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Genital Sensory Stimulation Shifts Estradiol Intraoviductal Signaling from Nongenomic to Genomic Pathways, Independently from Prolactin Surges[1]

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ABSTRACT

Estradiol (E_2) accelerates oviductal egg transport through nongenomic pathways involving oviductal protein phosphorylation in non-mated rats, and through genomic pathways in mated rats. Here we investigated the ability of cervico-vaginal stimulation (CVS) to switch the mode of action of E_2 in the absence of other male-associated components. Pro-estrous rats were subjected to CVS with a glass rod and 12 hours later were injected subcutaneously with E_2 and intrabursally with the RNA synthesis inhibitor Actinomycin D or the protein phosphorylation inhibitor H-89. The number of eggs in the oviduct, assessed 24 h later, showed that Actinomycin D, but not H-89 blocked the E_2-induced egg transport acceleration. This clearly indicates that CVS alone, without other mating-associated signals, is able to shift E_2 signaling from nongenomic to genomic pathways. Since mating and CVS activate a neuroendocrine reflex that causes iterative prolactin (PRL) surges, the involvement of PRL pathway in this phenomenon was evaluated. Prolactin receptor mRNA and protein expression in the rat oviduct was demonstrated by RT-PCR and Western blot, but their levels were not different on day 2 of the cycle (C2) or pregnancy (P2). Activated STAT 5a/b (phosphorylated) was detected by Western blot on P2 in the ovary, but not in the oviduct, showing that mating does not stimulate this PRL signalling pathway in the oviduct. Other rats subjected to CVS in the evening of pro-estrus were treated with bromoergocriptine to suppress PRL surges. In these rats, H-89 did not block the E_2-induced acceleration of egg transport suggesting that PRL surges are not essential to shift E_2 signaling pathways in the oviduct. We conclude that CVS is one of the components of mating that shifts E_2 signaling in the oviduct from nongenomic to genomic pathways, and this effect is independent of PRL surges elicited by mating.

Key terms: mating, oviduct, estradiol, signaling pathways, prolactin receptor, egg transport, rat.

INTRODUCTION

Estradiol acts through different signaling pathways in the oviduct of non-mated and mated rats. In mated rats, accelerated egg transport induced by E_2 is blocked by Actinomycin D, but not by the broad-spectrum protein kinase inhibitor H-89 (Orihuela et al., 2001). In contrast to mated rats, in non-mated females accelerated egg transport induced by E_2 is blocked by H-89, but takes place under conditions in which mRNA transcription and translation are totally suppressed by α-Amanitin or Actinomycin D (Orihuela and Croxatto, 2001; Orihuela et al., 2001). Thus, mating shifts intraoviductal signaling of E_2 from nongenomic to genomic pathways. This

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poses two main questions: 1) what are the postcoital changes in oviductal cells that lead to switch off one pathway and switch on the other? 2) what are the components of mating that elicit this response?. This paper addresses the second question.

Since one of the differences between mated and non-mated rats is that the latter have been subjected to coital stimulation, we postulate that factors associated with or consequent to mating affect the way the oviduct responds to E_2. The main components of mating that impinge on the female system are sensory stimulation (Peters et al., 1987), and interaction of seminal fluid components (Robertson, 2005) and sperm cells (Suarez and Pacey, 2006) with the female genital tract epithelium. Previously, we found that artificial insemination with epididymal spermatozoa shifts the response of the oviduct to E_2 from non-genomic to genomic pathways (Parada-Bustamante et al., 2003). Therefore, in this work we investigated the ability of cervico-vaginal stimulation (CVS) to shift the mode of action of E_2 in the absence of male pheromones, spermatozoa and seminal fluid provided by mating. The experiments were done in cycling rats in which RNA synthesis or protein phosphorylation in the oviduct was suppressed by local treatment with Actinomycin D or H-89, respectively. It was reasoned that, if CVS alone mimics the effect of mating one would expect decreased effectiveness of E_2 for accelerating egg transport when RNA synthesis is suppressed, but not when protein phosphorylation is inhibited. Since mating and CVS activate a neuroendocrine reflex that results in iterative prolactin (PRL) surges (Butcher et al., 1972; Smith et al., 1975; Erskin, 1995), we explored the participation of PRL on the switching of intraoviductal E_2 pathways induced by CVS. For this, we determined whether prolactin receptor (PRL-R) mRNA or protein are differentially expressed or activated in oviducts of mated and non-mated rats. The E_2 signaling pathway operating in rats in which CVS-induced PRL surges were suppressed by treatment with bromoergocriptine was also determined.

