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Minireview

PACAP, an Autocrine/Paracrine Regulator of Gonadotrophs[1]

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ABSTRACT

Hypothalamic-hypophysiotropic peptides are the proximate regulators of pituitary cells, but they cannot fully account for the complex functioning of these cells. Accordingly, awareness is growing that an array of peptides produced in the pituitary exert paracrine/autocrine functions. One such peptide, pituitary adenylate cyclase-activating polypeptide (PACAP), was originally identified as a hypothalamic activator of cAMP production in pituitary cells. Gonadotrophs and folliculostellate cells are the main source of pituitary PACAP, and each pituitary cell type expresses a PACAP receptor. PACAP increases alpha-subunit (*Cga*) and *Lhb* mRNAs, and it stimulates the transcription of follistatin (*Fst*) that, in turn, restrains activin signaling to repress *Fshb* and gonadotropin-releasing hormone-receptor (*Gnrhr*) expression as well as other activin-responsive genes. The PACAP (*Adcyap1*) promoter is activated by cAMP, and pituitary cells may communicate by a feed-forward, cAMP-dependent mechanism to maintain a high level of PACAP in the fetal pituitary. At birth, pituitary PACAP declines and pituitary follistatin levels decrease, which together with increased gonadotropin-releasing hormone secretion allow *Gnrhr* and *Fshb* to increase and facilitate activation of the newborn gonads. Changes in *Adcyap1* expression levels in the adult pituitary may contribute to the selective rise in follicle-stimulating hormone (FSH) from age 20–30 days to the midcycle surge and to the secondary increase in FSH that occurs before estrus. These results provide further support for the notion that PACAP is a key player in reproduction through its actions as a pituitary autocrine/paracrine hormone.

follicle-stimulating hormone (FSH/FSH receptor), follistatin, luteinizing hormone (LH/LH receptor), pituitary/pituitary hormones, pituitary adenylate cyclase-activating polypeptide

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) was isolated in 1989 from sheep hypothalamic extracts based on its action to stimulate cAMP production by rat pituitary cell cultures [1]. PACAP is the most highly conserved member of the VIP (vasoactive intestinal peptide)-secretin-glucagon peptide superfamily, with expression in tunicates, fish, amphibians, rodents, and higher mammals, including humans; a related protein, amnesiac, is found in drosophila [2]. This high degree of conservation across species suggests essential functions. Two isoforms of PACAP have been found: a 38-amino-acid form and a C-terminally truncated, 27-amino-acid form, with PACAP-38 accounting for 90% of the protein in most tissues.

Three distinct receptors are activated by PACAP: the $VPAC_1$ and $VPAC_2$ receptors (official symbols VIPR1 and VIPR2, respectively), which have relatively similar affinity for VIP and PACAP, and the specific PAC_1 receptor (PAC_1-R; official symbol ADCYAP1R1) [3]. Multiple splice variants of the *Adecyap1r1* (Fig. 1) result from the alternative splicing of two exons in the third intracellular loop (hip and hop) and were named null (neither hip nor hop), hip, hop1, hop2, hiphop1, and hiphop2 [4]. *Adcyap1r1* variants that differ from the null receptor in the amino-terminal extracellular domain have also been identified [5]. VIPR1, VIPR2, and all ADCYAP1R1 variants bind PACAP-38 and PACAP-27 with high affinity and, like other members of the group B, G protein-coupled receptor family of proteins, couple with GNAS to stimulate adenylate cyclase (although in certain systems, PACAP-38 is more potent than PACAP-27). In some cell types, PACAP also stimulates inositol phosphate (IP) production and increases intracellular calcium concentrations. Evidence suggests that the hip cassette reduces the calcium response [6] and that the hop cassette elevates intracellular calcium [7]. Thus, the variable expression of the ADCYAP1R1 variants would be expected to influence PACAP signaling and produce different transcriptomes. The interested reader is referred to a recent comprehensive review [3].

Consequent to the extensive distribution of PACAP and its receptors, PACAP exerts an array of functions on the nervous, immune, gastrointestinal, cardiac, and endocrine systems [8]. PACAP affects the exocrine and endocrine pancreas, hepatocytes, osteoblasts, adrenal medulla and cortex, testis and ovary, thyroid, pineal, neurohypophysis and pars tuberalis, as well as the anterior pituitary.

