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Biological characteristics of the peptides α and *β* isolated from bovine seminal plasma

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Abstract. The bull seminal plasma peptides α and *β* have been examined for their biological properties. While both the peptides were able to inhibit the human chorionic gonadotropin-dependent uterine response in the mouse, α alone exhibits the property of suppressing post-castrational rise in gonadotropin in appropriate animal models. This suggests that the peptide *β* must be acting directly on the ovary to suppress estrogen production and, consequently, the uterine weight increase. Such a possibility was confirmed when α and *β* were examined by the coupled bioassay which is capable of discriminating between pituitary feedback factors and those acting directly on the gonad. In a test system designed to examine chronic effects, both α and *β* showed evidence of acting directly on the ovary to inhibit human menopausal gonadotropin-induced estrogen production. Such a direct action could not be correlated with the relative potencies of these peptides when examined for their follicle stimulating hormone-receptor binding inhibitor and lutinizing hormone-receptor binding inhibitor activities.

Keywords. Seminal plasma peptides; inhibin activity; extra-pituitary action.

Introduction

Recent publications on inhibin encourage the belief that doubts as to the existence and physiological significance of this gonadal factor are not warranted. While the original postulation of this factor as well as initial attempts at its characterization were based on the male, advances in the past two years on the chemistry of this peptide hormone have stemmed from studies using ovarian follicular fluid as the source material (de Jong and Robertson, 1985). There is consensus among workers that atleast one form of inhibin activity associated with bovine, human, ovine and porcine follicular fluids is a 32 kD protein consisting of a 14 kD *A* and an 18 kD *B* chain. Considerable sequence homology for the two subunits isolated from different species has also been reported (Ling *et al.,* 1985; Miyamoto *et al.,* 1985; Rivier *et al.,* 1985; Robertson *et al.,* 1985; Fukuda *et al.,* 1986; Mason *et al.,* 1986; Leversha *et al.,* 1987).

Some effort has been made to introduce a direct radioimmunoassay (RIA) for inhibin and also to improve existing assays with a view to shortening the turn-around time (McLachlan *et al.,* 1986; Savoy-Moore *et al.,* 1986; Lee *et al.,* 1987).

The isolation from bovine seminal plasma of the peptides *α* and *β,* their physico-

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Abbreviations used: RIA, Radioimmunoassay; hFSH, human follicle stimulating hormone; FSH-BI, follicle stimulating hormone-receptor binding inhibitor; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin; LH-RBI, luteinizing hormone-receptor binding inhibitor.

chemical properties as well as an evaluation of their inhibin-like characteristics were reported in an earlier contribution (Mohapatra *et al.,* 1985). It was concluded that, while these two peptides correspond, in terms of physico-chemical characteristics, to the two bands seen in the Polyacrylamide gel electrophoresis described by Chari *et al.* (1978), they are not related to each other as isohormones, in as much as the peptide α alone shows true inhibin activity. It was considered of interest to examine in greater detail the biological properties of these two peptides. The results are reported in this paper.

Materials and methods

Samples for the biological studies

The two peptides were isolated as described earlier (Mohapatra *et al.,* 1985) and were stored at – 20°C as sucrose concentrates. Several batches were examined by the methods described below.

Coupled bioassay: Colony-bred 24 ± 1 day old immature Swiss albino mice were assessed for uterine response to graded doses (10, 20, 30 μg) of test samples in two parallel assays, in one of which the control and treated animals received a total dose of 40 IU of hCG (Pregnesin/Serono), while in the other they received a total dose of 5 IU of hMG (Pergonal/Serono). The injection and autopsy schedules as well as the routes of administration of injectibles were as described by Kharbanda *et al.* (1985).

Follicle stimulating hormone-receptor binding inhibition assay: The procedure of Darga and Reichert (1978) was adopted for preparing granulosa cell suspensions, using follicular fluid harvested from different-sized porcine ovarian follicles, excepting cystic follicles. The fluid was centrifuged at 3000 *g* for 10 min at 4°C and the supernatant discarded. The pellet was washed twice with and resuspended in 0·05 M Tris-HCl buffer pH 7·5 containing 5 mM $MgCl_2$, 0·1 M sucrose and 0·1% egg albumin (Sigma: 5 × crystallized). This was filtered through 3 layers of cheesecloth and the filtrate recentrifuged. The cells, recovered in the pellet, were resuspended in the assay buffer so as to give a concentration of 1 to 2 × 10^7 cells/ml.

