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Release of ovarian progesterone during the rat oestrous cycle by ganglionic cholinergic influence
The role of norepinephrine

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Abstract

The coeliac ganglion neurons, whose axons constitute the superior ovarian nerve (SON), contain cholinergic receptors. The aim of this work was to study the effect of cholinergic agents added to the coeliac ganglion on the release of ovarian progesterone in the coeliac ganglion–SON–ovary in vitro system. We also analyzed the release of norepinephrine in the ovarian compartment and its possible relationship with the release of progesterone. After the addition of cholinergic agents in the ganglion compartment, progesterone release was determined by radioimmuneassay (RIA) and norepinephrine by catecholamine assay (HPLC). The release of progesterone and norepinephrine in the ovary compartment was studied during period of 180 min in pre-oestrus (PE), oestrus (E), dioestrus day 1 (D1) and dioestrus day 2 (D2) rats.

The most relevant results concerning the action of acetylcholine were found on PE and dioestrus. On PE, the pre-ovulatory peak of progesterone, which is known to respond to the endocrine action, was not modified by neural effect of acetylcholine in our scheme. On the other hand, the progesterone peak occurs in the afternoon of D1, which has been described as independent of the gonadotrophic action but was inhibited by neural effect of acetylcholine in our experimental scheme. This action on D1 was accompanied by a decrease of norepinephrine release in the ovary compartment.

We conclude that the action of cholinergic agents varies according to the oestrous cycle stage and constitutes one of the factors governing the secretory activity of the ovarian steroids, in this case, progesterone.

Keywords: Coeliac ganglion; Progesterone; Oestrous cycle; Ovarian innervation

1. Introduction

Numerous studies support the notion of the importance of innervation on the regulation of the gonadal functions [1–5]. Recent studies by Gerendai et al. [6], using a viral tracing technique, have provided the first morphological evidence of the existence of a neuronal multi-synaptic way between the ovary and various centers of the central nervous system, through the sympathetic ganglionic pathway. On the other hand, experiments performed in our laboratory have shown that intracerebroventricular injection of epinephrine modifies, at short times, the concentration of progesterone in the ovarian vein of dioestrus day 1 (D1) and dioestrus day 2 (D2) rats. This may provide physiological evidence of the participation, at least in part, of the sympathetic ganglionic pathway, whose preganglionic neurotransmitter per excellence is acetylcholine. The superior ovarian nerve (SON) might be the neural way of the central adrenergic stimulation [7].

The superior ovarian nerve, whose fibers–mainly noradrenergic–originate mostly in the neurons of the coeliac ganglion, reaches the ovary through the suspensory ligament and enters it by the hilium [2,8]. Its varicosities form a net around the steroidogenic cells of the follicles and do not have a visible relationship with the corpora lutea [8,9]. In spite of this, the corpora lutea possess beta adrenergic receptors that respond to the sympathetic stimulus [10,11].

The coeliac ganglion is a component of the sympathetic prevertebral ganglionic pathway and norepinephrine constitutes the main postganglionic neurotransmitter released by it. The coeliac ganglion also has the specific structures to

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respond to cholinergic stimuli, such as nicotinic and muscarinic receptors. The former are mainly located in the postganglionic neurons called principal neurons and the latter are found in both principal neurons and interneurons [12,13].

The purpose of this work was to investigate whether the occupation of ganglionic cholinergic receptors produces variations in the release of ovarian progesterone during the oestrous cycle in the coeliac ganglion–SON–ovary integrated system, previously standardized in our laboratory [14,15]. Considering also that the SON is a nerve of the sympathetic ganglionic pathway [2,16], it was investigated whether in the system under study norepinephrine is released in the ovary compartment, and an attempt was made to establish what modifications are produced by ganglionic cholinergic action on the release of norepinephrine. Finally, in case such variations are produced, we intend to determine if they have an effect on the release of progesterone, since it is well known that, in vitro, catecholamines lead to progesterone secretion [17].

