抑えた．また，この培養液が示した抑制効果は，培養液にフォリスタチン抗体を添加しておくと中和され，アクチビン作用が復元した．以上の結果から，PACAPは濾胞星状細胞の性腺刺激ホルモン産生細胞に対するパラクリン作用を調節できることが示唆された．PACAPは下垂体の内分泌細胞に対する直接的な作用に加え，下垂体細胞間の相互作用の制御因子としての役割を有していると推察された．

一年以上の間，室温22度，14時間明：10時間暗の照明条件下で飼育されたシマリスを短日照明(10時間明：14時間暗)と低温条件に暴露することにより季節外冬眠を誘起した．我々は一年のどの時期でもこの季節外冬眠を誘起できた．この季節外冬眠は季節間において，冬眠—覚醒インターバルや，それぞれの冬眠や覚醒時間には有意な差を認めなかったが，冬眠に入るまでの期間の長さにおいて夏のみ約60日を要し，他の季節の平均30日より長かった．さらに，覚醒インターバルでの覚醒時刻は冬では明期に起こるのに対し，春では明期と暗期でほぼ等しい割合で起こった．これらの結果はシマリスの冬眠がサーカディアンリズム(概日リズム)とサーカニュアルリズム(概年リズム)の両者にリンクしていることを示唆している．夏の季節外冬眠において，セロトニン枯渇剤であるパラクロロフェニルアラニン(PCPA)の冬眠中での慢性投与は冬眠を阻止し，非冬眠動物への投与は逆に冬眠を誘発した．一方，オピオイドのアンタゴニストであるナロキソンの投与は覚醒時間の延長を起こした．これらの結果は，セロトニンによる冬眠誘起や維持機構がサーカニュアル(概年リズム)システムと独立したものであることを示唆している．

公衆衛生学：

生後4カ月の男児に下痢が認められ糞便から *Salmonella* Virchowが分離された．有効薬剤を投与したにもかかわらず1カ月以上に渡り同菌が分離された．乳児宅で飼育されていた室内犬3頭のうち，2頭から *S.* Virchowが分離された．乳児を入院させ治療したところサルモネラは陰性に転じた．乳児および犬から分離されたサルモネラの薬剤感受性および制限酵素 *Xba* Iを用いたPFGEパターンが完全に一致したことから，本事例は犬が感染源と考えられた．

外　科　学：

2歳の犬が食欲減少，腹囲膨満を主訴に来院した．開腹術により卵円形で長径31 cmの左側卵巣部腫瘤と直径11 cm・類円形の左側腹膜後腔部腫瘤を外科的に切除した．両腫瘤は，病理組織学的に管状構造をなす気管支および腸粘膜，毛包，皮脂腺，アポクリン汗腺，神経組織と，その間隙に介在する軟骨，骨，脂肪組織などからなり，奇形腫と診断された．本例は犬の腹膜後腔原発奇形腫の最初の報告例と思われる．

臨床繁殖学：

急性乳房炎を発症した泌乳期のホルスタイン種乳牛の乳房内にオゾンガスを注入し，その治療効果を評価した．オゾンガスは専用のオゾン発生装置を用いて発生させ，罹患分房の乳頭口から分房内に注入した．供試牛19頭のうち，15頭に対してオゾンガスの

FULL PAPER *Physiology*

A Role of Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) as a Regulator of Paracrine Interactions between Folliculo-Stellate Cells and Gonadotropes through the Control of Activin-Follistatin Interactions

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ABSTRACT. Pituitary folliculo-stellate (FS) cells were able to modify the effect of activin-A on gonadotropes through the paracrine factor, follistatin. The present study was aimed to examine whether a hypothalamic peptide, pituitary adenylate cyclase activating polypeptide (PACAP), could be a regulator of this paracrine interaction. Co-culture of FS cell-originated cell line TtT/GF cells with rat anterior pituitary cells showed faint inhibitory effect on the stimulatory action of activin-A on FSH secretion. When PACAP was added to the culture during the co-culture period, however, the presence of TtT/GF cells caused significant suppression of the effect of activin-A on FSH secretion. Conditioned-media (CM) from TtT/GF cells, obtained by incubation of TtT/GF cells in the presence or absence of PACAP, were next added to the cultures of anterior pituitary cells alone. CM from TtT/GF cells without PACAP treatment revealed slight, but not significant, suppressive effect on activin-induced increases in FSH secretion and the percentage of FSH cells. Meanwhile, CM from PACAP-treated TtT/GF cells attenuated both effects of activin-A. Furthermore, the inhibitory effect of the CM was neutralized when follistatin antibody was present in the culture. These results suggest that PACAP is able to regulate the paracrine action of FS cells on pituitary gonadotropes. Besides expressing direct actions on pituitary endocrine cells, PACAP may have roles as a regulator of cell-to-cell interactions within the pituitary gland.

