Is there a mucosal immune system associated with the mammalian oviduct?

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The oviduct is a key component of the reproductive system where essential stages such as spermatozoa capacitation, fertilization and early embryo development take place. Recently, an additional role for the entire female reproductive tract has emerged with important implications for our understanding and management of reproductive health, namely, its role in mounting local immune responses against microbial pathogens. Most of the evidence about mucosal immune responses in the female reproductive tract is related to the vagina, with less information available for the uterus. The less known segment in this regard is the oviduct, which prompted us to review and summarize the current state of knowledge on the immune system at the level of the mammalian oviduct. A comprehensive search was conducted in Medline and -for the last two years- also in Current Contents. Results demonstrate that the mammalian oviduct is endowed with many of the elements that characterize a mucosal immune system. However, there are gaps in our knowledge that, in addition to important interspecies differences, make it essential to explore further some fundamental questions regarding this system.

Key-terms: *Fallopian tube, immunoglobulins, local immune system, mucosal immune system, oviduct, review*

INTRODUCTION

There are several but not many papers demonstrating cellular and humoral components of the immune system in the oviduct of various species. The evidence suggests that there is an oviductal mucosal immune system different from the one described for the intestinal and respiratory tracts, as well as for the lower segments of the female reproductive tract. This putative oviductal immune system might contribute to maintain an aseptic *milieu*, free of the microorganisms that sporadically colonize the upper reproductive tract.

The purpose of this paper is to summarize the current knowledge about the existence of a local immune system associated with the oviductal mucosa in mammals. It is not intended to account for all the extensive information regarding the cytopathological changes induced in the oviduct by microorganisms causing sexually transmitted diseases, unless the data shed light on local immune mechanisms. For the same reason, a detailed account of all the evidence on oviductal cytokines was not intended. Interested readers on those topics may get that information in several excellent recent

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reviews (for example, see Cohen & Pollard, 1996; Cooper & Moticka, 1996).

This review is based on a search in Medline and Current Contents databases. The first was examined from 1970 to 1997, while Current Contents was used to locate the most recent papers and thus was only searched from 1996 to 1997. Keywords used were: Fallopian tube, oviduct, immune, immunoglobulins, secretory IgA, leukocytes, cytokines, salpingitis, major histocompatibility complex (MHC) class II, macrophages and lymphocytes. Over one hundred articles were found and our analysis is based on the papers considered strictly relevant to this review, that is, 28, plus a number of reviews and some general references.

The oviduct.

The mammalian oviduct (also commonly referred to as uterine or Fallopian tube) is a paired organ that extends bilaterally from the uterus towards the ovaries. One end of the oviduct is adjacent to the ovary and opens into the peritoneal cavity (human) or the *bursa ovarica* (rodents). The other end communicates with the uterine lumen. According to its structure and function, the oviduct may be divided into four segments, from the ovarian to the uterine end: infundibulum, ampulla, isthmus, and intramural or uterine portion. The infundibulum comprises the most distal portion of the oviduct and is formed by numerous finger-like projections, the fimbriae, whose ciliated cells facilitate the ovum "pick up" at the time of ovulation. The ampulla extends from the infundibulum toward the isthmus, is richer in ciliated cells than the isthmic portion and is endowed with numerous mucosal folds that project into its lumen. The isthmus is a narrower segment than the ampulla, whose muscular activity is more prominent than ciliary activity in the propulsion of the luminal ova towards the uterus. A small segment of the isthmus projects into the uterine wall before opening in the lumen of the uterus.

The reproductive roles of the oviduct include the timely transport of ova and early embryos toward the uterus, providing the appropriate environment to support the capacitation of spermatozoa, as well as fertilization and early embryo development. This organ has been demonstrated to be mainly under the regulation of sex steroids, that influence both the structure of the epithelium and composition of oviductal secretions (reviewed by Harper, 1994; Croxatto, 1996). The speed of ovum transport is also under the regulation of estradiol (Ortiz *et al*, 1979; Zenteno *et al*, 1989) and progesterone (Fuentealba *et al*, 1988) (more references in Croxatto *et al*, 1991). At least in the rat, the safety factor of ovum transport makes it highly resistant to strong stressful stimuli, despite important changes in circulating levels of sexual and adrenal steroids (Cardenas, 1992). Other aspects of oviductal function like its local immune system might be affected by the immunosuppressive effects of stress, but this problem apparently has not been addressed in the literature.

The mucosal immune system.

The mucosal surfaces are protected by both unspecific and specific defense mechanisms. The first group includes mechanical barriers, desquamation of epithelial cells, and humoral factors like lysozyme, lactoferrin and peroxidases. The specific mechanisms include lymphoid cells specifically located in or under the epithelia that locally produce antigen-specific antibodies (B lymphocytes), regulate the local immune response (T helper lymphocytes) or recognize and kill tumor and viral infected cells (cytotoxic T lymphocytes) (for a review see Kutteh & Mestecky, 1996).

The mucosal immune system is classically divided according to the site of antigen exposure and thereby initiation of the response. The bulk of the evidence about mucosal immunity has been obtained from the gastrointestinal and upper respiratory tracts, whose local immune systems are known as gut associated lymphoid tissue (GALT) and bronchus associated lymphoid tissue (BALT), respectively (Ada, 1993). More recent evidence about the mucosal immune system

in the female reproductive tract of several species has been obtained mostly from data available for the uterus and vagina.

The demonstration of a mucosal immune system distinct from the systemic one came from pioneer experiments providing evidence on the independence of the immune responses elicited by immunization throughout mucosal or systemic routes. In fact, mucosal immunization normally produces increased titters of specific antibodies in all the mucosae of the body without changes in circulating antibodies, as has been demonstrated for oral and vaginal immunization (reviewed by Ada, 1993; Mestecky & McGhee, 1993). This led to the model of a common immune system associated with the mucosae of the body.