MATERIALS AND METHODS

Animals: Sprague-Dawley rats (bred in house) weighing 200-260 g were used. The animals were kept under controlled temperature (21-24ºC), and lights were on from 0700 to 2100. Water and pelleted rat chow were supplied *ad libitum*. The phases of the estrous cycle were determined by daily vaginal smears (Turner, 1962). Rats were used after showing at least two consecutive 4-day cycles. Females in pro-estrus were either kept isolated or caged with fertile males. The following day (estrus) was considered day 1 of the cycle in the first instance and day 1 of pregnancy in the second, provided spermatozoa were found in the vaginal smear. The care and manipulation of the animals was made in accordance with the ethical guidelines of our institution.

Treatments

Local administration of Actinomycin D: The RNA synthesis inhibitor Actinomycin D (Calbiochem, La Jolla, CA, USA; Goldberg et al., 1962) was injected to intercept genomic pathways. One μg of Actinomycin D dissolved in 4 μL of saline solution was injected into each ovarian bursa (i.b.) as previously described (Orihuela and Croxatto, 2001). Control rats received the vehicle alone.

Local administration of H-89: A broad-spectrum protein kinase inhibitor was used to intercept nongenomic pathways. Fifteen μg of H-89 (N- [2-(*p* bromocinnamylamino) ethyl]-5- isoquinolinesulfonamide-dihydrochloride, Calbiochem, Combest et al., 1988) dissolved in 4 μL of saline solution was injected into each ovarian bursa (i.b.) as previously described (Orihuela and Croxatto, 2001). Control rats received the vehicle alone.

Systemic administration of E_2: Immediately after local administration of Actinomycin D, H-89 or vehicle, rats were injected subcutaneously with 10 μg of E_2 dissolved in 0.1 mL of propylene glycol. Control rats received the vehicle alone.

Systemic administration of Bromoergocriptine: On day 4 of the cycle (pro-estrus) at 2100 (1 h before CVS), rats were injected with 1 mg of Bromoergocriptine (BEC; Sigma Chemical Co., St. Louis, MO) dissolved in 0.2 ml 70 % ethyl alcohol. They received 3 more doses, at 12-hour intervals. Control rats received 0.2 ml 70 % ethyl alcohol alone. These doses of BEC suppress PRL surges that follow CVS (Gaytan et al., 1997; Bowen and Keyes, 1999), and we confirmed this in separate experiments (not shown).

Cervico-vaginal stimulation: Mechanical stimulation of the vagina and cervix was performed during the dark phase of pro-estrus. A glass rod was introduced deep in the vagina twice for 10-seconds each time with 5-seconds interval while the glass rod was vibrating. The following day was considered day 1 of pseudopregnancy. In a separate experiment we confirmed that this stimulus induces persistent diestrus, verified by daily vaginal smears during 7 days, in 100% of treated females (not shown).

Animal surgery: Intrabursal administration of drugs was performed in the morning of day 1 of pseudopregnancy as described by Orihuela et al., 2001. At this time ovulation has already taken place, so this treatment cannot affect the number of oocytes ovulated. Furthermore, we have previously demonstrated that drugs administered intrabursally act locally on the oviduct (Orihuela et al., 2001).

