^{3}H]-leucine into protein was unaffected (Table 3.3).

The role of cAMP in gonadotropin secretion

Dibutyryl cAMP (dbcAMP) caused a dose-dependent increase in LH and FSH secretion from cultured human pituitary cells (Table 3.4). The stimulatory effect of the cyclic nucleotide was not secondary to a toxic effect on the cells since incorporation of [^{3}H]-leucine into protein was not altered by cAMP at these doses (Table 3.3).

TABLE 3.4 Effect of dbcAMP on LH and FSH secretion from cultured human pituitary cells

	LH release (μg/ml)	FSH release (μg/ml)
Basal	1.88 ± 0.24	0.60 ± 0.11
10^{-6}M GnRH	8.56 ± 0.68*	2.94 ± 0.36*
0.1mM dbcAMP	3.24 ± 0.19**	1.70 ± 0.10*
1.0mM dbcAMP	6.20 ± 0.20*	2.74 ± 0.18*

All values represent mean ± SEM of triplicate 3 h incubations using pooled cells from 4 cadavers.
Significantly different from basal values * $p<0.01$. ** $p<0.05$. as determined by analysis of variance.

Desensitisation of human pituitary cells to subsequent stimulation with GnRH

GnRH-stimulated gonadotropes became desensitized to subsequent stimulations with GnRH. Following an initial 2 hour incubation with GnRH in doses ranging from 0.1 to 100 nM, the LH released during a second 2 hour incubation with GnRH in the same dose was reduced by 50-100% (Fig. 3.6A). The inhibition of FSH release ranged from 63-92% (Fig. 3.6B). The total LH and FSH cell content declined to 52% (Fig. 3.6A: Fig. 3.7A) and 51% (Fig. 3.6B: Fig. 3.7B) respectively. after 100 nM GnRH stimulation. When the initial incubation with GnRH was carried out under Ca^{2+}-free conditions, the cells still became desensitised to a further stimulation with GnRH (Fig. 3.7 A and B). The degree of

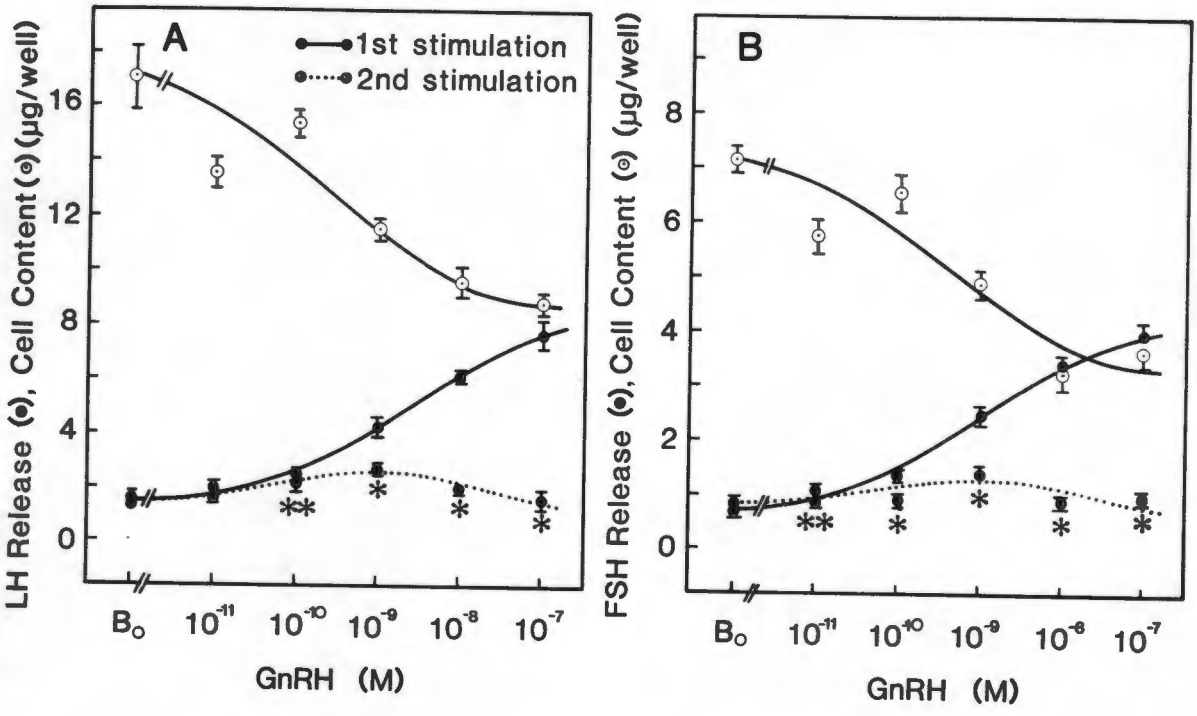

Fig. 3.6 The effect of two 2 h incubations with increasing doses of GnRH on LH (A) and FSH (B) secretion from cultured human pituitary cells. An initial 2 hour incubation with 0.01 to 100 nM GnRH (solid line) was followed by a second incubation (broken line) after which the cells were lysed and LH and FSH cell content measured (⊙).
All values represent mean ± SEM of triplicate incubations using cells from 3 cadavers.
Significantly different from initial 2h incubation * $p<0.01$, ** $p<0.05$ as determined by analysis of variance.
1 µg/well LH is equivalent to 0.277 IU/well and 1 µg/well FSH is equivalent to 0.053 IU/well.

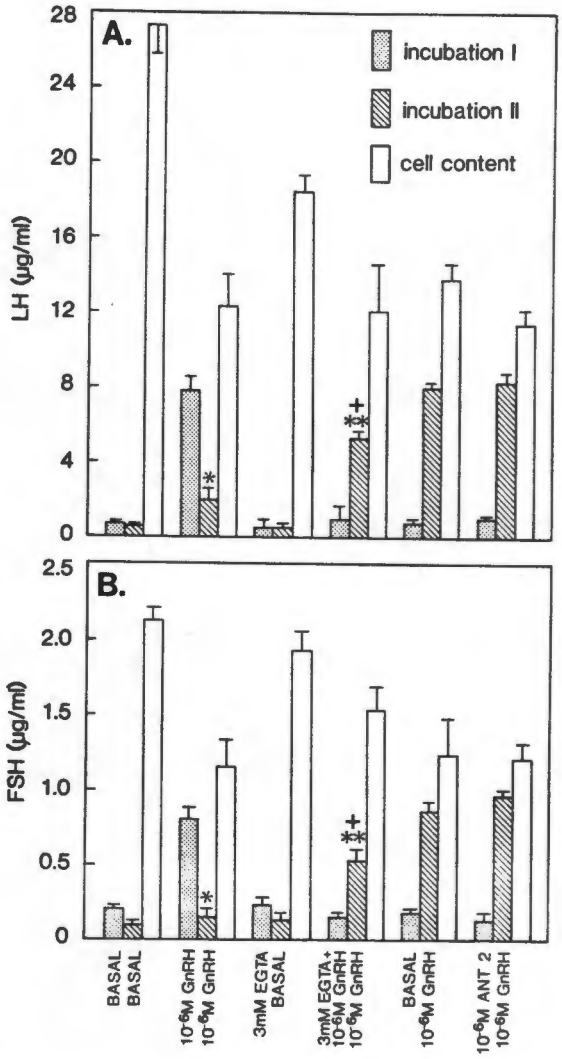

Fig. 3.7 Effect of an initial 2 h incubation with 10^{-6}M GnRH, in the presence or absence of Ca^{2+}, or 10^{-6}M GnRH antagonist 2, followed by a second 2 h incubation with 10^{-6}M GnRH, on LH (A) and FSH (B) secretion from cultured human pituitary cells. Incubation I is represented by the stippled bars, incubation II by the hatched bars and cellular LH and FSH content, at the end of the experiments, by the open bars.
All values represent mean ± SEM of triplicate incubations using pooled pituitary tissue from 3 cadavers.
Significantly different from gonadotropin release in response to 10^{-6}M GnRH from previously untreated cells;
* $p<0.001$, ** $p<0.01$.
Significantly different from gonadotropin release in response to a second 2 h stimulation with 10^{-6}M GnRH;
+ $p<0.001$
1 µg/ml LH is equivalent to 277 IU/l and 1 µg/ml FSH is equivalent to 53 IU/l.

desensitisation was, however, not as great as that in cells that were initially incubated with GnRH in the presence of Ca^{2+}. The human pituitary cells were not desensitised to GnRH when the initial incubation was carried out with a GnRH antagonist (Fig. 3.7 A and B).

Effect of pulsatile administration of GnRH on LH and FSH secretory profiles

The administration of 4 min pulses of GnRH at varying pulse intervals of 45 min, 90 min and 180 min resulted in similar profiles of LH (Fig. 3.8A) and FSH (Fig. 3.8B) secretion. After three repeated pulses of GnRH, however, the cells became desensitised and failed to respond to a fourth pulse.

