THE ROLE OF PACAP IN THE REGULATION
OF GONADOTROPH HORMONE SECRETION

Doctoral (Ph.D.) theses

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SUMMARY

The pituitary adenylate cyclase activating polypeptide (PACAP) was isolated from ovine hypothalamus based on its ability to activate adenylate cyclase. It is present in a number of places in the body, but it is synthesized in the greatest concentration in the hypothalamus. In the anterior pituitary PACAP mRNA was demonstrated in all stages of the estrous cycle. Numerous data reveal that PACAP plays a role in the regulation of gonadotroph hormone secretion.

In our experiments we used the cell immunoblot assay (CIBA) to show that PACAP is secreted by anterior pituitary cells not only on the day of proestrus, but also on other days of the estrous cycle (Szabó et al, 2002, 2004); however, PACAP can only be immunostained on the day of proestrus. The amount of PACAP released by the cells is less than the amount of LH released. This is demonstrated by the diameter of the blots formed around the cells and visualized by immunostaining. LH synthesized in a greater amount affects organs distant from the pituitary gland through the general circulation. Supposedly PACAP synthesized in a lesser amount has an auto- or paracrine effect on the anterior pituitary (Szabó et al, 2001, Köves et al, 2003). The anterior pituitary cells release PACAP in a daily rhythm, which differs in cultures of anterior pituitary cells taken from proestrous or diestrous females or from males. The number of PACAP blots after the LH surge, on the night of proestrus increases 12-13 times. Under in vitro conditions the LH release of gonadotroph cells changes to PACAP treatment. It changes depending on the time of sacrifice when the pituitaries were removed, that is the time of day, on the gender of the animal and in females on the stage of the estrous cycle. In proestrous females in the morning PACAP inhibited LH release compared to untreated cells, in the afternoon during the LH surge (an increased LH secretion) it stimulated it. In diestrous stage of females and in males PACAP did not considerably affect LH release of gonadotroph cells compared to untreated cells. Because the number of PACAP blots was highest in the night in cell cultures of proestrus female rats, it lets us suppose that PACAP plays a role in the terminating of the LH surge (Szabó et al., 2004). It may have an inhibiting effect through splice variants of PACAP receptor.
INTRODUCTION

A number of external stimuli, mainly light impulses regulate and harmonize the circadian and oscillatory functions of the neuroendocrine system through effecting an inner biological clock. According to Scharrer’s theory (1964), light impulses reach not only the center of sight through the optical nerve, but also the suprachiasmatic nucleus, the biological clock situated in the hypothalamus. Effecting the rhythm of this nucleus, light impulses regulate biological rhythms (see Klein et al, 1991) including the cyclic function of the hypothalamo-hypophyseal-gonad system of female rats.

A number of neuropeptides and neurotransmitters take part in regulating the hypothalmo-hypophyseal-gonad system. The most significant of these is the gonadotroph hormone releasing hormone (GnRH), which was isolated by Schally and his co-workers in 1971. Immunohistochemical studies revealed that this peptide in rat is primarily produced by neurons of the medial septum and the preoptic area (Baker et al, 1975; Sétáló et al, 1975). These are the cells of origin of the GnRH tract. Their fibers terminate in the superficial zone of the median eminence (Sétáló et al, 1976, Merchenthaler et al, 1980). This peptide consisting of ten aminoacids is synthethized in a greater amount on the morning of the day preceding ovulation. Together with the elevation of GnRH synthesis the level of estrogen in the peripheral circulation increases. Under constant lighting patterns (light period 5 a.m.- 7 p.m.) GnRH is secreted into the portal blood in the afternoon between 2-4 p.m. at the critical period. This increase of GnRH release is responsible for the elevated luteinizing hormone (LH) secretion, the so called LH surge, into the circulation, leading to ovulation.

In intact female rats ovulation occurs every four or five days, in other words the animal has a four or five day cycle. The cyclic release of GnRH is followed by an ovarian, then by a uterine cycle. The cytology of the vaginal smear also shows a cyclic change.

The pituitary adenylate cyclase activating polypeptide (PACAP) was isolated from ovine hypothalamus by Miyata and his co-workers in 1989 on the basis of its ability to activate adenylate cyclase. PACAP is present in the body in two bioactive amidated forms, one consisting of 27, the other of 38 aminoacids. PACAP is present in the central and peripheral nervous systems and in other tissues of the body. In greatest concentration it is found in the hypothalamus (Arimura et al, 1991). In ovine PACAP immunoreactivity can be seen in the supraoptic and paraventricular nuclei (Kovès et al, 1990). In rat, the PACAP immunoreactive fibers of neurons of
these nuclei ending in the median eminence can only be stained under special conditions. In 1994 Dow and his co-workers showed that PACAP is secreted into the hypophyseal portal capillaries and that its concentration here is higher than in the general circulation. These data suggest that the hypothalamic PACAP takes part in the regulation of the anterior pituitary function.

