Hypothalamic gonadotropin-releasing hormone is known to be critical for normal gonadotropin biosynthesis and secretion by the gonadotrope cells of the anterior pituitary gland. Additional regulation is provided by gonadal steroid feedback as well as by intrapituitary factors, such as activin and follistatin. Less well-appreciated is the role of pituitary adenylate-cyclase activating polypeptide (PACAP) as both a hypothalamic–pituitary releasing factor as well as an autocrine–paracrine factor within the pituitary. PACAP regulates gonadotropin expression alone and through modulation of GnRH responsiveness achieved by increases in GnRH receptor expression and interactions at the level of intracellular signaling pathways. In addition to direct effects on the gonadotrope, PACAP stimulates follistatin secretion by the folliculostellate cells and thereby contributes to differential expression of the gonadotropin subunits. Conversely, GnRH augments the ability of PACAP to regulate gonadotrope function by increasing pituitary PACAP and PACAP receptor expression. This review will summarize the current understanding of the mechanisms by which PACAP modulates gonadotrope function, with a focus on interactions with GnRH.
1. Introduction

Sexual maturation and normal adult reproductive function require precise temporal and quantitative regulation of gonadotropin gene expression as achieved through the complex interaction of multiple hormones arising from the hypothalamus, gonads, and anterior pituitary gland itself. The hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH) has been extensively studied for its effects on gonadotrope function. GnRH is released as discrete pulses into the pituitary portal vasculature, binds to specific G-protein coupled receptors on the gonadotrope cell surface, and thereby stimulates gonadotropin biosynthesis and secretion into the peripheral circulation. The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), act within the gonads to promote gamete maturation as well as the production of gonadal steroids (androgens, estrogens, and progesterone) and the peptide inhibin. These hormonal factors exert multiple functions including feedback at the hypothalamus and pituitary gland to modulate gonadotropin gene expression. Gonadotrope function is also known to be modulated further through the locally-derived activin-follistatin system.

Over 20 years ago, Arimura and colleagues isolated a novel neuropeptide from sheep hypothalamic extracts found to increase intracellular cAMP levels in anterior pituitary cells. Based on this activity, they named this peptide pituitary adenylate cyclase-activating polypeptide (PACAP or AdCYP1) (Koves et al., 1990; Miyata et al., 1989). In addition to independent effects on gonadotrope function, PACAP has been demonstrated to modulate GnRH effects on gonadotropin gene expression via alterations in hypothalamic GnRH release as well as through substantial cross-talk within the pituitary at the level of receptor expression, intracellular signaling, and transcriptional regulation. This review will summarize the current understanding of the mechanisms by which PACAP regulates gonadotropin biosynthesis and secretion, with a focus on interactions with GnRH.

2. PACAP and PACAP receptors

2.1. PACAP in the hypothalamic–pituitary axis

Based on sequence identity, PACAP is a member of the secretin/glucagon/vasoactive intestinal peptide/growth hormone releasing hormone polypeptide family, with PACAP and vasoactive intestinal peptide (VIP) showing the greatest degree of amino acid sequence homology within this family. Although closely related, PACAP is approximately 1000 times more potent than VIP in stimulating cyclic AMP production in rat pituitary cells. PACAP exists as either a 38-amino acid (PACAP38) or 27-amino acid (PACAP27) form, each derived from the same precursor protein encoded by the Adcyap1 gene. As a general rule, PACAP27 represents only a minor portion of total PACAP immunoreactivity in most tissues.

PACAP expression has been localized to the cell bodies in the hypothalamic paraventricular, supraoptic, and arcuate nuclei with dense fiber networks in the median eminence and pituitary stalk which abut the hypophysal portal capillaries (Hamnibal et al., 1995; Kimura et al., 1994; Piggins et al., 1996). Thus, PACAP neurons are ideally located to impact GnRH neuronal function and to act as a hypothalamic–pituitary releasing factor. Consistent with this function, the hypothalamic-portal blood concentration of PACAP in rats has been found to exceed the peripheral blood concentration by 2- to 4-fold (Dow et al., 1994). PACAP is believed to be secreted as pulses; however, the details of its secretion have not been well-characterized.

In addition to hypothalamic expression, PACAP has been shown to have widespread tissue expression including the male and female anterior pituitary gland, ovary, testes, and placenta (Arimura et al., 1991; Heinzlmann et al., 2008; Nemeth et al., 2006; Vaudry et al., 2009). While expression has been best characterized in the rat, where studied, data have suggested similar expression patterns in the mouse and in humans (Barberi et al., 2007; Daniel and Habener, 2000; Moore et al., 2012; Morelli et al., 2008; Nakamura et al., 2013; Scaldaferrri et al., 2000). Within the anterior pituitary gland, PACAP is expressed by both the gonadotrope and folliculosecreting cells, but not by the other hormone-producing cell types (Grafer et al., 2009; Koves et al., 1998a; Purwana et al., 2010; Radleff-Schlimme et al., 1998). Based on sandwich enzyme immunoassay, Szabo have calculated that adult male rat pituitary cell extracts contain between $10^{-10}$ and $10^{-9}$ M PACAP (Szabo et al., 2002a). Thus, PACAP regulates gonadotrope function both as a classical hypothalamic-releasing factor and as an autocrine–paracrine factor. The relative importance of these two sources of PACAP is currently poorly understood.

The number of PACAP-releasing cells and the amount of PACAP released per cell varies markedly depending on gender, time of day, and stage of the estrous cycle. Using an immunoblot technique, it has been reported that only a small number of pituitary cells secrete PACAP in male rats, although a doubling in number and a 6-fold increase in area of the blots is observed at 2000 h relative to earlier times in the day. In females animals, only a few PACAP-secreting cells are present on diestrus; however, this number increases substantially on proestrus with a peak in both number and area of the cell blots at 2000 h, suggesting that PACAP is an important modulator of gonadotropin expression during the peri-ovulatory period (Koves et al., 2003; Szabo et al., 2004). A similar late proestrus peak in PACAP mRNA levels has been reported by Moore and colleagues (Moore et al., 2005).