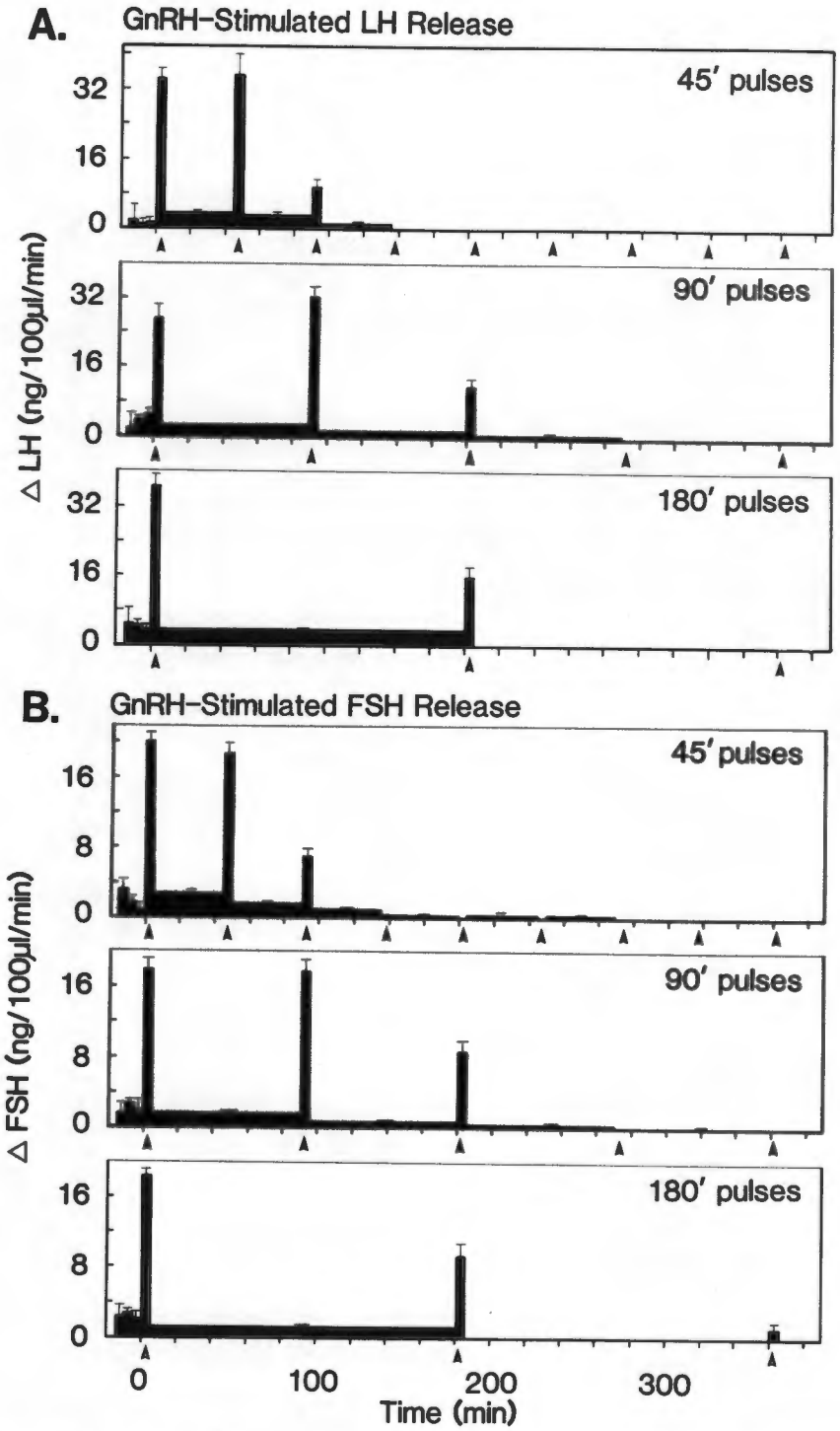

Fig. 3.8 Effect of 4 min pulses of GnRH, administered at pulse frequencies of 45 min, 90 min and 180 min, on LH (A) and FSH (B) secretion from cultured human pituitary cells. All values represent mean ± SEM of triplicate incubations using pooled pituitary tissue from 2 cadavers. Basal values were subtracted from GnRH-stimulated values in each case. 1 ng/100 µl/min LH is equivalent to 2.77 IU/l/min and 1 ng/100 µl/min FSH is equivalent to 0.53 IU/l/min.

DISCUSSION

Human pituitary cells dispersed from glands obtained at autopsy and cultured for 3 to 4 days released LH and FSH, TSH and PRL, GH and ACTH in response to 100 nM doses of GnRH, TRH, GHRH and CRH, respectively. When the releasing hormones were added simultaneously, the release of TSH was greater than that stimulated by TRH alone, suggesting that a releasing hormone other than TRH can augment the response to TRH. This was not investigated further as it is beyond the scope of this thesis.

GnRH stimulated LH release from human pituitary cells in a dose dependent manner, the EC_{50} being 0.95 nM, which is of the same order of magnitude as that for the rat (2.0 nM) in this and previous studies (Loumaye and Catt, 1983; Leiser et al., 1986). The human pituitary GnRH receptor has previously been found in our laboratory, to have a similar K_d for GnRH as the rat receptor (Wormald et al., 1985; chapter 2). GnRH agonists 1 and 2, chicken GnRH II were as active in stimulating LH release from human pituitary cells as from the rat. GnRH agonists 3 and 4, however, were more active in the human than in the rat, while GnRH antagonists 1, 2 and 3 were less active in the human pituitary bioassay than in the rat.

There are therefore definite differences between the human and rat GnRH receptors which involve not only the ability of the receptors to recognise and bind different GnRH analogues, but also their ability to be activated by these analogues. For example, chicken GnRH II and agonist 2 bound the human GnRH receptor with relatively poor affinities compared with the rat, but were as active in stimulating gonadotropin release in the human as in the rat. Recent studies carried out in our laboratory have demonstrated that the sheep GnRH receptor shares these characteristics of the human receptor for binding and being activated by these two particular analogues (unpublished data). Further differences between the human and rat receptors are evident from these bioassay data: GnRH agonists 3 and 4 bound both human and rat receptors with a similar affinities but were relatively poor agonists in the rat; GnRH antagonists 2 and 3 bound the human receptor with lower affinities than those of the rat and consequently were less active in inhibiting GnRH-stimulated gonadotropin secretion from human pituitary cells than from rat, although GnRH antagonist 2 was not as active in the rat as predicted from receptor studies; GnRH antagonist 1, although bound by the human and rat receptors with similar affinities, was a relatively poor antagonist in the human.

Receptor binding studies and bioassays using animal tissue are therefore not always good predictors of GnRH

analogue activity in man. The establishment of a system for culturing functionally active human pituitary cells may thus be of greater use in the selection of GnRH analogues for clinical use.

Stimulation with 100 nM GnRH resulted in a biphasic release of both LH and FSH from the cultured human pituitary cells. The lesser response of the human gonadotropes to GnRH after the initial brisk response was apparently not due entirely to depletion of gonadotropin stores since these cells then responded somewhat to Ca^{2+}-ionophore. The demonstration of decreased cellular LH and FSH content in the 4 h GnRH stimulation studies, however, suggests that gonadotropin depletion may have contributed to the diminished response in the 60 min study. The kinetics of LH secretion in the human are similar to what has been observed in the chicken and in the rat (Smith et al., 1987; Davidson et al., 1988). The kinetics of both LH and FSH release from human pituitary cells were identical, in contrast to in vivo studies where the rise in FSH in response to GnRH administration is slower but longer lasting than that of LH (Millar et al., 1982). This is most likely due to the modulatory effects of gonadal hormones and inhibin. Kinetic considerations, such as the longer half life of FSH, cannot account for the slower rise in FSH.

GnRH-stimulated gonadotropin secretion from the human pituitary was Ca^{2+}-dependent, since release was inhibited by the presence of 3 mM EGTA, as demonstrated earlier in rat (Bates and Conn, 1984; Limor et al., 1987), chicken (Davidson et al., 1987) and sheep (Magness et al., 1981) gonadotropes. The Ca^{2+}-ionophore, A23187, caused significant gonadotropin release, indicating that an influx of extracellular Ca^{2+} will elicit gonadotropin secretion by human, as well as rat (Bates and Conn, 1984; Limor et al., 1987) and chicken (Davidson et al., 1987) gonadotropes. The phorbol ester, TPA, an activator of protein kinase C, also caused significant LH and FSH release, suggesting that phospholipid turnover is a component in the mechanism of gonadotropin secretion. Limitations on availability of human material did not allow in depth studies but the data indicate that the human gonadotrope has similar signal transduction systems to those defined in the rat (Conn, 1986).

Incubation of the human pituitary cells for 3 h with dbcAMP resulted in a specific, dose-dependent increase in LH and FSH secretion. Although the kinetics of this effect were not investigated, this finding is in accordance with what has previously been found in the sheep (Magness et al., 1981) and more recently in the rat (Turgeon and Waring, 1986; Bourne and Baldwin, 1987a; Bourne and Baldwin, 1987b) that cAMP, while not involved in the acute phase of LH and

FSH release, appears to play a pivotal role in the second phase of release by stimulating de novo synthesis.

