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Intranasal application of PACAP and β-cyclodextrin before the "critical period of proestrous stage" can block ovulation

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Introduction: It was previously shown that intracerebroventricular administration of pituitary adenylate cyclase-activating polypeptide (PACAP) prior to GnRH mobilization in proestrus prevents ovulation in rats. In this study, we examined whether PACAP given intranasally could influence luteinizing hormone (LH) and prolactin (PRL) surges and ovulation. *Methods:* On the day of proestrus PACAP, β-cyclodextrin (modifier of blood–brain barrier) or PACAP + β-cyclodextrin was applied intranasally between 12:30 and 13:00. Blood samples were taken at 16:00, 18:00, and 20:00 for measuring plasma hormone levels. In the next morning, the expelled ova were counted. β-Cyclodextrin was also administered to male and diestrous female rats between 12:30 and 13:00 and blood was taken at 18:00. *Results:* PACAP prevented LH and PRL surges and ovulation in about half of the rats, β-cyclodextrin alone more effectively prevented ovulation. When PACAP and β-cyclodextrin were administered together, more rats ovulated like when PACAP given alone. β-Cyclodextrin did not influence LH and PRL levels in diestrous females; however, in males, it significantly enhanced PRL level. *Discussion:* Not only the intracerebroventricular, but the intranasal application of PACAP prevented ovulation. β-Cyclodextrin alone is more effective than PACAP and enhances PRL levels in male rats. PACAP and β-cyclodextrin given together weaken each other's effect. β-Cyclodextrin, as excipient of various drugs, has to be used carefully in human medications.

Original Article

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INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP or ADCYAP) was isolated from sheep's hypothalami and characterized by Miyata et al. (1989, 1990). It was named on its ability to stimulate adenylate cyclase in anterior pituitary cell culture. In the organism, PACAP is present in two amidated forms. PACAP38 is composed of 38 and PACAP27 is composed of 27 amino acids. The major form is PACAP38. Later, in this paper, we will cite data concerning the effect of PACAP38 and we will not indicate the number of amino acids. During the past three decades, many data have been accumulated about the receptors and role of PACAP in mammalian organisms. Three receptors were identified: PAC1 receptor, specific for PACAP, VPAC1, and VPAC2 receptors, which bind VIP and PACAP with equal affinity (Arimura, 2007; Arimura & Shioda, 1995; Bokaei et al., 2006; Gottschall et al., 1990, 1991; Ohtaki et al., 1990). PACAP has a multifunctional role in mammals and exerts its effect through different intracellular signal transduction pathways including the adenylate cyclase and posphopholipase C (Miyata et al., 1989; Rawlings, 1994). Its most important functions are the following: neurotransmitter, neuromodulator, and hypophyseotropic hormone (reviewed by Arimura, 2007; Arimura & Shioda, 1995). Neuroprotective role of PACAP was also demonstrated (Lee & Seo, 2014; Reglődi et al., 2000; Tamás et al., 2012). PACAP has an important role in many organ systems including the reproductive one (Köves et al., 2000, 2014; Reglődi et al., 2012; Sherwood et al., 2007). The lack of PACAP in PACAP knockout mice did not influence ovulation (Isaac & Sherwood, 2008); however, the enhanced PACAP level in the brain before the so-called "critical period" of proestrous stage [when the central nervous system prepares itself for the gonadotropin hormone-releasing hormone (GnRH) release to the hypophyseal portal circulation, identified by Barr & Barraclough, 1978] prevented ovulation (Kántor et al., 2000; Köves et al., 1996). In our previous experiments, PACAP was given

intracerebroventricularly (ICV) on the day of proestrus. About 10 μg, not a lower dose, prevented the luteinizing hormone (LH) surge and ovulation expected in the next morning. PACAP administered intravenously (IV) had no similar effect (Kántor et al., 2000). It means that the effect of PACAP was not exerted at the pituitary; rather, it was effective at the hypothalamic level. It was also shown that the inhibitory effect of ICV PACAP was mediated by corticotropin-releasing hormone (CRH) and endogenous opioids (Kántor et al., 2000).

