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Could hormone-induced loss of gonadotrophin receptors reduce the efficiency of superovulations stimulated by PMSG ?

par J. P. GOSLING, Mairead RYAN, Patricia MORGAN,

Department of Biochemistry, University College, Galway, Ireland.

Summary. Any future, large-scale, commercial application of embryo transfer techniques will require a continuous and reliable supply of embryos. The simplest potential method of meeting such a demand would be by superovulation using PMSG-*provided* treatments could be developed which are reliable *and* feasible in terms of cost and complexity. At present the exact requirements to modify ovulation rate in the cow are unknown. Neither are all the biological effects of treatment with PMSG understood. In an attempt to begin to clarify these areas, the literature on the biological effects of gonadotrophins, and the role of gonadotrophins in follicular development and ovulation is reviewed. In light of the very long half-life of PMSG, particular attention is paid to the ability of gonadotrophins to induce receptor loss and to desensitize target cells.

Introduction.

Superovulation and follicle stimulating activity

If embryo transfer techniques are to play a significant role in commercial cattle reproduction, reliable treatments to induce superovulation must be developed. Current superovulation treatments all rely on the use of gonadotrophins with follicle stimulating activity (Foote and Onuma, 1970 ; Sreenan and Beehan, 1976 ; Seidel *et al.*, 1978).

Pituitary follicle stimulating hormone (FSH) is unlikely to be ever available in large enough supply at a low enough cost. Since about 30 mg of crude FSH (Armour-P; Seidel *et al.*, 1978) is required for one superovulation treatment, FSH extracted from 100-500 ovine or bovine pituitaries (calculated from data in Stockell Hartree 1978 and Laster, 1972) would be necessary. The efficient purification scheme of Closset and Hennan (1978) which uses porcine pituitary acetone powder as starting material, may give a better yield. Equine or human pituitaries contain up to 20 times as much FSH as ovine or bovine glands (Stockell Hartree, 1978) but are not, of course, as readily available.

The serum of pregnant mares between day 40 and day 100 of pregnancy is the major source of follicle stimulating activity available commercially. Pregnant mare serum gonadotrophin (PMSG) is prepared by selective precipitation (Passeron, 1978)

to give a product with 1 500 to 3 000 IU/mg and which is approximately 20 p. 100 pure (Schams and Papkoff, 1972). The concentration of PMSG is 50-100 IU/ml of pregnant mare serum and therefore 25 to 60 ml of serum will yield sufficient partially purified PMSG (1 500-2 500 IU ; Sreenan and Beehan, 1976) to induce superovulation in one cow.

PMSG is, therefore, likely to be the sole source of follicle stimulating activity ever to be readily available in quantity ; and consequently, any future widespread application of superovulation treatments would be facilitated if PMSG rather than pituitary FSH were employed.

PMSG-properties and drawbacks.

PMSG is a unique gonadotrophin in at least two respects. It has a very long half-life (50-120 hrs in cattle ; Schams *et al.*, 1978) due to its sialic acid content, which at 21 p. 100 on the β-subunit (Papkoff, 1974) is easily a record for gonadotrophins. In addition, as well as FSH activity, it also acts as a luteinizing or interstitial cell stimulating hormone (Papkoff, 1978).

These properties contribute to the potency and usefulness of PMSG but may also be related to its undesirable side effects. For example, in cows treated with PMSG to induce multiple ovulations, PMSG is still detectable in the circulation 6 days after the time of the induced « superovulation » (Schams *et al.*, 1978). Therefore, in contrast to FSH, PMSG may continue to exert an effect long after the time of treatment, and not just an FSH effect but also an LH effect. The disadvantages of the superovulation treatments currently employed are well documented (Sreenan and Beehan, 1976). Most employ PMSG, and in the cow the major problem is that the ovulation rates obtained are highly variable. Variations in the LH : FSH activity ratios of different batches of PMSG were suggested as being important, but this now appears unlikely (Stewart *et al.*, 1976).

PMSG *in relation to endogenous gonadotrophins.*

It is our contention that a more complete understanding of the biological effects of treatment with PMSG may help in the design of improved superovulation treatments. But since PMSG treatment is intended to mimic, at a more potent level, the effects of the endogenous gonadotrophins, it is necessary to first understand the biological effects of the endogenous gonadotrophins.