EVIDENCE PACAP REGULATES GONADOTROPHS

At least one of the PACAP receptors is present in each of the anterior pituitary endocrine cell types and in pituitary folliculostellate (FS) cells [9, 10]. The null and hop ADCYAP1R1 forms predominate in the rat pituitary [11] and are expressed in mouse αT3–1 gonadotroph cells [12]. PACAP stimulates cAMP production in these cells and increases

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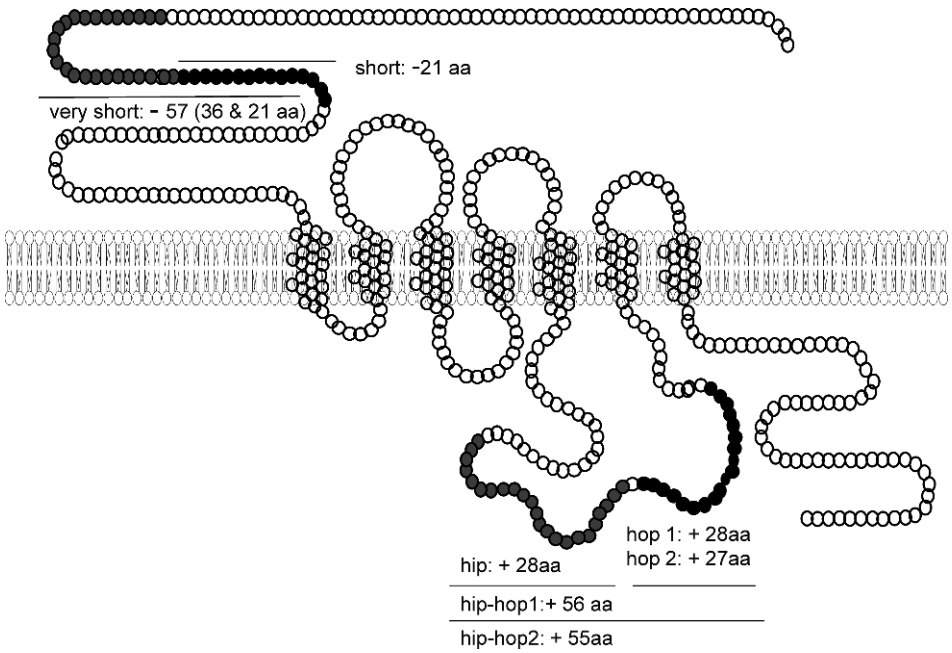

FIG. 1. Splice variants of the *Acyap1r1* as compared to *Adcyap1r1* null. For each cassette, the number of amino acids (aa) that are inserted (+) or deleted (−) is illustrated.

intracellular calcium concentrations independent of cAMP activation by stimulating IP turnover through phospholipase C [13]. Stimulation of cAMP occurs at a lower concentration (median effective concentration [EC_{50}] ~ 3 nM) than the rise in IP production (EC_{50} ~ 30 nM) [14], and PACAP-38 and PACAP-27 produce equipotent effects. Although the level of expression of ADCYAP1R1 is much lower in LβT-2 gonadotrophs than in αT3–1 cells [15], PACAP increases cAMP signaling in this more mature gonadotroph as well [16]. Information on signaling in normal gonadotrophs is limited; however, PACAP has been shown to increase intracellular calcium concentrations in rat gonadotrophs that were identified by reverse hemolytic plaque assay [17]. PACAP and gonadotropin-releasing hormone (GnRH) signaling interact in that PACAP-stimulated cAMP production is inhibited by GnRH through protein kinase C (PKC) in both αT3–1 [18] and LβT2 cells [19].