2.2. Gonadotrope cell lines

The study of gonadotrope physiology has been facilitated by the development of two immortalized mouse gonadotrope-like cell lines, αT3-1 and LjT2. LjT2 cells exhibit many functional characteristics consistent with those of mature, differentiated gonadotropes, including the expression of all three gonadotropin subunits and the GnRHR. In contrast, αT3-1 cells are considered to be less differentiated as they lack LHβ- and FSHβ-subunit expression (Turgeon et al., 1996c). PACAP and PACAP receptor mRNA and protein expression have been detected in both cell lines. Interestingly, PACAP mRNA is expressed constitutively in the relatively undifferentiated αT3-1 cells whereas PACAP mRNA is difficult to detect in LjT2 cells unless treated with an exogenous stimulus such as GnRH (Grafer et al., 2009; Purwana et al., 2010; Radleff-Schlimme et al., 1998). This observation is consistent with a report that pituitary PACAP levels decrease across development (Moore et al., 2009).

3. PACAP and GnRH intracellular signaling

3.1. PACAP receptor expression and function

PACAP binds to G-protein coupled receptors which have been categorized into three groups based primarily on their affinity for PACAP versus VIP. The PAC1 receptor (PAC1R) (Lozach et al., 1998) is relatively PACAP-specific, binding PACAP38 and PACAP27 with similar affinity but requiring 1000-fold greater concentrations in order to interact with VIP. In contrast, VPAC1 and VPAC2 receptors have approximately equal affinity for PACAP-38, PACAP-27, and VIP. Alternative splicing of the PAC1 receptor appears to have implications for both activation of intracellular signaling pathways as well as relative affinity for PACAP38 versus PACAP27, with most
tissues containing various proportions of each of the subtypes (Dickson and Finlayson, 2009; Vaudry et al., 2000).

The interaction of PACAP and its receptors generates a potent dose-dependent increase in intracellular cAMP levels and protein kinase A (PKA) activity in primary gonadotropes and gonadotrope cell lines. Although less pronounced than the response to GnRH, PACAP can also increase intracellular calcium concentration through activation of the phospholipase C/inositol phosphate (IP) pathway independent of cAMP levels (Canny et al., 1992; Rawlings et al., 1994; Schomerus et al., 1994; Tsuji et al., 1994). PACAP has also been shown to stimulate the mitogen-activated protein kinase (MAPK/3/1)extracellular signal-related kinase (ERK) pathway in gonadotrope cell lines (Fowkes et al., 2001; Harada et al., 2007). As demonstrated in sT3-1 gonadotropes, an increase in cAMP occurs at a lower concentration of PACAP than the rise in IP production (EC50 approximately 3 nM versus 30 nM) (Schomerus et al., 1994).

All three receptor subtypes have been detected in primary pituitary gonadotropes and gonadotrope cell lines with the PAC1R expressed at highest (Bresson-Bepoldin et al., 1998; Rawlings and Hezareh, 1996; Rawlings et al., 1995; Spengler et al., 1993). It is not known whether all gonadotropes express PACAP receptors. In one study, only 20% of gonadotropes were shown to bind biotinylated PACAP38 (Vigh et al., 1993). In contrast, functional studies have reported a PACAP-mediated increase in intracellular calcium levels in as many as 90% of gonadotropes, suggesting the presence of receptors in the majority of cells (Billiard, 1996; Canny et al., 1992; Perrin et al., 1993). While pituitary GnRH receptor expression is restricted to gonadotropes in the anterior pituitary, PACAP receptors are expressed by all five hormone-secreting cell types (i.e., gonadotropes, lactotropes, corticotropes, somatotropes, and thyrotropes) as well as by the supportive folliculostellate cells (Vigh et al., 1993). Although data is limited, PACAP has been shown to stimulate biosynthesis and secretion of growth hormone, prolactin, and adrenocorticotropic from dispersed pituitary cells (Vaudry et al., 2000). PACAP may therefore provide a link between the reproductive axis and other neuroendocrine systems.

3.2. GnRHR signaling cascades

GnRH, like PACAP, exerts its effects via binding to specific G-protein coupled receptors on gonadotrope cell membrane (Naor and Huhtaniemi, 2013). The GnRHR is coupled to the Gαq/11 member of the G-protein family which stimulates phosphoinositide-C and generation of diacylglycerols (DAGs), resulting in protein kinase C activation and inositol phospholipid (IP3) turnover (Kaiser et al., 1997a; Knollman and Conn, 2008; Melamed et al., 2012; Stojićkovic and Catt, 1995). Activated PKC stimulates the MAPK and ERK1/2 pathways and increases intracellular calcium level by promoting calcium mobilization from intracellular stores and calcium influx across the cell membrane. GnRH also increases the cAMP/PKA pathway although this response is less robust and delayed relative to the PACAP response. Lariviere et al. have reported that PACAP38 treatment increases intracellular cAMP by over 120-fold relative to basal levels within 30 min while GnRH treatment reaches a maximal 2.7-fold effect at 4 h in the LjT2 gonadotrope-derived cell line (Lariviere et al., 2006). Activation of these pathways ultimately results in the stimulation of gonadotropin biosynthesis and secretion.

In short, GnRH preferentially stimulates gonadotropin biosynthesis and secretion through the PKC/MAPK/calcium cascade and PACAP preferentially activates the cAMP/PKA pathway. Nevertheless, both peptides exert complex actions on an array of overlapping signaling molecules providing for multiple sites of functional convergence on second messenger, protein kinase, and transcription factor activity. As will be discussed in Section 3.3, simultaneous exposure to GnRH and PACAP has been observed to variously generate cooperative or competitive effects on second messenger pathways and gonadotropin biosynthesis and secretion.