Assessment of egg transport: Twenty-four hours after administration of Actinomycin D, H-89 or vehicle the animals were autopsied, the genital tract was removed and the oviducts were flushed separately with saline. Flushings were examined under low-power magnification (25X). The number of eggs recovered from the oviducts was recorded. We have previously determined from egg recovery experiments from uterus and vagina and from placing ligatures in the uterine horns that the reduction in the number of oviductal oocytes following treatment with E_2 corresponds to premature transport to the uterus (Ortiz et al., 1979; Villalón et al., 1982). Thus, we refer to it as E_2-induced accelerated egg transport.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Whole oviducts were removed and cleaned from fat tissue. Oocytes or embryos were flushed out of each oviduct to avoid contamination with their mRNA during oviductal RNA isolation. Oviducts in groups of two (from one rat) were homogenized and their total RNA was purified using the single-step method described by Chomczynski and Sacchi (1987) and RT was performed according to Brañes et al. (2005). In order to determine oviductal PRL-R transcript contents a 574 pb product related to PRL-R long (PRL-R_L) and a 485 bp product related to PRL-R short (PRL-R_S) gene were amplified by PCR. Three oligonucleotide primers were synthesized. Detection of both mRNAs was performed by combining a common sense strand oligonucleotide from the extracellular domain of the PRL-R that belongs to the coding region (5'GTCCCCACCCACCATAACTG 3') with either a PRL-R_L specific primer (5'GTGTGTGGGCTTAACACCTTG 3') or a PRL-R_S specific primer (5'GTTCCCCTG CATTGTCCAGT 3') for $PRLR_L$ and $PRLR_s$ isoforms, respectively. The PRL-R semiquantification was performed by amplification of a 353 bp product from the housekeeping gene β-actin, (primer sense 5'GCTCGTCGTCGACAACGGGTC 3', and antisense 5'CAAACATGATCTGGGT CATCTTCTC 3') used as an internal standard.

The thermal sequence used was: 92ºC for 1 min, 58ºC for 1 min and 72ºC for 45 s with a total of 29 cycles for PRL-R_L and 35 cycles for PRL-R_S and 23 cycles for β-actin. The PCR products were resolved in 12% acrylamide gels and subjected to silver staining (Winkler, Santiago-Chile). Bands were scanned and analyzed with the NIH Image software 1.61 to obtain net intensity values. Their identity was confirmed by automated sequencing using an ABI Prism310 sequencer as described by Muscillo et al. (2001). Total RNA of

ovarian tissues was used as positive control.

Western blot: Mouse anti-PRL-R, U5 (Affinity Bioreagents, Golden, CO, USA), mouse anti-phosphoSTAT5α/β (Upstate Cell Signalling, NY, USA), rabbit anti-ERK-2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) or rabbit anti-STAT5a (Santa Cruz) were used. Oviducts were removed, cleaned from fat tissue and flushed with saline in order to remove egg-associated proteins. Then, whole oviducts or muscle/serosa and mucosa layers were homogenized and samples were run on 10% SDS polyacrylamide slab gels according to Laemmli (1970). Proteins resolved in the gels were electro-blotted into nitrocellulose membranes (Towbin et al., 1979) and processed by western blot as described by Orihuela et al. (2003). Bands were scanned and analyzed with the NIH Image software 1.61 to obtain net intensity values.

Statistical analysis: The results are presented as mean ± SEM. Overall analysis was done by Kruskal-Wallis test, followed by Mann-Whitney test for pair-wise comparisons when overall significance was detected. Differences were considered significant when $p < 0.05$. We took the total number of eggs recovered from the 2 oviducts of a rat as a single data point, therefore the actual N in experiments done to determine the effect of drugs on oviductal egg transport is the total number of rats used in each group.

RESULTS

Experiment 1: This experiment was designed to determine whether CVS alone can shift E_2 signaling from nongenomic to genomic pathways. A total of 18 female rats were subjected to CVS in the evening of pro-estrus and 12 h later animals were divided into four treatment groups: 1) saline + propylene glycol; 2) Actinomycin D + propylene glycol; 3) saline + E_2 and 4) Actinomycin D + E_2. Egg transport was assessed 24 h after treatment, as described in Materials and Methods.

Treatment with E_2 alone lead to accelerated egg transport while local administration of Actinomycin D blocked E_2-induced oviductal egg loss (figure 1). Thus, just like mating does, CVS alone switched the action of E_2 in the oviduct to a genomic pathway.

Experiment 2: Here we determine whether CVS abrogates the requirement of protein phosphorylation for the induction of accelerated egg transport by E_2. A total of 22 female rats were subjected to CVS in the evening of pro-estrus and 12 h later they were divided into four treatment groups: 1) saline + propylene glycol; 2) H-89 + propylene glycol; 3) saline + E_2 and 4) H-89 + E_2. Egg transport was assessed 24 h after treatment, as described in Materials and Methods.

As expected, treatment with E_2 alone lead to accelerated egg transport. When rats were treated with H-89 + E_2, egg transport was also accelerated meaning that CVS eliminated the requirement of protein phosphorylation needed in non-mated rats (figure 2). Hence the results of the first two experiments clearly demonstrate that sensory stimulation of the cervico-vaginal area is able to shift the intraoviductal signaling through which E_2 accelerates egg transport, from non-genomic to genomic pathways.