PACAP and its mRNA are transiently expressed in rat's anterior pituitary (Heinzlmann et al., 2008; Köves et al., 2014; Szabó et al., 2004) and rat granulosa cells as well during the preovulatory period (Gräs et al., 1996). Later, Park et al. (2001) demonstrated that the treatment of preovulatory granulosa cells with GnRH agonist stimulated PACAP mRNA levels in a dose-dependent manner. *In situ* hybridization analysis of cultured preovulatory follicles revealed that GnRH-induced PACAP signals were detected in granulosa cells, but not thecal cells. In immature granulosa cells, co-treatment with GnRH agonist suppressed follicle-stimulating hormone (FSH) stimulated PACAP mRNA levels in a dose-dependent manner, whereas treatment with GnRH alone showed no effect. The effect of PACAP in the ovary and how PACAP influences female gonadal functions at the ovarian level were reviewed by Reglődi et al. (2012). It was found by their research group that PACAP level in the follicular fluid influences the number of the expelled ova after superovulation treatment in volunteer women (Koppan et al., 2012). Higher concentration of PACAP in follicular fluid associated with a lower number of expelled ova and the lower level of PACAP in the follicular fluid was associated with a higher number of expelled ova.

Ample evidence indicate that many drugs can exert an effect on the central nervous system when applied intranasally (IN; Born et al., 2002; Charlton et al., 2007; Guthoff et al., 2010; Heinzlmann et al., 2012; Illum, 2004; Kubek et al., 2009; Nonaka et al., 2012; Pietrowsky et al., 1996; Tiwari et al., 2012). This route of administration is not invasive because it avoids using needles as in the case of IV application or the opening of the skull as in the case of ICV administration. It seems that many drugs can enter the brain from the nose and in this case their level in the general circulation is very low, and thus the peripheral side effects are negligible (Illum, 2004). When a drug is delivered high enough into the nasal cavity, it can reach the olfactory region and the drug can be transported into the brain (Tiwari et al., 2012).

Cyclodextrins (CDs) are used as excipients (long-term stabilizator) in natural or synthetic substances. CDs enhance the absorption of drugs applied by various ways (Loftsson & Brewster, 2010). CDs are cyclic oligosaccharides prepared from starch by enzymatic cleavage of the amylose helix. The three most studied representatives consist of 6, 7, and 8 glucopyranose units called α-, β-, and γ-CDs, respectively (Szejtli, 1988). These CDs are considerably hydrophilic. The effect of CDs is due to the extraction of lipid constituents from cell membranes (Vecsernyés et al., 2014).

Nonaka et al. (2012) investigated whether PACAP administered IN was able to exert effect on the central nervous system. They used an animal model of Alzheimer's disease. It was found that PACAP given IN was effective in improving memory in a very low dose (0.01 μg). In the aforementioned work, it was also demonstrated that only the β-CD, not the other form of CDs, enhanced the accumulation of PACAP in the hypothalamus.

The ICV administration of PACAP is a very invasive way to prevent ovulation (Köves et al., 1996). The IN application only moderately influences the general condition of the rats. The aim of the present experiment was to examine whether PACAP entering the brain from the nasal cavity could efficiently influence the LH and the accompaning prolactin (PRL) surges and ovulation. Because β-CD in Nonaka's experiment was able to enhance the hypothalamic concentration of PACAP-administered IN, we also studied whether β-CD, co-administered with PACAP, could enhance the blocking effect of PACAP on LH and PRL surges and ovulation in female rats. There is no evidence in the literature on the neuroendocrine effect of β-CD administered alone. With the use of male and diestrous female rats, we also investigated how the β-CD (administered at the same daytime and the same way as in proestrous female rats) modifies the basal LH and PRL levels.

MATERIALS AND METHODS

Animals

Adult (2- to 3-month-old) male and female Wistar rats were used for the experiments (weight: 200–250 g of females and 350 g of males). The rats were kept in a light–dark cycle (lights on at 5:00 and lights off at 19:00) and temperature-controlled (22 ± 2 °C) vivarium. They were fed with standard lab chow (Gödöllő, Hungary) and water ad libitum.

From female rats, vaginal smears were taken daily, which were used for the experiment that showed at least two consecutive 4-day cycles.

Implantation of intravenous cannula

Under general anesthesia of female rats (chloral hydrate 35 mg/100 g bw, Reanal, Budapest, Hungary), a polyethylene cannula was implanted into the external jugular vein on the second day of diestrus. A ventral cervical skin incision was made right to midline with its caudal terminus at the level of the clavicle. The right external jugular vein was mobilized and a 25-mm-long soft cannula (Dow Corning, cat. no. 602-155) was inserted into the vessel and fixed in place. The cannula was elongated with 15-cm-long hard tube. Incision was made on the skin at the midline between the scapulae, and jugular cannula was pulled out under the skin through the scapular incision. The cannula was filled with heparinized saline and sealed with metal pin. Each rat was housed in an individual cage to prevent damage of cannula by another rat and in a separate rat room to minimalize the stress.