AIM OF OUR WORK

We used the cell immunoblot assay (CIBA) and the sandwich enzyme immunoassay (S-EIA) to answer the following questions:

1. Is PACAP secreted by the anterior pituitary cells \textit{in vitro}. If yes
2. What kind of cells secrete PACAP?
3. Measured by S-EIA what is the concentration of PACAP in the adenohypophysis under physiological conditions?
4. Does PACAP release \textit{in vitro} depend on the gender, in females on the day of the estrous cycle and on the time of day the animals were sacrificed?
5. Does LH release \textit{in vitro} depend on the gender, in females on the day of the estrous cycle and on the time of day the animals were sacrificed?
6. Does PACAP influence the LH release of gonadotrope cells and does the secreting ability of gonadotrope cells to PACAP depend on the gender, in females on the day of the estrous cycle and on the time of day the animals were sacrificed?
7. In what concentration does PACAP influence the LH release of gonadotrope cells and how does this concentration relate to the physiological one?
MATERIALS AND METHODS

Animals

We used 3-4-month-old Sprague-Dawley (CRL:CD) rats for our experiments. The animals were kept in temperature (22±2°C) and light (light period 5-19h) controlled vivarium. We used female rats in the stage of proestrous and diestrous and male rats for our experiments. The cyclic stage of the females was determined according to the vaginal smear taken daily. The samples were stained with methyl blue and the stage was determined. Only the animals with a regular four-day cycle were used. The males were chosen randomly.

Cell Immunoblot Assay (CIBA)

Cell culture:
The anterior pituitaries were used for CIBA (Arita et al, 1993, Cimini et al, 1994, modified by Nemeskéri). The animals were decapitated, the anterior pituitaries were removed under sterile conditions and after washing them several times in minimal essential medium (GIBCO EUROPE-Cat N° 072-1800) were cut into small pieces under stereomicroscope. They were placed in a Spinner flask (Bellco-Cat N° 1967-00025) containing 10ml minimal essential medium with 0,11% trypsin (Sigma-T4799) and 0,1% bovine serum albumin (Sigma-A8551). The anterior pituitaries were incubated on 37°C in the presence of Penicillin-Streptomycin until the cells separated, but remained viable. During the incubation the anterior pituitaries were treated with 5% CO₂ -95% air and agitated 50-50 times with Pasteur pipette. The cell suspension was centrifugated on 37°C with 1000 rpm for 10 minutes. The supernatant was removed and the cells were diluted with Dulbecco’s Modified Eagle’s Medium (GIBCO BRL-Cat N° 13016-0199) so every 15µl contain 7000 cells. We placed 5x5mm supported nitrocellulose membrane (BIO-RAD Laboratories-Cat N° 162-0091) on stainless metal grid in single-use sterile Petri dishes (Greiner Labortechnik-35/10mm). 650µl of Dulbecco’s Modified Eagle’s Medium was put in each Petri dish and 15µl of cell suspension was placed on each membrane. The cells were cultured for 22 hours on 37°C in the presence of 5% CO₂ -95% air mixture. The hormones and peptides released by the cells were bound by the membranes during the incubation period and were made visible by immunostaining.

Immunocytochemistry:

After culturing the membranes were fixed in 4% paraformaldehyde solution (pH 7,4), washed in Tris-buffer containing 1% Tween and in 10%
bovine serum albumin. We then added LH (1:10000) and PACAP (1:6000) antibodies. LH antibody was made in guinea pig (NIDDK-NHPP, University of Maryland, Baltimore). PACAP antiserum was made in rabbit in the laboratory of Arimura characterized by Köves et al, 1990, 1991. The next day the membranes were washed in 0,1M phosphate buffer, then treated with biotinylated antibody against guinea pig and rabbit antibodies (Vectastain ABC kit, Vector). The following day the membranes were again washed in 0,1M phosphate buffer and incubated with avidin-biotin-peroxidase complex. The binding of the antibody was visualized by diaminobenzidine tetrahydrochloride (DAB) and H$_2$O$_2$ or fluorescent staining. In case of double labeling PACAP was made visible by ABC technique and LH by indirect immunofluorescent staining using fluorescent isothiocyanate conjugate, secondary antibody. In this case the concentration of LH antiserum was 1:500.