The mucosal immune system differs from the systemic one by several features:

i- The predominant molecular form of immunoglobulin (Ig) produced is polymeric or secretory IgA (sIgA).

ii- This sIgA is produced by plasma cells located in subepithelial clusters of lymphoid cells, which in the gut form the Peyer's patches.

iii- Polymeric IgA released in the subepithelial tissue binds to the polymeric Ig receptors, located in the basal plasma membrane of epithelial cells.

iv- The polymeric IgA receptors trigger sIgA translocation throughout the epithelium into the lumen.

v- In the apical membrane, sIgA is released by proteases that cleave the polymeric receptor, releasing a fragment known as "secretory component", bound to the Ig.

vi- In the gut, the epithelial cells over the Peyer's patches are specialized to transport luminal antigens towards the subepithelium, where antigen presentation takes place.

vii- The function of sIgA is different from the predominant Igs in the circulation, namely, its effector functions are limited to inhibition of microorganisms adherence, induction of agglutination in the lumen and neutralization of intracellular viral particles (Mazanec *et al*, 1992; Kutteh & Mestecky, 1996).

viii- Lower amounts of IgG are steadily transported to the lumen by transudation, where they can trigger additional mechanisms, like complement-mediated lysis, but this process does not appear to be specifically related to the local induction of the immune response (Kutteh & Mestecky, 1996).

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Immune response.

A mucosal immune system at the level of the oviduct implies that local production of antibodies is induced after first exposure of antigens inside the oviductal lumen. Immune responses after local inoculation of microorganisms have been in fact demonstrated in animal models. *Chlamydia trachomatis* suspensions were inoculated into the ampullar segment in rabbits and specific antibodies appeared in serum after 1 to 2 weeks (Patton *et al*, 1982). Unfortunately, the levels of secretory IgA in oviductal flushes were not examined and precautions were not taken to avoid spilling over of *Chlamydia* into other segments of the reproductive tract or the peritoneal cavity. This last mechanism probably explains the appearance of circulating antibodies, because it is now known that immunization at the mucosal level does not induce systemic immune responses (Ada, 1993). Landers *et al* (1991) inoculated *C. trachomatis* into the bursa ovarica and uterine horns of mice, producing infection in all the reproductive tract, including the oviducts, but again IgG and IgM were only searched for in the peripheral circulation. In addition, the inoculation sites do not allow the conclusion that a local immune response was induced at the level of the oviduct. Patton *et al* (1983) studied the immune response in pig-tailed macaques after experimental induction of salpingitis by *C. trachomatis*, by examining the levels of IgA recovered from samples of cervical mucus. Higher levels of specific circulating IgG were detected after 2 weeks, as well as of IgA in the cervical mucus, which is a

demonstration of mucosal immune responses in the reproductive tract.

In humans, there are a few reports of increased specific IgG and IgA levels in the oviduct associated with antigen exposure. Ping (1979) recovered anti-spermatic IgG and IgA antibodies from oviducts flushed *in situ* during laparotomies for surgical sterilization. However, the migration of spermatozoa from the vagina towards the oviduct makes likely that antigen presentation leading to specific IgA production might have taken place at different levels of the reproductive tract. Furthermore, because of the migration of lymphocytes between the different mucosae of the organism (Mestecky & McGhee, 1993), the results of Ping (1979) also suggest that the oviductal mucosa is part of the mucosae-associated immune system of the body. A demonstration of local activation of the immune system in the human oviduct was provided by Cooper *et al* (1987), who cultured organs *in vitro* with *Neisseria* strains. Co-culture with the bacteria for up to 48 h increased the number of IgA-positive plasma cells in the subepithelial tissue, and also the immunostaining of the epithelium for secretory component and secretory IgA, with no changes in the number of IgG-positive cells. As stated by the authors, those changes do not necessarily result from specific activation after antigen presentation and probably reflects nonspecific response to lipopolysaccharide released by the Gram-negative bacteria (Cooper *et al*, 1987). Recently, in our laboratory, we (Cardenas *et al*, 1996) have demonstrated increased levels of IgA in oviductal flushings obtained *in vitro* from oviducts with salpingitis, as compared to non-inflammatory oviducts, without relation to the menstrual cycle; such difference was not detected in peripheral IgA levels.

The appearance of specific IgG and IgA in oviductal fluid collected through chronic intraoviductal cannulae in rhesus monkeys, after intramuscular immunization with the spermatozoal protein SP-10, was recently reported (Kurth *et al*, 1997). In this case, the passage of antibodies from the serum into the oviductal lumen is suggested by the correlation between plasmatic and intraoviductal levels of specific immunoglobulins, and by the observation that specific IgG levels in the oviductal fluid were at least one order of magnitude higher than IgA.

In conclusion, although evidence suggests that a local immune response could be induced in the oviduct, there is no report as yet demonstrating that antigen exposure restricted to the oviductal lumen induces a mucosal immune response.

Tissue and luminal IgA.

Histochemical studies have shown that the majority of plasma cells in the human oviduct produces IgA, with up to one third of those cells producing IgG (Kutteh *et al*, 1988, 1990; Kutteh & Mestecky, 1994). However, collection of luminal fluid in volunteers –by intraoviductal chronic cannulae exteriorized through the abdominal wall– yielded higher concentrations of IgG than IgA (Lippes *et al*, 1972). Similar findings were reported in rhesus monkeys with chronic cannulation for collection of intraoviductal fluid (Yang *et al*, 1983). Since IgG levels in the oviductal fluid were shown to be strongly correlated with serum IgG levels and albumin in the oviductal fluid of these monkeys, most of the IgG recovered from the oviductal fluid might have been originated from serum by transudation secondary to local inflammation and edema produced by the chronic cannulation. In the mouse oviduct, IgA producing plasma cells also predominate in the subepithelial tissue (Parr & Parr, 1985) and, in the rabbit, IgA levels were found to be higher than IgG levels in oviductal fluid (Oliphant *et al*, 1977). However, the secretion of IgA into the oviductal lumen might not occur in all mammalian species, since IgA was not detectable in oviductal flushings in the mare (Widders *et al*, 1984a,b).

Secretory component.

The presence of the secretory component (SC) bound to IgA is characteristic of mucosal immune systems (Mestecky &

McGhee, 1993). By immunohistochemistry, SC has been demonstrated in the epithelium of the human oviduct (Lippes *et al*, 1970; Tourville *et al*, 1970; Kutteh *et al*, 1988, 1990; Kutteh & Mestecky, 1994), but we have no information about similar reports for other species.

Leukocytes.