Drugs

(a) About 300 μg of PACAP (purchased from Sigma-Aldrich, St. Louis, MO) was dissolved in 15 μl acetic

acid + 60 µl distilled water. Then, 225 µl of physiological saline (0.9% sodium chloride, Reanal) containing 0.1% bovine serum albumin (BSA; Merck, Darmstadt, Germany) was added. The final concentration was 1 µg of PACAP/µl. (b) β-CD was purchased from Sigma and 5% solution was prepared with saline containing 1% BSA.

Administration of drugs

Under superficial ether anesthesia, IN application was carried out through a bougie. The needle of a Hamilton syringe (10 µl) was equipped with a piece of polyethylene tube (length: 10 mm, inner diameter: 0.037 mm, and outer diameter: 0.107 mm). About 10 µl of both drugs were sprayed through the left nostril onto the olfactory region by the syringe between 12:30 and 13:00 on the day of proestrus before the "critical period." We tried to inhibit the GnRH mobilization by applying PACAP IN.

Groups included in the experiment

Group 1: 11 proestrous females received 10 µl of saline containing 1% BSA (control group).
Group 2: 16 proestrous females received 10 µl PACAP.
Group 3: 6 proestrous rats received 10 µl of β-CD solution (see preparation of the drug).
Group 4: 10 proestrous females received 10 µl of PACAP + 10 µl of β-CD.
Group 5: 10 diestrous female received 10 µl of saline containing 1% BSA (control group).
Group 6: 8 diestrous female received 10 µl of β-CD.
Group 7: 7 males received 10 µl of saline containing 1% BSA (control group).
Group 8: 9 males received 10 µl of β-CD.

Blood sampling

In the afternoon, proestrus blood samples were taken at 16:00, 18:00, and 20:00 through the previously implanted cannula from the proestrous females. The rats were transferred to the surgery room one by one to avoid stress during this procedure. The diestrous female rats and the male rats were sacrificed by decapitation at 18:00 and the trunk blood was collected. The coagulation was prevented by ethylene diamine tetraacetic acid (EDTA sodium salt, Serva, Heidelberg, Germany) solution. The blood was centrifuged at 4 °C. Plasma was stored at −20 °C until determination of pituitary hormones.

Counting ova and measuring weight of organs

The proestrous rats were sacrificed on the next morning by decapitation and ova were looked for in the Fallopian tube. The weight of whole pituitaries and the cleaned ovaries were measured by torsion balance.

LH and PRL radioimmunoassays (RIA)

LH and PRL were measured using RIA kits provided by National Pituitary Agency, NIDDK and Dr. A. F. Parlow. The following instructions, supplied with the kit, were RIA procedures provided briefly:

Step 1. Iodination of LH and PRL: Chloramine-T method was used for iodination. Chloramin-T was purchased from Mallinckrodt Baker, Inc., Phillipsburg, NJ. Highly purified rat LH or PRL was iodinated using a modification of the procedure described by Greenwood et al. (1963). About 10 µl of phosphate buffer [0.1 M, pH 7.6 and containing 0.9% NaCl and 0.1% NaN_3 phosphate-buffered saline (PBS)] was added to 20 µl of LH or PRL (R-LH AFP71878 or R-PRL AFP105058 100 µg/ml) previously snap-frozen. Approximately, 1 mC of ^{125}I (Izotóp Intézet Kft. Budapest, Hungary) was then added to the vial, followed by 10 µl of Chloramine-T (10 mg/ml) to iodinate LH or PRL. Iodination was stopped after 45 s by adding 50 µl of sodium metabisulfite (10 mg/ml in PBS). The mixture was immediately transferred to PD-10 prepacked Sephadex™ G-25 M column and ½ ml fractions were eluted with PBS (0.01 M containing 1% BSA). The fraction containing the iodinated LH or PRL was diluted with approximately 50 ml of PBS containing 1% BSA (15,000–17,000 cpm). Binding probe was carried out.

Step 2. Each standard and unknown sample were measured in duplicate. In order to avoid cross-contamination, we used a clean disposable pipette tip for the addition of each reagent and sample. About 100 µl increasing amount of unlabelled standard hormone or 50 µl unknown sample were added to the vials containing 300 or 350 µl of 0.1% PBS, pH 7.6. About 100 µl of the appropriate dilution (1:120,000) of primary antiserum in PBS containing EDTA was added to each vial. ^{125}I-labeled hormone (100 µl; approximately 10,000–15,000 cpm) was then added and the assay mixture was incubated for 24 hr at room temperature. The next day, the second antiserum was added (200 µl, 1:12 dilution). After one, our incubation each vial received Polyethylenglykol 6000 solution (Fluka AG, CH-9470, Seelze, Germany).