The preabsorption of PACAP and LH antisera with the related antigens abolished the immunostaining indicating that our antisera are specific.

**Sandwhich Enzyme Immunoassay (S-EIA)**

Fifteen anterior pituitaries of male rats sacrificed at 10 a.m. were removed by decapitation under sterile conditions. The anterior pituitaries were measured and frozen on dry ice. They were stored on −70°C. Before S-EIA they were thawed, pooled by three and homogenized (Ultrasonic Homogenizer, Chicago) in trifluoroacetic acid. The homogenized pituitaries were centrifugated on 12000 rpm for 20 minutes on 4°C. The supernatant was dried by speedvac, was washed and dried again to remove the trifluoroacetic acid. The PACAP content was measured in four paralells. The PACAP concentration of the PACAP treated gonadotroph cell culture medium was measured after incubation. The samples and the standard peptide (PACAP38) were incubated in a microtest ELISA plate coated with PA-6N monoclonal antibody (antibody against the N-terminal end of PACAP38) for 96 hours on 4°C. Following incubation the Wells of the plate were washed by 0,02M phosphate buffer (pH7) containing 1% bovine serum albumin. The plate was treated with PA-2C monoclonal antibody (antibody against the C-terminal of PACAP38) marked with horseradish peroxidase for 24 hours on 4°C, then again washed in phosphate buffer. The horseradish peroxidase activation was measured by the TMB microwell peroxidase system (Kirkegaard and Perry Laboratory, USA). The antibodies were made and characterized by Suzuki and his coworkers (1993). This assay is only suitable to measure PACAP38.
Image analysis

The results were analysed by image analysis program. 10-10 viewfields were photographed on each membrane. The diameter and area of the blots were measured by Image ProPlus Program and the blots were counted. The data of each experimental group were statistically analysed using the the unpaired t-test of the Graphpad Prisma Program.

Experimental protocol

The experimental animals were sacrificed at 10 a.m. and at 4 and 20 p.m., at the beginning and the end of the LH surge. We used male and female rats for the experiment. The females being in proestrous or diestrous stage of the estrous cycle. A part of the membranes were stained for PACAP, the other part for LH and the third part were double stained for both PACAP and LH.

RESULTS

1. Similarly to LH blots PACAP blots were present in the anterior pituitary cultures of all groups.

   The secreted PACAP and LH are seen as round blots around the secreting cells bound to the nitrocellulose membrane. The difference in hormone secretion of the individual cells appears in the different diameter of the blots. In most blots the cells are not visible in the middle, but in some cases the cells can be seen. The contour of the non-secreting cells is sharp. In cultures the cells often loose their round or ovoid shape.

   The diameter of PACAP blots are smaller than of LH blots in case of similar culture periods, that is the cells secreted less PACAP in the same culture period (22 hrs) than LH.

2. The cells secreting PACAP are partially gonadotrope cells.

   There is partial colocalization between PACAP and LH immunoreactivity seen with double labeling. The difference in the size of the two types of blots can be well seen. During the 22 hour culture period there are many PACAP cells do not secrete the hormone synthetized, while the LH cells do.
3. The physiological concentration of PACAP present in the anterior pituitary can be measured in nMol. We made five pools of 3-3 anterior pituitaries of fifteen male rats. This was necessary because the amount of PACAP in each anterior pituitary is so little that it falls under the sensitivity of the S-EIA method. With this method we measured the amount of PACAP in the anterior pituitaries; it falls between $10^{-9}$M and $10^{-10}$M.

4. The number and the size of PACAP blots in each experimental group were different. We summed the number of blots on 6 nitrocellulose membranes of 2-2 animals.

In female rats of the proestrous stage the number of PACAP blots was higher than in diestrous, where only a few blots were seen. The number of blots was highest in the cultures of the proestrous animals sacrificed at 8 p.m. (440). In this estrous cycle the number of blots in the cultures of the animals sacrificed at 10 a.m. and at 4 p.m. were higher (37 and 73 respectively) than in those of any of the cultures of the diestrous cycle and there were a number of non-secreting only synthetizing cells. We have seen a number of PACAP positive but non-secreting cells in the cultures of the proestrous rats sacrificed at 8 p.m.