In the last 5 years, it has become clear that the biological effects of gonadotrophins are extremely complex (Hseuh, 1978 ; Catt and Dufau, 1976). For example, apart from stimulating steroidogenesis, LH is necessary for the maintenance of target cell integrity (Dym and Madhwa Raj, 1977) and it can also desensitize the target cells to its own effects (Dufau *et al.*, 1978*b*). The great majority of relevant studies have been performed using small laboratory animals but the findings are undoubtedly of general significance to our understanding of factors which may influence follicular growth and ovulation in the cow. However, very few investigations of the molecular mode of action of PMSG in comparison to LH and FSH have been carried out, even in laboratory animals.

The objective of this paper, therefore, is to review the recent literature on the mode of action of gonadotrophins, the regulation of cell sensitivity to gonadotrophins, and the role of gonadotrophins in follicular maturation and ovulation. In addition, the apparent ability of PMSG to regulate LH receptors willbe discussed.

Biological effects of gonadotrophins.

Mechanism of action.

Peptide and protein hormones interact with their target cells by binding to specific receptor molecules located on the outside of the cell membrane (Catt and Dufau, 1976; Shiu and Friesen, 1976). After binding, the hormone receptor complexes are internalized (Nordquist and Palmieri, 1974 ; Conn *et al.*, 1978) and degraded. Whether the hormone exerts any biological effect after internalization is unknown. Concomitant with occupation of the receptor, intracellular membrane bound adenyl cyclase is activated and cyclic adenosine 3′ : 5′-monophosphate (cAMP) synthesis from ATP is stimulated (Sutherland, 1972). Hormones having luteinizing hormone or follicle stimulating hormone activity both act in this way (Catt and Dufau, 1976) but each activity is associated with distinct and specific receptors (Kammerman *et al.*, 1972 ; Nimrod *et al.*, 1976). How the specific effects of different hormones are conveyed through a common second messenger is unknown ; but it may be due to specific compartmentalization of the cAMP synthesized (Dufau *et al.*, 1978*a*). Cyclic-AMP activates intracellular protein kinases which in turn catalyse the phosphorylation of proteins, the natures of which are at present unknown. The obligatory role of cAMP in the mechanism of gonadotrophin action was given strong experimental support by the work of Dufau *et al.* (1977).

Regulation of target cell sensitivity.

Catt and Dufau (1973) demonstrated that, while the majority of Leydig cell, LH-specific receptors are coupled to adenyl cyclase, occupation of less than 1 p. 100 of the receptors induced a maximal stimulation of testosterone synthesis. They postulated that the role of the « spare receptors » was to maximize sensitivity. This has been supported by Conti *et al.* (1977*b*) who found that the sensitivity of luteal cell progesterone synthesis to stimulation by human chorionic gonadotrophin (hCG) was directly related to the number of LH/hCG receptors per cell.

In the last three years many studies have demonstrated the ability of peptide hormones to influence or control the concentration of receptors specific for the same hormone or for another hormone. The number of LH/hCG receptors on rat Leydig cells may be dependent on, or be increased by treatment with FSH (Ketelslegers *et al.*, 1978), prolactin, growth hormone (Payne and Zipf, 1978) and insulin (Charreau *et al.*, 1978). However, treatment with LH or hCG can induce an active degradation of receptors (Hseuh *et al.*, 1976 ; Sharpe, 1976 ; Auclair *et al.*, 1977 ; Purvis *et al.* ; 1977 ; Tsuruhara *et al.*, 1977 ; Hseuh *et al.*, 1977 ; Haour *et al.*, 1977 ; Huhtaniemi *et al.*, 1978). Such treatments make testosterone synthesis by Leydig cells refractory to hCG stimulation (Hseuh *et al.*, 1977 ; Tsuruhara *et al.*, 1977). This occurs not only by a reduction in receptor number, but also by a desensitization of hCG-stimulatable

adenyl cyclase, and by desensitization of steroid synthesis to stimulation by cAMP ; whether induced by cholera toxin or in the form of dibutyryl cAMP (Tsuruhara *et al.*, 1977). The refractoriness is spontaneously reversible and normal functions return after 6 to 14 days depending on the dose of hCG administered (2 to 100 IU injected intravenously ; Tsuruhara *et al.*, 1977).