Step 3. After centrifugation at 3,000 rpm for 30 min, the supernatant was decanted and the precipitate was counted in an Automatic Gamma Counter (LKB Vertriebs GmbH, Vienna, Austria).

Statistical analysis

First, the mean of two determinations of the hormon levels was calculated. From the data concerning the known amount of hormons, the standard binding curves were drawn. The hormone level of each rat was calculated using LKB Clinigamma software (Biosurplus, San Diego, CA, USA) belonging to the Automatic Gamma Counter and this value was subjected to one-way analysis of variance. Statistical significance was defined as $p < .05$. Tukey's test was used as post-test. The sensitivity of the LH assay was 0.5 ng/ml of rat's plasma and the sensitivity of PRL assay was 1.0 ng/ml plasma.

Graphs were created by the GraphPad Prism 7 program (GraphPad Software, LLC, San Diego, CA, USA). All values in figures and tables are expressed as mean ± SEM.

RESULTS

Occurrence of ovulation in female rats

In the control rats (Group 1), which received physiological saline (applied onto the olfactory region), ovulation was observed. The mean value of the number of expelled ova was 7.4 ± 1.6. PACAP (Group 2) prevented ovulation in about half of the rats (7/16). In the rats, where ovulation occurred, the number of ova (7.2 ± 0.5) was similar to controls. β-CD alone (Group 3) was also able to prevent ovulation (5/6) and the number of ova in ovulating rat was only 5. When PACAP and β-CD were given together, ovulation was blocked only in one fifth (2/10) of the rats. The other eight rats ovulated. The number of ova did not differ significantly from that of the control rats (9.0 ± 0.5). Table 1 summarizes the occurrence of ovulation and the number of ova in the various experimental groups.

Weight of pituitaries and ovaries

Weight of the whole pituitaries did not show any significant difference in the four experimental groups; however, weight of the ovaries was significantly lower in the rats treated with β-CD alone (where ovulation did not occur) compared to the controls (Table 2).

LH plasma levels in the various groups of female rats in the afternoon of proestrus

In the control rats (Group 1), the LH levels increased by 16:00 and there was a gradual decrease by 20:00 and stayed further higher at 18:00 and 20:00 than the basal level (the diestrous level was considered as basal level; Figs 1A and 3). The difference was only significant between the level at 16:00 and level of diestrous ($p < .01$). From 16:00 till 20:00, the differences were not significant. PACAP administered before the critical period of the proestrus (Group 2) prevented the preovulatory LH surge and then ovulation in 7/16 rats. In these rats, the plasma LH levels were lower than the basal level, but the differences were not significant. If ovulation occurred, the LH levels elevated. The highest level was observed at 16:00, then the level of LH gradually decreased and reached a significantly lower level at 20:00 ($p < .05$; Fig. 1B). There was no significant difference between the plasma LH levels of the control and the blood sample of PACAP-treated rats at the same timepoint. β-CD alone (Group 3) also influenced the LH surge (Fig. 1C). In this group, ovulation occurred only in one rat; however, in 5/6 rats β-CD prevented it. In these latter rats, where ovulation did not occur, the LH levels were very low similar to the LH levels in PACAP-treated rats where ovulation was prevented. When PACAP and β-CD were administered together (Group 4), the LH levels showed similar pattern to that of treatment with PACAP or β-CD alone (Fig. 1D). In all rats, where ovulation was blocked, the LH levels remained extremely low without any significant difference. The ratio of the occurrence of ovulation in the case of the combined application of PACAP and β-CD was higher than that in the case of PACAP treatment alone.

PRL plasma levels in the various groups of female rats in the afternoon of proestrus

In the control rats (Group 1), the PRL levels were elevated at all the three examined timepoints (16:00, 18:00, and 20:00; Fig. 2A). The highest level was measured at 20:00, significantly higher than at 18:00. PACAP administered before the

Table 1. Occurrence of ovulation in the various groups

Group of rats	Treatment	Total number of rats	Block of ovulation	Ovulation	Number of ova
Group 1	Saline	11	–	11	7.4 ± 1.6
Group 2	PACAP	16	7	9	7.2 ± 0.5
Group 3	β-CD	6	5	1	5.0
Group 4	PACAP + β-CD	10	2	8	9.0 ± 0.5

Note. PACAP: pituitary adenylate cyclase-activating polypeptide; β-CD: β-cyclodextrin.