Experiment 3: This experiment was designed to compare the relative level of PRL-R mRNAs in oviducts from mated and non-mated rats. Animals were used on day 2 of the cycle (C2) and pregnancy (P2) because the differences in PRL secretion between mated and non-mated rats are well established on day 2 (Smith et al., 1975). A total of 6 animals on C2 or P2 were autopsied and 5 μg of their total RNA were reverse transcribed and amplified by PCR using primer pairs specific for the two forms of the PRL-R or β-actin. This experiment consisted of 3 replicas.

A band of 574 bp corresponding to PRL-R_L was detected in the oviduct of mated and non-mated rats. However, levels of mRNA PRL-R_L were not different between C2 and P2 (not shown). Since the primers used for

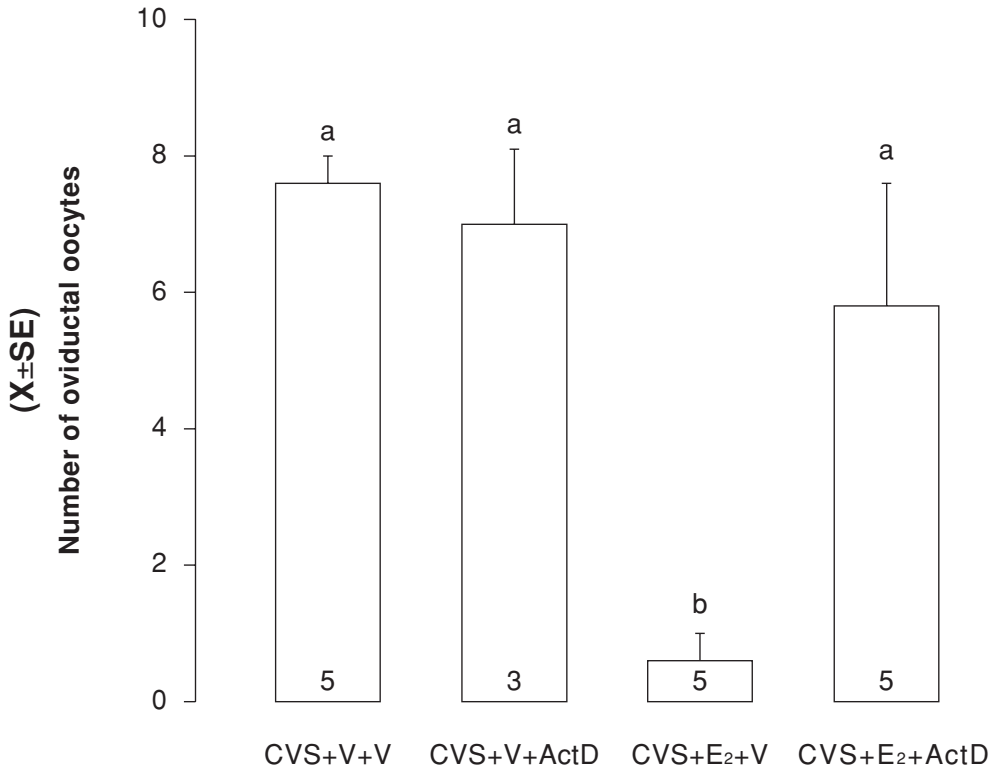

Figure 1: Number of oocytes recovered from the oviduct in rats on day 2 after cervico-vaginal stimulation (CVS). Rats were stimulated with a vibrating glass rod introduced in the vagina at 2100 of pro-oestrus. Twelve hours later, they received an intrabursal injection of Actinomycin D (ActD) 1 μg or vehicle (V), and a s.c. injection of estradiol (E_2) 10 μg or Vehicle (V). The number of oocytes was counted 24 h after ActD and E_2 treatment. Figures inside the bars indicate the number of animals used. Bars with different letters are significantly different ($p<0.05$).

RT-PCR were located in different exons, and since the RT-PCR was negative in the absence of reverse transcriptase in the ovarian and oviductal RNA samples, we can exclude possible amplification and contamination with genomic DNA. No band of 485 bp corresponding to PRL-R_S form was detected in oviductal samples, while it was detected in ovarian samples (not shown).