The human pituitary cells became less sensitive during prolonged stimulation with GnRH. This phenomenon of pituitary desensitization may be due to down-regulation of GnRH receptors after binding GnRH (Zilberstein et al., 1983), uncoupling of post-receptor mediating mechanisms (Keri et al., 1983; Smith et al., 1983), or due to depletion of certain cellular LH and FSH stores. It is likely that depletion of gonadotropin stores does contribute to this phenomenon, since following stimulation with 100 nM GnRH total cellular LH was reduced to 52% and FSH to 51% of that of the unstimulated cells. In a separate experiment, when the initial incubation with GnRH was carried out under Ca^{2+}-free conditions (i.e. under conditions where GnRH receptor binding occurs with no LH or FSH release), the cells still became desensitised to a subsequent stimulation with GnRH. The degree of desensitisation was, however, not as great as that in cells that were initially incubated with GnRH in the presence of Ca^{2+} suggesting that the desensitisation phenomenon under normal conditions is partly due to depletion of intracellular stores of LH and FSH, and partly due to some other Ca^{2+}-independent mechanism. The human pituitary cells were not desensitised to GnRH when the initial incubation was carried out with a GnRH antagonist, indicating that desensitisation requires receptor activation

in addition to receptor occupancy. Studies on GnRH-induced desensitisation in other mammalian (Smith and Vale, 1981; Keri et al., 1983; Smith and Conn, 1983; Conn et al., 1982a; Conn et al., 1982b) and chicken (King et al., 1986) pituitary cells have shown that desensitisation requires receptor microaggregation and internalisation and that receptor occupancy by GnRH antagonists is not sufficient to produce desensitisation.

It has been well documented that variations in GnRH pulse frequency in vivo alter the ratio of LH to FSH released from the pituitary (Wildt et al., 1981; Clarke et al., 1984; Gross et al., 1987). In order to determine whether LH and FSH can be controlled differentially by varying the pattern of GnRH stimulation alone, free of the effects of other regulatory hormones which are present in vivo, cultured human pituitary cells were pulsed with 4 min pulses of 3 nM GnRH at varying frequencies of 45 min, 90 min and 180 min. The cells became desensitised to GnRH after 2 pulses and failed to respond after the third. In rats it has been shown that pulsing perifused pituitary cells with low doses of GnRH results in stabilisation of the cells which are capable of responding to the releasing hormone for up to 24 h (Liu and Jackson, 1984). The human pituitary cells thus appear to be unable to synthesise new stores of LH and FSH. The cells are also not able to undergo self-priming by low doses of GnRH (data not shown) as has been described in

animal cells (Morris et al., 1986). This is possibly due to post-mortem changes in the tissue, since the human pituitary cells are not cultured immediately after death, as are animal cells, but anything from 6 to 24 h later. Alternatively, this phenomenon may merely be a characteristic of the static culture system.

These studies have established that human pituitary tissue, obtained within 24 hours of autopsy, may be cultured for 3 to 4 days and stimulated with the hypothalamic releasing hormones to secrete LH, FSH, TSH, PRL, GH and ACTH. Human gonadotropes are similar to those of the rat in their response to GnRH and a number of GnRH analogues, but differed in their response to two GnRH agonists and three GnRH antagonists. These results support our previous contention, from receptor binding studies (Wormald et al., 1985), that rat gonadotropes may not be useful for studying GnRH analogue activity to identify superactive analogs for use in human studies.

This system of culturing human pituitary cells from autopsy tissue is likely to be particularly useful for investigating the regulation of gonadotropic hormone secretion in man.

CHAPTER 4

OTHER PEPTIDE REGULATORS OF GONADOTROPIN SECRETION

SECTION 1

NOVEL PEPTIDE REGULATORS WITHIN THE HUMAN GnRH PROHORMONE

INTRODUCTION

GnRH is synthesised and secreted by hypothalamic neurons. Although in early reports an enzyme template mechanism was suggested to underlie the biosynthesis of GnRH (Johansson et al.,1972; Johansson et al., 1973), the identification of higher molecular weight immunoreactive GnRH in sheep and pig hypothalamus (Millar et al., 1977; Millar et al., 1978; Millar et al., 1981) and human placenta (Gautron et al., 1981) implicated conventional ribosomal biosynthesis of a prohormonal form. This was further supported by the in vivo demonstration of hypothalamic GnRH biosynthesis (Kochman et al., 1982) and by precipitation of the products of reticulocyte lysate translation of rat, mouse and human hypothalamic mRNA by GnRH antiserum (Curtis and Fink, 1983; Curtis et al., 1983). Millar et al. proposed that the amino-terminal pGlu residue of GnRH was derived from Gln while the Gly-NH_2 of the carboxyl terminus resulted from the enzymic processing of a Gly followed by a pair of basic amino acids which constitute the beginning of the carboxyl-terminal extension of the prohormonal precursor, as is the case with other amidated peptides (Douglass et al., 1984). This prediction has been confirmed by the elucidation of the nucleotide sequences of human placental GnRH cDNA (Seeburg and Adelman, 1984) and human hypothalamic GnRH cDNA (identical to placental cDNA) as well as rat hypothalamic cDNA (exibits 70% homology with human) (Adelman et al.,

1986). The GnRH sequence is followed by a cleavage and amidation site (Gly-Lys-Arg) and then a 53-amino acid sequence and a second potential cleavage site (Lys-Lys-Ile) (Fig. 1). In addition to cleavages at these pairs of basic amino acid residues, processing may occur at single basic amino acids, especially arginine (Fig. 1), as such cleavages occur in several peptide precursors (Douglass et al., 1984).

Since post-translational processing of several peptide precursors gives rise to different peptide fragments with various biological functions (Douglass et al., 1984), it was of interest to determine whether this is also true of the GnRH precursor. A number of overlapping peptide sequences, which lie between putative basic amino acid processing sites, were therefore synthesised in our laboratory and tested in the human pituitary bioassay system. These peptides have been designated pHGnRH for precursor Human GnRH, followed by the amino acid numbering as assigned by Seeburg and Adelman (1984).

MATERIALS AND METHODS

Peptides

Synthetic GnRH and precursor peptides: pHGnRH 14-26, pHGnRH 17-26, pHGnRH 14-36, pHGnRH 17-36, pHGnRH 14-37, pHGnRH 17-37, pHGnRH 28-36, pHGnRH 38-49, pHGnRH 51-66 and pHGnRH 54-66 were synthesised by Dr R deL Milton (Fig. 4.1.1) using conventional solid phase methodology (Stewart and Young, 1984); GnRH-associated peptide (GAP) was from Dr P Seeburg, Genentech Inc. (South San Francisco, CA).

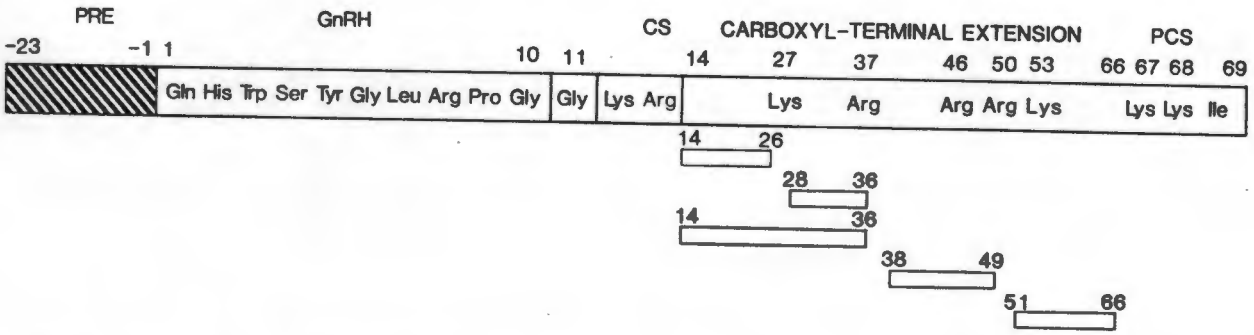

Fig. 4.1.1. Diagramatic representation of human pre-pro GnRH. The signal sequence (PRE) is followed by the GnRH sequence and the Lys-Arg cleavage site (CS). Single basic amino acid residues which may be additional post-translational processing sites are indicated in the carboxyl-terminal extension, and the second potential cleavage site (PCS) precedes the carboxyl-terminal isoleucine residue. Glu^{1} undergoes cyclisation to pGlu at the amino-terminus of GnRH, and Gly^{11} is the donor for amidation of Gly^{10} during the processing of the GnRH precursor. The open bars represent five of the pHGnRH peptides synthesised.

Preparation of cells

Human anterior pituitary glands were obtained at autopsy from healthy men (16-40 years old) between 11 and 18 h after traumatic accidental death and were processed as described in Chapter 3.

Incubation of cells

After 3 to 4 days in culture, the cells were washed 5 times with MEM, and test peptides added to duplicate or triplicate wells. After 3 hours of incubation the medium was removed, collected into tubes containing BSA, to prevent absorption of gonadotropins, such that the final concentration of BSA was 0.1%, centrifuged at 400xg for 5 min, and stored at -20°C until assay for LH, FSH and prolactin.

Receptor binding studies

Human anterior pituitaries were obtained from 1 woman (45 yr of age) and 2 men (20-26 yr of age) within 24 h of accidental death. Pituitary membranes were prepared as previously described (Wormald et al., 1985; Chapter 2), with the exception that the membrane suspension used was 2-fold more concentrated (0.2 human pituitary equivalents / ml).