For the assay, each tube contained 100 μl of the cell suspension, 50 μl of buffer or sample and 20 μl of [^{125}I] -human follicle stimulating hormone (hFSH) (Serono/ ≃11,000cpm). Incubation was at 37°C for 3 h in a metabolic shaker set at 110 oscillations per min. At the end of this period, 1 ml of the chilled assay buffer was added to each tube followed by centrifugation at 5,000 *g* for 10 min at 4°C. The supernatant was decanted off and the pellet counted in an autogamma counter (Beckman Gamma-4,000). Non-specific binding was determined in the presence of a 1000-fold excess of the cold hormone. Specific binding was computed from the equation: $B_s = (B_t\text{-}B_n)/B_t \times 100$, where B_s is specific binding, B_t is total radioactivity bound and B_n is non-specifically bound radioactivity. The follicle stimulating hormone-receptor binding inhibitor (FSH-BI) activities of samples tested are expressed in terms of per cent inhibition of binding (with respect to control) as a function of test sample added to the incubation mixture.

Luteinizing hormone-receptor binding inhibition assay: Using an identical protocol as above, the binding of [^{125}I]-hCG (Serono/ ≃11,000 cpm) and its inhibition by test samples was examined.

Effect of test samples on ovarian weight and serum estradiol levels in chronically treated female juvenile rats: The procedure of Chari *et al.* (1981a) was adopted. Wistar-derived colony-bred rats maintained under standard animal house conditions (Mohapatra *et al.,* 1985) were used. Appropriate doses of the test material(s) were administered as daily single intraperitoneal injections to 13 day-old females, till day 20. Two IU of human menopausal gonadotropin (hMG) (Pergonal/Serono) were administered subcutaneously once daily, 1 h after the injection of the test sample. The animals were sacrificed on day 21 and blood samples collected and processed for estimation of serum estradiol. Ovarian weights were also recorded.

The estradiol content of diethyl ether extracts of the sera was determined using commercial RIA kits supplied by Radio Isotopen Service, Eidg. Inst. For Reaktorforschung, 5303 Wurenlingen, Switzerland. The tracer was [^{125}I]-labelled estradiol. Dextran-coated charcoal was used to separate bound and free hormone. Intra- and inter-assay variations were less than 5%. The assay sensitivity was 0·5–1 Pg.

Results

Differential site of action of α and β in the mouse-based reversed Steelman-Pohley assay

In light of the observed difference between α and *β* in respect of a feedback effect on pituitary FSH secretion (Mohapatra *et al.,* 1985), evidence was sought for their differential effects using the coupled bioassay. The data summarized in figure 1 shows that, in this assay design, α suppressed the uterine response only in the hCG-primed

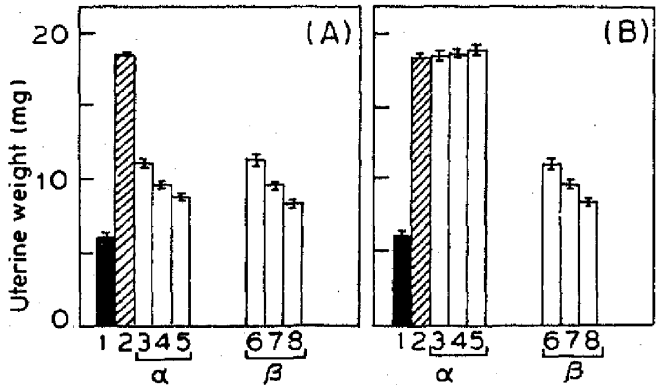

Figure 1. Discrimination between the sites of action of α and *β* in the mouse-based reversed Steelman-Pohley assay using hCG- and hMG-primed animals (coupled bioassay: see text for details). animals were treated with hCG**(A)** or hMG**(B).**
(1), Saline-injected control; (2), hCG/hMG-injected control; (3 5), 10, 20, 30 *μg* respectively of α; (6-8), 10, 20, 30*μ*g respectively of *β*.
Note that while *β* is able to suppress both the hCG- and hMG-induced uterine weight increase, α affects only the hCG-treated animals.

animals, whereas *β* exerted an inhibitory effect in both the hCG-primed and the hMG-primed animals.

Assessment of possible FSH-BI/LH-RBI activity

The peptides α and *β* were examined for their ability to prevent the binding of labelled hFSH and hCG, respectively, to porcine granulosa cells. Typical data are furnished as histograms in figure 2. When compared to *β,* the activity of both as FSH-BI and LH-RBI appears to be marginal. Even in the case of *β*, a dose of 1·7 mg per 10 million cells was required to effect 70% suppression of the binding of either FSH or hCG.