3.3. PACAP and GnRH interactions

GnRH and PACAP interact in an inhibitory manner at the level of intracellular cAMP levels. Despite the fact that both factors increase cAMP levels individually, GnRH blunts PACAP-induced cAMP accumulation by up to 70% as shown in both the sT3-1 and LjT2 gonadotrope-derived cell lines (Lariviere et al., 2006, 2008; Mc Ardle and Counis, 1996). The GnRH effect is calcium-independent and has been attributed to activation of a novel PKC isoform. In order to localize the GnRH effect within the PACAP/cAMP pathway, GnRH has been tested for its ability to alter cAMP accumulation in response to either forskolin, which acts directly on the adenylyl cyclase catalytic subunits, or to cholera toxin which activates adenylyl cyclase by ADP ribosylation of Gαs-subunits. GnRH fails to increase cAMP levels induced by either pharmacologic agent, suggesting that the effect might be even further “upstream” at the level of the PAC1R. In support of this mechanism, GnRH has been shown to phosphorylate the PAC1R via the PKC signaling pathway pathway (Lariviere et al., 2008).

In contrast, GnRH and PACAP cooperate to increase inositol phosphate (IP) production by sT3-1 gonadotropes. GnRH-stimulated IP production was increased synergistically by PACAP at low concentrations of GnRH (10−11 M) but was additive at higher concentrations of GnRH (Schomerus et al., 1994). This observation raises the possibility that PACAP may act to augment the GnRH response at times of low GnRH exposure.

The nitric oxide pathway pathway provides an additional example of interaction between GnRH and PACAP at the level of intracellular signaling. GnRH and PACAP increase nitric oxide synthase type I (NOS I; neuronal NOS) protein levels in rat gonadotropes via the cAMP sytem with a resultant increase in cyclic guanosine monophosphate (cGMP) (Garrel et al., 1998, 2010; Lozach et al., 1998). PACAP is twice as potent as GnRH in increasing NOS I levels in cultured rat anterior pituitary cells and stimulates cGMP levels through a rapid, as yet undetermined, mechanism as well as through a slower cAMP-dependent increase in NOS I. As NOS I levels peak on proestrus, nitric oxide signaling has been proposed to contribute to the LH surge in the rodent (Garrel et al., 1998; Lozach et al., 1998). Importantly, PACAP potentiates the cGMP response to GnRH in rat pituitary gonadotropes and may therefore help to augment GnRH-sensitivity on proestrus (Garrel et al., 2002). While the majority of studies suggest that nitric oxide and cGMP stimulate basal and hormonally-mediated gonadotropin secretion, conflicting results emphasize the need for further study in this area (Cecatelli et al., 1993; Gobetti and Zerani, 1998; Meints et al., 2012; Pinilla et al., 1998; Yu et al., 1997).

Table 1

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>α-Subunit-luc</th>
<th>LHβ-luc</th>
<th>FSHβ-luc</th>
</tr>
</thead>
<tbody>
<tr>
<td>LjT2 cells</td>
<td>Continuous</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PACAP</td>
<td>1&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>1&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>1&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
<tr>
<td>GnRH</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>1&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>1&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
<tr>
<td>PACAP + GnRH</td>
<td>1&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>1&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>1&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>1&lt;sup&gt;2,3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

| a Cells treated in static culture. |
| b Harada et al. (2007). |
| c Kanasaki et al. (2009). |
| d Purwana et al. (2010). |
| e Ferris et al. (2007). |
Either continuous PACAP or hourly PACAP pulses increased glyco-
script number by over 50% but did not change LH
expression. Conversely, continuous PACAP decreased FSH
response, pulsatile PACAP was found to significantly increase LH
mary rat anterior pituitary cells. In an elegant series of experi-
PACAP, pulsatile PACAP and/or pulsatile GnRH on perifused pri-

Table 2
Effect of PACAP and GnRH on gonadotropin mRNA levels.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>α-subunit</th>
<th>LHβ-subunit</th>
<th>FSHβ-subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary male rat pituitary</td>
<td>Continuous (a) PACAP</td>
<td>(1,2)</td>
<td>(1,2)</td>
<td>(1,2)</td>
</tr>
<tr>
<td></td>
<td>PACAP (b)</td>
<td>(1)</td>
<td>(2)</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>GnRH (b)</td>
<td>(1)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>Pulsatile (a) PACAP</td>
<td>(1)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td></td>
<td>GnRH (b)</td>
<td>(1)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td></td>
<td>Pulsatile GnRH + continuous PACAP (b)</td>
<td>(1)</td>
<td>(7)</td>
<td>(7)</td>
</tr>
<tr>
<td>LβT2 cells</td>
<td>Continuous PACAP (b)</td>
<td>(1)</td>
<td>(8)</td>
<td>(8)</td>
</tr>
<tr>
<td></td>
<td>Pulsatile (a) PACAP</td>
<td>(1)</td>
<td>(9)</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>GnRH (b)</td>
<td>(1)</td>
<td>(10)</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td>GnRH (low freq)</td>
<td>(1)</td>
<td>(11)</td>
<td>(11)</td>
</tr>
</tbody>
</table>

\(a\) Cells treated during perifusion.
\(b\) Cells in static culture.

Tsujii et al. (1994).
Tsujii et al. (1995).
Weiss et al. (1990).
Harada et al. (2007).
Kanasaki et al. (2009).