KEY WORDS: activin, conditioned-media, gonadotrope, PACAP, TtT/GF cell.

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Gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), play important roles in the regulation of reproduction and fertility. Studies on the regulatory mechanisms of pituitary gonadotropes, the source of gonadotropins, are therefore essential to understand the physiology, pathology, as well as to develop remedies, of reproductive functions and disorders. Hypothalamic gonadotropin-releasing hormone (GnRH) and gonadal steroid hormones are classically known regulators of the gonadotrope functions, and today the inhibin, activin and follistatin have also obtained recognition as the regulators [7]. Activin not only stimulates the secretion of FSH [22, 37] but also enlarges the population of FSH gonadotropes in primary cultures of rat anterior pituitary cells [17, 18]. Our previous study demonstrated that the latter effect of activin-A (one of three forms of dimeric activin; -A, -AB and -B) was negatively controlled by pituitary folliculo-stellate (FS) cells through a paracrine factor, follistatin [16]. This and other studies concerning interactions of activin and follistatin in various tissues [3, 6, 23] have led us to the understanding that the balance of these two factors are important in the regulation of various biological functions. In the anterior pituitary gland, follistatin expression has been shown to vary under different conditions [8, 12]. Although hypothalamic GnRH has been demonstrated to regulate the expression of follistatin in the anterior pituitary gland [2, 19], there is not enough information to conclude the overall regulatory mechanism of follistatin production, and thus of activin-follistatin cooperation system, by the hypothalamus that is a superior center of the regulation of endocrine phenomena.

Besides these major factors, there are still several candidates that could be involved in the regulation of gonadotrope functions. One of them is pituitary adenylate cyclase activating polypeptide (PACAP) which is produced in the hypothalamus [26], released into the hypophysial portal blood system [9], whose receptors have been detected in the anterior pituitary gland [11, 27, 34], and stimulates FSH and LH secretion [13]. In preliminary experiments using static cultures of rat pituitary cells, we found that the stimulatory effect of PACAP on FSH secretion, but not LH secretion, was dependent on cell density. This observation led us to the assumption that unknown cell-to-cell interaction might be involved in the effect of PACAP on FSH secretion. To assess this possibility, TtT/GF cells, a pituitary FS-like cell line [15] expressing PACAP type-I-like and type II-like receptors [24], or conditioned-media (CM) from TtT/GF cells were used, in combination with PACAP and activin-A, to examine whether PACAP would affect the paracrine interactions between FS cells and FSH gonadotropes within the anterior pituitary gland.

MATERIALS AND METHODS

Anterior pituitaries from 8–15 week-old female Sprague-Dawley rats at random estrous stages were enzymatically dispersed by the collagenase-based method reported previously [16]. Dispersed cells were suspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 20 mM HEPES, 50 U/m*l* penicillin, 50 μg/m*l* streptomycin and 10% (v/v) decomplemented fetal bovine serum (FBS) (DMEM-FBS) at the density of $2 \times$ or 4×10^5 cells/m*l*. A half m*l* of the cell suspension was placed in each well of 48-well multiplates and

were precultured for two days under a saturated atmosphere of 5% CO_2-95% air at 37°C. After intensive washes with DMEM, cultures were initiated in 0.5 m*l* of DMEM or DMEM-FBS with experimental treatments as below.

TtT/GF cells were cultured in DMEM/F-12 (1:1) supplemented with 2.5% FBS and 10% horse serum (HS) (DMEM/F-12-S). After a few subcultures, TtT/GF cells were suspended in DMEM-FBS at the density of 1×10^4 cells/m*l*. A half m*l* of DMEM-FBS or TtT/GF cell suspension was added to the primary pituitary cell cultures and were incubated for additional 48 hr to allow TtT/GF cells to regain activity. Then, cultures were washed two times with DMEM and were further cultured in 0.5 m*l* of DMEM-FBS in the presence or absence of activin-A (1 *n*g/m*l*) and PACAP (1, 10, 100 *n*M) for 48 hr.