2. Materials and methods

2.1. Animals

Virgin Holtzman strain female rats weighing 250 ± 50 g were used in all the experiments. The rats were kept in a light- (lights on from 0700 to 1900 h) and temperature (24 ± 2 °C)-controlled room. Animals had free access to food (Cargill, SACI, Saladillo, Buenos Aires, Argentina), and tap water were available ad libitum.Vaginal smears were taken daily and rats were used only after exhibiting at least two consecutive four-day oestrous cycles. Rats in proestrus (PE), oestrus (E), dioestrus day 1 (D1) and dioestrus day 2 (D2), were used for the experimental procedure. Groups of six animals were used by experiment.

The experiments were performed in accordance with the revised Guide for the Care and Use of Laboratory Animals [18] and the Guide for Animal Use and Handling of the National University of San Luis.

2.2. Drugs

The following drugs: L-Acetylcholine hydrochloride (Ach), L-hexametonium (C_6), atropine (At), ascorbic acid, bovine serum albumin fraction V (BSA), were purchased by the Sigma Chemical Co. (St. Louis, Mo, USA). Other reagents and chemicals were of analytical grade. (1,2,6,7-^{3}H) Progesterone was provided by the New England Nuclear (Boston, MA, USA).

2.3. Surgical procedures

Rats on pro-oestrus, oestrus, D1 and D2 were anesthetized with ether under bell, as the anaesthetic for the surgery. The surgery was carried out between 1500 and 1600 h. The system removal, histological control, characterization and standardization of incubation conditions were performed as described previously [14]. Briefly, the system removed by dissection is conformed by the left ovary, the fibers that constitute the SON, inserted in the suspensory ligament, and the coeliac ganglion accompanied by some small ganglia that surround it. The total surgical procedure was completed in 1–2 min.

2.4. Experimental procedure

The coeliac ganglion–SON–ovary system was washed with the incubation medium and placed immediately in the cuvette consisting of two compartments. Each cuvette contained 2 ml of Krebs–Ringer bicarbonate buffer, pH 7.4 solution, in the presence of dextrose (0.1 mg/ml) and BSA (0.1 mg/ml), as has been described for incubation of ovaries in other in vitro systems [10,19].

The ganglion was placed in a compartment and the ovary in the other one, both joined by the SON, which had to remain humid with the work solution. The system was stabilized by preincubation in metabolic bath at 37 °C for 30 min in an atmosphere composed by 95% O_2 and 5% CO_2. The end of the preincubation period was considered as incubation time 0.

At this time, the buffer was changed in both compartments; ascorbic acid (0.1 mg/ml in Krebs Ringer) was added as an antioxidant agent to the ganglionic compartment.

The values of the progesterone released under these conditions was considered as a control value (control groups). The cholinergic agents employed in this investigation were acetylcholine (Ach) as an cholinergic agonist, hexametonium (C_6) antagonist nicotinic and atropine (At) as a muscarinic antagonist. The different substances were dissolved in equal concentrations, 10^{-6} M [20] and volumes (2 ml) of Krebs–Ringer solution plus ascorbic acid. The samples of liquid from the ovarian compartment (250 μl) were collected at 30, 60, 120 and 180 min since the incubation had started. These were kept at −20 °C until the determination of progesterone by radioimmuneassay (RIA).

For norepinephrine determinations, incubation liquid was collected at 180 min and the samples were maintained at −70 °C until determination by HPLC.

The respective corrections were made in all cases considering the volume extracted in each period tested.

2.5. Progesterone assay

Progesterone was measured by RIA as previously described [21]. The sensitivity of the assay is less than 5 ng/ml serum, and the inter- and intraassay coefficients of variation were less than 10% the variability and cross-reaction of this RIA has been reported previously validated [22]. The results were expressed as nanograms of progesterone/milligram of ovarian tissue (*P* ng/mg tissue) against time of incubation.