Experiment 4: Here PRL-R protein levels in oviducts of mated and non-mated rats were compared. A total of 6 animals on C2 or P2 were autopsied and their oviducts were excised and homogenized. Aliquots of the clarified homogenates containing the same amount of protein (25 μg) were subjected to electrophoresis and Western blot using antibody U5 that recognizes the extracellular domain of both PRL-R isoforms. This experiment consisted of 3 replicas.

An immunoreactive band of 42 KDa corresponding to PRL-R (Katoh et al., 1987; Okamura et al., 1989) was found in both conditions, but no differences were found between C2 and P2 (not shown).

Experiment 5: Here we assessed whether the distribution of PRL-R in the oviduct changes after mating. A total of 24 animals on C2 or P2 were autopsied and their total protein was extracted separately from mucosa or muscle/serosa layers of isthmus and ampulla. Aliquots of the clarified homogenates containing the same amount of protein (15 μg) were subjected to electrophoresis and Western blot to determine levels of PRL-R as described. This experiment consisted of 2 replicas.

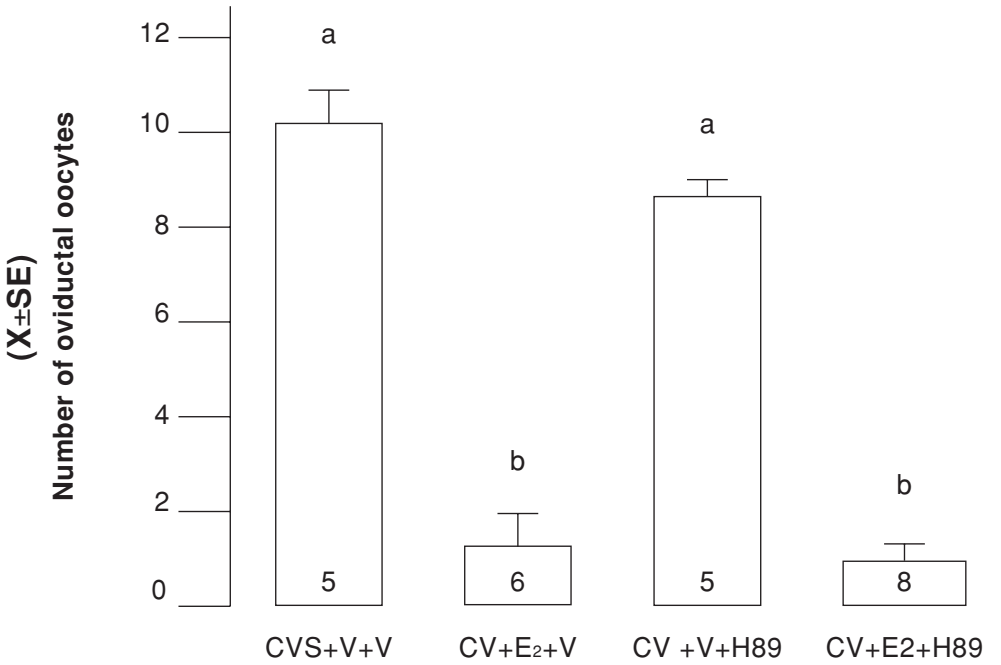

Figure 2: Number of oocytes recovered from the oviduct in rats on day 2 after cervico-vaginal stimulation (CVS). Rats were stimulated with a vibrating glass rod introduced in the vagina at 2100 of pro-oestrus. Twelve hours later, they received an intrabursal injection of H-89 15 μg or vehicle (V), and a s.c. injection of estradiol (E_2) 10 μg or vehicle (V). The number of oocytes was counted 24 h after H-89 and E_2 treatment. Figures inside the bars indicate the number of animals used. Bars with different letters are significantly different ($p<0.05$).

PRL-R was found in the muscle layer of isthmus and ampulla in C2 and P2, while no immunoreactive band was found in the mucosa of these segments. As positive control of the technique and to verify that mucosal samples were not degraded, aliquots of all samples were incubated with an antibody against ERK and all showed a positive band (not shown). Since PRL-R was found in the muscle and knowing that the isthmic segment bears the largest amount of muscle, PRL-R levels in the isthmus on C2 and P2 were compared. Other animals were autopsied on C2 (N = 3) or P2 (N = 3) to determine whether PRL-R protein level in isthmus changes after mating. There was no significant difference in the levels of PRL-R in the isthmus between the two conditions (not shown).