CM from TtT/GF cells were yielded by incubation of TtT/GF cells in DMEM/F-12-S with or without PACAP (1, 10, 100 *n*M) for 24 hr. Anterior pituitary cells were cultured in the presence or absence of activin-A (1 *n*g/m*l*), CM (1/5 volume) and follistatin antibody (1/50 volume) for 48 hr.

For RIAs, NIDDK-rFSH-I-8 and NIDDK-rLH-I-9 were iodinated by a modification of the chloramine-T method [14], and NIDDK-anti-rFSH-S-11 and NIDDK-anti-rLH-S-11 were used as primary antibodies. Bound and free hormones were separated with anti-rabbit precipitating antibody (sheep anti-rabbit immunoglobulins immobilized on silica beads). FSH and LH determinations are expressed in terms of NIDDK-rFSH-RP-2 and NIDDK-rLH-RP-3, respectively.

For immunocytochemistry, cells were fixed with Bouin's solution (without acetic acid) for 10 min at room temperature (23 ± 2°C). The cells were immunostained as previously reported [16] using the streptavidin-biotin-peroxidase method (a kit from ZYMED Laboratories, South San Francisco, CA, U.S.A.). The incubation with the primary antibody, anti-rat FSH (1:1,500), was performed for 1 hr at room temperature. The specificity of anti-rat FSH was examined by pre-incubation of the antibody with excess amount (12.5 μg/m*l*) of rat FSH (NIDDK-r-FSH-I-8) or rat LH (NIDDK-r-LH-I-9). The percentage of immunostained cells was determined by counting more than 300 pituitary cells for a slide while noting the number of stained cells under a light microscope.

Data are expressed as the mean ± SEM of three experiments, except those in Fig. 1 that are the mean ± SEM of triplicate determinations from one of two similar experiments. Differences were statistically evaluated by ANOVA, and Student-Newman-Keuls test was used as a post-hoc test. $P<0.05$ was considered significant.

Human recombinant activin-A was from Dr. Y. Eto, Central Research Laboratory of Ajinomoto Co., Kawasaki, Japan. Anti-rat follistatin (lot #FP22) and the RIA reagents were provided by NIDDK's National Hormone & Pituitary Program and Dr. A. F. Parlow. DMEM, DMEM/F-12 (1:1), HEPES buffer solution, trypsin, FBS and HS were purchased from GIBCO BRL (Grand Island, NY, U.S.A.); PACAP-38, A23187 and penicillin-streptomycin solution were from Sigma (St. Louis, MO, U.S.A.); rat FSH antibody (rabbit) for immunocytochemistry and anti-rabbit precipitating antibody were from Biogenesis (Poole, England); TtT/GF cells were from RIKEN CELL BANK (Wako, Japan); and other general reagents were from Nacalai Tesque (Kyoto, Japan).

RESULTS

As preliminary experiments, anterior pituitary cells were cultured in 48-well multiplates at the density of $1 \times$ or 2×10^5 cells/well, and were incubated in DMEM in the presence or absence of 10 *n*M PACAP for 4 hr. LH secretion was significantly augmented by PACAP at either cell density (Fig. 1B). In contrast, FSH secretion was significantly enhanced by PACAP at the density of 1×10^5 cells/well, but not at 2×10^5 cells/well (Fig. 1A). Since treatment of cells with 100 μM A23187, a Ca^{2+} ionophore, for 4 hr yielded FSH secretion of approximately 4.4 *n*g/100 μl medium (not shown in Fig. 1), the ineffectiveness of PACAP on FSH secretion at 2×10^5 cells/well is not due to the exhaustion of releasable FSH or saturation of FSH in the medium.

DMEM-FBS alone or TtT/GF cell suspension (5×10^3 cells/well in DMEM-FBS) was added to the primary pituitary cell cultures (1×10^5 cells/well in DMEM-FBS). In the absence of TtT/GF cells, activin-A-stimulated increases in FSH secretion were not significantly affected by simultaneous addition of PACAP (Fig. 2A). In the presence of TtT/GF cells, however, simultaneous addition of PACAP revealed tendency to attenuate the stimulatory effect of activin-A on FSH secretion; 1 or 100 *n*M PACAP significantly attenuated the effect of activin-A, although the effect of 10 nM PACAP was not statistically significant (Fig. 2B).