Whereas PACAP has variable and species-specific effects on the secretion of adrenocorticotropin hormone, growth hormone, prolactin, and thyroid-stimulating hormone, evidence is growing that PACAP is an important regulator of gonadotropin secretion and subunit gene expression [20]. PACAP stimulates the release of luteinizing hormone (LH) and uncombined glycoprotein α-subunit from primary pituitary cell cultures [21]. The effect of PACAP on LH secretion is modest compared to that of GnRH, however, and PACAP-stimulated LH secretion desensitizes rapidly [22]. PACAP effects are abolished by the protein kinase A inhibitor H-89, by the PKC inhibitor bisindolylmaleimide, and by decreasing the extracellular calcium concentration with ethylene glycol tetraacetic acid [23]. In addition to stimulating LH secretion directly, PACAP augments the response to GnRH stimulation by shifting the GnRH-LH secretion dose-response curve to the left [24], although peak LH secretion appears to be unaffected [25]. When pituitary cells are perifused with pulses of GnRH, a more physiological model of the hypothalamic-pituitary unit, continuous treatment with PACAP markedly enhances GnRH-stimulated LH secretion [25].

In addition, PACAP affects the expression of each gonadotropin subunit gene. PACAP increases *Cga* mRNA levels by stimulating transcription primarily through the protein kinase A (PKA)-cAMP signaling pathway [26]. PACAP lengthens *Lhb* mRNA transcripts in primary pituitary cultures [25] and activates the *Lhb* promoter in LβT2 cells, partly by increasing EGR1 [27]. PACAP stimulates transcription of the *Gnrhr* in LβT2 cells through CREB and NR5A1 (SF-1) [28], and *Gnrhr* mRNA in αT3–1 cells is increased by PKA activation. In other cell types, PACAP increases transcription of fos and c-jun. The ADCYAP1R1 influences cell growth and development through the extracellular signal-related kinase (ERK)-mitogen-activated protein kinase (MAPK) pathway in a variety of tissues [29, 30], and whereas PACAP stimulates ERK in αT3–1 gonadotrophs [31], to our knowledge no studies of PACAP effects on gonadotroph development or proliferation have been published. In contrast to its stimulation of the *Cga*, *Lhb*, and *Gnrhr* genes, PACAP reduces *Fshb* mRNA levels in primary rat pituitary cells [25], although not in LβT-2 pituitary cells [32, 33]. These observations imply that PACAP may enhance responsiveness to GnRH and regulate the differential secretion of LH and follicle-stimulating hormone (FSH). The reported effects of PACAP on gonadotrophs are summarized in Table 1.

FOLLISTATIN PLAYS A PIVOTAL ROLE IN THE PITUITARY ACTIONS OF PACAP

An elaborate mechanism selectively regulates *Fshb* gene expression and, thereby, FSH production, independently of

TABLE 1. Effects of PACAP on gonadotrophs.

Effect	Reference
Stimulates α-subunit and LH secretion	[21, 24]
Increases responsiveness to GnRH	[24, 25]
Lengthens *Lhb* mRNA transcripts	[25]
Stimulates *Cga* transcription	[26]
Stimulates *Lhb* transcription	[27]
Stimulates *Fst* transcription	[36]
Decreases *Fshb* mRNA expression	[25]
Stimulates *Gnrhr* transcription	[28]
Increases PAC1-receptor (*Adcyap1r1*) mRNA levels	[33]
Stimulates nitric oxide synthase	[23]

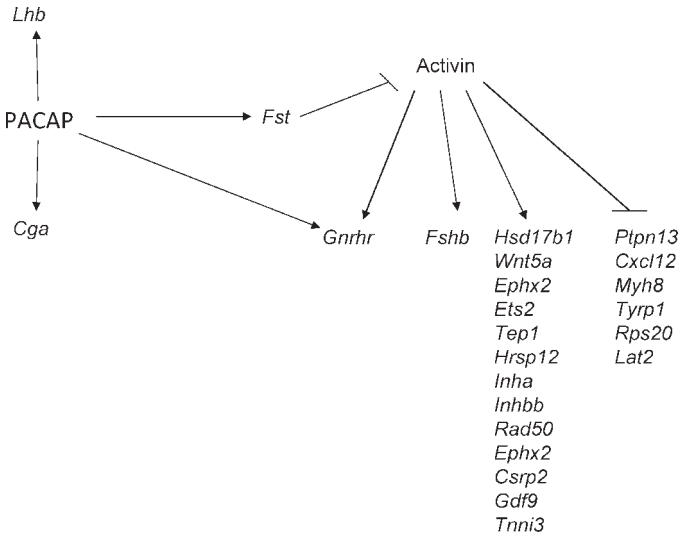

FIG. 2. A schematic diagram to illustrate the direct effects of PACAP on gonadotropins and effects that may be mediated through regulation of follistatin. Expression of other activin-regulated genes is from studies with LβT2 gonadotroph cells by Mazhawidza et al. [85] and Zhang et al. [86].