Experiment 6: Here we tested whether mating activates the PRL signaling pathway in the rat oviduct. Prolactin-induced phosphorylation of the transcription factor STAT 5 a/b in its target organs is consistent with a functional PRL pathway (Hair et al., 2002; Kabotyanski and Rosen, 2003). Therefore, levels of phospho-STAT 5a/b in oviducts from C2 and P2 were determined. The ovary was used as a positive control since it is a known target organ of PRL in the rat. Eight oviducts or ovaries from 4 rats on C2 or P2 were homogenized and aliquots containing the same amount of protein (100 μg) were processed to determine the level of phopho-STAT5a/b as described.

Immunoblots of phospho-STAT 5a/b of whole oviducts revealed no specific band on C2 and P2, while a prominent band was detected in the ovarian sample taken of P2 (Figure 3). Thus, mating does not activate this PRL signaling pathway in the oviduct.

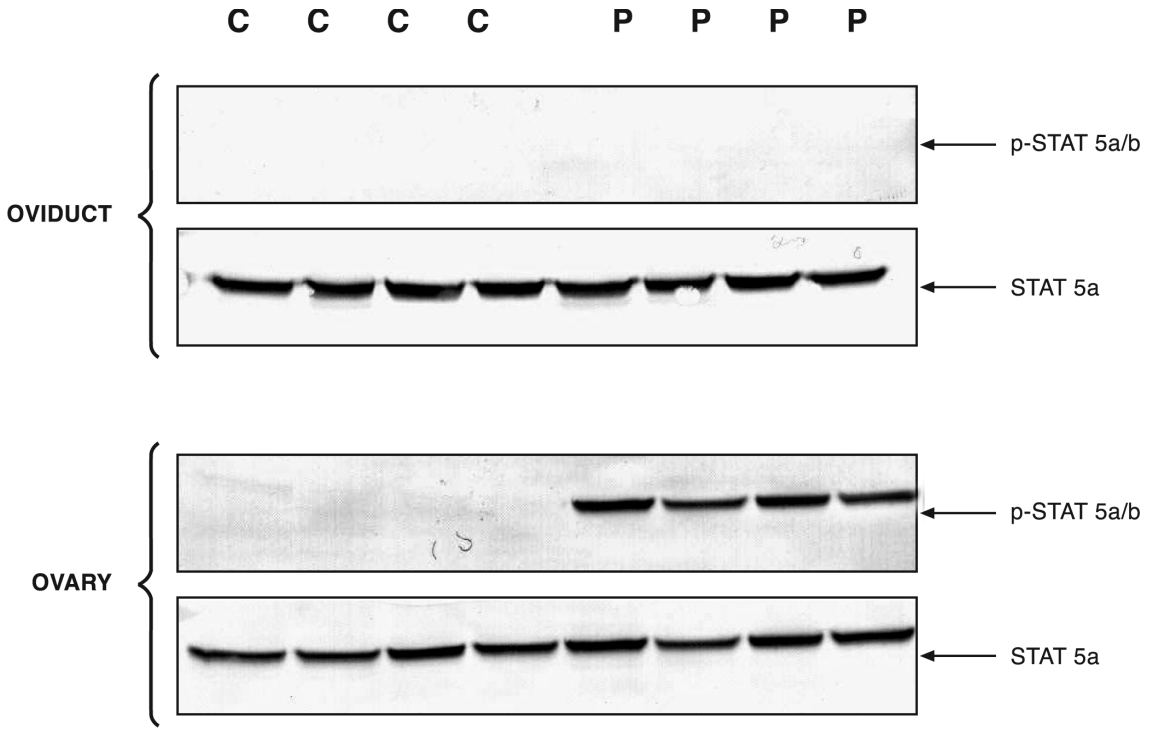

Figure 3: Immunoblot of phosphorylated STAT 5a/b (p-STAT 5a/b) or STAT 5a of rat oviducts or ovaries on day 2 of the cycle (C) or pregnancy (P). Organs were removed and their total proteins (100 μg) were subjected to SDS-PAGE followed by Western blot with anti p- STAT 5a/b or STAT 5a antibodies. Note that the immunoblot revealed a specific band for p-STAT 5a/b only in the ovary of P whereas STAT 5a was present in both organs in C and P.