The LH/hCG receptors of corpus luteum cells are also subject to regulation by hormones. In the rat, prolactin is apparently necessary for the synthesis of a normal amount of receptor by the differentiating luteal cells (Holt *et al.*, 1976 ; Richards and Williams, 1976). Treatment with prostaglandin $F_2 \alpha$ causes a decrease in receptor concentration and this is prevented by simultaneous application of prolactin (Grinwich *et al.*, 1976). While treatment with small amounts of hCG may have a stimulatory effect, which reflects the role of LH in maintaining luteal function ; treatment with larger amounts causes a dramatic decrease in the number of measurable receptors on luteal cells (Conti *et al.*, 1976 ; Conti *et al.*, 1977*a* and 1977*b*). The detailed studies of Conti *et al.* (1977*a*) indicate that, as in rat testis, this is a process of active receptor degradation. They found that occupation of 1-2 p. 100 of the receptors 5 hr after treatment was associated with loss of 40-50 p. 100 of receptors 24 hr after treatment. As with Leydig cells, these treatments also induce a refractoriness in the steroidogenic response, in this case hCG stimulated progesterone synthesis (Conti *et al.*, 1977*b*).

Receptors and follicular development.

The investigations of Richards and coworkers (Richards and Midgley, 1976 ; Richards *et al.*, 1978) and others (Lindner *et al.*, 1977 ; Lamprecht *et al.*, 1977 ; Ryan *et al.*, 1977 ; Lee, 1976) have added a new dimension to our knowledge of the control of follicular development ; namely the role of cellular sensitivity to hormone stimulation. Most of these studies involved hypophysectomy or hormone replacement therapy and, therefore, have been carried out with laboratory animals, but, since the general conclusions agree with such data as has been obtained with large animals (e.g. Lee, 1976) and since the conclusions complement and extend, rather than contradict, the classical concepts of the roles of estrogens, FSH, LH and prolactin ; they are probably relevant to the situation in humans or even cows. To quote Richards *et al.* (1978) ; « once the mechanisms are more clearly understood, similarities rather than differences may be revealed ».

FSH binds specifically to the granulosa cells of primordial follicles in the rat (Presl *et al.*, 1974) and in the presence of estradiol causes granulosa cell proliferation and an increase in the number of FSH receptors on granulosa cells (Richards and Midgley, 1976). Treatment with FSH induces an increase in the density of LH specific receptors on granulosa cells (Zeleznik *et al.*, 1974 ; Richards and Midgley, 1976), and pretreatment with hCG, while having no effect by itself, potentiates and prolongs this effect (Ireland and Richards, 1978). Accordingly, we now have a picture of follicle development in which hormones interact with follicles to increase the ability of cells to respond later to stimulation by the same and/or other hormones.

The most dramatic change that occurs is the increase in the number of LH specific receptors per cell, on the granulosa cells. This increase is clearly correlated with stage of cycle in rats (Nimrod *et al.*, 1977) and with follicle size in pigs (Channing and

Kammerman, 1973 ; Kammerman and Ross, 1975 ; Stouffer *et al.*, 1976 ; Lee, 1976 ; Nakano *et al.*, 1977), sheep (Carson *et al.*, 1978) and cows (Gosling *et al.*, 1977) and may therefore be regarded as a general feature of follicle growth.

Table 1 is taken from Gosling *et al.*, (1978) and shows the results of an experiment in which granulosa cells were incubated with increasing concentrations of ^{125}I-hCG in order to characterize the LH/hCG binding properties of the cells. The granulosa cells were obtained from antral follicles of three size classes from normal cows at slaughter. For further details see the legend of table 1. These results and the results of other subsequent experiments (Morgan and Gosling, unpublished results), indicate that as follicles develop, their binding capacity for hCG or LH increases but that the binding affinity does not change. The most dramatic increase in the number of binding sites per cell occurs as the follicles approach preovulatory size.