Lymphoid cells have been examined in the oviduct of several species (for example, see Otsuki *et al*, 1989, for rabbit; Parr & Parr, 1985, for mouse). However, the most complete characterization has been made for the human oviduct (van Bogaert & Maldague, 1978; Haney *et al*, 1983; Peters, 1986; Otsuki *et al*, 1989; Boehme & Donat, 1992; Wollen *et al*, 1994; Givan *et al*, 1997), where helper T cells (CD4+), cytotoxic/suppressor T cells (CD8+) and B lymphocytes have been found, in addition to granulocytes and macrophages. Most of the oviductal lymphocytes are scattered all over the *lamina propria*, but few accumulations of lymphocytes –resembling follicles without germinal center– have been described in the isthmic portion of the organ (Otsuki *et al*, 1989). There are also intraepithelial cytotoxic/suppressor T cells and a smaller number of helper T cells (Peters, 1986; Boehme & Donat, 1992). Cytotoxic/suppressor T lymphocytes have not been functionally characterized, *i.e.*, it is not known whether these cells have a classical cytotoxic activity against virus infected cells or if they are suppressor T cells. In the first case, a highly heterogeneous set of T cell receptors (TCR) would recognize a viral peptide specifically bound to MHC class I molecules on the surface of infected cells (Zhang *et al*, 1992; Shibata *et al*, 1992; Imarai *et al*, 1995). The binding of TCR and the viral peptide bound to MHC class I induces the cytotoxic activity and lysis of infected cells. If CD8+ T lymphocytes have suppressor activity (Bloom *et al*, 1992), local immunosuppression will be the most likely outcome after their activation (reviewed by Strobel & Mowat, 1998).

Lymphoid cells were not found in the rabbit oviduct (Otsuki *et al*, 1989) and only plasma cells have been described in the mouse oviduct (Parr & Parr, 1985). According to Parr and Parr (1996), there is no report yet dealing with the identification of lymphocyte populations in the oviduct of rodents.

Cytokines and growth factors.

Because many of the functions of the immune system are regulated by soluble molecules collectively known as cytokines, it is pertinent to look for these molecules in the oviduct in the context of this review. Inflammatory cytokines such as interleukin-1 (IL-1), interferon γ, IL-6, IL-8 and tumor necrosis factor α (TNF-α) and high levels of the immunosuppressor cytokines IL-10 and TGF-β are detected in the lumen of the organ (Hunt *et al*, 1993; Srivastava *et al*, 1996; reviewed by Chegini, 1996). Cellular sources of these molecules probably include the lymphoid cells that migrate into the organ during local infection (van Voorhis *et al*, 1996). However, the epithelium of the oviduct has been shown to be a main source of several cytokines. For example, the oviductal epithelium of the mouse synthesizes TNF-α (reviewed by Hunt, 1993), which is known to trigger the inflammatory cascade that involves induction of other cytokines from several target cells. The epithelium of the human oviduct is also the primary site for the synthesis of granulocyte-macrophage colony stimulating factor (Zhao & Chegini, 1994) and transforming growth factor β (TGF-β) (Zhao *et al*, 1994).

We must have in mind that the pleiotropism of cytokine effects implies that they might not necessarily be involved in immune regulation. For example, it has been suggested that oviductal TGF-α and epidermal growth factor influence early embryonic development (Chegini, 1996). These epithelial cytokines might be directly regulated by pathogens and they may exert influence on the course of the infection, as described for *Chlamydia trachomatis*. This strongly induces the expression of TNF-α in the oviductal epithelium, a response that might play a role in the development of the ensuing salpingitis (Ault *et al*, 1996) and should inhibit the growth of this bacterium (Shemer-Avni *et al*, 1988).

An immune role for the oviductal epithelium?

Because the epithelium of the human oviduct is endowed with MHC class II molecules that may vary with the menstrual cycle (Bulmer & Earl, 1987; Edelstam *et al*, 1992; Imarai *et al*, 1998), it has been suggested that it might play a role in antigen presentation. Traditionally, MHC class II proteins are regarded as markers of antigen-presenting cells (APC), such as macrophages and Langerhans cells (Abbas *et al*, 1994). These cells –in addition to other *non professional* APC's– endocyte and breakdown protein antigens within specialized vesicles, where the binding of peptides of the foreign proteins to the MHC class II molecule occurs. The new complex MHC class II-peptide is presented to the external surface of the plasma membrane of APC's, where it binds to T lymphocytes which might then be activated. However, antigen presentation might also lead to suppression of the immune response directed towards the antigen presented if co-stimulatory molecules –like intercellular adhesion molecule 1 (ICAM-1) and B7, required for lymphocyte activation– are absent in the APC (Van Parijs & Abbas, 1998). In fact, despite the oviductal epithelium expresses ICAM-1, it appears to be restricted to the luminal border (Cardenas *et al*, 1997), where it could not bind its receptor in epithelial and subepithelial lymphocytes. Furthermore, B7 was found in none of more than 20 human oviducts analyzed by immunohistochemistry (Cardenas *et al*, 1997).

Endocytosis is an additional property of APC's that has been demonstrated for oviductal epithelial cells of the human (Croxatto *et al*, 1996; Imarai *et al*, 1998), the rat (Imarai *et al*, 1996) and the cat (Murakami *et al*, 1985). In the human, this endocytotic activity was demonstrated *in vitro* after inoculation of antigens into the lumen of small oviductal segments and it had no relationship with the menstrual cycle (Imarai *et al*, 1998).

CONCLUDING REMARKS

The mammalian oviduct contains several of the cellular and humoral elements required to elicit a local immune response: plasma cells, secretory component, secretory IgA, cytotoxic-suppressor and helper T lymphocytes, MHC class II-positive cells, like macrophages and the epithelium itself. However, there is no demonstration yet of local production of antibodies after intraoviductal immunization in any species. Thus, the question about the existence of intraoviductal induction of immune responses remains open.

The increased levels of luminal IgA during salpingitis in humans suggest local activation of a mucosal immune system, reinforcing the hypothesis that an additional role for this system might be the development of tolerance to foreign antigens, such as those that normally reach the oviductal lumen as result of sexual intercourse and also with the early embryo. This could be achieved by clonal anergy secondary to activation without co-stimulatory signals, like ICAM-1 and B7 in the epithelial cells, and/or immunosuppression suggested by the presence of cytotoxic-suppressor and helper T lymphocytes, as well as high levels of immunosuppressor cytokines.