Then, CM from TtT/GF cells were added to the primary pituitary cell cultures. CM from TtT/GF cells that had been treated without PACAP weakened the stimulatory effect of activin-A on FSH secretion, compared with that in cultures

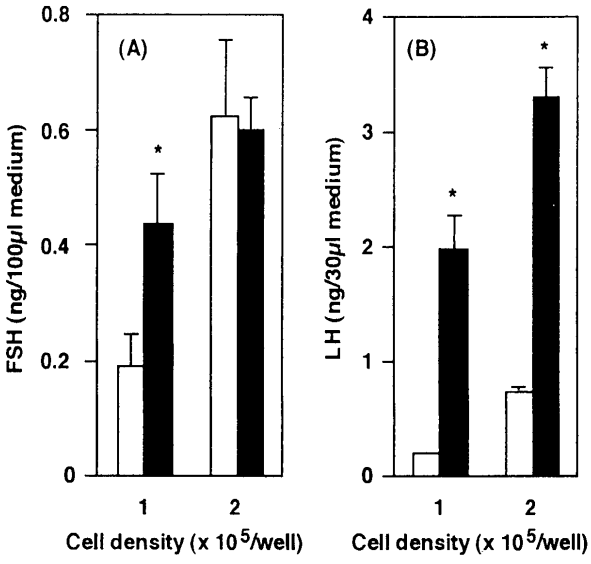

Fig. 1. Cell density-dependency of PACAP action on gonadotropin secretion. Anterior pituitary cells were cultured at the density of $1 \times$ or 2×10^5 cells/well, and were incubated in the presence (filled columns) or absence (open columns) of 10 *n*M PACAP for 4 hr. *, $P<0.05$ vs. corresponding open columns

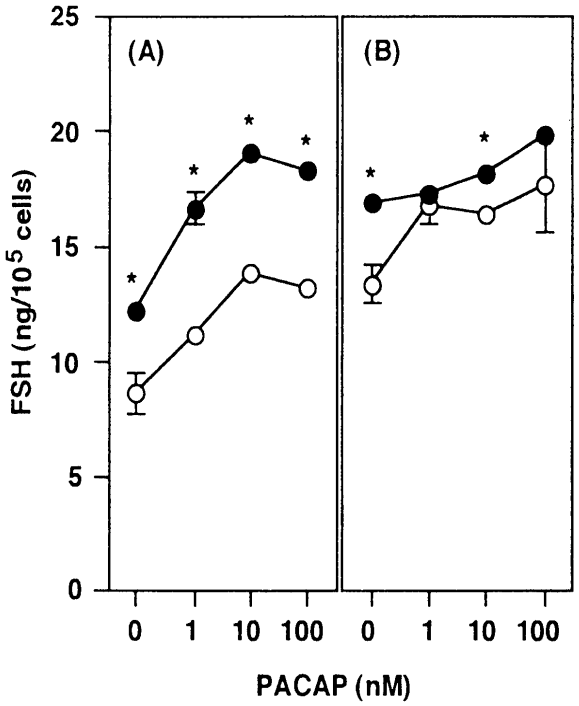

Fig. 2. Activin-A-induced increases in FSH secretion are suppressed by PACAP in co-cultures with TtT/GF cells. Medium alone (A) or TtT/GF cells (B) were added to the primary pituitary cell cultures. Then, the cells were cultured with (filled circles) or without (open circles) 1 *n*g/m*l* activin-A in the presence or absence of PACAP (1, 10, 100 *n*M) for 48 hr. *, P<0.05 vs. corresponding open circles.