4. PACAP regulation of gonadotropin biosynthesis

4.1. Transcription

LH and FSH are dimeric proteins encoded by the glycoprotein α-
subunit and an LHβ- or FSHβ-subunit, respectively. PACAP and
GnRH have also been investigated for their ability to alter expres-
sion of all three of these subunits, with the greatest amount known
about effects on the common α-subunit. GnRH has been found to
differentially regulate gonadotropin subunit transcription, mRNA
levels and secretion depending on whether exposure is continuous
or pulsatile, with further modulation obtained by alterations in
pulse frequency (Bedecarrats and Kaiser, 2003; Kaiser et al.,
1997b; Katt et al., 1985) Similar functional distinctions have been
reported for PACAP responses. The effects of PACAP and/or GnRH
on gonadotropin promoter activity and steady-state mRNA levels
are outlined in Tables 1 and 2, respectively.

Tsujii and colleagues have investigated the effects of continuous
PACAP, pulsatile PACAP and/or pulsatile GnRH on perifused pri-
mary rat anterior pituitary cells. In an elegant series of experi-
mments, pulsatile PACAP was found to significantly increase LHβ
mRNA levels (albeit by a modest 20%) without altering FSHβ
expression. Conversely, continuous PACAP decreased FSHβ tran-
script number by over 50% but did not change LHβ expression.
Either continuous PACAP or hourly PACAP pulses increased glyco-
protein α-subunit mRNA levels by approximately 50%. PACAP was
found to partially block the well-described stimulatory effect of
pulsatile GnRH on expression of the FSHβ transcript. Both PACAP
and GnRH increased LHβ transcript length as previously reported
for GnRH and correlated with increased transcript stability (Tsujii
et al., 1995; Tsujii and Winters, 1995). Based on studies in the
LβT2 cell line, high frequency PACAP pulses preferentially increase
LHβ gene expression while low frequency pulses preferentially
stimulate FSHβ gene expression (Kanasaki et al., 2009). These re-
sults suggest that PACAP, like GnRH, specifically regulates LHβ
and FSHβ subunit gene expression in a pulse frequency specific
manner. Furthermore, the mode of PACAP secretion in vivo may be
an important determinant of the differential expression of the
gonadotropin subunit genes.

As LβT2 cells have been reported to express low levels of PAC1R,
Purwana and colleagues transfected cells with an expression
vector for this receptor prior to hormonal treatment. In this model,
simultaneous treatment with GnRH and PACAP increased LHβ- and
FSHβ-gene promoter activity to a degree that far exceeded the re-
response to either hormone alone, demonstrating a cooperative
interaction at the level of transcriptional activation for these genes
(Purwana et al., 2010). Furthermore, overexpression of PAC1R was
observed to sensitize the cells to GnRH, decreasing the EC50 and
increasing the magnitude of the LHβ and FSHβ-luc response to
GnRH alone. This effect was attributed to augmentation of GnRH-
mediated stimulation of downstream signaling molecules in the
ERK and cAMP pathways based on enhanced phosphorylation of
pERK and activation of SRE-luc and CRE-luc constructs. As treat-
ment with a PACAP antagonist, PACAP 6-38, did not alter the GnRH
response, the authors concluded that induction of local PACAP
secretion by GnRH was not important for the augmented respon-
siveness observed in PAC1R-overexpressing cells (Purwana et al.,
2011). Nevertheless, as reported by the same group, the PACAP
antagonist was able to blunt GnRH-stimulated FSHβ mRNA expres-
sion, suggesting a role for secreted PACAP in these static cultures
(Kanasaki et al., 2011). As a whole, these studies strongly support
a role for PACAP, or at least its receptor, in mediating GnRH effects
on gonadotropin transcript expression.

4.2. Cis-elements

Based on the observation that PACAP and GnRH increase stea-
dy-state mRNA levels of the glycoprotein hormone α-subunit,
additional studies aimed to define the GnRH- and PACAP-responsive
region(s) in the promoter region of this gene. The mouse and
human α-subunit gene promoters contain critical differences in
nucleotide sequences and, as a result, are regulated via distinct
mechanisms.

The mouse glycoprotein α-subunit promoter has been found to
contain two regions which impart GnRH-responsiveness, a so-
called GnRH-response element (GnRH-RE) at position −406/−399
and the pituitary glycoprotein hormone basal element (PGBE) at
positions −337/−330 (Schoderbek et al., 1993). These regulatory
regions provide a point for convergence of GnRH and PACAP signal-
ing as clustered point mutations in the GnRH-RE and PGBE de-
crease or increase PACAP-mediated stimulation of promoter activity,
respectively (Attardi and Winters, 1998).
GnRH and PACAP have also been shown to increase transcriptional activity of the human α-subunit gene, despite the lack of either GnRH-RE or PGBe sequences in this promoter. Co-treatment generates an additive rather than synergistic response, suggesting independent regulatory mechanisms. Full PACAP responsiveness is achieved via two CRE cis-elements in region –147/-116 known to bind CREB and mediate cAMP signaling, as well as a second, uncharacterized region at position –244/-195 in the promoter. PACAP effects can be mimicked by activation of adenylate cyclase with forskolin or by protein kinase A (PKA) overexpression and can be partially blocked by inhibition of either the PKA or MEK1/2 (MAPK) pathways, clearly implicating these signaling systems in the observed transcriptional responses (Harada et al., 2007; Tsujii et al., 1995). In contrast, GnRH-mediated stimulation requires calcium signaling but is independent of the CRE sites (Burris et al., 1998). The –244/-195 promoter region has been found to contain a gonadotrope-specific element (GSE) which binds the transcription factor steroidogenic factor-1 and confers both basal and GnRH responsiveness (Holdstock et al., 1996). Therefore, unlike effects on the mouse α-subunit gene, PACAP and GnRH appear to act via distinct cis-elements on the human gene.