2.6. Catecholamine assay (HPLC)

The norepinephrine in 20-μl aliquots of liquid from the ovarian cuvette (180 min), was partially purified by batch alumina extraction, separated by reverse-phase high-pressure liquid chromatography using a 4.6 mm × 250 mm Zorbax R_xC_{18} column (Du Pont, USA) and quantified by current produced upon exposure of the column effluent to oxidizing and then reducing potentials in series using a triple-electrode system (Coulochem II, ESA, Bedford, MA) [23]. Recovery through the alumina extraction step averaged 70–80% for catecholamines. Catecholamine concentration, in each sample, was corrected for recovery of an internal standard dihydroxybenzylamine. The results were expressed as picograms of norepinephrine/milligram of ovarian tissue/ml of incubation medium (NE pg/mg ovary/180 min incubation).

2.7. Statistical analysis

Results are presented as mean ± S.E.M. in each group. Student's *t*-test was used to assay significant differences between means of two groups. Analysis of the variances (ANOVA) followed by Tau's multiple range test was used for several comparisons. A value of $P < 0.05$ was accepted as statistically significant [24].

3. Results

3.1. Coeliac ganglion–SON–ovary system: effect of the addition of cholinergic agents in the ganglion compartment on the progesterone release by the ovary in the estrous cycle

The cholinergic effect on the release of progesterone in the coeliac ganglion–SON–ovary system was studied by addition to the ganglion compartment of 10^{-6} M acetylcholine and its antagonists.

The results of each experiment are expressed with respect to the corresponding control group. On pro-oestrus, no differences in progesterone release were observed at any of the studied times, when acetylcholine or hexametonium were used. On the other hand, atropine increased progesterone release at all the studied times (30 min, $^{\bullet}P < 0.05$; after 60 min, $^{*}P < 0.001$) (Fig. 1A).

On oestrus, addition of acetylcholine produced a dual effect on the release of ovarian progesterone: inhibition at 30 min ($^{*}P < 0.001$) and stimulation after 120 min ($^{*}P < 0.001$). On the other hand, both atropine or hexametonium stimulated progesterone release, after 60 min in the case of atropine (60 min, $^{\bullet}P < 0.05$; 120 and 180 min, $^{*}P < 0.001$), and throughout the whole experiment in the case of hexametonium (30 min, $^{\bullet}P < 0.05$; 60, 120 and 180 min, $^{*}P < 0.001$) (Fig. 1B).

On dioestrus day 1, both acetylcholine and its antagonists decreased the release of ovarian progesterone. Acetylcholine inhibited progesterone release at all the studied times ($^{*}P < 0.001$), while the inhibiting action of atropine and hexametonium was observed after 60 min ($^{*}P < 0.001$) and 120 min ($^{\bullet}P < 0.05$), respectively (Fig. 1C).

On dioestrus day 2, acetylcholine produced a decrease in progesterone release at 30 min ($^{\bullet}P < 0.05$) and 180 min ($^{*}P < 0.001$) as compared to the control, while atropine and hexametonium increased progesterone release after 60 min ($^{\bullet}P < 0.05$), with values over three times greater after 120 min in both cases ($^{*}P < 0.001$) (Fig. 1D).

3.2. Effect of the addition of acetylcholine in the ganglion compartment on the release of norepinephrine in the ovarian incubation liquid in the integrated in vitro coeliac ganglion–SON–ovary system

Under basal conditions, the level of catecholamine in the ovarian incubation liquid was significantly lower on D2 than on D1 (58.1 ± 6 versus 6.32 ± 1) (columns with the same letter differed significantly; a, $P < 0.001$).

When acetylcholine was added to the ganglion compartment, the release of norepinephrine in the ovarian compartment decreased significantly on D1 (Ach D1 versus control D1) ($^{*}P < 0.001$) but showed no variations on D2 (Ach D2 versus control D2). On the other hand, significant differences in norepinephrine release in the ovarian compartment were observed between D1 and D2 when acetylcholine was added to the ganglion compartment (6.37 ± 0.69 versus 37.5 ± 2.1) (Ach D2 versus Ach D1) (columns with the same letter differed significantly; b, $P < 0.001$) (Fig. 2).