Experiment 7: This experiment was designed to determine whether PRL surges induced by mating are involved in switching the E_2 pathways in the oviduct. Pro-estrous rats were treated with either BEC (N = 25) or vehicle (N = 6). After 1 hour, they were subjected to CVS and 12 h later at 0900 on day 1 of pseudopregnancy, animals received the second injection of BEC or its respective vehicle. Three hours later, vehicle treated animals were given 15 μg H-89 i.b + 10 μg E_2 s.c while BEC-treated animals were divided into three treatment groups: 1) saline + propylene glycol, 2) saline + E_2, 3) H89 + E_2. On the same day at 2100 animals received the third injection of BEC or its vehicle. Twenty-four hours after H-89 + E_2 treatment, animals were autopsied to assess egg transport as described in Material and Methods.

Egg transport was similarly accelerated following treatment with BEC+H-89+ E_2 as with vehicle of BEC + H89+ E_2 (figure 4). Thus, suppression of PRL surges induced by CVS did not prevent CVS from switching E_2 signaling pathways in the oviduct.

DISCUSSION

It was previously described that the presence of spermatozoa or foreign protein in the uterus is a component of mating capable of switching the signaling pathway of E_2 in the oviduct from nongenomic to genomic in the absence of CVS and seminal fluid (Parada-Bustamante et al., 2003). Here, we showed that CVS alone is also an effective signal to shift E_2 signaling pathway in oviduct. Thus, all three components provided by mating: CVS, foreign protein or sperm cells acting on the female genital tract can independently switch the mode of action of E_2 in the rat oviduct. This redundancy suggests that mating-induced shifting of E_2 signaling in