The involvement of the oviductal epithelium in the processing of luminal antigens, taking place in several forms, is suggested by the available evidence and summarized in Figure 1. First, the internalized antigens could bind to the MHC class II complex and be presented at the plasma membrane to neighboring lymphocytes (step 1 in fig 1). Whether this would lead to antigen production will depend on the presence of co-stimulatory molecules, whose differential expression might contribute to the discrimination between pathogens and spermatozoal or embryonic antigens. Second, translocation of antigens towards the subepithelial tissue –such as demonstrated for some intestinal epithelial cells– would expose them to macrophages and other APC's which could then trigger the immune response (step 2 in fig 1). Two additional mechanisms might be the physical elimination of foreign antigens by the phagosome (step 3 in fig 1), and the production of cytokines (step 4 in fig 1) having direct inhibitory effects upon some bacteria. Because the epithelial cells

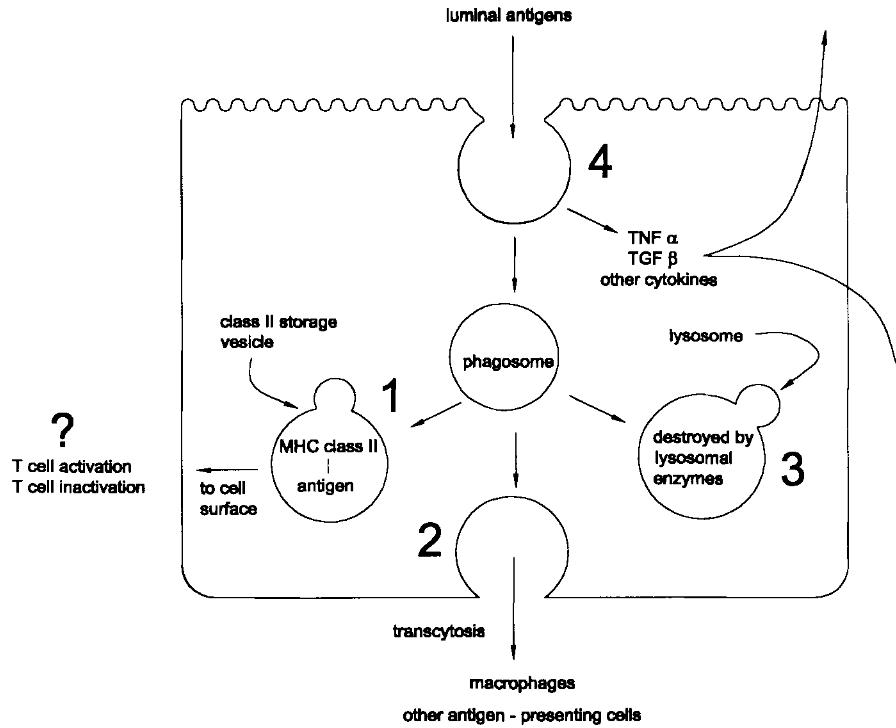

Fig 1. Model for the roles of oviductal epithelium in antigen processing and local immune responses. Model built by gathering information obtained from different species, suggesting that this epithelium might either present antigens to neighboring lymphocytes (step 1), translocate antigens towards the stroma (step 2), destroy them by combination with lysosomes (step 3) and/or modulate the local immune response by cytokines (step 4).

are the first oviductal cells facing the foreign antigens, their cytokines might also provide the initial signaling to trigger and coordinate the ensuing immune responses from epithelial and subepithelial lymphocytes. These different putative roles of the oviductal epithelium do not need to be present all the time in all the epithelial cells or in the entire organ. In fact, they could appear as part of responses to different antigenic challenges, since different cytokines have been shown to modify the immune properties of non professional APC's.

The model here proposed for the role of the oviductal epithelium in the response against foreign antigens reaching the lumen was built by gathering information from different sources and species. The important differences between species, regarding components of the putative local immune system in the oviduct, must be considered when searching for animal models to explore the important question that prompted us to write this review.

Finally, in order to disclose the clusters or areas of research defined by the original articles included in this review and their evolution, they were submitted to a citation analysis based on their references (Small, 1987). Figure 2 shows the relationships between the articles, where the lines relate each paper with the references it cites, and the time course is represented in a vertical imaginary axis from 1970 to 1998. The animal species examined in each case is indicated by the abbreviations after the reference (see legend of the figure). It is apparent that there are two main clusters, the first one (italic uppercase), with most

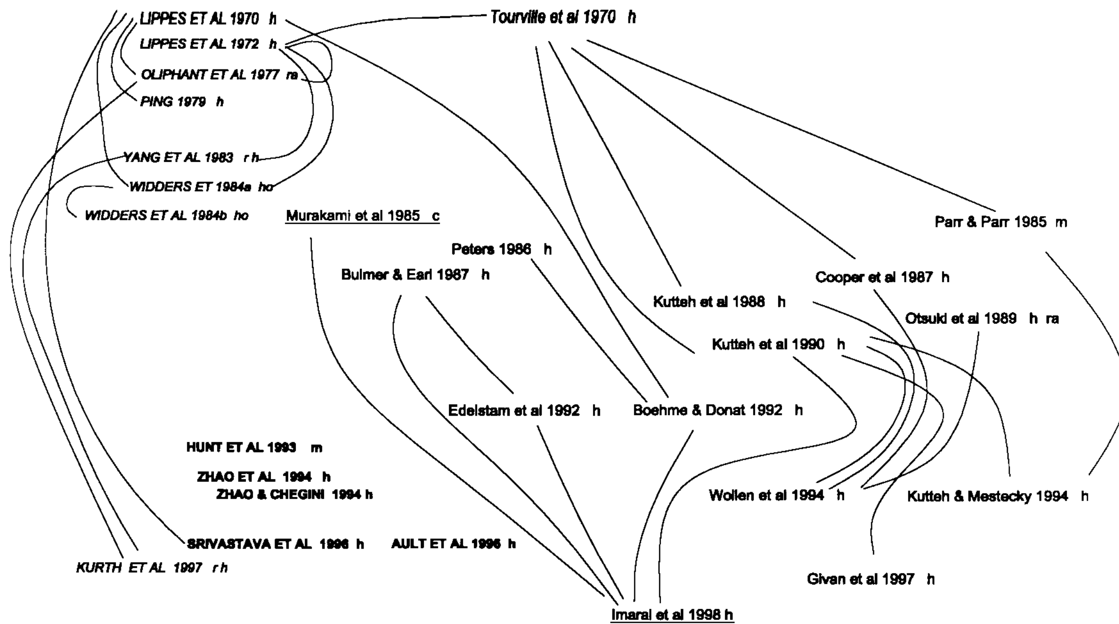

Fig 2. Citation analysis of original papers dealing with different aspects of local immunity in the oviduct. Clusters identified by letter type. First cluster (italic uppercase) deals with oviductal IgA production; second, with identification of cell types related with immune roles (lowercase); third, with oviductal cytokines (bold uppercase). A small additional group comprises two papers dealing with phagocytic properties of oviductal epithelium (underlined). Some papers may be included in more than one cluster. Animal species examined in each article indicated by single or two letter abbreviations after reference: **h**, human; **ra**, rabbit; **rh**, rhesus monkey; **ho**, horse; **m**, mouse; **c**, cat.

