Peptides of postulated inhibin activity

Lack of in vitro inhibin activity of a 94-residue peptide isolated from human seminal plasma, and of a synthetic replicate of its C-terminal 28-residue segment

Sergio Kohan, Berit Fröysa, Ella Cederlund*, Thomas Fairwell°, Richard Lerner†, Jan Johansson*, Shafiq Khan+, Martin Ritzen+, Hans Jörnvall*, Sten Cekan and Egon Diczfalusy

*Reproductive Endocrinology Research Unit, +Pediatric Endocrinology Unit, and *Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden, °Molecular Disease Branch, NHLBI, NIH, Bethesda, MD 20892* and †*Research Institute of Scripps Clinic, La Jolla, CA 92037, USA*

Received 22 January 1986; revised version received 25 February 1986

A 94-residue polypeptide isolated from human seminal plasma and its chemically synthesized C-terminal 28-residue segment were studied in an in vitro inhibin bioassay utilizing rat pituitary cell cultures. Both peptides have previously been claimed to have inhibin activities, and the effects on the secretion and cellular content of gonadotrophins (FSH and LH) were now assessed in the in vitro assay. No inhibition was found. After 72 h of culture, both the cellular content and the spontaneous as well as the LHRH-stimulated release of bioactive or immunoactive FSH and LH remained unaffected. Similarly, no effects were found on the storage and/or release of prolactin, growth hormone, or thyrotropin. We conclude that both the native 94-residue peptide and the synthetic replicate of its C-terminal 28-residue segment, do not influence the pituitary FSH secretion when assessed in this in vitro system.

Inhibin *Follicle-stimulating hormone* *Luteinizing hormone* *Seminal plasma* *Synthetic peptide* *Pituitary cell culture*

1. INTRODUCTION

It is established that the regulation of the follicle stimulating hormone (FSH) secretion from the pituitary is partially under the control of a non-steroidal gonadal hormone called inhibin [1,2]. Since the first report of this activity by McCullagh [3], many have tried to isolate the active principle from different sources [4–6]. Two types of peptide have been purified from human seminal plasma [7,8], the smaller one also occurring in multiple size forms apparently because of different fragmentations from a parent molecule [9], and the larger one with a postulated inhibin activity in the C-terminal segment [10]. Their amino acid sequences [7,11–13] revealed them to be unrelated. The amino acid sequences also permitted later identification of the smaller peptide as one related to a major degradation product of a high-M_r precursor secreted by the seminal vesicles [14], and of the larger peptide as a sperm-coating antigen [12] originating from prostatic epithelium [13,15]. Evidence was also presented for the prostatic origin of a closely related immunoreactive material [16].

The inhibin activity of the larger peptide (with 94 residues) was measured by in vivo assays using castrated rats and by in vitro incubations of mouse pituitaries [11]. It was also reported that this peptide exhibits inhibin activity in a rat pituitary cell culture assay. However, this assay was found to be less sensitive than the mouse pituitary assay [10], in

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

contrast to previous reports from others showing high sensitivity for the rat pituitary cell culture with different crude preparations of inhibin [17]. Consequently, the postulated inhibin activity of the 94-residue peptide appeared to require further analysis.

Because of all these questions, we decided to investigate the activity of the 94-residue peptide, and its C-terminal 28-residue synthetic replicate [10], in the rat pituitary cell culture bioassay and to compare the results with the known inhibin activity of ovine rete testis fluid, bovine follicular fluid and unfractionated human seminal plasma. The effect of the peptides on the storage and release of LH, FSH and other pituitary hormones was measured. No specific effects were detected. However, signs of non-specific cell toxicity were recorded in certain instances.

2. MATERIALS AND METHODS

2.1. *Preparations studied*

2.1.1. Native peptide

The 94-residue peptide was purified by reverse-phase high-performance liquid chromatography of a partially purified preparation from human seminal plasma as described by Sheth et al. [8] and identified by analysis of amino acid composition and N-terminal residues [8].