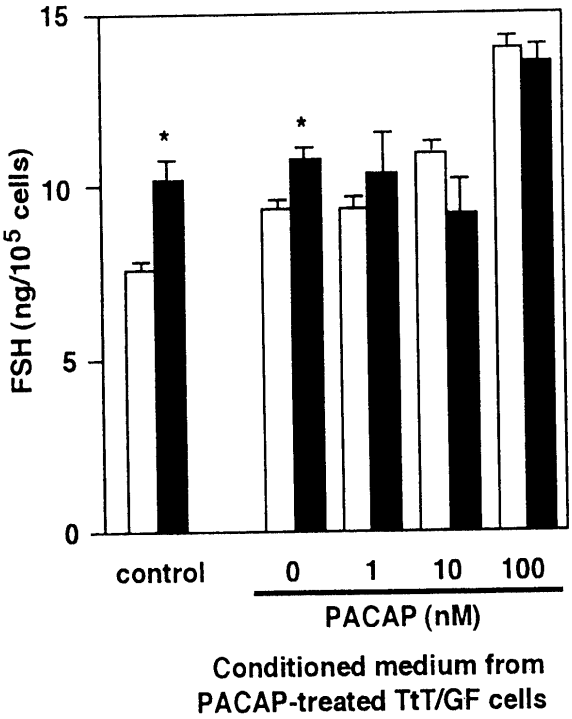

Fig. 3. Conditioned-media (CM) from PACAP-treated TtT/GF cells attenuate the stimulatory effect of activin-A on FSH secretion. CM were yielded by incubation of TtT/GF cells in the presence or absence of PACAP (1, 10, 100 *n*M) for 24 hr. Anterior pituitary cells were cultured with or without (control) CM in the presence (filled columns) or absence (open columns) of 1 *n*g/m*l* activin-A for 48 hr. *, P<0.05 vs. corresponding open columns.

without CM (Fig. 3; control), but the effect of activin-A remained significant (Fig. 3; 0 *n*M PACAP). When CM had been collected from TtT/GF cells under the stimulation of PACAP, addition of CM attenuated the stimulatory effect of activin-A on FSH secretion (Fig. 3; 1, 10 and 100 *n*M PACAP). Similarly, activin-A-induced increases in the percentage of immunoreactive FSH cells tended to be suppressed, although not significantly, by CM without PACAP treatment (Fig. 4; 0 nM PACAP), and blocked by CM that had been yielded under PACAP treatment (Fig. 4; 100 *n*M PACAP). The immunoreaction of the antibody was abolished by pre-incubation with rat FSH, whereas it was not significantly affected by pre-incubation with rat LH (data not shown), indicating that the FSH antibody showed little cross-reaction with LH and, therefore, virtually detected FSH cells specifically. Furthermore, the inhibitory effect of CM from PACAP-treated TtT/GF cells was neutralized by simultaneous addition of follistatin antibody (Fig. 4; dotted column).

DISCUSSION

Studies using static culture systems of rat pituitary cells have presented controversial results with respect to the effect of PACAP on gonadotropin secretion; some demonstrated weak but significant stimulation [13, 29] and others ineffectiveness [26]. In contrast, those using perfusion systems have demonstrated consistent stimulatory effects of PACAP on gonadotropin secretion [26, 36]. Since perfusion systems generally exclude the influence of internally produced substances and reflect the net action of externally added materials, it was possible that PACAP might affect the secretion of unknown substances from pituitary cells that had an ability to interfere with the hormone secreting activity of gonadotropes. This view is supported by the results in the preliminary experiments, demonstrating that the stimulatory effect of PACAP on FSH secretion, but not LH secretion, was attenuated in high cell density. These results suggest that, besides direct stimulation of gonadotropin secretion, PACAP also induces the release of some material(s) that specifically suppresses FSH secretion via a paracrine action.

There is information that leads us to speculate that PACAP is involved in paracrine interactions between FS cells and hormone-producing cells in the anterior pituitary gland. For instance, PACAP stimulates the release of interleukin-6 (IL-6) from FS cells [24, 35], and IL-6 itself has been shown to stimulate GH, PRL, LH and FSH secretion from rat pituitary cells [10]. The paracrine factor involved in the preliminary experiments, however, does not appear to be IL-6 since the effect was inhibitory, not stimulatory, and was selective on FSH secretion.

Regulation of the secretion of FSH, but not LH, is thought

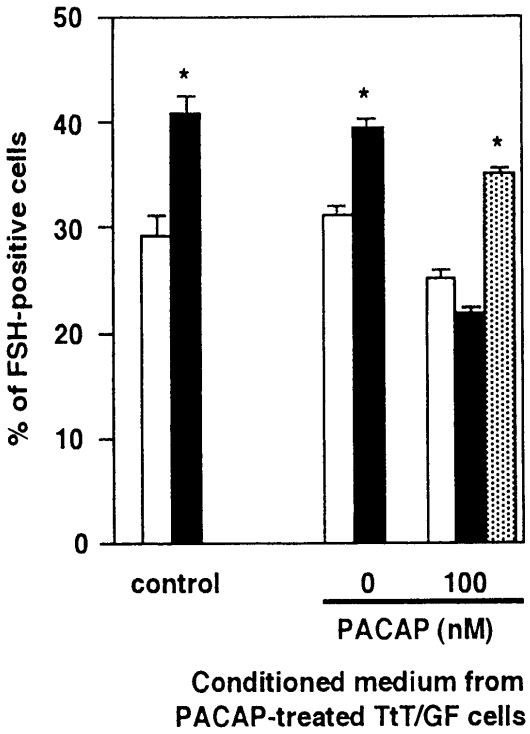

Fig. 4. Conditioned-media from PACAP-treated TtT/GF cells attenuate activin-A-induced increases in the percentage of FSH cells. Anterior pituitary cells were cultured with or without (control) CM in the presence (filled columns) or absence (open columns) of 1 *ng/ml* activin-A for 48 hr. Follistatin antibody was simultaneously added to some cultures (CM plus activin-A plus follistatin antibody; dotted column). *, $P<0.05$ vs. corresponding open columns.