papers published between 1970 and 1985, deals with oviductal production of total and/or specific IgA in different species. The last member of this group is a recent paper dealing with the appearance of specific antibodies in the oviductal lumen of rhesus monkeys after systemic immunization. The second main cluster (lowercase) deals with the characterization of the oviductal local immune system, mostly by histochemical methods, its first member being a pioneer work by Tourville *et al* (1970) that identified IgA, IgG and secretory component in the mucosa of the human oviduct. A third cluster (bold uppercase) includes papers dealing with cytokines. A small additional group comprises two papers (underlined) dealing with the phagocytic properties of the oviductal epithelium.

It is apparent that most groups have been primarily interested in the human oviduct, and that early works were concerned with the local production of antibodies, providing evidence that the oviduct in most species is endowed with the capacity to produce IgA. Later, when it was technically feasible, the identification of cell populations expressing markers of cells involved in the immune response became the predominant interest of the scientific community. Understandably, papers on oviductal cytokines formed the last cluster to appear, because it followed the identification of an ever increasing number of cytokines in all tissues, including the reproductive tract.

The previous analysis points to functional studies as the most probably approach of future research in this area. The examination of specific antibodies in the oviductal lumen after systemic and mucosal immunization is of recent appearance in the specialized literature (Kurth *et al*, 1997), and needs to be further addressed to explore the feasibility of developing either protective levels of secretory IgA against microbial pathogens and/or new anticonceptive strategies. The emerging role of cytokines in the regulation of the local responses will probably involve an increasing number of researchers in this area.

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Lactation inhibits the potentiating effect of galanin upon the GnRH-induced LH release observed in diestrous-1 rat

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Recent demonstrations of no changes in hypothalamic gonadotropin releasing hormone (GnRH) gene expression and GnRH levels detected at the pituitary gland in diestrous and lactating rats, indicate that lactational hypogonadotropism in this species is not associated with inhibition of hypothalamic GnRH synthesis and secretion. Hypothalamic galanin potentiates GnRH effects on luteinizing hormone (LH) secretion in male and cycling rats. To explore the interaction between GnRH and galanin during lactation, we studied ***in vitro*** *the effects of pulsatile stimulation with those peptides upon LH synthesis and secretion from rat pituitaries on diestrous 1 or day 10 of lactation. Hemipituitaries were separately incubated in 1 ml Dulbecco's Minimal Essential Medium supplemented with 1% penicillin-streptomycin and fetal calf serum, at 37°C in 5%* CO_2*-air. The hemipituitaries were stimulated during 12 h with hourly pulses, 6 min each, of (a) gonadotropin releasing hormone (GnRH 25 ng/pulse), (b) rat galanin (600 ng/pulse), (c) GnRH plus galanin, or (d) saline. Medium was collected before each pulse to determine LH by radioimmunoassay. After the 12 h pulsatile regime total RNA was extracted and both actin and β-LH mRNA were determined by reverse transcriptase polymerase chain reaction. There was a significant stimulation of LH secretion by GnRH (ANOVA,* $p<0.001$*) without significant differences between diestrous and lactation pituitaries. Galanin alone did not modify LH secretion but it potentiated the effect of GnRH upon pituitaries from diestrous (*$p=0.036$*) but not lactating rats. Neither peptide alone or its combination modified pituitary β-LH mRNA levels. Results show that galanin regulates differently the secretion and synthesis of LH at the pituitary level. The disappearance of galanin-induced potentiation of GnRH effects upon LH secretion during lactation might contribute to the hypogonadotropism of lactation in the rat.*

Key terms: *lactation, LH, GnRH, galanin, pituitary, rat*

INTRODUCTION

There is substantial evidence in different species indicating a major role for hypothalamic gonadotropin releasing hormone (GnRH) in the regulation of LH secretion. Destruction of central GnRHergic neurons discharging in the median eminence and interruption of hypothalamus-pituitary connections (Knobil, 1980), as well as

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pharmacological antagonism of GnRH actions (McCormack *et al*, 1977; Cetel *et al*, 1983; Hall *et al*, 1990) suppress both LH secretion and ovulation. LH pulses are coincident with the synchronized discharge of at least two populations of mediobasal hypothalamic neurons (Cardenas *et al*, 1993) releasing boluses of GnRH into the pituitary-portal circulation (for example see Clarke & Cummins, 1982; Levine *et al*, 1982; Terasawa *et al*, 1988; Ramirez *et al*, 1991).

However, a variety of peptides including galanin, neurotensin, leucine-enkephalin, β-endorphin, and substance P are discharged into the pituitary portal circulation by hypothalamic neurons (refs. in Leiva & Croxatto, 1994). Some of those peptides stimulate the secretion of LH *in vitro* (Leiva & Croxatto, 1994) but their roles in the intact animal have not been established.

On the other hand, lactation in the rat, as in most mammals, is associated with inhibition of reproduction accounted for by decreased secretion of luteinizing hormone (LH) (McNeilly, 1994). Based on indirect evidence it was postulated that lactational hypogonadotropism in the rat is secondary to diminished GnRH secretion by the mediobasal hypothalamic GnRHergic neurons controlling LH secretion (reviewed by McNeilly, 1994). However, the recent demonstration of similar (a) hypothalamic GnRH gene expression in diestrous and lactating rats, assessed by measurements of GnRH mRNA (Marks *et al*, 1993), and (b) GnRH levels impinging upon the pituitary gland in diestrous and lactating rats (Cardenas & Ramirez, 1996), indicates that lactational hypogonadotropism in this species is not associated with inhibition of hypothalamic GnRH synthesis and secretion. Other factors then must be searched for to explain the lactational inhibition of reproductive function in this species.