2.1.2. Synthetic peptides

Two syntheses of the 28-residue C-terminal fragment of the previously suggested inhibin native peptide [10] were investigated. In one preparation (peptide I below), the peptide was synthesized by the Merrifield solid-phase method [18], using a Beckman 990B peptide synthesizer. After cleavage and deprotection, the peptide was desalted on a Biogel P-2 column and the crude peptide reduced and reoxidized to form the correct disulfide bonds [19]. Final purification was achieved by reverse-phase high-performance liquid chromatography. An adjacent pool containing a minor fraction of the synthetic material was also collected (peptide Ia). The correct structure of the pure peptide I was verified by complete sequence analysis in a liquid-phase sequencer and by amino acid composition after hydrolysis. Both analyses gave the expected results, proving the correct structure.

In the other preparation (peptide II), a Merrifield solid-phase synthesis was carried out as above, but the product obtained was not further purified.

2.1.3. Inhibin preparations

The control preparations used (bovine follicular fluid, diluted 1:1000, and ovine rete testis fluid, 20.8 U/ml) were gifts from Dr F.J. de Jong (Department of Biochemistry, Erasmus University, Rotterdam, The Netherlands) and Dr D.M. Robertson (Department of Anatomy, Monash University, Clayton, Victoria, Australia), respectively. Human seminal plasma was treated with charcoal [20].

2.2. *Pituitary cell culture*

All procedures were performed under sterile conditions. Glassware was siliconized and heat-sterilized (60 min, 100°C). Solutions were sterilized by ultrafiltration through Millipore filters (Millex-GS 0.22 μm).

Anterior pituitary cells from adult male rats were prepared using digestion with trypsin [21] and diluted with the incubation medium [Dulbecco's modified Eagles medium (Flow Lab., UK), supplemented with 5% horse serum and 2.5% fetal calf serum (Flow Lab.)]. Aliquots (200 μl) of about 150000 cells were cultured in multiwell plates (Falcon 3070) at 37°C in a water-saturated atmosphere of 95% air/5% CO_2 for 72 h before the addition of any test substance to the medium.

2.3. *Methods for inhibin assays*

2.3.1. Spontaneous release of FSH and LH from pituitary cells

After the 72 h pre-culture, the medium was removed and discarded. Fresh aliquots (250 μl) of culture medium with and without different amounts of the test substances were added and the cells cultured for another 72 h. The medium was then carefully removed and stored at −20°C before assay for FSH and LH.

2.3.2. FSH and LH contents of cells

After washing the cells once with 0.1% bovine serum albumin in Dulbecco's modified Eagles medium, they were lysed by addition of 250 μl of 0.05 M phosphate-buffered saline containing 0.1% Triton X-100. The lysate was stored at −20°C.