RESULTS

Biological activity of pHGnRH peptides

Gonadotropin-releasing activity was associated with five of the pHGnRH peptides, viz. pHGnRH 14-26, pHGnRH 14-36, pHGnRH 17-36, [$Arg^{37}NH_2$]pHGnRH 14-37 and [$Arg^{37}NH_2$]pHGnRH 17-37 (Fig. 4.1.2. expt. 1). These peptides consistently gave rise to a significantly ($p<0.05$) greater than basal LH and FSH release (in seven experiments). pHGnRH 28-36 consistently (in five experiments) failed to stimulate greater than basal LH and FSH release (Fig. 4.1.2. expt. 1). pHGnRH 17-26 significantly stimulated LH ($p<0.01$) and FSH ($p<0.05$) in two out of four experiments (Fig. 4.1.2. expt. 2). The active pHGnRH peptides did not stimulate secretion of prolactin or TSH from the human pituitary cell cultures (Table 4.1.1).

pHGnRH 14-36 stimulated LH and FSH release in a dose dependent manner, the EC_{50} for LH being 178nM (Fig. 4.1.3 A). and for FSH 200nM (Fig. 4.1.3 B). which is more than 200-fold greater than that of GnRH.

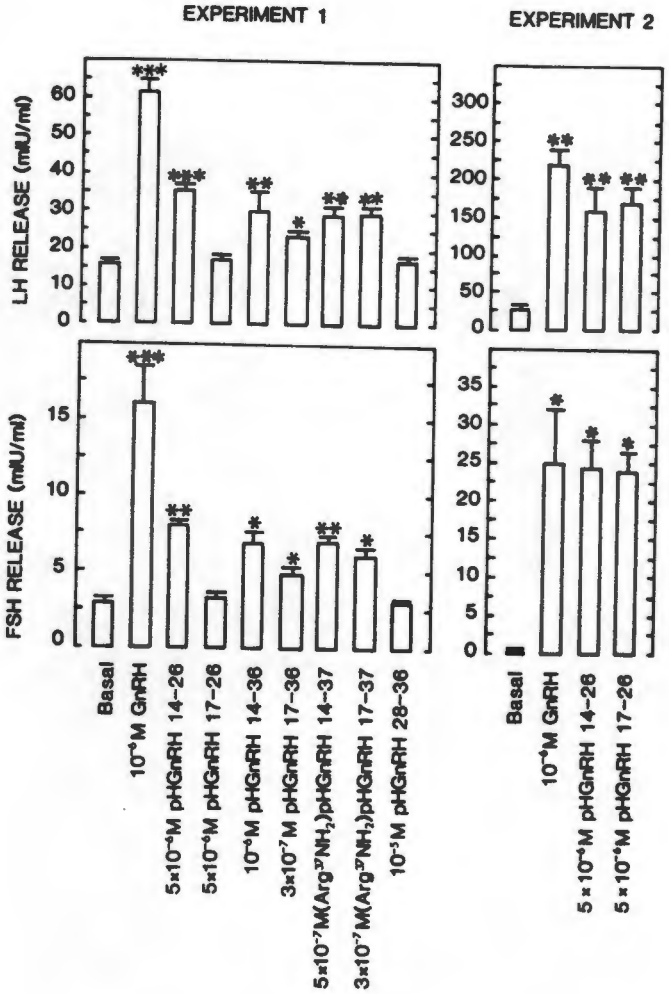

Fig. 4.1.2. Gonadotropin release from cultured human pituitary cells stimulated with pHGnRH peptides. Data from two experiments are presented. All the pHGnRH peptides tested, except pHGnRH 17-26 and pHGnRH 28-36, were consistently associated with significant gonadotropin release (expt. 1). The longer sequences (>20-amino acid residues) were approximately 10-fold more active than the shorter peptides (<15-amino acid residues). pHGnRH 17-26 stimulated gonadotropin secretion from certain pituitary cell cultures (expt. 2) but not from others (expt. 1), while pHGnRH 28-36 was consistently unable to stimulate gonadotropin release (expt. 1).
All values represent mean ± SEM of triplicate 3 h incubations using pooled pituitary tissue from 4 cadavers for expt. 1, and from 5 cadavers for expt. 2.
Significantly different from basal values; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as determined by analysis of variance.

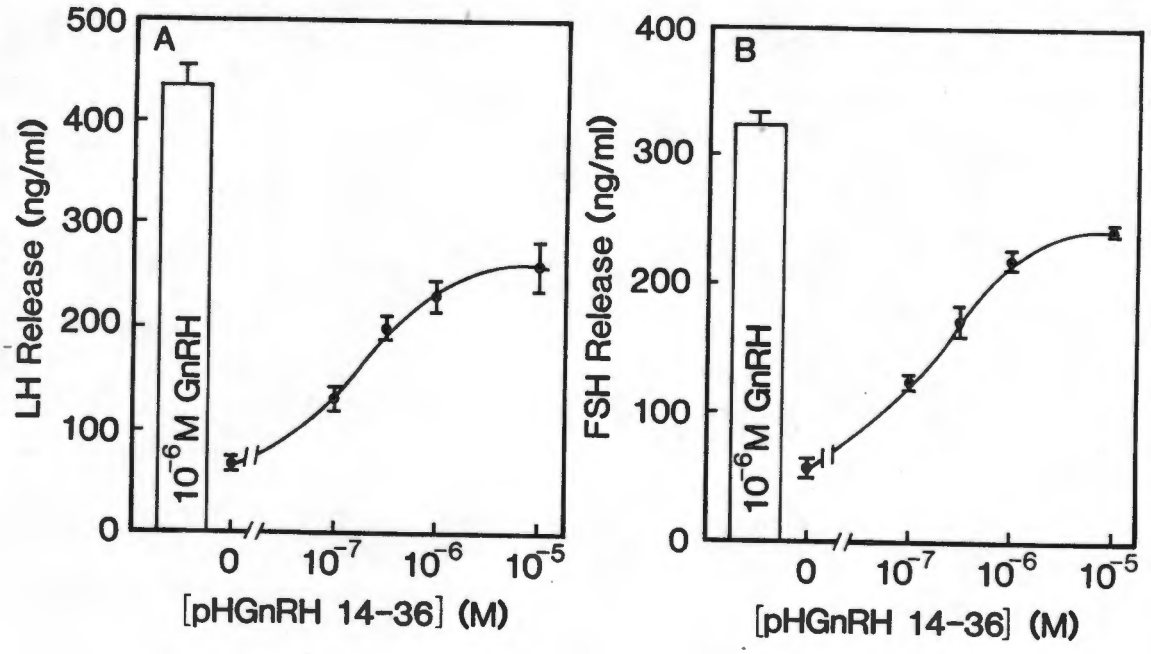

Fig. 4.1.3. Dose-responsive gonadotropin secretion from cultured human pituitary cells stimulated with pHGnRH 14-36. pHGnRH 14-36 stimulated LH (A) and FSH (B) release in a dose-dependent manner, the EC_{50} for LH release being 178 nM and for FSH 200 nM.
All values represent mean ± SEM of duplicate 3 h incubations using pooled pituitary tissue from 2 cadavers.

TABLE 4.1.1. Effect of pHGnRH peptides on prolactin and TSH secretion from cultured human pituitary cells

	Prolactin secretion (ng/ml)	TSH secretion (μU/ml)
Basal	11.3 ± 0.45	3.1 ± 0.75
10^{-6}M GnRH	11.2 ± 0.75	2.9 ± 0.30
10^{-6}M pHGnRH 14-26	10.2 ± 0.50	3.2 ± 0.65
10^{-6}M pHGnRH 14-36	10.8 ± 0.92	2.8 ± 0.64
10^{-6}M pHGnRH 17-36	9.0 ± 0.21	3.1 ± 0.26

All values represent mean ± SEM of duplicate or triplicate 3 h incubations using pooled cells from 4 cadavers.

Ca^{2+}-dependence of pHGnRH-stimulated gonadotropin release

pHGnRH 14-36 - stimulated LH and FSH release was Ca^{2+}-dependent (Fig. 4.1.4). In the presence of extracellular Ca^{2+} both GnRH and pHGnRH 14-36 stimulated significant ($p<0.01$) gonadotropin secretion while in the presence of 3mM EGTA the response was almost entirely inhibited.