to be under the control of locally produced activin and follistatin in the pituitary gland [4, 20, 28]. Evidence of a paracrine interaction between FS cells and gonadotropes was presented in our previous study, showing that follistatin in the CM of FS cells was able to attenuate a stimulatory effect of activin-A on FSH gonadotropes [16]. Considering that the physiological regulators of FS cells remain uncertain, it was possible to assume that PACAP produced in the hypothalamus, a superior center of the regulation of reproductive functions, could be a regulator of the paracrine interactions between FS cells and gonadotropes in the anterior pituitary gland. To assess this hypothesis, PACAP-stimulated TtT/GF cells or CM from PACAP-treated TtT/GF cells were added to primary cultures of rat anterior pituitary cells, and it was examined whether they would affect the effects of activin-A on FSH secretion and on the percentage of immunoreactive FSH cells. Results in the present study demonstrated that co-culture of TtT/GF cells with pituitary cells, as well as addition of TtT/GF CM, revealed tendency to suppress the stimulatory effects of activin-A, which confirms our previous results [16]. In addition, treatment with PACAP significantly strengthened the inhibitory effects of TtT/GF cells, both in co-culture and CM addition experiments, on both effects of activin-A. Furthermore, follistatin was suggested to be the paracrine factor whose secretion from TtT/GF cells was enhanced by PACAP, which is in good agreement with the results by Winters *et al.* [40] that showed PACAP stimulation of follistatin gene expression in anterior pituitary cells. These results indicate that the paracrine interaction of FS cells and FSH gonadotropes through activin-follistatin cooperation is, at least in part, regulated by PACAP.

Although PACAP has been reported to stimulate proliferation of TtT/GF cells [24], we did not investigate this effect and its influence, if any, in the co-culture experiments. FS cells appear to have capacity to form gap junctions in the anterior pituitary gland [33], which should increase intercellular communications. Therefore, it is not reasonable to rule out the possibility that increased physical connection between TtT/GF cells and gonadotropes in PACAP-treated co-cultures underlay the paracrine action of PACAP-treated TtT/GF cells. However, this possibility appears minimal because of the results from experiments using CM and by analogy that physical connection between FS cells and growth hormone/prolactin-secreting cells does not mediate the functional communications between these cell types [1]. On the other hand, the effect of PACAP on normal gonadotrope cell growth is not clear, although PACAP stimulates proliferation of αT3–1 cells [32], a transformed cell line that express α-subunit, but not β-subunit, genes of gonadotropins [39]. Further studies are necessary to determine whether a direct effect of PACAP on gonadotrope proliferation is involved or not and such an effect, if any, underlies the suppressive effect of PACAP on activin-A action.

Another unsolved question is why addition of TtT/GF cells or TtT/GF CM without PACAP treatment caused increases in baseline FSH secretion. Since TtT/GF cells also produce several physiological materials possessing ability to affect the function of gonadotropes, including IL-6 as mentioned above, such materials might be involved in this phenomenon.

Besides direct effects on the secretion and synthesis of gonadotropins, PACAP has been demonstrated to modulate cellular responses to various factors. For example, PACAP appears to modulate pulsatile GnRH release from the hypothalamus, which in turn affects the secretion and synthesis of pituitary gonadotropins [31, 38]. Cross-talk between the actions of PACAP and GnRH at different intracellular levels in gonadotropes is also suggested [5, 25, 32]. Furthermore, recent studies have demonstrated the localization of PACAP in gonadotropes and thus suggest its autocrine roles in the regulation of this cell type [21, 30]. Including the effect demonstrated in the present study, it remains uncertain which of these effects of PACAP predominates, or whether all of them are equally significant, in the regulation of gonadotrope functions and thus of reproduction and fertility.

In conclusion, the present study suggests a physiological role of PACAP as a regulator of paracrine interactions between FS cells and gonadotropes. PACAP appears to regulate the paracrine interactions through the control of follistatin production and/or release from FS cells.

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