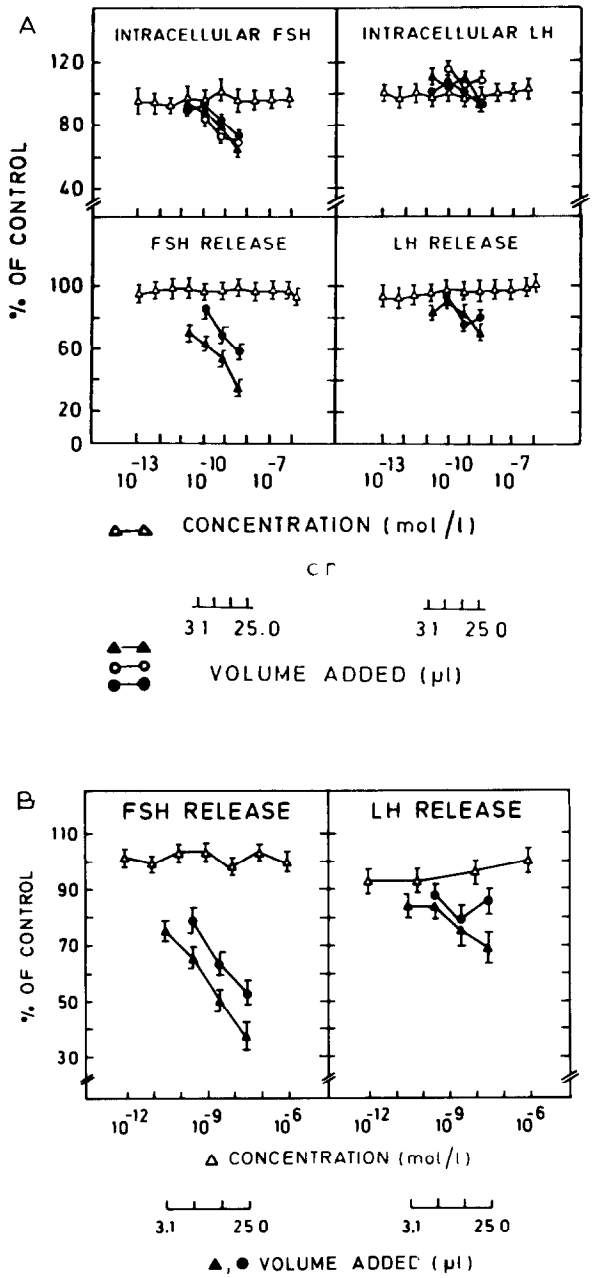

Fig.1. Lack of effect of the 94-residue peptide on the LHRH-stimulated release and intracellular content of immunoreactive (A) and bioactive (B) FSH and LH. Cells were cultured in the presence of different amounts of the 94-residue peptide (Δ—Δ), human seminal plasma (○—○), ovine rete testis fluid (▲—▲) and bovine follicular fluid (●—●), for 72 h, followed by 6 h in the presence of added LHRH (10 nmol/l). The intracellular content of FSH and LH was measured after the first culture period in parallel experiments. Levels of FSH and LH were measured by RIA (A) or bioassay (B) and are expressed as percentages of mean values (mean ± SD, $n = 9$) found in control wells without any test preparation. The upper scale refers to the peptide concentration in log units, the lower scale to the volume of reference preparations added.

2.3.3. LHRH-stimulated release of FSH and LH

After the 72 h culture with the test substance, cells were washed and fresh aliquots (250 µl) of 0.1% BSA-Dulbecco's modified Eagles medium added, with or without LHRH (Hoechst, 10 nmol/l) and containing the same concentrations of the test substances as those during the previous 72 h cultures. The cells were then incubated for another 6 h before removal of the medium for FSH and LH assays.

2.4. *Assays of pituitary hormones*

2.4.1. Radioimmunoassays

Immunoreactive hormones (FSH, LH, GH, TSH, PRL) were measured by a double antibody radioimmunoassay (RIA) using reagents provided by the National Institute of Arthritis, Metabolism and Digestive Diseases, National Institute of Health, Bethesda, USA. The reference preparations used were coded: NIAMDD-rat-FSH-RP-1, NIAMDD-rat-LH-RP-1, NIADDK-rGH-RP-2 (AFP-3190B), NIADDK-rTSH-RP-2 (AFP-5153B) and NIADDK-rPRL-RP-3 (AFP-4459B).

2.4.2. In vitro bioassay of FSH and LH

Activities were measured, using previously described systems [22,23].

2.5. *Cell toxicity*

Cells were assessed for viability by measurement of the retention of ^{51}Cr (sodium chromate, Amersham, England) according to Robertson et al. [24].

3. RESULTS

3.1. *Native 94-residue peptide*

No influence on the LHRH-stimulated immunoreactive or bioactive FSH release was observed when the 94-residue native peptide was applied in final concentrations ranging from

10^{-6} mol/l to 2.6×10^{-13} mol/l (fig.1). Shorter culture times than 72 h before the application of LHRH (24 and 48 h) also failed to show any influence. Similarly, the peptide did not influence the LH release (fig.1). However, control preparations of ovine rete testis fluid and bovine follicular fluid employed for the validation of the assay exhibited an inhibitory effect (fig.1). The spontaneous release of immunoreactive or bioactive FSH or LH was also not affected by the presence of the 94-residue peptide (not shown), although the control preparations produced a clear effect, less pronounced on LH than on FSH. Finally, no suppression of the intracellular content of FSH was obtained after 72 h of culture with different amounts of the 94-residue peptide, although a marked inhibition was found with the control preparations (fig.1A). The cellular LH content was not significantly influenced by any of the substances studied (fig.1A).