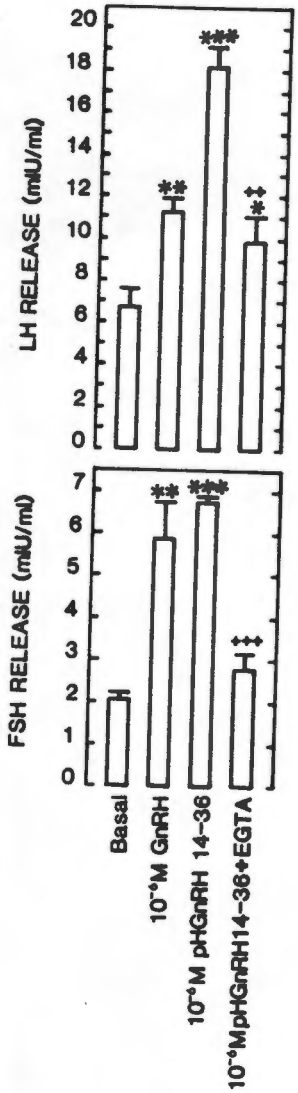

Fig. 4.1.4. The effect of EGTA on the gonadotropin release from cultured human pituitary cells stimulated by pHGnRH 14-36. The presence of 3 mM EGTA significantly reduced both the LH and FSH release stimulated by pHGnRH 14-36 to near basal values.
All values represent mean ± SEM of duplicate 3 h incubations using pooled pituitary tissue from 3 cadavers.
Significantly different from basal values; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as determined by analysis of variance.
Significantly different from pHGnRH 14-36 - stimulated gonadotropin release; ++ $p<0.01$, +++ $p<0.001$ as determined by analysis of variance.

Effect of pHGnRH peptides on [^{3}H]-leucine incorporation

The precursor peptides, pHGnRH 14-26, pHGnRH 14-36 and pHGnRH 17-36, had no effect on [^{3}H]-leucine incorporation in cultured human pituitary cells when compared with basal or unstimulated cells (Table 4.1.2).

TABLE 4.1.2. Effect of pHGnRH peptides on [^{3}H]-leucine incorporation in cultured human pituitary cells

Treatment	[^{3}H]-Leu incorporation (% basal)
Basal	100.0 ± 3.8
10^{-5}M pHGnRH 14-26	106.2 ± 3.2
10^{-5}M pHGnRH 14-36	99.9 ± 2.6
10^{-5}M pHGnRH 17-36	93.4 ± 2.7

All values represent mean ± SEM of triplicate 3 h incubations using pooled cells from 2 cadavers.

Effect of a GnRH antagonist on pHGnRH-stimulated gonadotropin release

GnRH antagonist 3 (see Chapters 2 and 3), at a dose of 10^{-6}M, completely inhibited both 10^{-5}M pHGnRH 14-26 - (Fig. 4.1.5, expt. 1) and 10^{-6}M pHGnRH 14-36 - stimulated LH and FSH release (Fig. 4.1.5, expt. 2).

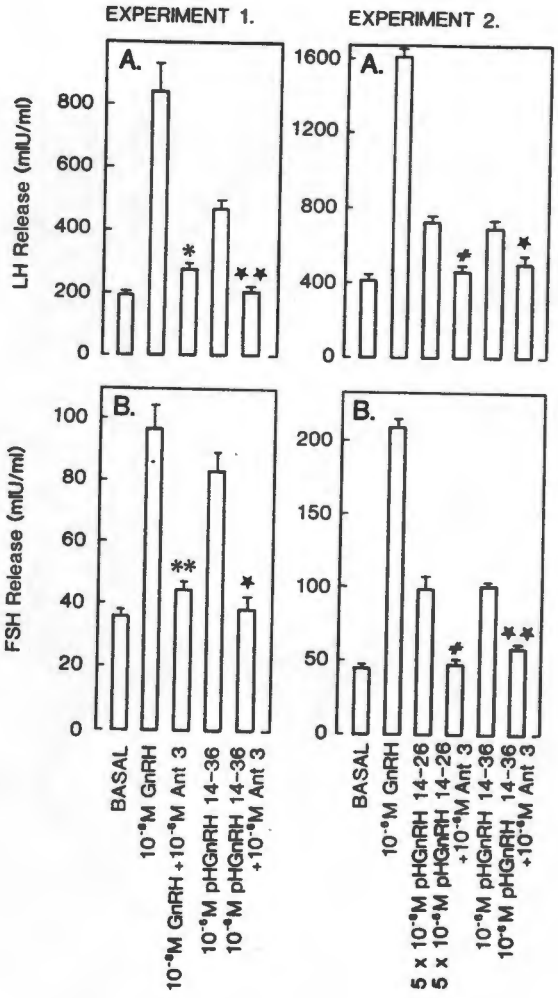

Fig. 4.1.5. The effect of a GnRH antagonist on pHGnRH 14-26 and pHGnRH 14-36 - stimulated gonadotropin secretion from cultured human pituitary cells. Data from two experiments are presented. GnRH antagonist 3, at a dose of 10^{-6}M, inhibited 10^{-8}M GnRH- and 10^{-8}M pHGnRH 14-36 - stimulated gonadotropin secretion (expt. 1) and $5x10^{-8}$M pHGnRH 14-26 - stimulated release (expt. 2).
All values represent mean ± SEM of duplicate and triplicate 3 h incubations using pooled pituitary tissue from 2 cadavers for expt. 1, and 5 cadavers for expt. 2.
Significantly different from GnRH-stimulated gonadotropin secretion; * $p<0.01$, ** $p<0.001$ as determined by analysis of variance.
Significantly different from pHGnRH 14-36 - stimulated gonadotropin secretion; (1 star) $p<0.01$, (2 stars) $p<0.001$ as determined by analysis of variance.
Significantly different from pHGnRH 14-26 - stimulated gonadotropin secretion; # $p<0.01$ as determined by analysis of variance.

Effect of pHGnRH 14–36 on [^{125}I]–GnRH agonist binding to human pituitary membranes

pHGnRH 14–36 competed with the [^{125}I]-GnRH agonist, [D-Ala^{6},N$^{\alpha}$MeLeu^{7},Pro^{9}NEt]-GnRH, for binding to human pituitary cell membranes (Fig. 4.1.6). The presence of 10^{-4}M pHGnRH 14–36 in the human GnRH receptor binding assay reduced binding of the labelled GnRH agonist to 32.8 $\pm$ 0.2 % of control values. pHGnRH 14–26 (5×10^{-5}M) also competed for binding to the GnRH receptor (Fig. 4.1.6), although to a much lesser degree than pHGnRH 14–36.

The combined effect of pHGnRH 14–36 and GnRH on gonadotropin secretion

10^{-6}M GnRH and 10^{-6}M pHGnRH 14–36 significantly stimulated LH (Fig. 4.1.7 A) and FSH (Fig. 4.1.7 B) release. Co-stimulation of the cultured human pituitary cells with GnRH and pHGnRH 14–36 did not result in any additional release of gonadotropins, above that stimulated by GnRH alone (Fig. 4.1.7).

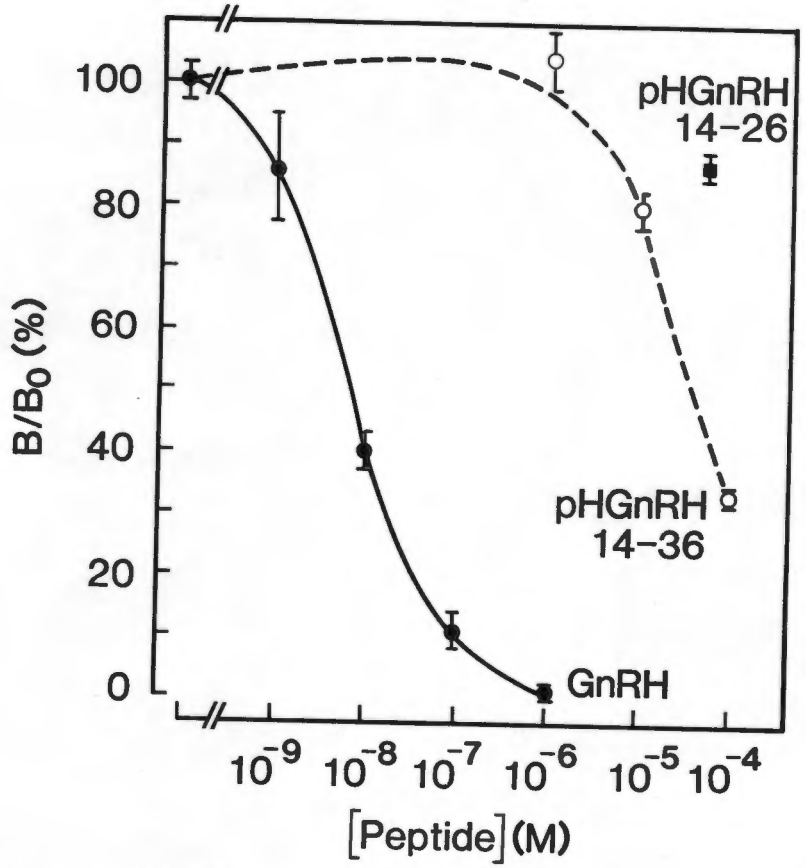

Fig. 4.1.6. Displacement of [^{125}I]-GnRH agonist from human pituitary membranes by increasing concentrations of GnRH, pHGnRH 14-36 and pHGnRH 14-26. pHGnRH 14-36 displaced the labelled GnRH agonist with an ED_{50} of 3.98×10^{-9}M. pHGnRH 14-26 was less active than pHGnRH 14-36, displacing only 10% of the label at a dose of 5×10^{-5}M.
All values represent mean ± SEM of triplicate incubations using pooled pituitary membranes from 3 cadavers.