Table 2. Weight of organs in various groups

Group of rats	Treatment	Total number of rats	Weight of whole pituitaries (mg/rat)		Weight of the two ovaries (mg/rat)	
			Block of ovulation	Ovulation	Block of ovulation	Ovulation
Group 1	Saline	11	–	17.3 ± 0.5		109.4 ± 3.7
Group 2	PACAP	16	16.6 ± 0.6	17.2 ± 0.5	98.6 ± 4.30	109.9 ± 4.1
Group 3	β-CD	6	17.9 ± 0.6	18.5 ± 0.0	91.6 ± 6.08*	99.0 ± 0.0
Group 4	PACAP + β-CD	10	16.0 ± 0.7	16.9 ± 1.1	106.3 ± 12.9	100.3 ± 6.4

Note. PACAP: pituitary adenylate cyclase-activating polypeptide; β-CD: β-cyclodextrin.
*$p < .05$ saline vs. β-CD (block of ovulation).

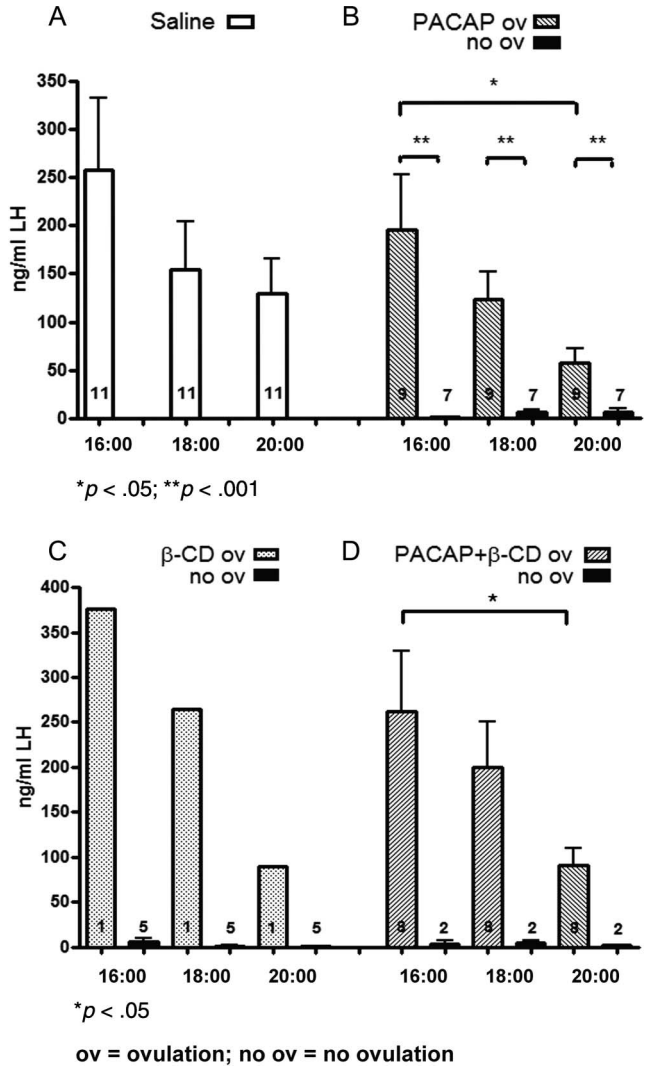

Fig. 1. Effect of intranasal application of PACAP, β-CD, and combination of PACAP + β-CD on the preovulatory LH surge. The plasma LH levels were measured in the samples taken in the afternoon of proestrus at 16:00, 18:00, and 20:00. (A) LH levels in saline-treated control rats. (B) LH levels in PACAP-treated rats. (C) LH levels in β-CD-treated rats. (D) LH levels in PACAP + β-CD-treated rats. The number in or above the columns indicate the number of blood samples. In the rats where ovulation occurred, the LH levels gradually decreased from 16:00 till 20:00, the difference was significant in PACAP and PACAP + β-CD-treated rats between levels taken at 16:00 and 20:00 (*$p < .05$). In the cases where ovulation was blocked, the LH levels were significantly lower than in ovulating rats at the corresponding timepoints (**$p < .001$)