Hypothalamic galanin has been shown to act as modulator of GnRH function in the male and the cycling rat (Merchenthaler *et al*, 1990;1991; Lopez *et al*, 1991; Leiva & Croxatto, 1994) so that it might have a role in lactational hypogonadotropism in this species. To explore the interaction between GnRH and galanin on LH synthesis and secretion during lactation we studied *in vitro* the effects of pulsatile stimulation with these peptides in pituitaries obtained from rats on diestrous 1 or day 10 of lactation. Partial results of this paper were previously communicated at the meeting of the Sociedad Chilena de Ciencias Fisiológicas (Imarai *et al*, 1997).

MATERIAL AND METHODS

Sprague-Dawley rats were obtained from the animal facilities of the Facultad de Ciencias Biológicas of the P. Universidad Católica de Chile. Rats on day 10 of lactation (8 pups/rat) or diestrous 1 were sacrificed by decapitation and the pituitaries dissected. Hemipituitaries from each rat were separately incubated in 1 ml Dulbecco´s Minimal Essential Medium supplemented with 1% penicillin-streptomycin (DMEM) and fetal calf serum, at 37°C in 5% CO_2-air. Hemipituitaries were submitted to pulsatile stimulation with (a) gonadotropin releasing hormone (GnRH 25 ng/pulse, Sigma), (b) rat galanin (600 ng/pulse, Sigma), (c) GnRH plus galanin, or (d) saline. Each experimental group consisted of 6 hemipituitaries from 6 rats. The other halves were submitted to saline stimulation as contemporaneous control group. Preliminary experiments indicate that a pre-stimulation incubation of at least 1 hour was necessary for stabilization of the basal and GnRH-stimulated LH release.

Hourly pulses were applied during 4, 8 or 12 h. Each pulse consisted of 6 min exposure to the peptide which was added to the well in a volume of 10 µl. Afterwards the incubation medium was removed and replaced by 1 ml DMEM. The incubation medium of the well was removed before each pulse to determine the cumulative LH secretion and replaced by fresh medium. Samples were kept at -20°C until the assay.

LH concentration was determined by radioimmunoassay using rat luteinizing hormone (NIDDK-rLH-9, AFP-102250C), rat luteinizing hormone antiserum (rabbit, NIDDK-anti-rLH-11) and rat luteinizing hormone reference preparation (NIDDK-rLH-RP-3) provided by the National

Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases, NICHHD, USA. Intra- e inter-assay coefficients of variation were lower than 10%. Sensitivity was about 10 pg per tube.

Gene expression was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from the pooled pituitary glands after the 12 hours pulsatile stimulation using guanidinium thiocyanate (Chomczynski & Sacchi, 1987). Gene specific expression was characterized by PCR using oligonucleotides designed to amplify the beta chain of rat LH transcript. Oligonucleotides specific for actin were used to standardize the amplification of β-LH mRNA regarding the total amount of mRNA included in each sample. The sequences were obtained from the web page of the National Center for Biotechnology Information (www2.ncbi.-nlm.nhi.gov). The primers for LH were: (1) 5´-GCT-CCA-GGG-GCT-GCT-GCT-GTG (LH-2), designed to hybridize to the junction between exon 1 and exon 2, and (2) 5´-ACA-GGT-CAT-TGG-TTG-AGT-CCT-GGG-ACC-CCC-ACA-GTC (LH-3) designed to hybridize to exon 3. The primers for actin were (1) 5´-CTC-ATC-GTA-CTC-CTG-CTT-GCT-G (ACT-3) designed to hybridize to exon 6, and (2) 5´-GCT-GTG-CTA-TGT-TGC-CCT-AGA-C (ACT-4) designed to hybridize to exon 4. The cDNA was generated from 30 ng of total RNA in the presence of 10 mM dNTPs (Promega), 50 pmol polidT (Promega, Madison, WI), 100 mM DTT, 25 mM $MgCl_2$ (Promega) and 200 units of M-MLV reverse transcriptase (Promega, Madison, WI). PCR was performed from 5 µl of cDNA mixture using standard buffer (Promega), 50 pM primers, 50 mM $MgCl_2$ (Gibco BRL), 2 mM dNTPs (Promega) and 2.5 units of Taq polymerase (Promega, Madison, WI), in a total volume of 25 µl. Protocol for amplification during 35 cycles was 95°C for 1 min, 65°C for 1 min and 72°C for 1 min, ending with 10 min extension at 72°C. PCR reactions were analyzed by electrophoresis on a 1.5% agarose gel. Pituitary total RNA obtained from 2 weeks ovariectomized rats was used as additional control for the PCR amplification.

Data are presented as the arithmetic mean ± SE. Differences between groups were assessed by analysis of variance after log-transformation of the data to correct for heterogeneity of variance and lack of normality of LH values. Multiple comparisons were made by Duncan's Multiple Range tests only after the overall analysis of variance showed significant effects of main factors. Statistical analysis was performed using the Apple Macintosh version of Quick Statistica Rel. 3.0b (Statsoft, Inc.) software. Significance level was set at $p<0.05$.

RESULTS

The effects of GnRH and galanin upon LH secretion are presented in Table I. The analysis of variance, including the condition of the rat (diestrus 1 or lactation), peptide pulsatile treatment (GnRH, galanin, GnRH + galanin or saline) and incubation time (4, 8 or 12 h) as main factors, demonstrated that there was no effect of incubation time upon LH responses to GnRH or galanin. There was a significant effect of peptide treatment ($p<0.001$) accounted for by the stimulations of LH secretion induced by (a) GnRH, without significant differences between diestrous and lactation pituitaries, and (b) GnRH plus galanin (Table I). Galanin alone did not modify LH secretion, but it potentiated the effect of GnRH upon diestrous pituitaries ($p=0.036$), but not on pituitaries from lactating rats (*i.e.*, there was a significant interaction between the condition of the rat and the peptide treatment; $p<0.001$). The mean of the pooled data corresponding to LH levels obtained at 4, 8 and 12 h after peptide stimulation is presented in Figure 1.