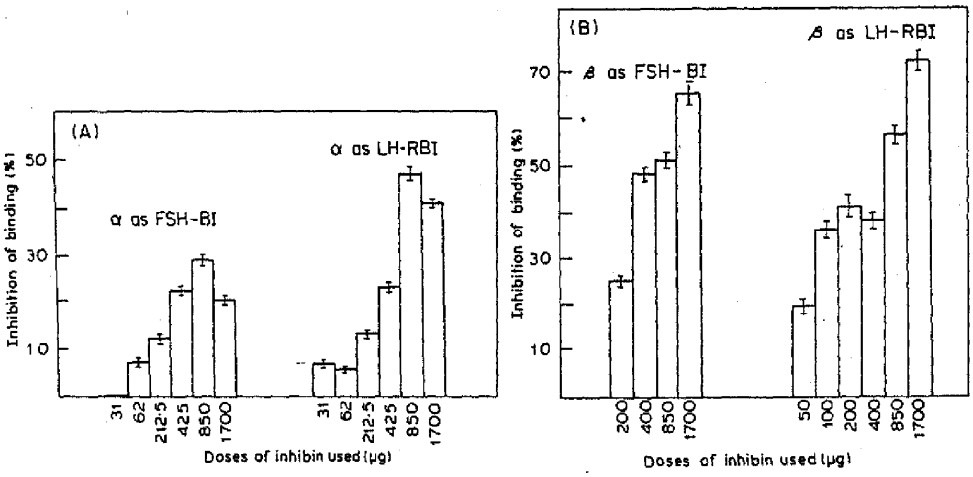

Figure 2. Assay of the peptides **(A)** and *β* **(B)** for FSH-BI and LH-RBI activities using porcine granulosa cells.

Long-term effects of the peptides α and β

The effects of chronic treatment of juvenile female rats with α and *β* are summarized in figure 3. Treatment with either peptide has only a marginal effect on hMG-induced ovarian weight increase. However, there is a readily discernible inhibitory influence on estrogen production. Two points are of interest. Firstly, there is no clear dose-dependence in the response at the levels tested. Secondly, *β* is more effective in intervening directly at the ovarian level and suppressing hMG-stimulated estrogen production.

Discussion

Relationship between α and β

The results presented in this paper, in conjunction with the data of the earlier contribution (Mohapatra *et al.,* 1985), clearly rule out α and *β* being isohormones of bull seminal plasma inhibin.

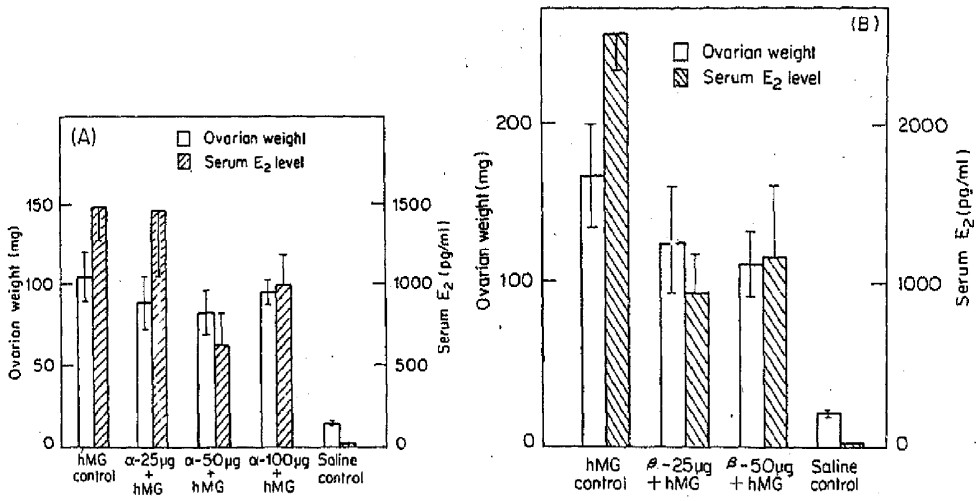

Figure 3. Effect of chronic treatment of juvenile female rats with the peptides α (**A**) and *β*(**B**).
See text for details of assay design. Data of **A** and **B** were obtained on independent occasions, hence the marked difference in baseline values. The dose-dependent fall in ovarian weight is not statistically significant cither for α or for *β.* As for inhibition of ovarian steroidogenesis, *β* appears to be more effective— however, there is no clear dose-dependent effect.