In male rats the number of PACAP blots was very scarce. In the cultures of the animals sacrificed at 10 a.m. and at 4 p.m. the number of blots was six and five and of the animals sacrificed at 8 p.m. it was eleven. Non-secreting cells can rarely be seen.

It seems like the number of blots in each experimental group was highest in animals sacrificed at 8 p.m. than in the other two groups of animals sacrificed at 10 a.m. or at 4 p.m.

There was no difference in the diameter and area of the blots of proestrous females sacrificed at 10 a.m. and 8 p.m., however at 4 p.m. both parameters were significantly lower. In diestrous females the mean number of blots was 1, 1 and 3. There was no significant difference in the diameter and area of the blots. This can be the result of the extremely low number of blots.

In males the 11 blots seen in the animals sacrificed at 8 p.m. were apparently large compared to blots at 10 a.m. and 4 p.m. and compared to blots seen in proestrous females at any time.

5. We have seen a great number of LH blots in the anterior pituitary cultures of animals sacrificed at different times; however, their number and size varied.
There was LH secretion in every culture, but the amount of LH secreted was different in each group. The number of LH blots was much higher than the number of PACAP blots in all experimental animals except on the night of proestrus.

In proestrous stage the number of secreting and blot-forming cells was high at 10 a.m. and 4 p.m., but it significantly decreased by night following the LH surge. In diestrous in the morning we found a high number of blots similarly to the blot number in the morning cultures of proestrous rats; however, the size of the blots was significantly smaller. At 4 p.m. there were fewer blots, but somewhat larger, still not being as big as the ones in proestrus at 4 p.m. The diameter and area of the blots in diestrous stage were obviously the largest at 8 p.m., being significantly greater than the ones in proestrus at 8 p.m.

In males the number of hormone secreting cells was higher at 10 a.m. and 8 p.m. than at 4 p.m. The diameter and area of LH blots formed by the cells were similar at 10 a.m. and 8 p.m. without significant difference in size, however at 4 p.m. the blots were dotlike and significantly smaller than the ones in the morning and the evening.

6. The effect of PACAP on LH secretion varied according to the time of sacrifice.

Treating the anterior pituitary cultures with 10^{-10}M concentration of PACAP did not influence LH secretion, therefore this treatment was not used again.

In females 10^{-7}M concentration of PACAP given to the medium influenced the LH secretion of the cells in a different way at each time of sacrifice in both proestrus and diestrous rats. At 10 a.m. the number of blots in the PACAP treated cultures was lower than in the treated cultures; however, at 4 p.m. and at 8 p.m. in the treated cultures the number of blots was higher than in the control ones. In females of the proestrous stage at 10 a.m. the diameter and the area of the blots showed an opposite change, they were significantly greater than the untreated ones. At 4 p.m. and 8 p.m. the amount of hormone secreted by the individual cells was not different due to PACAP treatment. In diestrous rats the diameter and area of the LH blots significantly increased in the morning to PACAP treatment and significantly decreased in the afternoon and the evening.

In males PACAP treatment did not alter the number of LH blots; however, PACAP significantly decreased the hormone secretion of individual cells in cultures of 10 a.m. and 8 p.m., while at 4 p.m. it did not have an effect.
7. PACAP given to the culture medium influenced the LH secretion in a concentration similar to the physiological level.

At the end of the culturing period the PACAP present in the medium was regained and measured by S-EIA. In the Petri dish containing the culture medium to which $10^{-7}$M concentration of PACAP was added only $10^{-10}$M could be measured. This amount is similar to the amount measurable in anterior pituitaries, so it can be considered physiological. PACAP added to the Petri dish containing culture medium in $10^{-10}$M concentration could not be retrieved in a measurable amount at the end of the culturing period, probably that is the reason why it had not influenced the LH secretion of the cells.

CONCLUSION

The data in the literature and our experimental results support the theory that PACAP plays a role in the regulation of the hypothalamo-hypophyseal-gonad system (Köves et al, 2003, Fahrenkrug et al, 1996). There are data about PACAP being transiently expressed in the granulosa cells of the preovulatory follicles in rat ovarium and not in any other stages of the estrous cycle (Lee et al, 1999, Ko et al, 1999). We have found that the synthesis of PACAP shows a great variability also in the anterior pituitary, and it has a level during proestrous stage that is enough for immunostaining in pituitary slices (Köves et al, 1998). Our results correlate with the work of Wuttke and his coworkers (1994) and Radleff-Schlimme and his coworkers (1998) in which they found that the amount of PACAP mRNA in the hypophysis is raised by estrogen in proestrus (having a high enough concentration for immunostaining) and not in the other stages of the estrous cycle. The CIBA method used in our experimental work is able to detect the hormone release of a few cells. The amount of hormone secreted by an individual cell is very small; however, with this technique even this amount can be detected.