Table II shows the β-LH mRNA/actin mRNA ratio in cycling and ovariectomized rats, and in the same pituitaries exposed to 12-h pulsatile stimulation whose secretion was described in the preceding paragraph. As reported (Haisenleder *et al*, 1994), long term ovariectomy increased the ratio of the hormone over actin mRNA (Fig 2), showing that the method detected the expected increase in the expression of the

Table I

Effect of pulsatile stimulation with GnRH and galanin upon in vitro LH (ng/ml) release from rat hemipituitaries.

	Incubation time 4 h	8 h	12 h	All
Day 10 of lactation:				
Saline	2.1 ± 0.6 (17)	1.1 ± 0.4 (17)	2.1 ± 0.9 (15)	1.8 ± 0.4
GnRH	4.8 ± 1.7 (5)	9.1 ± 1.2 (5)	4.2 ± 2.5 (3)	6.2 ± 1.1[a]
Galanin	2.6 ± 1.7 (6)	1.6 ± 0.9 (6)	1.1 ± 0.5 (6)	1.7 ± 0.6
GnRH + Galanin	4.5 ± 0.9 (6)	3.5 ± 2.2 (6)	4.6 ± 2.9 (6)	4.2 ± 1.2[a]
Diestrus 1:				
Saline	0.8 ± 0.2 (17)	1.2 ± 0.3 (17)	1.1 ± 0.3 (16)	1.1 ± 0.2
GnRH	4.3 ± 0.8 (6)	5.9 ± 1.7 (6)	2.3 ± 0.5 (6)	4.1 ± 0.7[b]
Galanin	1.1 ± 0.2 (5)	0.7 ± 0.1 (5)	1.3 ± 0.7 (5)	1.0 ± 0.2
GnRH + Galanin	10.7 ± 2.4 (6)	11.2 ± 3.4 (6)	3.6 ± 1.1 (5)	8.8 ± 1.7[bc]

Means ± SEMs (n). **a,b:** different from their respective control groups stimulated with saline ($p<0.001$, ANOVA). **c:** different from GnRH-stimulated hemipituitaries of diestrus rats ($p=0.036$). See text for further details.

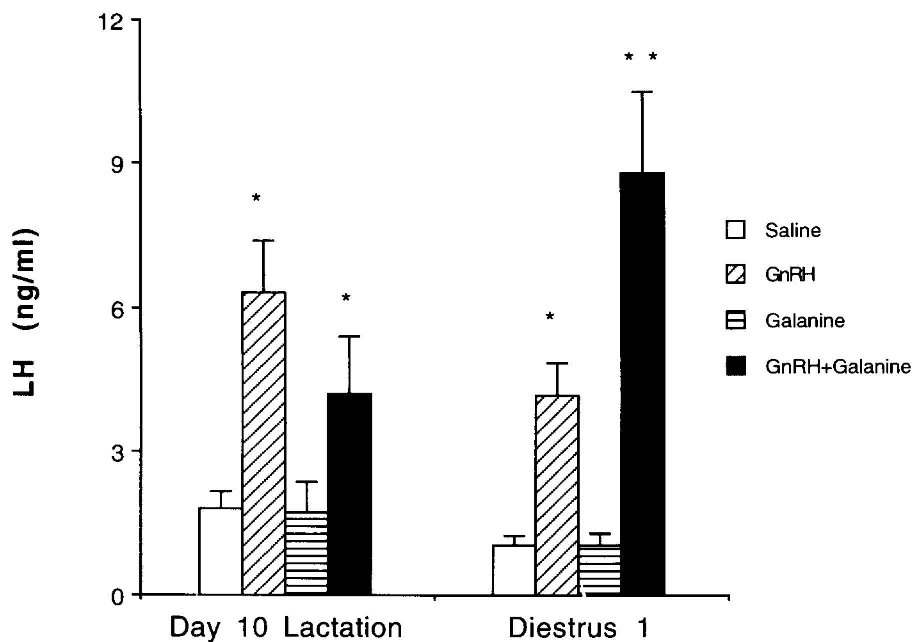

Fig 1. Main effects of pulsatile stimulation with GnRH and galanin upon *in vitro* LH secretion from rat hemipituitaries. Because ANOVA disclosed no time effect, the grand means ± SEM of pooled data obtained from stimulations at 4, 8 and 12 h are presented. *: different from the respective saline stimulated control. **: different from GnRH stimulated hemipituitaries of diestrous 1 rats.

β-LH gene. Similarly, the capability of the RT-PCR to detect biologically meaningful differences in the β-LH gene expression was also evidenced by the two- to three-fold decrease in the β-LH mRNA/actin mRNA ratio during lactation compared to diestrus (Lee *et al*, 1989a,b). None of the pulsatile regimes changed significantly the β-LH mRNA/actin mRNA ratio. The bands for β-LH and actin mRNAs are shown in Figure 3, which illustrates one out of two runs of RT-PCR amplification.

Table II

Ratio of optical densities corresponding to the specific bands of βLH mRNA over actin mRNA obtained by RT-PCR from pituitaries after 12-h pulsatile stimulation.

	βLH mRNA/ actin mRNA ratio
Control experiment	
Cycling	0.31
Ovariectomized (2 weeks)	2.17
Day 10 of lactation	
Saline	0.88
GnRH	0.62
Galanin	1.01
GnRH + Galanin	0.77
Diestrus 1	
Saline	2.27
GnRH	1.99
Galanin	2.19
GnRH + Galanin	2.11

DISCUSSION

The results presented in this paper disclose a previously unknown modulatory effect of lactation upon the effects of galanin on the anterior pituitary gland. As previously reported, galanin potentiated the GnRH-induced LH secretion in the cycling rat (see below), but this effect disappeared during lactation. Galanin and/or GnRH did not modify β-LH gene expression after 12 h of pulsatile stimulation in our experimental conditions. Because, in this species, the suppression of LH associated with lactation is not correlated with inhibition of the GnRH pulse generator activity (Cardenas & Ramirez, 1996) or changes in GnRH gene expression (Marks *et al*, 1993), this modification in the effects of hypothalamic galanin impinging upon the pituitary gland might contribute to the lactational unresponsiveness of the pituitary to GnRH.