Transcriptional activity of the LHβ- and FSHβ-subunits can also be stimulated by treatment with PACAP or by activation of the cAMP/PKA signaling system; although by a modest 2-fold relative to the 10-fold response observed for the α-subunit promoter (Ferris et al., 2007; Harada et al., 2007; Horton and Halvorson, 2004; Tsujii et al., 1995). The rat LHβ gene contains two composite DNA-regulatory regions which bind the transcription factors SF-1 and Egr-1 and are critical for mediating the GnRH response, at least in part via increases in Egr-1 protein levels (Halvorson et al., 1999; Kaiser et al., 2000; Tremblay and Drouin, 1999). Using cultured primary pituitary cells from mice expressing an LHβ promoter-luciferase transgene, Ferris and coworkers reported an increase in LHβ promoter activity in response to PACAP, forskolin, or GnRH (Ferris et al., 2007). In LβT2 cells, the forskolin response was lost with mutation of the 3′SF-1 cis-element but not the 5′SF-1 or Egr-1 sites. Furthermore, forskolin did not alter SF-1 or Egr-1 binding to the endogenous LHβ promoter, suggesting that PKA-mediated transcription was achieved via post-translational protein modifications leading to increased association with coactivators or decreased interaction with corepressors (Ferris et al., 2007). In contrast, using a somatolactotrope GH3 cell line model, Horton and colleagues observed a PKA-mediated increase in Egr-1 protein levels and loss of the cAMP/PKA response with mutation of the Egr-1 but not the SF-1 cis-elements (Horton and Halvorson, 2004). Assuming that PACAP acts as the major hormonal stimulator of intracellular cAMP levels, these studies suggest that the SF-1 and/or Egr-1 DNA-regulatory regions in the LHβ gene may be critical for conferring both PACAP and GnRH responsiveness. Unfortunately, as of yet, no studies have investigated the importance of these sites in the presence of both hormonal factors.

4.3. Follistatin

While PACAP (and GnRH) decrease FSHβ mRNA levels in rat primary cells in static culture, it has been noted that PACAP increased FSHβ mRNA levels in a pure population of mouse gonadotrope cells LβT2 cells (Fujii et al., 2002). This apparent discrepancy has now been attributed to the ability of PACAP to stimulate follistatin production by rat gonadotropes and supportive folliculostellate cells in primary pituitary cell cultures but not in the follistatin-deficient LβT2 cell line (Fujii et al., 2002; Katayama et al., 2000; Winters et al., 1997). Follistatin binds and, thereby, prevents the ability of activin to stimulate FSHβ biosynthesis. In support of this mechanism, conditioned medium from PACAP-treated TT/GF folliculostellate cells has been shown to attenuate the ability of activin A to increase FSH secretion from primary pituitary cell cultures (Katayama et al., 2000). GnRH also increases follistatin expression with an amplified response in the presence of PACAP (Fuji et al., 2002). Hormonal regulation occurs, at least in part, via activation of the follistatin promoter as PACAP, GnRH, and constitutively active PKA or MEK kinase expression vectors increase activity of a follistatin-promoter reporter construct (Mutia et al., 2009). Taken together, these results suggest that PACAP and GnRH may act cooperatively to achieve differential regulation of LHβ and FSHβ by increasing intrapituitary follistatin levels, at least in the rodent. Of note, studies in the non-human primate suggest that species-specific differences exist in the hormonal regulation of follistatin and FSHβ gene expression. Using cultured primary pituitary cells from rhesus monkeys, Kawakami and colleagues found that follistatin expression was stimulated by PACAP but not by GnRH and that FSHβ gene expression was increased by both hormones (Kawakami et al., 2002).

In vivo studies have reported a direct correlation between PACAP and follistatin expression and inverse correlation with FSHβ expression in fetal and perinatal rodent pituitaries (Moore et al., 2009, 2012). Furthermore, targeted overexpression of pituitary PACAP has been shown to enhance follistatin expression with an associated reduction in gonadotropin subunit and GnRH receptor mRNA levels as well as a decrease in circulating gonadotropin and testosterone levels and resultant delayed puberty. These data strongly support a critical role for PACAP on pituitary function during development and sexual maturation.

5. PACAP effects on gonadotropin secretion

As for GnRH, hypothalamic PACAP is believed to be secreted as discrete pulses into the portal vasculature, although the pulse frequency and amplitude of this release has yet to be determined. Pituitary-derived PACAP likely provides a more continuous source of stimulation on the gonadotropes and other pituitary cell types. The relative importance of these two sources of PACAP for pituitary function is currently unknown.

A limited number of in vivo investigations have clearly demonstrated the ability of PACAP infusions to regulate LH secretion in rats, although studies in sheep and humans have been less conclusive (Hammond et al., 1993; Osuga et al., 1992; Radlleff-Schlimme et al., 1998; Sawangjaroen et al., 1997). As reported by Radlleff-Schlimme and colleagues, intravenous administration of 10 mcg of PACAP38 at 40 min intervals over 6 h increases serum LH levels by an impressive 45-fold (Radlleff-Schlimme et al., 1998).