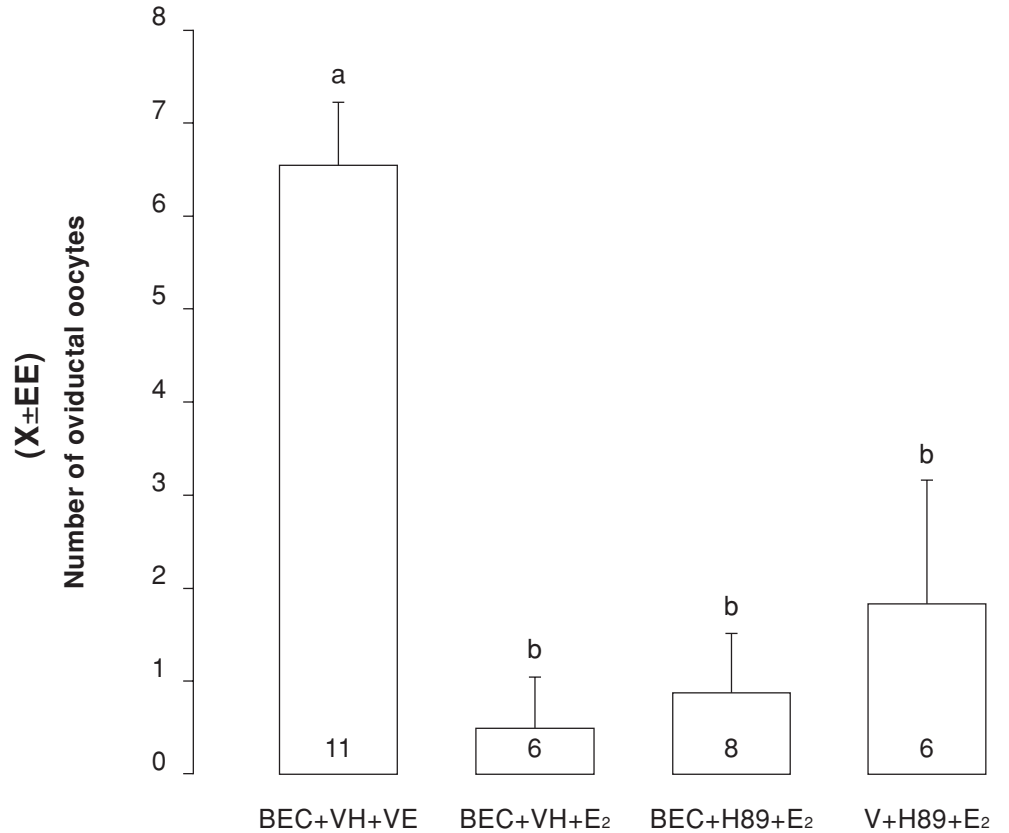

Figure 4: Number of oocytes recovered from the oviduct of rats on day 2 after cervico-vaginal stimulation (CVS). Rats were treated with 1 mg of Bromoergocriptine (BEC) or vehicle (V) at 2100 of pro-oestrus and 1 hour later were stimulated with a vibrating glass rod introduced in the vagina. At 0900 on day 1 of pseudopregnancy animals received the second injection of BEC or V and 3 h later V-treated animals were given H-89 i.b 15 μg + oestradiol (E_2) s.c. 10 μg while BEC-treated animals were separated into three treatment groups: 1) vehicle of H-89 (VH) + vehicle of E_2 (VE), 2) VH + E_2 and 3) H-89 + E_2. The third injection of BEC or V was given on the same day at 2100. Animals were autopsied to assess egg transport 24 h after V, H-89 or E_2 treatment. Figures inside the bars indicate the number of animals used. Bars with different letters are significantly different ($p<0.05$).

the oviduct from non-genomic to genomic pathways in a species in which, under normal conditions, mating invariably leads to pregnancy, must be an important element of the reproductive strategy. Since previous works (Müller et al., 2005) showed that mating modifies the *modus operandi*, as far as ovum transport is concerned under basal conditions and in response to an E_2 pulse, it is clear that mating-associated signals induce a wide and profound change in oviductal physiology.

Cervico-vaginal stimulation activates a neuroendocrine reflex that results in iterative PRL surges (Butcher et al., 1972; Smith et al., 1975). We hypothesized that just as these surges divert the corpus luteum from undergoing luteolysis and sustain progesterone secretion, they could be responsible for switching on and off genomic and non-genomic E_2 signaling pathways in the oviduct. Therefore, we investigated a possible involvement of PRL-R and the effect of suppressing PRL surges on E_2 signaling in the oviduct. In the rat two distinct PRL-R mRNAs, PRL-R_S and PRL-R_L, which code for proteins between 40-100 kDa, have been identified

(Katoh et al., 1987; Boutin et al., 1988; Okamura et al., 1989). Our results show that PRL-R_L mRNA and a PRL-R protein of 42 kDa are present in the oviduct and that their tissue level is not different in mated and non-mated rats.

Upon binding to its receptor, PRL activates different intracellular signalling pathways principally the JAK2/STAT 5 cascade (Campbell et al., 1994; Lebrun et al., 1994; Rui et al., 1994; Goffin et al., 2002). CVS did not activate STAT 5 in the oviduct although it did in the ovary. Moreover, when PRL surges were suppressed by treatment with a dopamine agonist, CVS was still able to switch the mode of action of E_2 in the oviduct. Altogether these results clearly show that CVS shifts E_2 pathways in the rat oviduct by a PRL-surge independent mechanism. Probably, other CVS-induced neuroendocrine or neural reflexes not yet identified are responsible for this phenomenon, but this remains to be determined.

Long and short isoforms of PRL-R mRNA coexist in ovary, uterus and mammary gland (Ouhtit et al., 1993) and their ratio of expression varies depending on the tissue and the physiologic stage of the animal, PRL-R_L being the predominant form in the majority of tissues (Nagano and Kelly, 1994). PRL-R mRNA has been detected by RT-PCR in different rat tissues such as corpus luteum (Telleria et al., 1997), testis (Zhang et al., 1995), decidua (Gu et al., 1996), liver (Kloehn et al., 2001) and ovary (Zhang et al., 1995). To our knowledge, this is the first report on the presence of PRL-R_L mRNA and PRL-R protein in the rat oviduct. The fact that PRL-R is expressed in oviductal muscle/serosa layer may indicate participation in muscle metabolism or maintenance, although this remains to be determined. On the other hand, because STAT 5 was not activated in the oviduct when plasmatic PRL levels were elevated suggests that PRL acts in the oviduct through a cascade independent of JAK2/STAT 5. Further studies are necessary to determine the signaling cascades that participate in the PRL pathway in the myosalpinx.

In summary, CVS is one of the components of mating that is capable of shifting the response of the oviduct to E_2 from nongenomic to genomic pathways. This effect is independent from PRL surges elicited by CVS or mating. The presence of PRL-R_L mRNA and PRL-R protein in the oviduct suggests that PRL may have some biological role in this organ.

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