In anterior pituitary cultures the number and the size of PACAP blots were significantly smaller than LH blots. This suggests that PACAP is secreted in a lesser amount than LH, which is emptied into the general circulation from the anterior pituitary having an effect on other organs. On the basis of this we suppose that PACAP synthesized locally in the pituitary gland affects the response of LH cells to GnRH by autocrine and paracrine manners. Radleff-Schlimme and his coworkers (1998) have supposed the autocrine role of PACAP earlier. Experimental animals were treated repeatedly intravenously with PACAP. This increased PACAP gene
expression in the anterior pituitary six times. They supposed that PACAP acted via PAC1 receptor, specific to PACAP, on the cell membrane because VIP did not alter PACAP gene expression.

When the number and area of the blots are multiplied together, we get a picture of the dynamics of PACAP and LH secretion.

The secretion of PACAP shows a typical daily rhythm. In every experimental group the most PACAP secreted is found in the evening. At this time it is dark in the animalhouse. This well correlates with the previous work of our laboratory where strong immunoreactive PACAP cells were found in the anterior pituitary three weeks after enucleation in female rats being in the stage of the estrous cycle when in intact rats no PACAP immunoreactivity was found. If an animal in the proestrous stage is sacrificed at 8 p.m., following the LH surge, the anterior pituitary culture shows a twelve-thirteen time greater PACAP secretion, while in cultures of diestrous females and males sacrificed at 8 p.m. PACAP secreting cells can rarely be seen. This allows us to conclude that the increase of PACAP secretion is a result of changes in the hormonal milieu and not of the short period of time without light. This is the time, 8 p.m., by when the LH surge has finished and the LH secretion has decreased to its minimum after a time.

The dynamics of LH secretion clearly shows that the gonadotroph cells of the anterior pituitary on the day of proestrus received different information in vivo than on the day of diestrus or the gonadotroph cells of male animals. In the diestrous stage of females and in males the pattern of LH secretion is similar, while in proestrus it is totally different. The LH secretion of the gonadotroph cells taken from proestrous females at 8 p.m. after the LH surge has significantly decreased showing that the cells have already emptied the hormone in vivo, while in the morning and the afternoon before the LH surge the cells had a significant hormone reserve.

It is a well known fact that the endocrine organs function rhythmically, they show circannular, circadian and circoral rhythms. The pineal gland from a bird in vitro in a superfusion system has kept the circadian rhythm of its melatonin secretion (Csernus et Mess, 2003). It is an interesting fact that the 24 hour period cannot be abolished in vitro, but a phase shift can be produced if the light-dark periods are shifted. The daily rhythm is also influenced by the wavelength of light. The cell cultures in our experiments were done on 37°C for 22 hours in a dark environment. The values gotten were therefore influenced the same way by the outer environment. The differences between the values observed according to our hypothesis are due to the different information of the cells imprinted into them and brought to their in vivo environment.
PACAP is able to influence the LH secretion of gonadotroph cells directly without other hypothalamic factors. The effect of PACAP, according to our experimental results, depends on the gender, on the time of day the animals are sacrificed and in females on the stage of the estrous cycle at the time of the experiment. The responsiveness of gonadotroph cells to PACAP was also different depending on the time of sacrifice. In the anterior pituitary cell cultures of proestrous females it was inhibiting in the morning, it decreased the total blot area, then it stimulated the LH release compared to the untreated cultures. In diestrous females and in males there was no valuable difference between the PACAP treated and untreated cell cultures. This is due to the very low number of blots, that is the released amount of hormone in these two experimental groups.

Two years after the discovery of PACAP, Culler and Paschall (1991) found that it potentiates the effect of GnRH on LH release in gonadotroph cell line. Tsuji and his coworkers (1994) got similar answers in perfusion system. A french research group (Lariviere et al, 2005) found in the past year that there is "cross-talk" between PACAP and GnRH inside an LH cell. GnRH affects LH release through the IP3/PKC pathway, while PACAP through the cAMP/PKA pathway. When the two peptides are given together there is "cross-talk" inside the cell between the two "second messenger" mechanisms. The stimulating or inhibiting effect of PACAP on LH release depends on the amount of GnRH and PACAP compared to each other.