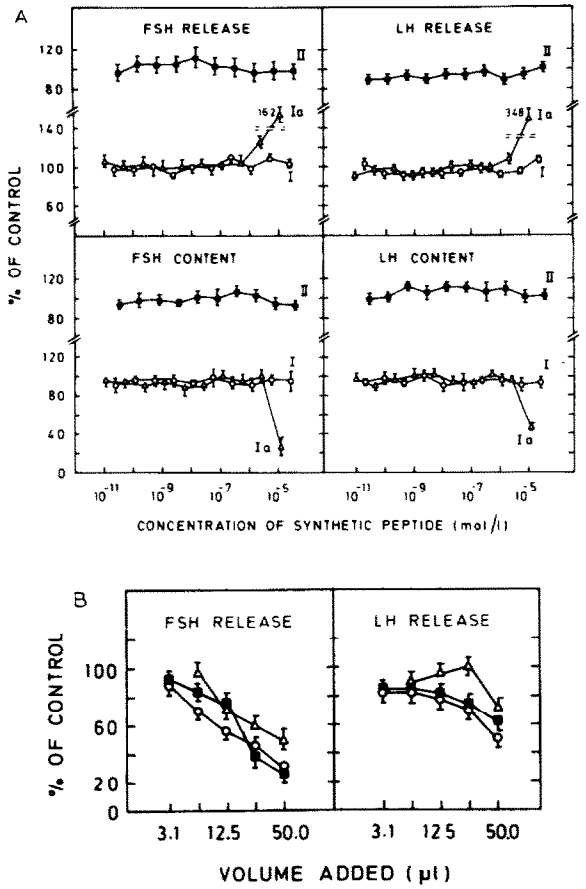

Fig.2. Effect of (A) the 28-residue peptide preparations and (B) the standard inhibin preparations on intracellular content and spontaneous release of gonadotrophins. (A) Cultured pituitary cells were treated for 72 h with different doses of the 28-residue peptides. (B) Similar experiment, but in this case different doses of ovine rete testis fluid (○—○), human seminal plasma (△—△) and bovine follicular fluid (■—■) were used for 72 h. Values are expressed as in fig.1.

3.2. *Synthetic 28-residue peptide*

None of the synthetic peptide preparations showed any inhibin activity when they were applied to the pituitary cell culture system (fig.2), except for preparation Ia at the largest dose tested, 13×10^{-6} mol/l (fig.2A). However, at this concentration, preparation Ia was markedly toxic as demonstrated by the poor retention of ^{51}Cr (fig.3). Since Ia is a side fraction from the purification, it is also the material with least amount of authentic synthetic peptide. As in the experiments with the

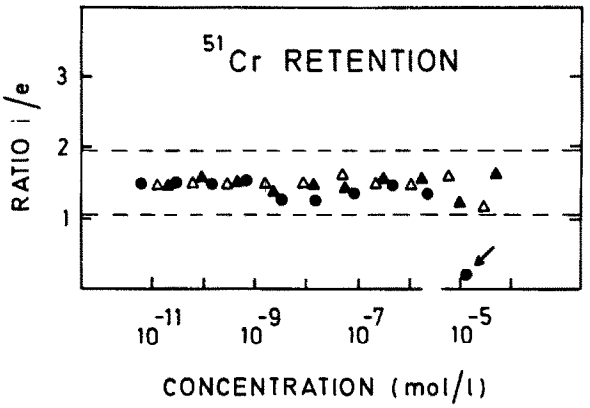

Fig.3. Test of possible toxic effects of the three 28-residue peptide preparations on pituitary cells in culture. Pituitary cells were cultured with ^{51}Cr for 48 h, the medium was then removed and fresh medium with different doses of the synthetic preparations [I (△), Ia (●) and II (▲)] was added to appropriated wells and the culture continued for another 72 h. At the end of this culture period ^{51}Cr was measured in the medium and in the cells. Levels of ^{51}Cr are expressed as the ratio between intra- and extracellular radioactivity (cpm) of ^{51}Cr [$^{51}Cr(i)/(e)$]. Dotted lines represent the limits of 2 SD, from the mean. Note that the highest concentration of preparation Ia was the only one showing toxicity to the cells (arrow).