Validity of the reversed Steelman -Pohley assay for inhibin

Reference has been made to the characteristic difference between *α* and *β* with respect to inhibin activity. While both peptides suppress the hCG-induced uterine weight increase in immature mice in a dose-dependent manner, *α* alone has true inhibin activity (Mohapatra *et al.,* 1985). This suggests that these two peptides must be affecting uterine response to hCG through different mechanisms. While α acts to suppress endogenous FSH release, *β* appears to act directly on the ovary to prevent its response to circulating gonadotropin. Viewed in this context, the limitation of the assay for inhibin proposed by Ramasharma *et al.* (1979) becomes evident.

Coupled bioassay for inhibin

The search for additional evidence for a direct ovarian inhibitory action of *β* in the reversed Steelman-Pohley assay led to the use of the coupled bioassay (Kharbanda *et al.,* 1985). The rationale for this assay design is to be found in the observations of Lamond and Emmens (1959), Using intact as well as hypophysectomized immature female mice, the latter 24 h after surgery, these workers assessed uterine responses to graded doses of pituitary as well as placental gonadotropins. They noted that the effect of hCG was virtually abolished by the surgery suggesting that, being deficient in FSH activity, it needed endogenous secretion of FSH to cause ovarian estrogen production. Ramasharma *et al.* (1979) have shown that there is indeed an hCG-induced surge of FSH in the intact immature mouse and that this is necessary for stimulation, *via* the ovary, of uterine growth.

On the other hand, the response to hMG was hardly affected by hypophysectomy (Lamond and Emmens, 1959). According to the authors, this is because hMG has both FSH- and LH-like activities, their levels falling within a range where endogenous gonadotropin, either FSH or LH, adds little to their action in the normal immature mouse (Lamond and Emmens, 1959). In other words, while hMG can directly stimulate estrogen production in the immature mouse ovary in these short-term assays, the action of hCG is dependent upon the availability of endogenous FSH.

The coupled bioassay was designed taking these observations into account. The doses of hMG and hCG, respectively were such as to effect, in the 24 h period of the assay, a 3-fold increase in uterine weight over saline controls. Identical test doses were then used to evaluate suppression of the gonadotropin effect. Interestingly, *β* caused a dose-dependent inhibition of uterine response in the hCG-treated as well as the hMG-treated mice, whereas α was effective only in those animals receiving hCG, a finding fully in consonance with the interpretation of Lamond and Emmens (1959). Thus, it is clear that the peptides α and *β* differ in the mechanism by which they elicit response in the hCG-primed, immature female mouse model.

It may be mentioned that the coupled bioassay meets the requirement for a simple, quick, relatively inexpensive and reliable diagnosis of preparations suspected to possess inhibin activity and distinguishing them from those acting directly at the level of the gonad.

Significance of the FSH-BI/LH-RBI activities of α and β

Given the results of the coupled bioassay, it was a natural step to proceed to an examination of the two peptides for possible FSH-BI and LH-RBI activities. It is evident that the ability of α to suppress either FSH or hCG binding to ovarian target cell receptors is at best marginal. Even with *β,* as much as 1·7 mg of the material was required to effect a 70% reduction in the binding of either gonadotropin to the respective receptors. It is to be noted that in these *in vitro* assays, approximately 10 million porcine granulosa cells were used in each incubation. Since peptide hormones are considered capable of eliciting full biological response even with limited receptor occupancy (Seelig and Sayers, 1973; Catt and Dufau, 1978), the assessment of the physiological significance of the observed FSH-BI/LH-RBI activities of these two peptides is problematical.

Long term effects of α and β

It is significant that the results of the long-term study using juvenile female rats points to a direct ovarian action not only of *β* but of *α* as well. Chari *et al.* (1981a, b, 1982) have accumulated data in support of the contention that inhibin from human follicular fluid, while exhibiting the characteristic ability to suppress post-castrational rise in FSH, also acts to interfere directly with ovarian response to FSH. Along the same lines, Franchimont *et al.* (1979) have reported that low molecular weight preparations of inhibin from human seminal plasma and ram rete testis fluid are capable of inhibiting the multiplication of testicular germinal cells *in vitro,* as judged by the incorporation of tritiated thymidine into DNA.

In conclusion, the results presented in this paper demonstrate that the peptide α is truly inhibin in nature, acting as it does at the level of the pituitary to suppress FSH output, whereas β exerts its effect directly at the gonadal level and has no influence on the pituitary.

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