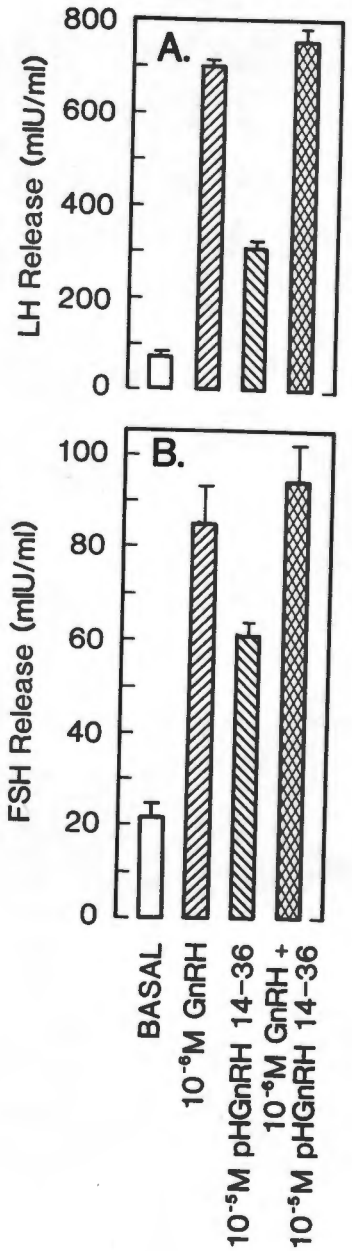

Fig. 4.1.7. Effect of GnRH and pHGnRH 14-36 on LH and FSH release from cultured human pituitary cells, when added alone or in combination. pHGnRH 14-36, at a dose of 10^{-5}M, did not stimulate any additional release of LH (A) or FSH (B) above that stimulated by 10^{-6}M GnRH.
All values represent mean ± SEM of triplicate 3 h incubations using pooled pituitary tissue from 2 cadavers.

DISCUSSION

The elucidation of the structure of the human GnRH precursor (Seeburg and Adelman, 1984) has provided information for a wide range of studies on the regulation of the reproductive system. In view of the precedent of some hormone precursors giving rise to several processed peptides with different biological activities (Douglass et al., 1984) it was of interest to determine whether the same is true for the GnRH precursor.

A series of ten overlapping peptide sequences, which are potentially processed from the human GnRH precursor, were synthesised and tested in the human pituitary bioassay system for biological activity (Fig. 4.1.1). Five of these peptides (pHGnRH 14-26, pHGnRH 14-36, pHGnRH 17-36, [Arg^{37}.NH_2]-pHGnRH 14-37 and [Arg^{37}.NH_2]-pHGnRH 17-37) consistently, on seven independent occasions, gave rise to significantly greater than basal LH and FSH release. The peptide fragment, pHGnRH 28-36, consistently (in five experiments) failed to stimulate gonadotropin secretion. Each of the active overlapping peptides incorporates the decapeptide sequence, Asn-Leu-Ile-Asp-Ser-Phe-Gln-Glu-Ile-Val, corresponding to pHGnRH 17-26 which is therefore considered to represent a defined region of the GnRH precursor with intrinsic gonadotropin-releasing activity. This peptide fragment was therefore also synthesised and

tested for gonadotropin-releasing activity. pHGnRH 17-26 significantly stimulated LH and FSH release from different cell cultures in two out of four experiments. In the remaining two experiments this decapeptide fragment did not significantly stimulate gonadotropin release, although GnRH and pHGnRH 14-26 included in the same experiments did stimulate gonadotropin secretion. A 5-fold higher concentration of pHGnRH 17-26 (and pHGnRH 14-26), in comparison to the other active peptides, was required to stimulate gonadotropin release. The relatively low and inconsistent activity of pHGnRH 17-26 suggests that this peptide fragment approaches the minimal sequence required for gonadotropin release. The increased activity associated with the longer peptides, which incorporated carboxyl-terminal extensions of this minimal sequence, stresses the enhancing effect that the flanking sequence has on gonadotropin-releasing activity. This is also in accordance with that which has been reported by Nikolics et al. (1985); that the entire C-terminal extension, pHGnRH 14-69 or GAP (GnRH associated peptide), stimulated LH and FSH release from rat pituitary cells with a similar or greater potency than GnRH. Other synthetic peptides covering the remainder of the GnRH precursor sequence (pHGnRH 38-49, pHGnRH 51-66 and pHGnRH 54-66) did not exhibit gonadotropin releasing activity (data not shown), although a complete series of overlapping fragments has not been synthesised.

pHGnRH-stimulated gonadotropin secretion from the cultured human pituitary cells was dose-dependent. The active peptides were, however, at least 300-fold less active than GnRH and in most experiments did not achieve the same maximum release of LH and FSH as GnRH. This effect of these peptides was specific in that prolactin and thyrotropin concentrations in the medium were unaffected. Also, LH and FSH release in response to these peptides did not occur in the absence of extracellular calcium. The removal of calcium ions by chelation with EGTA significantly reduced the LH and FSH release, stimulated by pHGnRH 14-36, to near basal levels. Further evidence that these effects of the pHGnRH peptides are specific comes from the study showing that incorporation of [^{3}H]-Leu into protein by the cells was unaffected by the presence of the peptides.

In view of the lack of structural homology between the active pHGnRH peptide fragments and GnRH itself, it was of interest to determine whether the gonadotropin release stimulated by these different peptides was mediated by a common receptor on the pituitary gonadotrophs. The GnRH antagonist, [Ac-D-Nal(2)^{1},D-α-Me-ClPhe^{2},D-Trp^{3},D-Arg^{6},D-Ala^{10}NH$_{2}$]-GnRH, which competetively inhibited GnRH-stimulated gonadotropin secretion from the cultured human pituitary cells, also blocked the release of LH and FSH associated with both pHGnRH 14-26 and pHGnRH 14-36. This indicates that the active pHGnRH peptides stimulate the

gonadotrophs via the GnRH receptor. We initially reported that the GnRH antagonist was not able to prevent pHGnRH-stimulated gonadotropin secretion (Millar et al., 1986b; Milton et al., 1986). These early experiments, however, were carried out under sub-optimum conditions, whereby, following stimulation of the cultured cells, BSA was not added to the collection tubes. This resulted in approximately 90% loss of LH and FSH from the medium due to nonspecific adherence of the hormones to the walls of the tubes.

Further evidence that active pHGnRH peptides stimulate gonadotropin release via the GnRH receptor is provided by GnRH receptor binding studies. pHGnRH 14-36 displaced [^{125}I]-labelled GnRH agonist, from human pituitary membranes, with approximately 10,000-fold lower affinity than GnRH. pHGnRH 14-26, however, at a dose of $5x10^{-5}M$ displaced only 10% of the label from the receptor, while pHGnRH 14-36, at the same dose, displaced almost 60%. pHGnRH 14-26 was similarly less active than the longer peptides in the bioassay. There is an apparent discrepency between the ability of pHGnRH 14-36 to bind the GnRH receptor (10,000-fold less active than GnRH) and its ability to release LH and FSH (300-fold less active than GnRH). This phenomenon has also been observed with weak GnRH agonists (Hasegawa et al., 1984; Millar et al., 1986a) and may merely reflect the poor ability of these weak agonists to compete with the labelled superactive agonist for binding, whereas in the

bioassay, which is a noncompetitive system, activity is directly dependent on receptor affinity.

In view of the fact that the active pHGnRH peptides stimulate gonadotropin secretion via a similar mechanism to GnRH, it was important to exclude the possibility of contamination of these peptides by GnRH or GnRH agonist. Analytical reverse phase high performance liquid chromatography fractionation of pHGnRH 14-36, under conditions in which pHGnRH 14-36 eluted later than GnRH and GnRH analogues synthesised in our laboratory, showed that the LH-releasing activity in rat cells and receptor binding activity to rat pituitary membranes coincided with the pHGnRH 14-36 UV absorption peak (Milton et al., 1987). Further evidence against contamination is provided by the fact that pHGnRH 14-26 was active in the human bioassay but not in the rat (Milton et al., 1987). Only pHGnRH 14-36 and longer peptides were active in stimulating LH release from cultured rat pituitary cells (data not shown).

pHGnRH 14-36, when added to the human pituitay cells with GnRH, could not stimulate gonadotropin release above the maximum released by GnRH. No evidence of synergistic or antagonistic effects was observed. Similarly, there was no additive effect with GAP (pHGnRH 14-69) and GnRH in rat pituitary cell cultures (Nikolics et al., 1985). These observations indicate that the same cell was activated by

both GnRH and the pHGnRH peptides, and that pHGnRH 14-36 stimulated LH and FSH release from a common intracellular pool. The biphasic nature of gonadotropin secretion in response to GnRH (see Chapter 3, Fig. 3.4; Naor et al., 1982; King et al., 1986), however, suggests that there may be different cellular pools of LH and FSH, and since the pHGnRH peptides (in most experiments) did not stimulate the same maximum release of gonadotropins as that acheived by GnRH, this suggests that these peptides are not capable of activating all the cellular LH and FSH pools which are activated by GnRH. This provides further evidence against the argument for GnRH contamination of the active pHGnRH peptides.