LH, that involves activin and follistatin from the pituitary as well as inhibin from the gonads [34]. In this schema, follistatin binds activin with high affinity to form a biologically inactive complex that restrains activin signaling. Follistatin is highly regulated, and with its rapid half-life [35], the effect of follistatin can begin and terminate abruptly. PACAP increases follistatin (*Fst*) mRNA levels in primary pituitary cell cultures and activates the *Fst* promoter by stimulating cAMP-PKA signaling [16, 36]. Quantitative in situ hybridization coupled to immunostaining revealed that *Fst* expression in both gonadotrophs and FS cells is increased by PACAP [32]. Two alternatively spliced mRNAs are produced from the *Fst* gene, with follistatin-288 having no exon 6 sequence and the greater activin-neutralizing activity [37], and PACAP may have its greatest effect on the production of follistatin-288. Thus, PACAP induction of *Fst* expression is likely to explain, at least in part, its suppression of *Fshb* mRNA and may regulate many of the downstream targets of activin signaling. A diagram of the proposed role of follistatin in PACAP control of gonadotropin subunit gene expression is shown in Figure 2.

Nitric oxide synthase type 1 (NOS1; neuronal NOS) is expressed in gonadotrophs and FS cells, and PACAP increases NOS in pituitary cells and potentiates the effect of GnRH to increase nitric oxide-dependent cGMP production [23, 38]. PACAP and NO/GMP enhance the action of GnRH to stimulate LH release in some systems. NOS1 has been linked to the control of gene expression by activin, as has GnRH that is administered continuously [39], and NO increases the expression of *Fst* in myoblasts [40]. Thus, NO might play a role in the actions of PACAP and in the regulation of follistatin and FSH by PACAP.

PACAP IN VIVO

Less is known about the role of PACAP in vivo. PACAP and its receptors are widely expressed in rodents, and they are found in high concentration throughout the central nervous system, with the highest concentration in the hypothalamus [41, 42]. PACAP increases LH secretion when administered in vivo to rats [43], although not to ovariectomized ewes [44], and is thought to be a hypophysiotropic hormone because its concentration in rat hypothalamic portal blood exceeds that of peripheral blood [45]. PACAP protein levels in most regions of the rat brain increase from low levels at birth to peak levels by age 30–60 days and are maintained throughout adulthood [46]. *Adcyap1* mRNA expression within the paraventricular nucleus (PVN) declines in the male rat between 20 and 30 days of age and then increases, with reciprocal changes in *Fshb* and *Gnrhr* mRNA levels [47]. Levels of *Adcyap1* mRNA in the PVN vary during the rat estrous cycle, increasing 3 h before the proestrous LH/FSH surge and then declining [48]. Because the PVN projects axons to the median eminence, the increase in PVN PACAP may play a role in the LH surge, in the increase in anterior pituitary cAMP that occurs at the time of the surge [49], and in the developmental changes in *Fshb* and *Gnrhr* that occur in the rat between 20 and 30 days of age. Evidence also indicates that PACAP enhances progesterone-mediated female sexual behavior [50, 51].

In addition, PACAP may regulate GnRH secretion. PACAP injected s.c. on Day 1 of life delayed vaginal opening and reduced GnRH immunoreactivity in the preoptic region of 9- and 30-day-old rats [52]. On the other hand, PACAP-specific receptors are also expressed in GT1–7 GnRH neuronal cells, but PACAP stimulates, rather than inhibits, cAMP production and GnRH secretion by these cells [53].