4. Discussion

Ovarian innervation includes not only the neural components that enter the ovary such as the SON and the ovarian plexus, but also intermediate structures such as the coeliac ganglion, which constitutes a link of rapid information between the central nervous system and the ovary [6].

The autonomous ganglia such as the coeliac ganglion, a component of the sympathetic ganglionic pathway, have been considered as "small brains" capable of receiving and integrating signals and organizing a response [25]. Eccles [26] confirmed that the after-potential following the ganglion cell discharge is an intrinsic reaction of the ganglion, which regulates in this way its own excitatory cycle.

Without attempting to oversimplify the complex problem of ganglionic functioning and regulation, the aim of this work was to study whether the action of cholinergic agents on the coeliac ganglion modifies the release of ovarian progesterone during the oestrous cycle. We also investigated whether in this process the norepinephrine release in the ovary compartment is modified, since most of the fibers constituting the SON are noradrenergic [2,9,10]. For this purpose, we used a coeliac ganglion–SON–ovary system previously standardized in our laboratory [14,15]. This

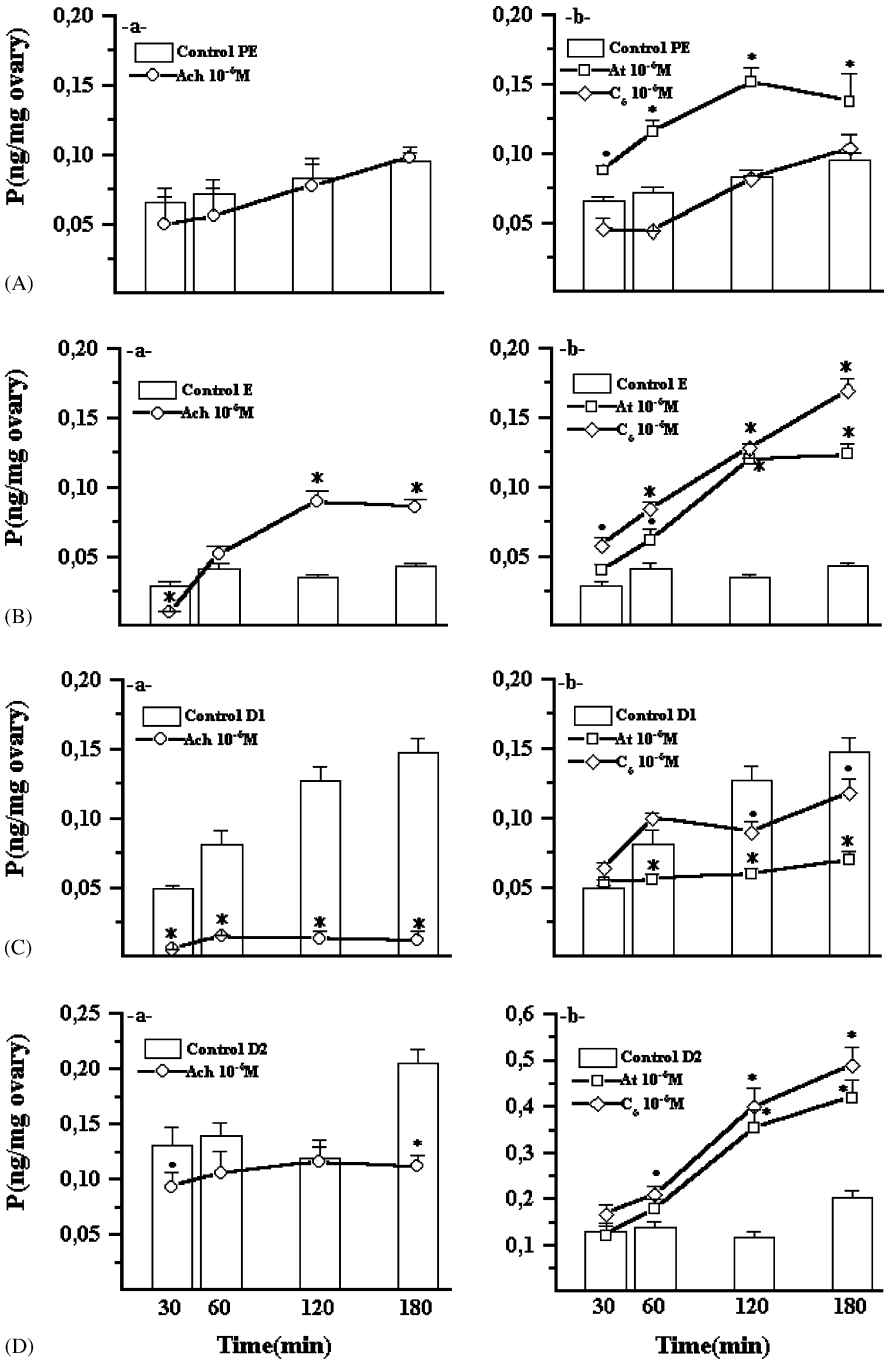

Fig. 1. Effect of agonist (a) and antagonist (b) colinergic in ganglion compartment on ovarian progesterone (P) release in the coeliac ganglion–SON–ovary system obtained from rats on pro-oestrus-PE (A), oestrus-E (B), dioestrus day 1-D1 (C), dioestrus day 2-D2 (D). The system was incubated in buffer Krebs–Ringer, plus ascorbic acid (0.1 mg/ml in Krebs–Ringer) solution, at 37 °C in an atmosphere of 95% O_2–5% CO_2 for 180 min without (control) and with colinergic agents in 10^{-6} M concentration added to the ganglionic compartment (experimental group). Ach: acetilcholine; C_6: hexametonium; At: atropine. Results are expressed as mean ± S.E.M. of six animals per experimental group from respective control. $^{*}P < 0.001$ and $^{\bullet}P < 0.05$.