TABLE 1

^{125}I-hCG *binding to bovine granulosa cells*

Size of follicles	Ka (M^{-1})	Ka ($\times 10^{-10}$ M) $\pm$ SEM	Bniding sites per cell $\pm$ SEM
2-6 mm	10.9×10^9	0.9 ± 0.9	140 ± 50
6-10 mm	9.2×10^9	1.1 ± 0.2	$2\,063 \pm 164$
> 10 mm	9.3×10^9	1.1 ± 0.3	$4\,025 \pm 510$

(from Gosling *et al.*, 1978)

Ovaries were obtained fresh at the abattoir and placed immediately on ice. The animals were mostly heifers and non-pregnant. The granulosa cells were harvested by the method of Channing and Kammerman (1973) with 1 p. 100 egg albumin (Sigma grade II), 0.1 M Sucrose, 5mM $MgCl_2$, 0.05M tris-HCl, pH 7.5 as buffer. Follicles which appeared in any way atretic were not used. The incubation was in a shaking water bath at 37 °C for 6 hrs. Each tube contained 3.5×10^6, 3.5×10^6 or 3.0×10^6 cells from small medium or large follicles respectively. ^{125}I-hCG was prepared by lactoperoxidase catalysed iodination of hCG (Cr 119) supplied gratis by NIAMDD. The specific activity of the label was 60 μCi/μg and it was 40 p. 100 bindable by excess rat testis LH receptors. Each tube contained between 0.2 to 5.0 $\times 10^{-10}$M bindable ^{125}I-hCG. Specific binding was estimated by including tubes corresponding to each set of conditions which also contained 5 μg of hCG. Nonspecifically bound counts (less than 2 p. 100 of total label) were subtracted to give specific binding. Free label was removed after incubation by 3 centrifugal washings, and the bound fraction counted. ^{125}I-hCG bound was plotted against total bindable ^{125}I-hCG present, and fitted to a hyperbola by a least squares method to give estimates of binding per cell and binding affinity.

The exact biological purpose of this widespread property of granulosa cells from preovulatory follicles is unknown. That the receptors are biologically active is supported by the finding that hCG stimulatable adenyl cyclase increases in parallel with receptor number (Lee, 1976). There is now evidence to indicate that the preovulatory LH surge, or treatment with hCG causes the granulosa cells of preovulatory follicles to be desensitized to further stimulation by hCG (Marsh *et al.*, 1973 ; Hunzicker-Dunn *et al.*, 1976). The hCG stimulatable adenyl cyclase activity falls (Hunzicker-Dunn *et al.*,

1976) and so does the number of LH/hCG receptors (Richards *et al.*, 1976 ; Bockaert *et al.*, 1976).

In the bovine the number of receptors per cell form the corpus luteum of mid-pregnancy is about 50.000 (Papaionannou and Gospodarowicz, 1975) which is considerably greater than the number per granulosa cell from bovine (4.000/cell ; Gosling *et al.*, 1978) or porcine (10.000/cell ; Kammerman and Ross, 1975 ; Lee, 1976) large follicles. Whether these receptor molecules are the same molecular species as the granulosa cell receptors is unknown.

Biological effects of PMSG.

PMSG cross-reacts in specific receptor assays for both LH and FSH (Stewart *et al.*, 1976), and such systems have been employed to measure the ratio of LH to FSH activity in various commercial batches of PMSG. (Stewart *et al.*, 1976 ; Schams *et al.*, 1978). The α subunit of PMSG will combine with the β subunit of pituitary LH or FSH (Papkoff, 1974). Therefore, PMSG is closely related to LH and FSH in general molecular structure and probably all evolved from an ancestral gonadotrophin. However, in spite of the widespread use of commercial PMSG in endocrine laboratories to stimulate ovarian activity, the biological effects of PMSG as compared to LH or FSH have been little studied. In addition, results obtained in *in vivo* experiments are not directly comparable because of the very long half-life of PMSG. Combarnous, Ketelslegers and Hennan (1978) examined the relative affinities of porcine FSH (pFSH), pLH and PMSG for porcine testicular FSH specific and LH specific receptors. They found, that though the relative affinities of LH and PMSG for LH specific receptors were similar, the affinity of FSH specific receptor for pFSH was 4-fold greater than for PMSG. They concluded that PMSG is much more effective as a luteinizing hormone than it is as a follicle stimulating hormone.