Several groups have developed PACAP-deficient mice, but unfortunately, most of these pups die at birth or by the second week of life with wasting, ketosis, and dyslipidemia [54]. Survival improves if the environmental temperature is maintained at 25–28°C [55]. PACAP-deficient females are subfertile [56], and males are testosterone-deficient because of gonadotropin insufficiency [57]. Because reproduction is sensitive to environmental stress and nutritional deficiency, additional models of PACAP deficiency are needed before definitive conclusions about the effects of PACAP in these models are possible.

PITUITARY PRODUCTION OF PACAP

Although initially viewed as a hypothalamic-hypophysiotropic neuropeptide, evidence is increasing that PACAP is produced in the pituitary and has a paracrine/autocrine mechanism of action. Early immunoassays detected PACAP in adult rat pituitary, although at much lower levels than in hypothalamus, as well as in human pituitary tissue [41, 42]. Koves et al. [58] identified immunoreactive PACAP in rat gonadotrophs at proestrus, and Jin et al. [59] used laser-capture microdissection to demonstrate *Adcyap1* mRNA in FS cells. PACAP secretion by pituitary cell cultures rises in late proestrus [60], and a transient increase in pituitary *Adcyap1* expression occurs in female rats during the overnight hours between proestrus and estrus when FSH levels are elevated [48]. Accordingly, pituitary PACAP could contribute to the termination of the secondary FSH surge during the early hours of estrus. Pituitary PACAP levels are lower in males [61]; however, a significant decline in pituitary *Adcyap1* mRNA in male rats between 17 and 21 days of age coincides with a pronounced rise in *Fshb* compared to *Lhb* mRNA [47].

PITUITARY PACAP IN THE FETUS

Moore et al. [62] reported recently that *Adcyap1* mRNA and PACAP protein levels are high in the embryonic (Embryonic Day 19) rat pituitary, then decline strikingly and abruptly at birth (Fig. 3A). Photomicrographs in previous studies have suggested the expression of *Adcyap1* mRNA within the embryonic rat pituitary [63], and *Adcyap1* mRNA is expressed at higher levels in the fetal human pituitary (gestational age, 16–20 wk) than in the adult human pituitary [64].