Further work is required to establish whether any of the pHGnRH peptide fragments have a physiological role in gonadotropin secretion. In particular, it is necessary to demonstrate that the active peptide/s is secreted into the hypophyseal-portal vessels at concentrations capable of stimulating gonadotropin secretion, and that the administration of an antiserum to the peptide inhibits gonadotropin secretion in vivo. Whether these peptides fulfill a physiological role or not, it is evident that synthetic pHGnRH peptides incorporating the sequence 17-26 and the full 56-amino acid peptide (pHGnRH 14-69 or GAP) (Nikolics et al., 1985) do release gonadotropin and so offer the potential for the development of a new range of

synthetic analogues for experimental studies and clinical applications.

SECTION 2

SUBSTANCE P

INTRODUCTION

Substance P (SP) is an undecapeptide which is widely distributed within the peripheral and central nervous systems of mammals (Nicholl et al., 1980; Aronin et al., 1983; Pernow, 1983), and has been implicated as a regulatory peptide in the control of reproductive function (Kerdelhue et al., 1978; Vijayan and McCann, 1979; Eckstein et al., 1980). SP has been found to inhibit GnRH-stimulated LH and FSH release from cultured rat anterior pituitary cells (Kerdelhue et al., 1979; Kerdelhue et al., 1982) and to bind to specific high affinity binding sites in rat anterior membranes (Kerdelhue et al., 1982; Kerdelhue et al., 1985), suggesting a direct modulatory role for SP on GnRH stimulation of gonadotropin secretion. More direct evidence for a physiological role of SP is provided by the observation of an increase in LH and FSH after administration of SP antisera to rats (Kerdelhue et al., 1979; Kerdelhue et al., 1982). SP, when administered to female rhesus monkeys, however, had no effect on gonadotropins (Eckstein et al., 1980), suggesting that the peptide may not play a role in the control of reproduction in primates.

In view of these findings, I have investigated the presence of specific SP receptors in the human pituitary and

the effect of SP on GnRH-stimulation of LH and FSH release from cultured human anterior pituitary cells.

MATERIALS AND METHODS

Hormones

Synthetic SP was obtained from UCB s.a., (Bruxelles, Belgium); SP analogues [D-Tyr^{0}]-SP, [D-Tyr^{0},NorLeu^{11}]-SP, SP(3-11), SP(7-10), SP(5-8) and SP(1-4) were from Dr B Kerdelhue, INRA CNRS (Jouy-en-Josas, France); and synthetic TRH and GnRH were from Dr R deL Milton, University of Cape Town Medical School (Cape Town, South Africa).

Binding studies

Human anterior pituitaries were obtained from twelve healthy men (20-40 years old) within 24 h after accidental death and stored at -70°C until use. The tissue was homogenised in buffer A (10mM HEPES, 1mM EDTA, 1mM dithiothreitol, 0.1% BSA, 10^{-5}M bacitracin, pH 7.4) centrifuged at 1,000 x g for 15 min and the resulting supernatant at 10,000 x g for 30 min. The membrane pellet was resuspended in the same buffer. All binding studies were performed in triplicate as previously described in Chapter 2. Total binding (5.33-11.64%) was determined in the absence of unlabelled peptide and nonspecific binding (3.73-8.90%) by the addition of 10^{-6}M SP or analogue.

[D-Tyr^{6},NorLeu^{11}]-SP was labelled with Na^{125}I (Kerdelhue et al., 1973) and purified by QAE Sephadex A-25 chromatography (Nett and Adams, 1977) (specific activity 800 μCi/μg). K_ds were estimated from Scatchard analysis and from ED_{50} values using the formula of Cheng and Prusoff (1973).

Preparation of the cells

Human anterior pituitaries were obtained at autopsy from 16 healthy men (25-35 years old) between 6 and 24 h after accidental death, and processed as previously described in Chapter 3.

Incubation of the cells

After 3 days in culture, the cells were washed 5 times with minimal essential medium (MEM) and the test substances added in triplicate. After a 3 h incubation the medium was removed, centrifuged at 400 x g for 5 min and the supernatant assayed for LH and FSH.

Radioimmunoassays

Assays were performed in duplicate using a human LH radioimmunoassay (Amerlex LH RIA kit - code IM 2081, Amersham; intra-assay c.v. 3.2%) and a human FSH

radioimmunoassay (Amerlex FSH RIA kit - code IM 2071, Amersham; intra-assay c.v. 6.2%)

RESULTS

SP binding to human pituitary cell membranes

Binding of ^{125}I-[D-Tyr^{o},NorLeu^{11}]-SP to human anterior pituitary membranes was progressively inhibited by the addition of increasing concentrations of unlabelled SP (Fig.

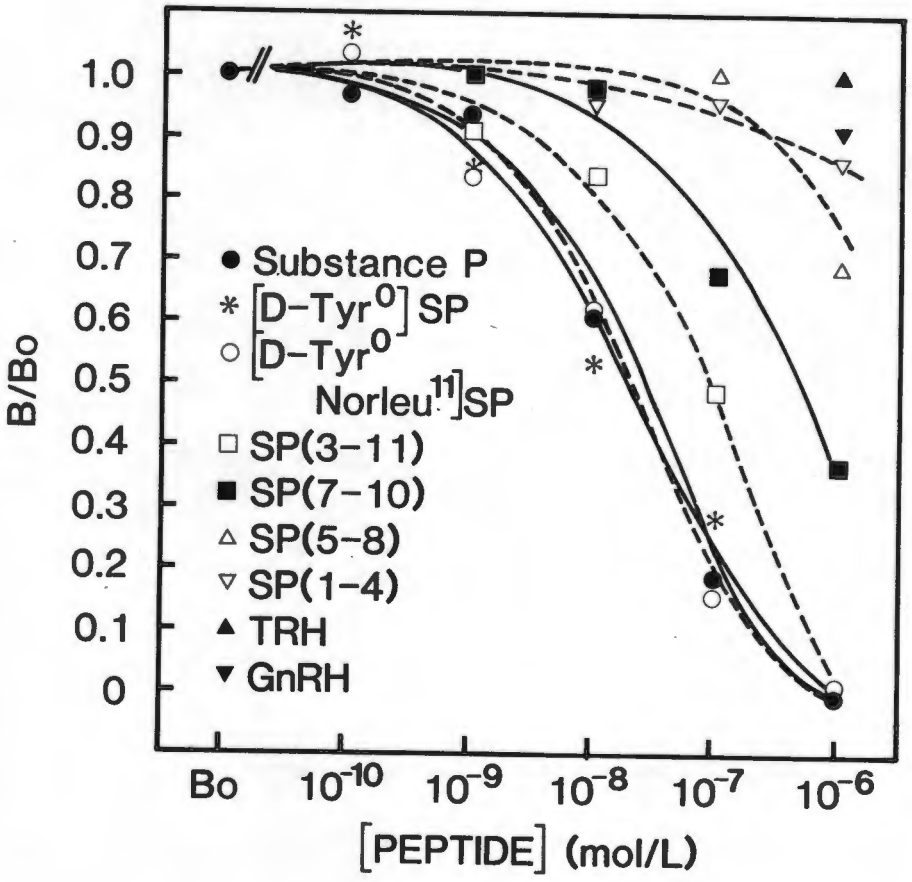

Fig. 4.2.1 Displacement of [^{125}I]-labelled [D-Tyr^{o}, NorLeu^{11}]-SP binding to human pituitary membranes by unlabelled SP (●), [D-Tyr^{o}]-SP (*), [D-Tyr^{o},NorLeu^{11}]-SP (O) SP(3-11) (□), SP(7-10) (■), SP(5-8) (△), SP(1-4) (▽), TRH (▲) and GnRH (▼).

4.2.1). Scatchard analysis (Fig. 4.2.2) indicated that the binding was saturable and involved a single class of high affinity sites (K_d of 19.3 nM). The binding affinities of SP, [D-Tyr^{0}]-SP and [D-Tyr^{0},NorLeu^{11}]-SP were similar (K_ds of 17.6 nM, 35.3 nM and 19.3 nM respectively) while the shorter fragments of SP exhibited lower binding affinities (Fig. 4.2.1, Table 4.2.1).