Can PMSG *induce* LH-*receptor loss ?*

Unlike the dynamic tissues of the active ovary, rat testis interstitial cells are a relatively stable, and very well characterized, target for LH action. For this reason much of the work on the role of hormones in the regulation of LH receptors has been done with adult male rats (Dufau *et al.*, 1978*b*). Therefore, in our preliminary study to examine the effects of PMSG treatment on LH receptors, we employed adult male rats (Gosling *et al.*, 1979). Rats of similar weight were injected subcutaneously at a single site. Two days later the rats were killed and the hCG binding capacity of the interstitial cells estimated. For further details see the caption of table 2. A two day period was chosen because from the data of others (Tsuruhara *et al.*, 1977) receptor loss, when it occurs, is well established at this time. Table 2 (taken from Gosling *et al.*, 1979) shows the mean amounts of hCG bound per mg interstitial cell homogenate for each treatment. The treatments were : injection with saline, 5 IU PMSG, 50 IU PMSG or with 500 μg LH. The data were subjected to a single factor analysis of variance and the differences between treatments were significant ($p < 0.05$). For rats treated with 50 IU PMSG the mean was significantly lower than the control ($p < 0.05$) and for LH treated animals the difference was highly significant ($P < 0.01$). Treatment with 5 IU PMSG had no effect. Papkoff *et al.*, (1978) reported that pure PMSG was equipotent

with pure LH in a radio-receptor assay system. This would indicate that 50 IU of PMSG is equivalent to about 5 μg of the ovine LH employed in the above experiment. The results of a radio-receptor analysis of the PMSG batch actually used agreed with this.

TABLE 2

Mean amounts of choriogonadotropin specifically bound to testis homogenates prepared after 2 days from treated rats

No. of rats	Infection	Choriogonadotropin bound (fmol/mg of protein)
5	Buffer	6.21 ± 0.35
6	5 IU PMSG	5.37 ± 0.89
5	50 IU PMSG	3.88 ± 0.78 *
5	500 μg oLH	2.01 ± 0.23 **

(from Gosling *et al.*, 1979)

The incubation was for 3 hrs. The rats were killed by cervical dislocation and decapsulated testes were gently homogenized in buffer and filtered through glass wool to give a solution rich in interstitial cells and cell fragments. Known amounts of homogenate (5-6 mg of protein) were added to each tube. The concentration of hCG was 600 pM which included 20,000 cpm of ^{125}I-hCG. The incubation was for 3 hrs. For further details see the legend of table 1. Results above are means ± SEM. The ovine LH (NIH-LH-518) was supplied gratis by NIAMDD. *Significantly different from control ($p < 0.05$). **Significantly different from control ($p < 0.01$).

Therefore it appears that PMSG as well as hCG and LH can induce LH receptor loss. However, these preliminary results need to be extended. For example the extent to which the apparent loss may have been due to receptor occupancy was not determined. And to determine the relative potencies of these hormones would require a system uncomplicated by differences in clearance rates ; for example an *in vitro* system like that proposed by Chen and Payne (1977). Neither was the time course of the effects of each treatment determined.

The degree of relevance of these results to the induction of multiple ovulation in the cow must remain for the moment indeterminate. However, the ability of treatment with PMSG to induce LH receptor loss, coupled to the susceptibility of granulosa cell and luteal cell LH receptors to desensitization plus the fact that PMSG remains in the bovine circulation for up to 12 days after treatment (Schams *et al.*, 1978) ; suggest that the poor results obtained in general with superovulation treatment employing PMSG may in part be due to interference by PMSG in the normal processes of ovulation and luteinization. The finding that shortening the time of PMSG action by treatment with anti-PMSG serum tends to improve the results of superovulation treatment (Dhondt *et al.*, 1978) is evidence in favour of this hypothesis. However, a greater understanding of the molecular processes involved may allow the development of simple treatments which are practicable on a large scale and which give reliable results.

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Résumé. Toute application commerciale à grande échelle dans le futur des techniques de transfert d'embryons nécessitera un approvisionnement continu et sûr d'embryons. La méthode potentielle la plus simple pour faire face à une telle demande serait par superovulation, en utilisant PMSG — à condition que des traitements sûrs et réalisables en termes de coût et de complexité puissent être développés. Actuellement les exigences précises pour modifier le taux d'ovulation chez la vache sont inconnues. Ne sont également pas connus tous les effets biologiques du traitement avec PMSG. Afin de commencer à clarifier ces domaines, la bibliographie sur les effets biologiques des gonadotrophines et le rôle des gonadotrophines dans le développement folliculaire et l'ovulation est passée en revue. En raison de la très longue demi-vie de PMSG, une attention particulière a été portée à la possibilité d'induction de perte de réceptivité et de désensibilisation des cellules-cibles par les gonadotrophines.

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