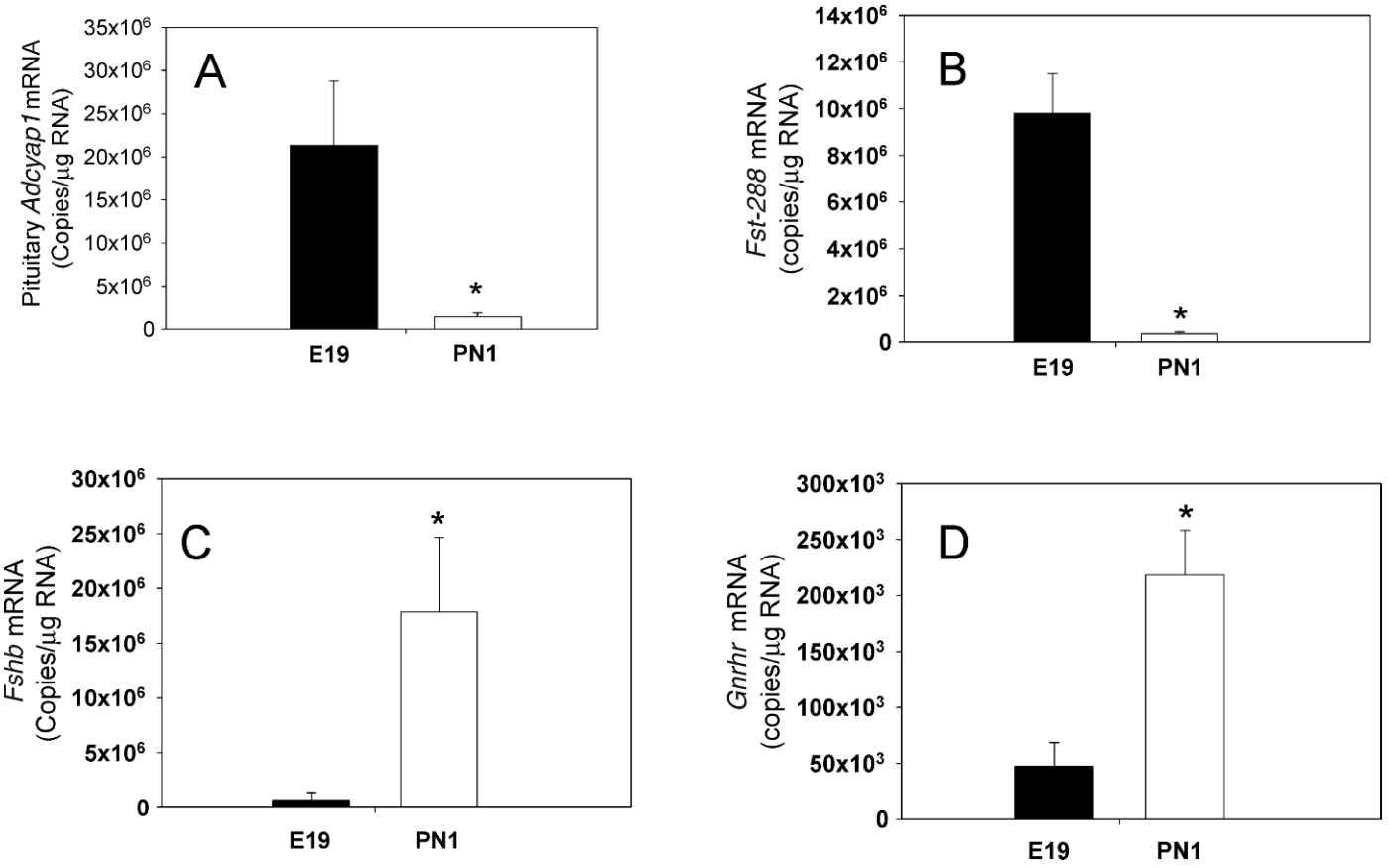

FIG. 3. Levels of *Adcyap1* (**A**), *Fst*-288 (**B**), *Fshb* (**C**), and *Gnrhr* (**D**) mRNAs in the male rat pituitary on Embryonic Day 19 (E19) and Postnatal Day 1 (PN1). Results were determined by quantitative RT-PCR and represent the mean ± SEM (n = 6–8 animals/group). Pituitary *Adcyap1* mRNA levels declined 94% from E19 to PN1 and were accompanied by a comparable decrease in *Fst*-288 mRNA. The fall in follistatin is thought to release activin signaling and allow expression of *Fshb* and *Gnrhr* as well as other genes to increase. *$P < 0.05$ vs. E19. (Adapted from Moore et al. [62], with permission from The Endocrine Society, Copyright 2009.)

The level of pituitary *Fst* mRNA falls sharply (Fig. 3B) at birth, in parallel with the decrease in PACAP; this is in keeping with the idea that PACAP is a major regulator of *Fst* expression. Interestingly, the magnitude of the decline in follistatin-288 exceeds the change in total pituitary *Fst*, suggesting that PACAP influences the splicing of this mRNA. Moreover, the fall in pituitary *Fst*-288 at birth is accompanied by a substantial increase in *Fshb* (Fig. 3C) and *Gnrhr* (Fig. 3D) mRNA levels, a change that could be explained by increased activin signaling because follistatin has decreased. These and previous results suggest that the high level of PACAP in the embryonic anterior pituitary facilitates the early appearance of gonadotropin *Cga* and delays the ontogeny of *Fshb* relative to *Lhb* by stimulating *Fst* transcription.

REGULATION OF PACAP (*Adcyap1*) GENE EXPRESSION

The *Adcyap1* promoter contains sequences that are homologous to the CRE and is activated by both PACAP and forskolin [65, 66]. Furthermore, treatment of rats with PACAP-38 increased pituitary *Adcyap1* mRNA levels in vivo [67], and PACAP increased ADCYAP1R1 expression in LβT2 gonadotroph cells [33]. Together, these experiments support the idea of a feed-forward mechanism through which PACAP increases cAMP production, which in turn increases pituitary *Adcyap1* expression.