Table 3 PACAP and GnRH effects on gonadotropin secretion: PACAP augments GnRH-mediated stimulation of gonadotropin secretion by primary pituitary cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>α-Subunit</th>
<th>LH</th>
<th>FSH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary male rat pituitary</strong></td>
<td>Continuous&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PACAP</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>GnRH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PACAP + GnRH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsatile GnRH&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>PACAP + Pulsatile GnRH&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><strong>PACAP</strong></td>
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<sup>a</sup> Cells in static culture.
<sup>b</sup> Cells treated during perfusion.
<sup>1</sup> Tsujii et al. (1994).
<sup>2</sup> Culler and Paschall (1991).
<sup>3</sup> Tsujii et al. (1995).
Multiple *in vitro* studies have confirmed PACAP-stimulated release of dimeric LH and FSH as well as the free α-subunit from primary pituitary cell cultures and gonadotrope-derived αT3-1 cells, although to a more modest extent than observed for GnRH (Culler and Paschall, 1991; Hart et al., 1992; Ortmann et al., 1999; Schomerus et al., 1994). A subset of these studies is shown in Table 3. In one study, treatment of dispersed rat pituitary cells in static culture with 10 nM PACAP38 for 4 h increased LH and α-subunit secretion by 2-fold and 3.5-fold, respectively (Hart et al., 1992). Similarly, Tsuji and colleagues reported a 2-fold increase in LH secretion following 24 h of treatment with 10 nM PACAP38 (Tsuji et al., 1995). They observed a greater increase in response to 10 nM GnRH treatment (over 3-fold), but no additive effect in the presence of both hormonal factors when tested at a single dose and timepoint. Nevertheless, subsequent experiments using submaximal concentrations of PACAP and GnRH have clearly demonstrated the ability of PACAP38 to augment GnRH-stimulated LH and FSH secretion and inositol phosphate (IP) production in static cultures of primary rat pituitary cells (Culler and Paschall, 1991; Ortmann et al., 1999; Schomerus et al., 1994; Tsuji et al., 1994). Using dispersed adult male pituitary cells in static culture, Culler and coworkers reported a concentration dependent increase in LH and FSH secretion following 3 h of PACAP treatment. Furthermore, they observed reciprocal potentiation of the effects of PACAP and GnRH on LH and FSH secretion (Culler and Paschall, 1991).

PACAP has also been shown to enhance the GnRH response in a perifusion system which allows hormones to be administered continuously and modulates downstream intracellular signaling pathway(s) (Tsuji and Winters, 1995). Although minimally active itself, continuous PACAP38 markedly enhances the amplitude of GnRH-induced LH, FSH, and α-subunit secretion from these cells with a gradual increase in interpulse secretion of LH and α-subunit but a decline in the FSH interpulse levels. This amplification persisted for at least 3 h following withdrawal of PACAP38 from the perifusion medium, consistent with a PACAP-mediated alteration in either GnRHR expression or downstream intracellular signaling pathway(s) (Tsuji et al., 1994). In summary, although PACAP itself is only weakly stimulatory, it has a strong sensitizing effect on GnRH-induced LH secretion.

6. Reciprocal receptor regulation

It has been proposed that functional interaction between PACAP and GnRH may be achieved in part via reciprocal effects on their associated receptors. Stated differently, substantial evidence points to the ability of PACAP to upregulate GnRHR receptor (GnRHR) gene expression and, conversely, the ability of GnRH to regulate PAC1R expression.

6.1. PACAP and GnRHR

Fast frequency PACAP pulses have been shown to increase endogenous GnRHR mRNA levels in LpT2 gonadotropes (Kanasaki et al., 2009). PACAP has been shown to stimulate GnRHR gene promoter activity in the human, mouse, and rat although substantial species-specific differences exist in terms of the cis-elements reported to mediate this response. Pulsatile GnRHR is a highly effective activator of GnRH receptor expression; however, this response is achieved via different cis-elements and signaling pathways than are utilized by PACAP and studies of combinatorial GnRH and PACAP on GnRHR receptor expression are lacking.

As reported by Cheng et al., PACAP38 induces a dose-dependent increase in human GnRHR promoter activity in αT3-1 gonadotropes. This response is effectively blocked by co-treatment with an adenylate cyclase inhibitor, implicating the cAMP/PKA signaling system. Mutation of AP-1/CRE sites at position −568/−561 and/or −340/−333 inhibited forskolin-mediated increases in GnRHR gene promoter activity, although the effect of these mutations on the PACAP response was not tested (Cheng and Leung, 2001). A second group identified a more distal region in the human GnRHR gene at position −1676/−1648 which confers PACAP-responsiveness and interacts with a neighboring silencer element (Ngan et al., 2001).

In the mouse gene, PACAP was observed to stimulate GnRHR transcription within 30 min with a peak 5-fold induction by 8 h in αT3-1 cell transfection experiments (Sadie et al., 2003). Although forskolin also increased mGnRHR transcription, the response required 16 h of treatment suggesting an indirect effect through an intermediate protein, likely SF-1, as forskolin increases SF-1 mRNA levels and phosphorylation. The authors concluded that PACAP increased mouse GnRHR transcription via both unstimulated burst and a slower, cAMP-mediated response. Activity of the rat GnRHR gene is also increased via PACAP activation of the PKA signaling pathway. Using deletion and mutational analyses, Pincus and co-workers characterized two PACAP response elements which they termed PACAP-RE I (PARE I) and PARE II. These regulatory regions contain an SF-1 binding site and an imperfect cAMP response element, respectively, implicating both SF-1 and CREB in the PACAP response in the rat gene (Pincas et al., 2001).

6.2. GnRH and PAC1R

Both pulsatile and static GnRH treatment have been shown to augment PAC1R mRNA and protein levels in LpT2 gonadotropes (Grafer et al., 2009; Kanasaki et al., 2009, 2011; Purwana et al., 2010). In addition, GnRHmodulates PAC1R signaling via increased phosphorylation in this cell line (Kanasaki et al., 2009; Lariviere et al., 2008). In contrast, in primary pituitary cell cultures, PAC1R transcript number is unchanged following 6 h of GnRH treatment and decreases by a small but statistically significant margin with 24 h of treatment (Zheng et al., 2013). While these differences may be due to the immortalization process in LpT2 cells, it seems more likely that the altered response is due to the presence of a mixed primary cell population and accumulation of inhibitory hormonal factor(s) with prolonged time in culture.