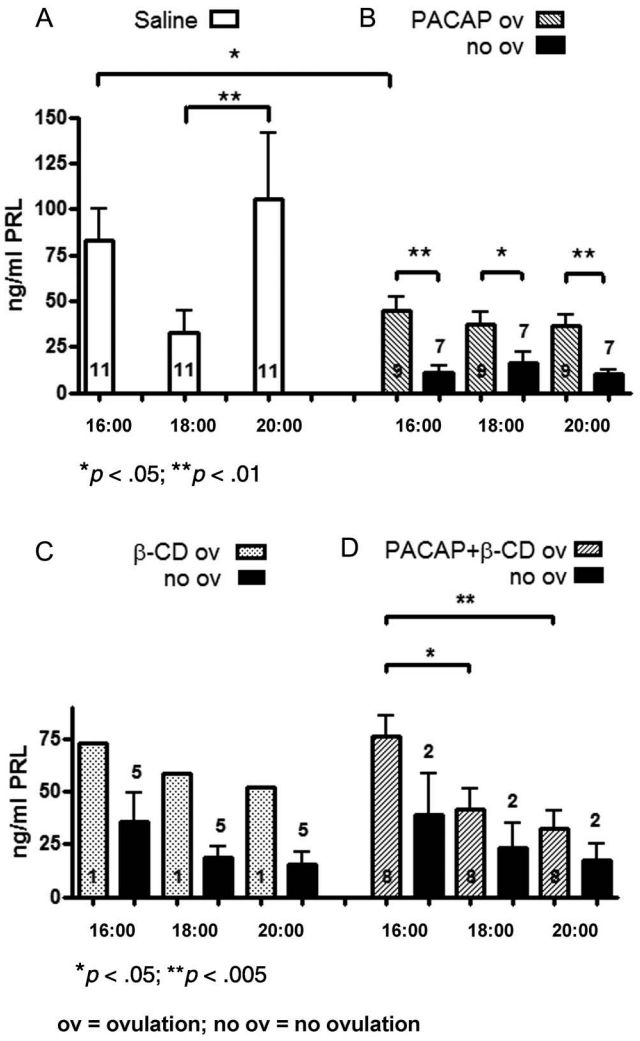

Fig. 2. Effect of intranasal application of PACAP, β-CD, and combination of PACAP + β-CD on the preovulatory PRL surge. The plasma PRL levels were measured in the samples taken in the afternoon of proestrus at 16:00, 18:00, and 20:00. (A) PRL levels in saline-treated control rats. (B) PRL levels in PACAP-treated rats. (C) PRL levels in β-CD-treated rats. (D) PRL levels in PACAP + β-CD-treated rats. The number in or above the columns indicate the number of blood samples. In the control rats, the PRL levels were highest at 20:00, significantly higher than at 18:00 (**$p < .01$). In the other three groups, the pattern of the PRL levels was similar to the pattern of LH levels, highest at 16:00 and gradually decreased by 20:00. The differences in the PRL levels at different timepoints were only significant in PACAP + β-CD-treated rats (*$p < .05$, 16:00 vs. 18:00 and **$p < .005$, 16:00 vs. 20:00). In the PACAP-treated rats, where ovulation did not occur, the PRL levels were significantly lower at each timepoint than in those where ovulation was verified ($p < .01$ and $p < .05$, respectively)

critical period of the proestrus (Group 2) prevented the preovulatory PRL surge in the rats where ovulation was blocked. The plasma PRL levels were very low, significantly lower than in the ovulating rats at the same timepoint ($p < .01$, $p < .05$, and $p < .01$, respectively). If ovulation occurred, the PRL levels were moderately enhanced and did not reach the control level. Significant difference was only observed between the samples taken at 16:00 ($p < .05$; Fig. 2B). β-CD alone (Group 3) prevented ovulation in 5/6 rats. There was no significant difference between the PRL levels taken at 16:00, 18:00, or 20:00. The PRL levels were elevated in one rat where ovulation occurred (Fig. 2C). When PACAP and β-CD were administered together (Group 4) in those rats, where ovulation occurred, the PRL level elevated at 16:00 similarly to controls and gradually and significantly decreased from 16:00 to 20:00 ($p < .05$ and $p < .005$, respectively), the values and pattern were similar to that of β-CD alone. In the non-ovulating rats, the PRL levels were lower than in ovulating rats (Fig. 1D).