system permits an in vitro emulation of in vivo conditions, preserving innervation and paracrine and autocrine regulations without the humoral influence. A further advantage of this system is that it possesses its own neural tone, which permits addition of the antagonists in absence of the agonist [14]. In the present experimental scheme, we used acetylcholine as cholinergic agonist because it is considered to be the classical preganglionic neurotransmitter of the sympathetic ganglionic pathway [25–27]. On the other hand, ovarian progesterone has proved to be the most sensitive steroid to neural influence in in vitro studies [1]. Furthermore, experiments performed in our laboratory have provided evidence that the stimulation of the coeliac ganglion with adrenergic agents in the coeliac ganglion–SON–ovary integrated sys-

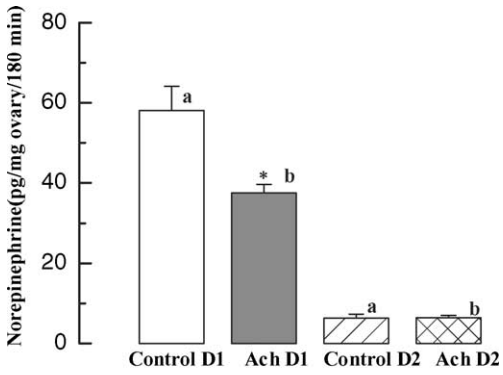

Fig. 2. Concentration of norepinephrine (NE), expressed as pg/mg ovary/180 min incubation, in the ovary incubation medium, in the coeliac ganglion–SON–ovary system, obtained from rats on dioestrus day 1 (D1), and dioestrus day 2 (D2). The system was incubated in buffer Krebs–Ringer, plus ascorbic acid (0.1 mg/ml in Krebs–Ringer) solution, at 37 °C in an atmosphere of 95% O_2–5% CO_2 for 180 min without (control) and with acetylcholine (Ach) in 10^{-6} M concentration added to the ganglionic compartment (experimental group). Results are expressed as mean ± S.E.M. of six animals per experimental group from respective control. *$P < 0.001$. Columns with the same letter differ significantly, a, b, $P < 0.001$.

tem modifies the release of progesterone both in the different stages of the oestrous cycle [14] and in pregnancy [15].