Gonadotropin-releasing hormone stimulates *Adcyap1* expression via the PKA, PKC, and MAPK pathways in LβT2 cells through CREB, JUNB, and FOS [68] and increases the mRNA for *Adcyap1r1* [33]. Thus, GnRH may play a role in PACAP activation. Receptors for dopamine are found in αT3–1 [69] and LβT2 gonadotroph cells [70], and when dopamine activates Gαi-coupled D2 receptors, adenylate cyclase 5 activity is blocked and cAMP levels decline [71]. Thus, an increase in dopamine signaling at birth [72] might suppress cAMP and, thereby, *Adcyap1* mRNA levels. *Adcyap1* mRNA in PC12 rat pheochromocytoma cells is increased by dexamethasone [73]. In addition, estrogens stimulate PACAP in the ventromedial nucleus and arcuate nucleus [50], whereas progesterone increases *Adcyap1* and *Adacyap1r1* mRNAs in the rat hypothalamus [74]. Thus, these steroid hormones may also regulate pituitary PACAP. Because gonadotrophs and FS cells are the major sources of pituitary PACAP [75, 76] and both cell types express PACAP receptors [10], it is interesting to propose that these cells communicate [77] to maintain the high level of PACAP in the fetal pituitary, as diagrammed in Figure 4. Experiments are underway to understand the factors that maintain PACAP in the fetal pituitary and to unravel the mechanism for the dramatic decline that occurs in *Adacyap1* expression near the time of birth.

CONCLUSIONS

It has been more than 15 years since PACAP was hypothesized to be a key player in reproduction [78]. PACAP may be a hypophysotropic regulator with a role in the midcycle

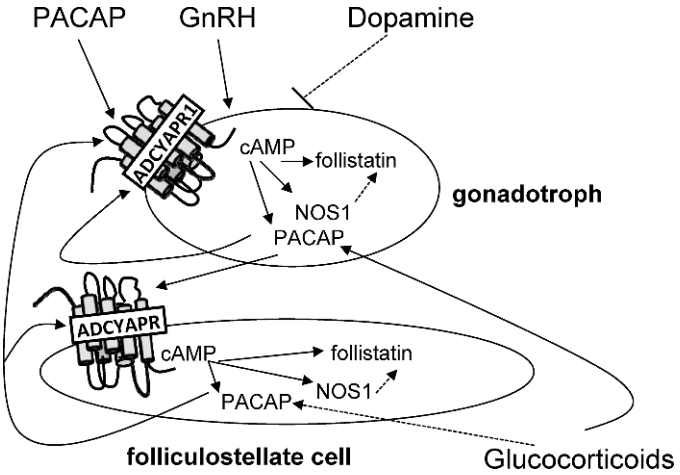

FIG. 4. A proposed mechanism for the up-regulation of pituitary *Adcyap1* expression through cAMP signaling in gonadotrophs and folliculostellate cells. PACAP stimulates ADCYAP1R1 to increase cAMP production, which induces *Adcyap1* and *Adcyap1r1* as well as *Nos1* and *Fst* gene expression. PACAP secreted by gonadotrophs may activate folliculostellate cells, which likewise secrete PACAP that stimulates gonadotrophs. Dopamine, GnRH, central nervous system PACAP, and glucocorticoids may influence *Adcyap1* expression. nNOS, neuronal NOS.

surge, and PACAP in the pituitary may control the differential expression of the gonadotropin subunit genes in the fetus and contribute to this control mechanism in adults. The decline in pituitary PACAP at birth may initiate a series of steps that activate gonadal function in the neonate and help explain the finding that mice deficient in PACAP [79, 80] or in ADACYAP1R1 [81] expression have reduced fertility.

It is important to emphasize that the relevance of these findings to reproduction in humans is not yet known. However, as in rodents, the level of FSH in human preterm infants is less than the level of LH [82], and PACAP increases *Fst* mRNA levels in primate FS cell-enriched pituitary cultures [83]. Thus, PACAP might regulate gonadotropin gene expression in the human fetal pituitary and might play a role in the differential secretion of LH and FSH that occurs in normal adolescence, in hypogonadotropic hypogonadism including hyperprolactinemia, during fasting, in women with polycystic ovary syndrome, and in some pituitary tumors in which functional, high-affinity PACAP-binding sites have been found [84]. Further research is needed to determine the role of PACAP in reproduction and to understand whether dysfunction of this peptide is important in disorders of the human pituitary.

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