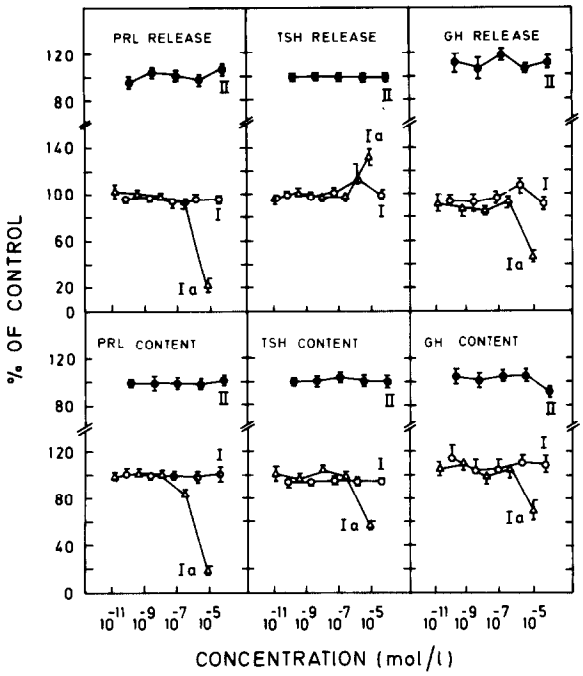

Fig.4. Effect of the three 28-residue peptide preparations on the prolactin (PRL), thyrotrophin (TSH) and growth hormone (GH) content of, and spontaneous release from pituitary cells in culture. Cultured cells were treated for 72 h with different doses of the 28-residue peptides. Values are expressed as in fig.1.

94-residue peptide, those with the three crude preparations of inhibin all showed the expected inhibin effects, with a preferential suppression of the FSH rather than the LH release (fig.2B).

Furthermore, there was no effect of the peptides on the release and cellular content of any of the other pituitary hormones studied (PRL, TSH and GH), except again the toxic effect of preparation Ia at the highest concentration (fig.4). In separate experiments, as expected, this monolayer pituitary cell system was responsive to thyrotropin releasing hormone and growth hormone releasing factor (not shown) by releasing thyrotropin, prolactin, and growth hormone, respectively, in a dose-related manner.

4. DISCUSSION

Inhibition of spontaneous and LHRH-stimulated release of FSH [25] and the suppression of the cellular content of FSH [26] are established effects of inhibin preparations when they are applied to a pituitary cell culture system. In this study, preparations used as positive controls in the assay (bovine follicular fluid, ovine rete testis fluid and human seminal plasma) showed the expected [24–28] suppression of FSH cell content and release (figs 1,2). However, none of the synthetic peptide preparations (I, Ia, II), corresponding to the C-terminal segment of the seminal 94-residue peptide or the native 94-residue peptide, showed any of these properties. Other pituitary hormones (GH, TSH, PRL) were also measured in the culture medium and in the cells as an index of specificity.

A biphasic dose response relation has been reported [10] concerning the influence of peptides on the LHRH-stimulated release of FSH. However, considering the large range of concentrations tested here, the possible existence of a biphasic response cannot account for the observed absence of inhibin activity.

The sensitivity of the rat pituitary cell culture assay has been debated in relation to the assay with whole pituitaries or in vivo studies [17]. However, this debate also seems to be irrelevant for the present study because of the absence of inhibin activity of the 94-residue peptide at a wide range of concentrations, at the same time as a marked inhibin activity of crude preparations from various sources was demonstrated.

The present assay was highly sensitive to LHRH stimulation in the sense that the basal secretion of FSH was increased 3–5 times (4.4 ± 1.3, $n = 9$) after the application of 10 nmol/l of LHRH. This is in agreement with previous reports [21]. When inhibin activity of the 94-residue peptide and its C-terminal segment in a rat pituitary cell culture was reported [10], no description was given of details on culture conditions, FSH and LH RIAs or number of experiments. Consequently, no detailed comparison can be made to find the possible reasons for the discrepancy between that study and the present one. However, as shown by the present data, we consistently find lack of activity under the assay conditions described.