The results obtained show that acetylcholine provoked a typical progesterone release profile, depending on the oestrous cycle stage. In PE, when follicle maturation and ovulation takes place, the secretion of ovarian progesterone was not modified by the addition of acetylcholine to the ganglion compartment. This may be because the main peak of progesterone secretion in this stage of the cycle [28,29] is mainly due to the action of gonadotrophines rather to a direct neural influence. However, the existence of neural input is demonstrated by the effect of the antagonists, mainly atropine, which is an indication of the ganglionic receptors participation. The nicotinic receptors appear to participate in the basal release of ovarian progesterone, as shown by the results obtained with acetylcholine and hexametonium. On the other hand, the presence of atropine in the ganglion led to a stimulating effect, probably by inhibition of the inhibitory postsynaptic potential generated in the ganglion, as previously described [27].

In E, a stage characterized by a reordering and structural organization that involves the formation of corpora lutea, addition of acetylcholine to the ganglion had a stimulating unspecific effect on the release of ovarian progesterone at longer times. This may indicate a neural participation along with luteal development. On the other hand, on D1, for which a new smaller progesterone peak has been described, probably of luteal origin and independent of the hormonal action [28], we observed a marked neural modulation of inhibitory type caused by the addition of acetylcholine in ganglion. The effect of the antagonists atropine and hexametonium suggests that the impact of acetylcholine is produced on the ganglionic nicotinic receptors. Finally, the same inhibitory response was observed on D2, but only at longer times and of an unspecific nature.

Our most relevant results relate to D1 and D2. At these stages the corpora lutea are the functionally active structures and progesterone is the main secretion product. In spite of this, the neural input produced by acetylcholine in ganglion was inhibitory. This led us to investigate whether in our coeliac ganglion–SON–ovary integrated system under basal conditions, norepinephrine is liberated in the ovarian compartment; secondly, whether the secretion rate of catecholamine is modified by the ganglionic cholinergic action; and thirdly, whether variations in the release of norepinephrine in the ovary compartment are responsible for the modifications in the release of ovarian progesterone observed on D1 and D2.

In this respect, it is well known that there exists a stimulating effect on the release of progesterone caused by catecholamines when they impact on the beta adregenergic receptors on the corpora lutea [30–32]. In turn, previous studies in our laboratory have showed that addition of norepinephrine to the ganglion compartment has a stimulating effect on progesterone release on D1. These results led us to propose that the neurotransmitter responsible for such effect was norepinephrine released from the SON to the ovary [14].

In the present work, it was possible to demonstrate that in the system under study in basal conditions norepinephrine is liberated in the ovarian compartment at values significantly higher on D1 as compared to D2. On the other hand, when we performed cholinergic stimulation in ganglion, a marked decrease of norepinephrine in the ovarian compartment with respect to the control was observed on D1, but not on D2.

The results obtained seem to indicate that on D1 the fall in the release of ovarian progesterone due to addition of acetylcholine in ganglion is caused by a lower availability of norepinephrine in the ovarian compartment. However, on D2, the same effect observed at longer times does not appear to be due to modifications in norepinephrine release, but probably to the action of some other neurotransmitter with inhibiting characteristics [33]. These findings show the prevalence of the neural sympathetic effect over the endocrine one on dioestrus.

This paper has provided a physiological demonstration of the modifications in the release of ovarian progesterone by effect of cholinergic agents in the ganglion throughout the oestrous cycle. Possible interactions with other factors such as steroids and steroid-metabolizing enzymes genes will be the focus of future studies.

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