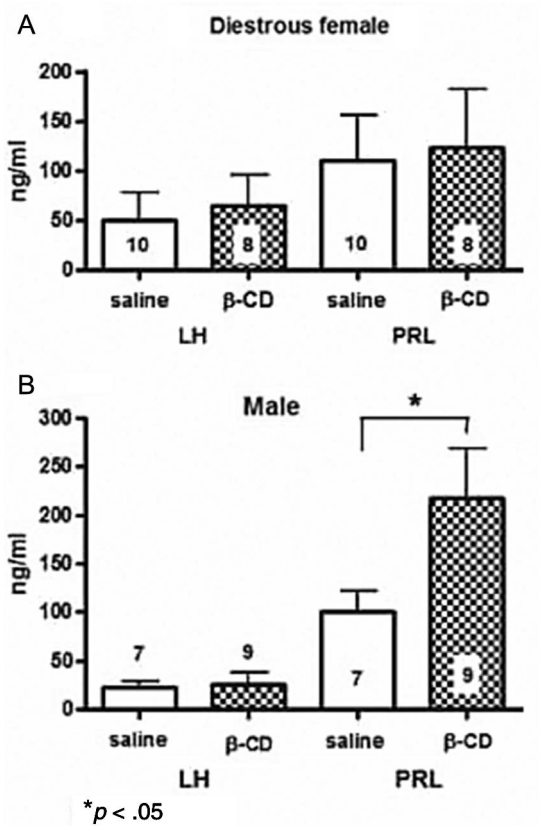

Fig. 3. Effect of intranasal application of β-CD on the LH and PRL levels in diestrous female (A) and in male rats (B). The plasma LH and PRL levels were measured in the samples taken at 18.00. The number in or above the columns indicate the number of blood samples. Both in female and male rats, the LH levels did not differ in saline- and β-CD-treated rats; however, the PRL levels were significantly higher in male rats compared to saline-treated rats upon the β-CD treatment (*$p < .05$)

The significance was not calculated because the number of rats in this group was low.

LH and PRL levels in diestrous rats

Diestrous rats were treated with saline or β-CD in the same time of day as the proestrous rats. LH and PRL levels did not change significantly upon the β-CD treatment in this stage of the estrous cycle (Fig. 3A).

LH and PRL levels in male rats

Male rats were similarly treated with saline or β-CD in the same time of day as the diestrous rats. The LH levels did not change upon the β-CD treatment; however, the PRL levels significantly enhanced in the β-CD treated rats (Fig. 3B).

DISCUSSION

It was demonstrated more than 65 years ago that several neuroactive drugs could prevent ovulation. These drugs were applied before the critical period of proestrous stage of the ovarian cycle (Everett & Sawyer, 1950). Our previous experiments (Kántor et al., 2000; Köves et al., 2014, 1981, 1996) clearly show that endogenous opioids, PACAP, and CRH, when applied ICV, were also effective in this model. Recently, we have used a non-invasive approach to test the effect of PACAP on ovulation. PACAP was administered IN in the same dose (10 μg/rat) as it was applied ICV in our previous experiment. Several ways are described how the drugs enter the brain from the nasal cavity and then they act similarly to the ICV administration. The first possibility is a way through the olfactory pathway. When a drug is delivered high enough into the nasal cavity, the olfactory mucosa may be reached and drug transport into the brain and the cerebrospinal fluid may occur through the olfactory receptor neurons. Paracellular and transcellular passive absorption, carrier-mediated transport, and absorption via trancytosis through the nasal epithelium are not excluded (Arora et al., 2002; Lochhead et al., 2015; Tiwari et al., 2012).

How can we explain the blocking effect of PACAP on ovulation administered just before the critical period? The expression of PACAP in the hypothalamus and anterior pituitary fluctuates during the estrous cycle. It was demonstrated by Moore et al. (2005) that in female rats hypothalamic PACAP is upregulated in the morning of proestrus, then its level decreases prior the GnRH release into the portal circulation (reviewed by Köves et al., 2014). In contrast, the PACAP level in the anterior pituitary enhances on the late of proestrus. Cell immunoblot assay revealed that the number of PACAP-releasing cells tremendously increased in a culture containing the pituitary sample from the proestrous rats at 20:00; however, the number of PACAP releasing cells is very low in a culture containing the pituitary sample from diestrous rats (Szabó et al., 2004). It was supposed that the enhanced number of PACAP-secreting cells in proestrus is involved in stopping the LH surge at late evening. In our experiments, we enhanced the hypothalamic level of PACAP by ICV or IN application, and it supposedly prevented the GnRH release and the consequential LH surge and ovulation. There is no evidence how the pituitary PACAP level changes in these rats in the lack of LH surge. We supposed that the number of PACAP-releasing cells remains similarly lower than in diestrous rats. It seems that PACAP is involved in the dynamic control of the gonadotropic hormone secretion (Counis et al., 2007).