7. Hormonal regulation of PACAP expression

7.1. By GnRH

It has been clearly demonstrated that GnRH stimulates PACAP mRNA and protein levels in both primary pituitary gonadotropes and gonadotrope cell lines (Grafer et al., 2009; Kanasaki et al., 2009, 2011; Purwana et al., 2010; Zheng et al., 2013). The GnRH response is mediated via the PKA, PKC and MAPK pathways and the subsequent binding of AP-1 and CREB transcription factors to regulatory elements identified within the proximal PACAP gene promoter (Grafer et al., 2009). Recent data suggest that GATA transcription factors also contribute to GnRH-mediated stimulation of PACAP gene expression (Thomas et al., 2013). The GnRH-stimulated increase in locally-secreted PACAP then may modulate gonadotrope sensitivity to GnRH via increased GnRHR expression and alterations in overlapping intracellular signaling pathways as already described.
PACAP expression is preferentially stimulated by slow GnRH pulse frequencies (every 120 min) relative to fast pulse frequencies (every 30 min) (Kanasaki et al., 2009). Of note, the magnitude of the GnRH-mediated increase in PACAP mRNA levels in static culture greatly exceeds the response in perfusion in which secreted factors are washed away. GnRH treatment for 24 h increased PACAP mRNA levels by 25-fold versus 3.5-fold following perfusion for 16 h at low frequency (Kanasaki et al., 2011; Purwana et al., 2010). As PACAP itself increases both PACAP and PAC1R expression in the pituitary (see Section 7.2), these results are consistent with the loss of cooperative interaction between GnRH and locally-secreted PACAP in the perfusion model.

7.2. By PACAP

PACAP has been shown to increase its own expression as well as expression of its receptor, thereby creating a positive “feed forward” loop to further regulate gonadotropin gene expression. Intravenous PACAP infusion in male rats increases pituitary PACAP mRNA levels by approximately 1.5-fold (Radloff-Schlimme et al., 1998). PACAP stimulates mouse PACAP gene promoter activity by greater than 10-fold and increases PACAP immunoreactivity by 2-fold in a human neuroblastoma cell line (Radloff-Schlimme et al., 1998; Suzuki et al., 1994; Yamamoto et al., 1998). PACAP also markedly stimulates PAC1R gene expression with low pulse frequencies increasing mRNA levels by over 15-fold in LjT2 cells (Kanasaki et al., 2009).

7.3. By steroids

The impact of gonadal steroids on hypothalamic and pituitary PACAP expression has been evaluated indirectly via measurement across the estrous cycle and following gonadectomy. As both GnRH pulsatility and gonadal steroid levels fluctuate in these models, it is not possible to determine which of these factors is responsible for observed changes in the pituitary. Nevertheless, PACAP mRNA and protein levels have been found to increase late on proestrus in the rat following endogenous increases in estradiol and progesterone (Koves et al., 2003; Moore et al., 2005; Szabo et al., 2004). Furthermore, PACAP protein levels have been found to drop precipitously in both the hypothalamus and anterior pituitary gland following gonadectomy in both male and female rats, with a slow return to baseline by 2–3 weeks (Nemeth et al., 2006). These studies suggest a generally positive effect of steroids on PACAP expression in the hypothalamic–pituitary axis, at least in a complex in vivo setting.

A limited number of studies have looked at the direct effects of steroid treatment on central PACAP expression. Ha and colleagues have shown that progesterone, alone or in combination with estradiol, induces significant increases in PACAP and PAC1 receptor mRNA levels in the medial basal hypothalamus of ovariectomized rats (Ha et al., 2000). It has been postulated that the post-ovulatory rise in progesterone may suppress GnRH pulsatility in part via induced central PACAP expression. Our group has investigated the effects of gonadal steroids in adult male rat pituitary cell cultures. Estradiol treatment was found to blunt PACAP gene expression but did not alter GnRH-mediated increases in PACAP mRNA levels (Zheng et al., 2013). Conversely, treatment with DHT or progesterone for 6 or 24 h was found to augment the GnRH-mediated increase in PACAP mRNA levels and blunt GnRHR transcript number, although neither steroid had an effect alone levels (Zheng et al., 2013). We have subsequently identified two cis-elements within the rat PACAP promoter which mediate androgen-responsiveness (Grafer and Halvorson, 2013). As a group, these results suggest that alterations in circulating steroid levels likely impact the relative influence of PACAP and GnRH on gonadotropin expression.

8. GnRH neuronal function

Current data suggest that PACAP may regulate gonadotropin secretion by modulation of pulsatile GnRH release in addition to direct effects on the pituitary gonadotropes. Although contradictory results have been reported, the bulk of studies suggest that PACAP inhibits GnRH expression in contrast to its general augmentation of GnRH action at the pituitary level. PACAP intracerebroventricular (ICV) injection or injection directly into the medial basal hypothalamus housing the GnRH neurons suppressed GnRH pulsatility, as measured by decreases in mean LH concentration, pulse frequency and pulse amplitude in ewes (Anderson et al., 1996; Sawangjaroen et al., 1997). In contrast, ICV injection of PACAP in male rats increased GnRH mRNA levels by 12.5%, an effect that was abrogated by concomitant administration of the PACAP antagonist, PACAP 6–38 (Li et al., 1996). Injection of the antagonist itself produced a similar reduction in GnRH expression, consistent with the presence of endogenous PACAP activity. Intravenous PACAP treatment has been variously found to increase, decrease or have no effect on gonadotropin secretion (Chiodera et al., 1996; Li et al., 1996; Osuga et al., 1992; Sawangjaroen et al., 1997). It is possible that inhibitory central effects are masked by direct stimulatory effects at the pituitary, but this possibility remains to be formally tested.

The ability of PACAP to impact ovulation and pubertal timing has also been investigated. Supporting an inhibitory effect, ICV administration of PACAP38 during early proestrus prevents the LH surge and blocks ovulation in female rats (Kantora et al., 2000; Kantora et al., 2000; Koves et al., 1998b, 2003, 1996). Intravenous administration of PACAP38 does not influence ovulation, suggesting a central action at the hypothalamus (Koves et al., 1996). Interestingly, PACAP27 was not observed to inhibit ovulation in the single study in which its effects were tested (Kantora et al., 2000).