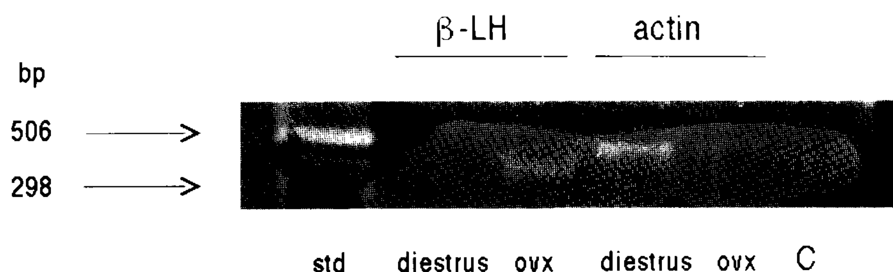

Fig 2. Agarose gel electrophoresis of reverse polymerase chain reaction products after amplification of pituitary β-LH mRNA and actin mRNA from diestrous and 2 weeks ovariectomized rats. Arrows indicate 298 and 506/517 bp standards (std). **ovx:** ovariectomized rat. **C:** control lane without pituitary RNA.

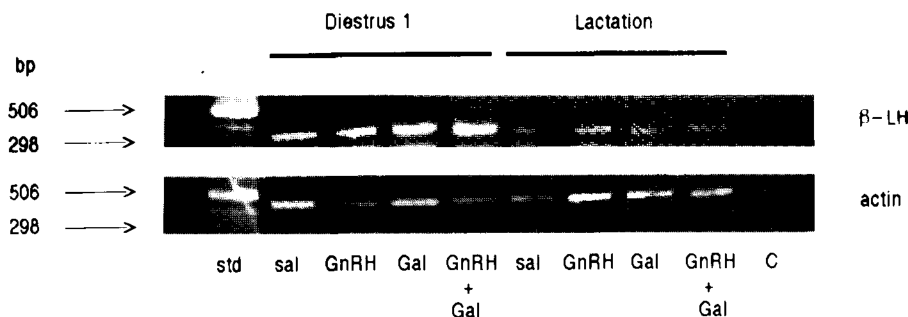

Fig 3. Agarose gel electrophoresis of reverse polymerase chain reaction products after amplification of pituitary β-LH mRNA and actin mRNA from diestrous and lactating rats. Pituitaries were stimulated for 12 h with hourly pulses of saline (sal), 25 ng/pulse gonadotropin releasing hormone (GnRH), 600 ng/pulse rat galanin (Gal), or GnRH plus Gal. Arrows indicate 298 and 506/517 bp standards (std) for each gel. **C:** control lane without pituitary RNA.

The requirement of pulsatile GnRH stimulation at a fixed and optimal frequency for normal pituitary secretion of gonadotropins was first demonstrated by Knobil *et al* in the Rhesus monkey (Knobil, 1980). Recently, it was shown that a pulsatile stimulation with GnRH is a requirement for increased transcription of gonadotropin subunit genes in the male rat (Haisenleder *et al*, 1991), the minimal stimulation time for inducing increased levels of β-LH mRNA being 4 h. Thus, we decided to stimulate with pulses during at least 4 h and up to 12 h to better evaluate the effects of galanin. The lack of effect of incubation time on LH responses in our experiments indicate that the experimental conditions allowed maintenance of the hemipituitaries secretory activity during the duration of the experiments. In this regard, the pituitary of the diestrous 1 rat appears to be different from that of male rats (Haisenleder *et al*, 1991), because we did not observe increased β-LH mRNA levels after 12 h of GnRH pulses.

Hypothalamic galanin has been recently shown to act as modulator of GnRH function. There are galanin-immunoreactive neurons in the mediobasal hypothalamus projecting towards the median eminence (Merchenthaler *et al*, 1990) and 63% of GnRH-positive neurons in the proestrus rat are also immunopositive for galanin (Merchenthaler *et al*, 1991). This peptide is released in pulses coincident with or preceding GnRH pulses into the pituitary-portal circulation (Lopez *et al*, 1991) and produces a dose-dependent release of GnRH from hypothalamic fragments *in vitro* (Merchenthaler *et al*, 1990). In addition, it stimulates LH secretion by itself and potentiates GnRH-induced release of LH from anterior pituitary cells *in vitro* (Lopez *et al*, 1991; Leiva & Croxatto, 1994). Contrary to what was reported for GnRH mRNA, galanin mRNA levels in GnRH neurons are inhibited by roughly 50% in lactating rats (Marks *et al*, 1993).

There is evidence of endogenous galanin involvement in LH regulation: levels of hypothalamic galanin and its mRNA increase during the preovulatory LH surge (Merchenthaler *et al*, 1991; Brann *et al*, 1993; Marks *et al*, 1993) and anti-galanin antibodies decrease by about 30% the preovulatory LH surge (Lopez *et al*, 1993). Galantide, the specific pharmacological antagonist of galanin, also inhibits the preovulatory LH surge (Sahu *et al*, 1994).

Regarding the mechanism(s) of the changing role of galanin during lactation, we can only speculate at this point about possible modifications of the population of galanin receptors in the anterior pituitary. Two types of galanin receptors have been characterized (Fisone *et al*, 1989; Land *et al*, 1991; Wynick *et al*, 1993), but there is no evidence yet regarding their regulation during lactation. A decrease in pituitary GnRH receptors has been demonstrated in the lactating rat (Smith & Lee, 1989) and similar changes in the receptors for galanin might be expected at the pituitary level.

We did not observe *in vitro* the diminished responsiveness of pituitary LH to exogenous GnRH that is correlated with a decreased number of pituitary GnRH receptors, previously reported *in vivo* in the lactating rat (Lee *et al*, 1989c). Because our experimental conditions allow to conclude that the peptides directly affected the pituitary gland, the comparison with the *in vivo* experiments of Lee *et al* (1989c) suggests that actions of GnRH at levels other than the pituitary might play a role in the *in vivo* experiments. Exogenous GnRH could inhibit the release of endogenous GnRH acting on GnRH receptors located in the median eminence, as well as affecting the release of other neurotransmitters known to affect LH release, like GABA and glutamate (for example, see Feleder *et al*, 1996).

In summary, we demonstrated a modulatory role of lactation on the effects of galanin upon the pituitary gland in the rat. The potentiating effect of galanin on GnRH-induced LH secretion, that occurs during the cycle, disappears during lactation, providing further support for the emerging role of galanin in the hypothalamic regulation of pituitary LH secretion.

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