According to our studies it is most likely that in vivo on the night of proestrus the increasing amount of PACAP in the anterior pituitary together with the decreasing amount of GnRH inhibits LH release. Supposedly it plays a role in terminating the LH surge in an auto- or paracrine manner. Our in vitro studies do not give an answer to how hypothalamic PACAP plays a role in this process. According to the earlier results of our laboratory (Köves et al, 1996, Kántor et al, 2000, Kántor et al, 2001), it is possible that the effect of hypothalamic PACAP also depends on the stage of the estrous cycle. PACAP given intracerebroventricularly on the day of proestrus before the critical period inhibits the LH surge in 70-75%, thus inhibiting ovulation that would have occurred the next day. PACAP given intravenously does not have an effect on the LH surge. This means that this effect is mediated by hypothalamic factors. The observations of Moore and his coworkers (2005) support our hypothesis. They found that the amount of PACAP in the hypothalamic paraventricular nucleus and the anterior pituitary changes with the ovarian cycle. With the dark-light period used in our laboratory the LH surge occurs between 4 and 8 p.m. on the day of proestrus. This is preceded by the release of GnRH into the portal circulation. The research group found
that the amount of PACAP mRNA in the PV is highest at noon, then it significantly decreases before the surge. This was verified by the quantitative analysis of in situ hybridization. The increase of the amount of PACAP mRNA is characteristic of the proestrous stage of the cycle and of the median subdivision of the PV. The expression of PACAP in the anterior pituitary also changes with the cycle and shows characteristic changes. PACAP mRNA was determined and the amount of mRNA was amplified by RT-PCR. The results suggest that the low amount of PACAP mRNA on the day of meta- and diestrus further decrease during the LH surge then on the night of proestrus it increases and on the morning of estrus it is again very low. Directly preceding this the increase of PACAP mRNA is followed by the increase of follistatin mRNA, which results in the selective suppression of FSH.

PACAP in cell culture is able to influence LH secretion in a concentration close to the physiological one. We reached a physiological concentration in the culture when $10^{-7}$M concentration of P38 was given to the medium. At the end of the culture period we measured $10^{-10}$M concentration by S-EIA. The reason for this is that a major part of PACAP might bind to the wall of the Petri dish thus significantly decreasing the concentration of PACAP in the medium. When PACAP concentration was $10^{-10}$M at the beginning of the culture period, at the end PACAP was not measurable by the available method, it fell under the physiological level and probably this gave reason for why it did not influence LH secretion.

The question may arise concerning our work why we chose a 22 hour culture period. The studies of the research group at Pécs reveal that the anterior pituitary cells treated with releasing hormones react in seconds and secrete the classical hormones (Csernus et al, 1994). We primarily studied the release of PACAP and the LH release parallel. Our technique is basically different from the superfusion system. The amount of PACAP secreted by an individual cell is very little, the hormone secreted by the cells cannot be summed. The nitrocellulose membrane binds the hormone around the cell by which it was released; the hormone was stained by immunostaining. The greater amount of hormone released for a longer period of time is easier to detect by immunostaining. It would have been enough to culture the cells for a shorter time to stain LH, but the staining was carried out in parallel cultures, thus we needed the same culture period.

According to our experimental results we conclude:
1. CIBA is appropriate for detecting PACAP and LH release of a few cells individually.
2. PACAP is released from anterior pituitary cells in vitro without the influence of hypothalamic factors.
3. The amount of PACAP released is less than the amount of LH. This lets us to conclude that the hypophyseal PACAP possibly effects LH secretion in an auto- or paracrine manner and does not effect endocrine function through the general circulation.

4. PACAP release of the anterior pituitary cells depends on the gender, in females on the stage of the estrous cycle and on the time of sacrifice. The highest amount of PACAP release was detected in proestrous females in the evening at 8 p.m. \textit{in vivo}. This means that the amount of locally secreted PACAP depends on the hormonal milieu at the time of the removal of the pituitaries from the animals.

5. The effect of PACAP on LH secretion differs depending on the experimental conditions. PACAP was inhibitory in cultures of anterior pituitaries taken from proestrous females in the morning. It decreased the total blot area, but was stimulatory at the time of the proestrous surge. In diestrous females and in males the summed area of LH blots did not differ significantly in treated and untreated cultures.

6. According to our studies the anterior pituitary cells in cultures keep the information brought by them before the removal of the organ without the presence of hypothalamic factors. This information "imprinted" influences the responsiveness of the cells to PACAP.
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