PACAP treatment of neonatal rats has been observed to delay puberty, decrease the weight of the anterior pituitary, decrease the intensity of LHRH immunostaining, and decrease the number of eggs released at first ovulation (Koves et al., 1998a; Szabo et al., 2002b). Choi and co-workers observed a decline in endogenous PACAP and PAC1R mRNA in the medial basal hypothalamus and preoptic areas of female rats preceding first ovulation, consistent with de-repression of GnRH neuronal activity (Choi et al., 2000). Of note, however, ICV injection of an antisense oligodeoxynucleotide against PAC1R in juvenile female rats (28-days old) delayed vaginal opening and decreased LHβ and GnRHR mRNA expression in the anterior pituitary, suggesting that PAC1 synthesis and, presumably PACAP signaling, are important positive regulators of final pubertal maturation (Choi et al., 2000). Taken as a whole, these data strongly suggest that PACAP signaling plays an important role in the pubertal process via alteration of GnRH neuronal activity, although the timing and direction of this effect require further study.

Alterations in GnRH pulsatility may be due to innervation of GnRH cells by PACAP-containing neurons and/or indirect actions via interneurons. In support of direct GnRH neuronal activation, PAC1 and VPAC2 receptors have been demonstrated in an immortalized GnRH cell line (Olcice et al., 1997). A number of studies have implicated corticotropin-releasing factor and endogenous opioids as intermediaries between PACAP and GnRH neuronal function, although the role of opioids remains controversial (Kantor et al., 2000, 2001, 1996). Central effects could theoretically also occur in response to circulating PACAP levels. Indeed, both PACAP27 and PACAP38 have been shown to cross the blood–brain barrier.
barrier through a saturable transport mechanism with the smaller PACAP27 also gaining access via transmembrane diffusion. Nevertheless, it has been estimated that only 0.1% of intravenously administered PACAP crosses into the CNS. This degree of transfer has been estimated to be adequate for conferring protective effects against hippocampal ischemia, but it is not known whether it is adequate for altering hypothalamic function (Banks et al., 1993, 1996).

9. Transgenic models

Transgenic models provide a potentially powerful approach for analyzing the importance of PACAP and its receptor in hypothalmic–pituitary function. Global PACAP- and PAC1R-deficient mouse models have been generated and have suggested a role for PACAP in maintenance of normal fertility (Isaac and Sherwood, 2008; Jamen et al., 2000b; Shintani et al., 2002). It should be noted that interpretation of these models is complicated by at least two factors: (1) overlap in expression of PACAP and VIP and their receptors in reproductive tissues which may provide redundant function and, (2) widespread expression in other organ systems leading to abnormalities in thermogenesis, carbohydrate and lipid metabolism, and neurologic function which may indirectly impact reproductive capacity (Gray et al., 2001; Hashimoto et al., 2001; Jamen et al., 2000a; Sherwood et al., 2007).

Jamen and colleagues have reported decreased fertility in female, but not in male, mice null for expression of the PAC1 receptor (Jamen et al., 2000b). This was not felt to be attributable to changes in gonadotropin synthesis and turnover based on quantitation of pituitary LH and FSH by immunohistochemistry. However, no serum measurements were obtained and the animals were observed to have slightly prolonged estrous cycles with an irregular diestrous phase, perhaps suggesting subtle abnormalities in the hypothalamic–pituitary axis.

In comparison, PACAP null female mice were reported to have normal estrous cyclicity but decreased mating frequency (Shintani et al., 2002). A second PACAP knockout mouse model was more fully characterized and found to have normal puberty, estrous cyclicity, folliculogenesis, mating frequency and fertilization rates, but impaired implantation rates (Isaac and Sherwood, 2008). The implantation defect was postulated to arise from decreased PACAP-mediated prolactin production by the pituitary and, thereby, lower progesterone production.

Although loss of PACAP signaling has not been clearly shown to impact gonadotrope function in global knockout models, overexpression of PACAP specifically targeted to the pituitary is associated with delayed puberty and increased gonadotropin and GnRHR expression in male animals (Moore et al., 2012). Interestingly, gene array demonstrated that pituitary expression of multiple factors associated with GnRH signaling were altered in the transgenic animals, supporting a role for PACAP in mediating the GnRH response in gonadotropes.

10. Conclusion

As reviewed in this article, a substantial amount of information has accumulated regarding the impact of PACAP on gonadotrope function, both alone and in conjunction with GnRH. In addition to modulation of GnRH neuronal activity, PACAP sensitizes the gonadotrope to GnRH by increasing GnRHR number as well as through interactions at the level of intracellular signaling. GnRH and PACAP may both increase PACAP and PAC1R expression forming a positive feed forward loop to further sensitize the cells. At least in rodents, PACAP also provides a mechanism for divergent gonadotropin expression via stimulation of follistatin secretion by the pituitary folliculostellate cells. Further complexity is suggested by recent experiments demonstrating gonadal steroid modulation of pituitary PACAP and PAC1R expression. Fig. 1 outlines a number of points of interaction between these two neuropeptides.

Despite these advances, the majority of studies have utilized culture systems and substantial gaps remain in our understanding of PACAP function in vivo. For example, additional information is needed regarding the relative contribution of hypothalamic and pituitary PACAP on the regulation of gonadotrope physiology. Mice with pituitary- or hypothalamic-specific loss of PACAP or PAC1R expression could be informative in this regard. Further studies will provide important new insights on the role of this novel factor as a mediator of gonadotrope function, including modulation of GnRH-dependent effects in these cells.

References