Both direct measurements of cell toxicity by determination of ^{51}Cr retention and the release of hormones showed that peptide Ia damaged the cells at the highest concentration. Such evaluation of cell damage was not performed in the previous

investigations claiming inhibin activity of the peptides studied [10,11]. Furthermore, synthetic peptides [11,29], closely related to the 94-residue peptide and the 28-residue peptide presently studied, failed to influence the spontaneous release of FSH when they were applied to dispersed pituitary cells in culture [2].

We conclude therefore that the 94-residue peptide and its fragment lack inhibin activity. This is consistent with present knowledge of the structure and origin of the 94-residue peptide. Thus, the amino acid sequence shows the peptide to be the same as the sperm-coating antigen in human seminal plasma [12,13,15]. The site of origin has been shown to be prostatic epithelium [13,15,16]. In addition, the peptide does not disappear from peripheral blood after gonadectomy [16,30], and is present at high concentrations in gastric secretions [31]. None of these properties is expected for a hormonal peptide that would regulate FSH secretion in an inhibin-like manner. Consequently, all the properties of the 94-residue peptide, and its 28-residue C-terminal fragment, harmonize with the present demonstration of the absence of inhibin activity. (After the preparation of this report, another complete structure was shown for inhibin [32], in agreement with several recent isolations [33–36]. This structure also questions previous data by demonstrating another structure for inhibin, thus confirming our conclusions derived from direct measurements with the previous peptides.)

ACKNOWLEDGEMENTS

We are grateful to Dr A.R. Sheth (Institute for Research in Reproduction, Bombay, India) for supplying the seminal plasma preparation. The bovine follicular fluid was obtained by courtesy of Dr F.H. de Jong (Department of Biochemistry, Erasmus University, Rotterdam, The Netherlands), the ovine rete testis reference preparation by courtesy of Dr D.M. Robertson (Department of Anatomy, Monash University, Clayton, Victoria, Australia). We are also grateful to Dr G. Bialy, NICHD, Bethesda, MD, USA, for the generous supply of the rat RIA reagents. This investigation received financial support from the World Health Organization's Special Programme of Research in Human Reproduction, the Swedish Cancer Society, the Swedish Medical Research Council, and the LEO Research Foundation. S.K. was a recipient of a fellowship from the Swedish Institute.

REFERENCES

[1] Franchimont, P., Chari, S., Hazee-Hagelstein, M.T., Debruche, M.L. and Duraiswami, S. (1977) in: The Testis in Normal and Infertile Men (Troen, P. and Nankin, H.R. eds) pp.253–270, Raven, New York.
[2] De Jong, F.H. and Robertson, D.M. (1985) Mol. Cell. Endocrinol. 42, 95–103.
[3] McCullagh, D.R. (1932) Science 76, 19–20.
[4] Nandini, S.G., Lipner, H. and Moudgal, N.R. (1976) Endocrinology 98, 1960–1965.
[5] Chari, S., Duraiswari, S. and Franchimont, P. (1978) Acta Endocrinol. 87, 434–448.
[6] Sairam, M.R., Ranganathan, M.R., Ramasharma, K. and Lamothe, P. (1981) Mol. Cell. Endocrinol. 22, 231–250.
[7] Seidah, N.G., Ramasharma, K., Sairam, M.R. and Chrétien, M. (1984) FEBS Lett. 167, 98–102.
[8] Sheth, A.R., Arbatti, N., Carlquist, M. and Jörnvall, H. (1984) FEBS Lett. 165, 11–15.
[9] Li, C.H., Hammonds, R.G. jr, Ramasharma, K. and Chung, D. (1985) Proc. Natl. Acad. Sci. USA 82, 4041–4044.
[10] Arbatti, N.J., Seidah, N.G., Rochemont, J., Escher, E., Sheth, A.R. and Chrétien, M. (1985) FEBS Lett. 181, 57–63.
[11] Seidah, N.G., Arbatti, N.J., Rochemont, J., Sheth, A.R. and Chrétien, M. (1984) FEBS Lett. 175, 349–355.
[12] Johansson, J., Sheth, A.R., Cederlund, E. and Jörnvall, H. (1984) FEBS Lett. 176, 21–26.
[13] Akiyama, K., Yoshioka, Y., Schmid, K., Offner, G.D., Troxler, R.F., Tsuda, R. and Hara, M. (1985) Biochim. Biophys. Acta 829, 288–294.
[14] Lilja, H. and Jeppsson, J.-O. (1985) FEBS Lett. 182, 181–184.
[15] Yoshioka, Y., Akiyama, K., Tsuda, R., Hara, M., Schmid, K., Offner, G.D. and Troxler, R.F. (1984) Fed. Proc. 43, 1633.
[16] Beksac, M.S., Khan, S.A., Eliasson, R., Skakkebaek, N.E., Sheth, A.R. and Diczfalusy, E. (1984) Int. J. Androl. 7, 389–397.
[17] Hudson, B., Baker, H.W.G., Eddie, L.W., Higginson, R.E., Burger, H.G., De Kretser, D.M., Dobos, M. and Lee, V.W.K. (1979) J. Reprod. Fert. Suppl. 26, 17–29.
[18] Merrifield, R.B. (1969) Advances Enzym. 32, 221–296.