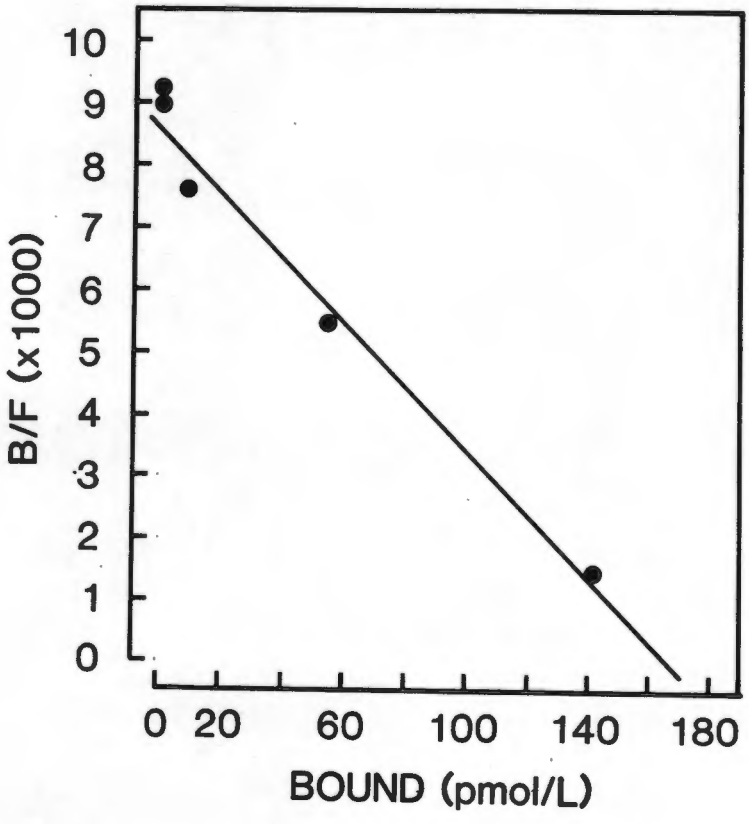

Fig. 4.2.2 Scatchard analysis of the binding of [D-Tyr^{0}, NorLeu^{11}]-SP to human pituitary membranes.

TABLE 4.2.1. Apparent dissociation constants of SP analogues and fragments, and other unrelated peptides, binding to human pituitary membranes

	K_d (nM)
Substance P	17.6 ± 2.9
[D-Tyr^0]-SP	35.3 ± 18.5
[D-Tyr^0,NorLeu^{11}]-SP	19.3 ± 3.0
SP(3-11)	92.4 ± 8.4
SP(7-10)	286.0 ± 42.0
SP(5-8)	> 1000
SP(1-4)	> 1000
GnRH	> 1000
TRH	> 1000

All values represent mean ± SEM of binding studies carried out in triplicate using pooled membranes from 6 cadavers. K_ds were calculated by Scatchard analysis for [D-Tyr^0,NorLeu^{11}]-SP and by the method of Cheng and Prusoff (1973) for SP, [D-Tyr^0]-SP, SP(3-11) and SP(7-10).

Effect of SP on GnRH-stimulated LH and FSH release

Cultured human pituitary cells secreted both LH and FSH when stimulated with 10^{-8}M GnRH (Fig. 4.2.3). In one experiment, GnRH-stimulated LH secretion was progressively inhibited ($p<0.05$) by increasing concentrations of SP (Fig. 4.2.3A). FSH release was also reduced in the presence of SP, but the difference was not significant (Fig 4.2.3B). In a second experiment 10^{-6}M SP inhibited 3nM GnRH-stimulated LH

secretion (data not shown), while in three further experiments SP had no effect on LH and FSH secretion from these cells.

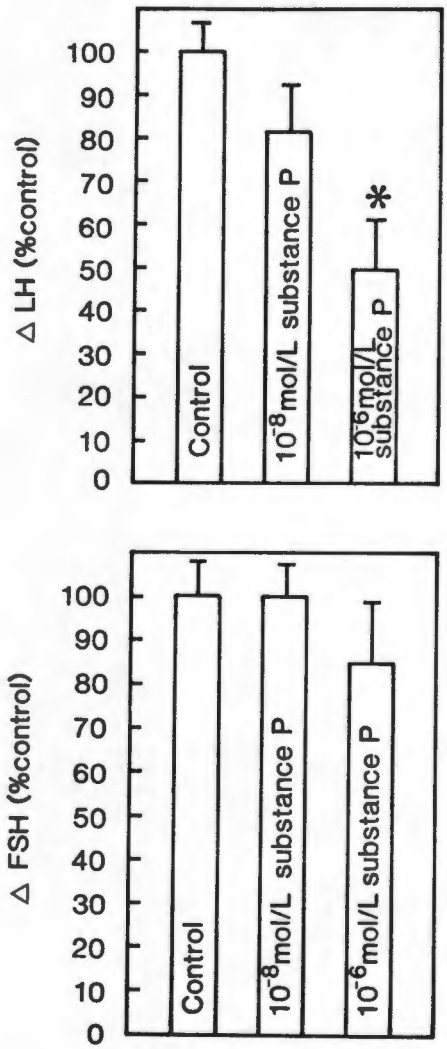

Fig. 4.2.3 The effect of 10^{-8}M and 10^{-6}M SP on 10^{-6}M GnRH-stimulated LH and FSH secretion from cultured human pituitary cells.
All values represent mean ± SEM of a triplicate 3 h incubation using pooled pituitary tissue from 3 cadavers, and were calculated as a percent of the specific increase in LH and FSH induced by GnRH.
Control represents the specific increase over basal values, of LH and FSH, stimulated by 10^{-6}M GnRH alone.
* Significantly different from control value as determined by analysis of variance.

DISCUSSION

Previous reports have suggested a modulatory role for SP in regulating gonadotropin secretion during the oestrus cycle of the rat (Kerdelhue et al., 1982). SP has been found to affect the release of gonadotropins and prolactin in the rat, both in vivo (Kerdelhue et al., 1978; Vijayan and McCann, 1979) and in vitro (Kerdelhue et al., 1979). This has been further supported by the demonstration of SP binding sites in rat anterior pituitary membranes (Kerdelhue et al., 1985) and by the observation that administration of SP antisera to rats results in elevated LH and FSH levels (Kerdelhue et al., 1979 and 1982). The administration of SP to female rhesus monkeys induced a significant increase in prolactin secretion but had no effect on gonadotropins (Eckstein et al., 1980), suggesting that the peptide does not play a significant role in the control of reproduction in primates.

The present study has demonstrated the presence of a single class of saturable, specific, high affinity binding sites or receptors for SP in the human anterior pituitary. The binding characteristics of the human pituitary SP receptor were similar to those of the rat (Kerdelhue et al., 1985). SP, [D-Tyr^{0}]-SP and [D-Tyr^{0},NorLeu^{11}]-SP had similar affinities (K_{d}s of 17.6 nM, 35.3 nM and 19.3 nM respectively) for the receptor, while the shorter fragments,

SP(3-11). SP(7-10). SP(5-8) and SP(1-4) had progressively lower potencies (K_is of 92.4 nM. 286 nM. >1 μM and >1 μM respectively). As amino acids are removed from the N- or C-terminus of the molecule the binding affinity of the receptor for the peptide is diminished.

SP directly inhibited LH secretion from the cultured human pituitary cells. This effect was observed on only two out of five occassions which is perhaps not surprising, since previous in vitro studies carried out in the rat showed that the inhibitory effect of SP was most pronounced in female pituitaries obtained at pro-oestrus when SP receptor number is high. suggesting that the phenomenon is steroid-dependent (Kerdelhue et al.. 1982). This study has nevertheless demonstrated that SP is capable of inhibiting gonadotropin secretion in the primate.

The demonstration of a single class of high affinity, specific binding sites for SP and the inhibition by SP of gonadotropin secretion from cultured human pituitary cells, indicates that SP can act directly at a pituitary level in man and thus may play a regulatory role in the human reproductive cycle. The mechanism by which SP exerts its effect, however, has yet to be elucidated.

CHAPTER 5

CONCLUDING REMARKS AND DIRECTIONS FOR FUTURE STUDIES

CONCLUSIONS

This thesis has investigated the binding characteristics of the human GnRH receptor and has demonstrated that it differs from the rat receptor in its interaction with at least four GnRH analogues, its ionic requirements for monovalent and divalent cations, and in the molecular size of the GnRH binding protein. This may have important clinical implications, since these data suggest that rat studies are not a valid index for GnRH analogue activity in man.

A system for culturing human pituitary cells has been established and the data from these studies have further demonstrated that there is poor correlation, not only between the biological potencies of five GnRH analogues in humans and rats, but also between the receptor assay and the bioassay within a species (particularly in the human). The mechanism of GnRH action and the regulation of gonadotropin secretion, from the human pituitary cells, by substance P and a series of novel peptides putatively processed from the human GnRH precursor, however, was found to closely parallel that observed in the rat.

These data support the previous contention, from receptor binding studies, that rat gonadotrophs are not

appropriate for investigating the activities of GnRH analogues for use in human studies.

FUTURE STUDIES

In order to characterise the binding site of the human GnRH receptor in more detail, human receptor binding studies need to be carried out using a library of different GnRH analogues. In this way the region of the GnRH molecule required for binding can be approximated, and some idea of the chemical nature of the binding site gained.

The human bioassay system can also be exploited in order to investigate the regulation of gonadotropin secretion, in man, by other hormones and neurotransmitters. In particular the effects of the gonadal steroids, which have been shown in a number of animal models to affect gonadotropin secretion at the pituitary level by altering the responsiveness of the gonadotropes to GnRH; inhibin, which specifically inhibits FSH secretion from rat pituitary cells; the opioid peptides, which are capable of directly inhibiting GnRH-stimulated gonadotropin secretion in rats; and the catecholamines, which have been shown to potentiate the effect of GnRH on rat gonadotropes, should be investigated, thus providing more insight into the regulation of the human reproductive cycle.

In addition to these studies, the human pituitary cell bioassay system can be extended to investigate the direct

regulation of the other pituitary hormones; TSH, prolactin, ACTH and GH, in man.

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PUBLICATIONS ARISING FROM PhD RESEARCH

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