The question arises: why was ovulation blocked only in half of the rats? In the case of ICV application the injected amount has to be the same in each rat. In the case of nasal application several physiological factors may influence the drug absorption (Arora et al., 2002). These are the following: nasal mucus secretion, cycle of nasal epithelium, pH of the nasal cavity, mucocilliary clearance and cilliary beat frequency. These factors may show individual difference.

Nonaka et al. (2012) in collaboration with Banks (2015), who is an expert in studying the drugs passing through the blood–brain barrier (BBB), convincingly testified that only β-CD, not the other forms of CDs, enhanced the accumulation of PACAP in the hypothalamus. As it was mentioned above (Moore et al., 2005) in the hypothalamic paraventricular nucleus, PACAP mRNA is upregulated in the morning of proestrus 3 hr before the gonadotropin surge and then declines. We supposed that β-CD added with

PACAP could maintain the high PACAP level during the critical period and it could enhance the accumulation of PACAP in the hypothalamus increasing its level above the physiological one, and in this way PACAP could prevent ovulation more potently. Unexpectedly, β-CD alone was the most effective, only one rat ovulated from six. When PACAP and β-CD were added together, they weakened each other's effectiveness. In this group, the block of ovulation was observed only in 2 of 10 rats. It seems that there is a competition between the two drugs. However, the molecular background of this competition is not clear during that time.

CDs are more and more frequently used in human pharmacotherapy (reviewed by Vecsernyés et al., 2014). The use of CDs is widespread for nasal, oral, parenteral, pulmonary, or skin delivery of drugs. Experimental research revealed the potential therapeutic use of CDs and CD nanoparticles in neurodegenerative diseases, stroke, neuroinfections, and brain tumors. CDs are used to improve the nasal absorption of these drugs by extraction of different lipids from plasma membranes, thus increasing their aqueous solubility. The CDs are highly potent at the level of the BBB (Merkus et al., 1999). The effect of CDs on the nasal absorption of estradiol was investigated 25 years ago (Hermens et al., 1990). It was found the β-CD enhanced the nasal absorption of 17 β-estradiol. Szejtli and Szente (2005) investigated the effects of CDs on the taste of foods and on the stabilization of flavors. The bitter taste of drugs or food components can be reduced or eliminated by CDs. Adstringent components of foods, beverages, or cigarette smoke (nicotine) can also be complexed and their taste reduced or fully eliminated. Another research group (Numanoğlu et al., 2007) described that CDs enhanced the stability and water solubility of fragrance materials in cosmetics. Inspite of the expansive use of CDs, the effect of CDs on endocrine status of humans was not examined to date. What may be the mechanism of how β-CD can block ovulation and decreases the weight of ovaries? The most obvious way is that β-CD inhibits the GnRH release. However, how it is carried out needs a complex further experiment. When we examined the mechanism how ICV PACAP inhibits ovulation, we injected antagonists of those molecules that were candidates for mediating the blocking effect of PACAP (CRH and endogenous opioids; Kántor et al., 2000). A direct effect of β-CD on GnRH neurons is also not excluded. In this moment, there is no acceptable explanation of how β-CD decreases the ovarian weight a day after its administration.

The dynamism of LH level changes during the proestrous afternoon was similar in control and treated rats (Fig. 1). It means that both PACAP and β-CD did not result in a significant difference in the pattern of LH in the rats where ovulation occurred. When ovulation was blocked, the LH levels were extremely low, even lower than in diestrous rats. There is evidence that, during the proestrous afternoon, LH surge is accompanied by a PRL surge (Butcher et al., 1974). In the rats where ovulation was blocked, the PRL levels were lower than in those where ovulation occurred.

In diestrous females, rats β-CD did not influence either the LH or the PRL levels; however, in male rats, β-CD did not influence the LH, but significantly enhanced the PRL levels.

The major conclusions of this present work

(a) In the case of intranasal application, PACAP alone prevented ovulation in about half of the rats. (b) β-CD alone potently prevented ovulation and enhanced the PRL levels in male rats. (c) PACAP and β-CD given together weakened each other's blocking effect on ovulation. (d) β-CD, as excipient of various drugs, has to be used carefully in human medications.

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Ethical Statement: The rats were treated according to the rules of "European convention for the protection of vertebrate rats used for experimental and other scientific purposes," Strasbourg, 1986 and Hungarian Government Directive 243/98. Our protocol was approved by the Local Animal Care and Use Committee (permission no.: 22.1/1158/3/2010).

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Authors' Contributions: AH contributed in design, acquisition, and analysis of data. MO contributed in analysis of data. KK contributed in conceptualization, design, interpretation of the results, participation in the drafting, and revising the article.

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