[19] Gutte, B. and Merrifield, R.B (1971) J. Biol. Chem. 246, 1922–1941.
[20] Baker, H.W.G., Burger, H.G., De Kretser, D.M., Eddie, L.W., Higginson, R.E., Hudson, B. and Lee, V.W.K. (1978) Int. J. Androl. Suppl. 2, 115–124.
[21] Eddie, L.W., Baker, H.W.G., Higginson, R.E. and Hudson, B. (1979) J. Endocrinol. 81, 49–60.
[22] Ritzén, E.M., Fröysa, B., Gustafsson, B., Westerholm, G. and Diczfalusy, E. (1982) Hormone Res. 16, 42–48.
[23] Van Damme, M.-P., Robertson, D.M. and Diczfalusy, E. (1974) Acta Endocrinol. 77, 655–671.
[24] Robertson, D.M., Au, C.L. and De Kretser, D.M. (1982) Mol. Cell. Endocrinol. 26, 119–127.
[25] De Jong, F.H., Smith, S.D. and Van Der Molen, H.J. (1979) J. Endocrinol. 80, 91–102.
[26] Scott, R.S., Burger, H.G. and Quigg, H. (1980) Endocrinology 107, 1536–1542.
[27] Main, S.J., Davies, R.V. and Setchell, B.P. (1979) J. Reprod. Fert. Suppl. 26, 3–14.
[28] De Jong, F.H. (1979) Mol. Cell. Endocrinol. 13, 1–10.
[29] Ramasharma, K., Sairam, M.R., Seidah, N.G., Chrétien, M., Manjunath, P., Schiller, P.W., Yamashiro, D. and Li, C.H. (1984) Science 223, 1199–1202.
[30] Sheth, A.R., Moodbidri, S.B., Bandivdekar, A.H., Vanage, G.R., Hurkadli, K.S. and Arbatti, N.J. (1984) in: Gonadal Proteins and their Biological Significance (Sairam, M.R. and Atkinson, L.E. eds) pp.39–48, World Scientific, Singapore.
[31] Sheth, N.A., Vaze, A.Y. and Sheth, A.R. (1982) Clin. Endocrinol. 17, 157–163.
[32] Mason, A.J., Hayflick, J.S., Ling, N., Esch, F., Ueno, N., Ying, S.-Y., Guillemin, R., Niall, H. and Seeburg, P.H. (1985) Nature 318, 659–663.
[33] Robertson, D.M., Foulds, L.M., Leversha, L., Morgan, F.J., Hearn, M.T.W., Burger, H.G., Wettenhall, R.E.H. and De Kretsen, D.M.V. (1985) Biochem. Biophys. Res. Commun. 126, 220–226.
[34] Miyamoto, K., Hasegawa, Y., Fukuda, M., Nomura, M., Igarashi, M., Kangawa, K. and Matsuo, H. (1985) Biochem. Biophys. Res. Commun. 129, 396–403.
[35] Rivier, J., Spiess, J., McClintock, R., Vaughan, J. and Vale, W. (1985) Biochem. Biophys. Res. Commun. 133, 120–127.
[36] Ling, N., Ying, S.-Y., Ueno, N., Esch, F., Denoroy, L. and Guillemin, R. (1985) Proc. Natl. Acad. Sci. USA 82, 7217–7221.