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The Role of Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) in Gonadotrope Function

Mini Review

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Pituitary adenylate cyclase activating polypeptide (PACAP) was first isolated in 1989 from ovine hypothalamus due to its potent ability to stimulate cAMP accumulation in rat anterior pituitary cell culture. Two PACAP isoforms have been found: a 38 amino-acid form (PACAP38) accounting for 90% of the protein in most tissues and a C-terminally truncated 27-amino-acid form (PACAP27). The PACAP gene has five exons and four introns, and the introns are bounded by the consensus splicing sequences 5'-GT and 3'-AG. In mammals three receptors for PACAP are recognized: VIPR1 and VIPR2 which present a similar affinity for vasoactive intestinal peptide and PACAP, and the specific PAC1 receptor which preponderantly couples to membrane Gαs protein, which results in rapid cAMP/PKA pathway activation. The physiological importance of PACAP is underscored by the consequences of knocking-out its gene. In PACAP-deficient mice, most of the pups died at birth or by the second week of life with wasting, ketosis and dyslipidemia. At least one of the PACAP receptors is present in each of the anterior pituitary endocrine cell types, and in pituitary folliculostellate (FS) cells GnRH was shown to stimulate PACAP and its receptor expression in gonadotropes as well as in folliculostellate cells, and also PACAP acting via cAMP/PKA pathway induction increased the GnRH receptor level. Both GnRH and PACAP signaling interact in gonadotropes and GnRH was shown to stimulate PACAP and its receptor expression in gonadotropes as well as in FS cells. In turn, PACAP acting via cAMP/PKA pathway induction increased the level of GnRH receptor expression. A regulatory effect of PACAP on gonadotropin activity is also exerted at each gonadotropin subunit gene level. PACAP was shown to stimulate α and LHβ subunits and mRNA expression and to suppress the FSHβ mRNA level via activation of follistatin gene and protein expression.

Key words: PACAP, PACAP gene, PACAP receptors, cAMP/PKA signaling, follistatin, anterior pituitary

INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP) was first isolated in 1989 from ovine hypothalamus due to its potent ability to stimulate cAMP accumulation in rat anterior pituitary cell culture (Miyata et al., 1989; Arimura, 1992). In the hypothalamus the PACAP-immunoreactive fibers are localized mainly in the supraoptic (SON) and paraventricular (PVN) nuclei which

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are connected with the anterior pituitary gland through the hypophyseal portal blood vessels (Vigh et al., 1991). Two naturally existing PACAP isoforms have been found: a 38 amino-acid form (PACAP38) accounting for 90% of the protein in most tissues and a C-terminally trunctated 27-amino-acid form (PACAP27). As the most highly conserved member of the VIP (vasoactive intestinal peptide)/secretin/glucagon peptide superfamily, PACAP is expressed in tunicate, fish, amphibians, rodents and mammals, whereas a related protein, amnesiac, is found in Drosophila (Sherwood et al., 2000). PACAP is detectable and biologically active in many tissues, including brain, pituitary, adrenal, testis, ovary, placenta and nerve fibers of both the gut and the lung (Vaudry et al., 2009) where it is considered as a neurohormone, neuromodulator, neurotransmitter, and vasoregulator (Rawlings and Hezarch, 1996). PACAP has also been shown to function as a growth and developmental factor promoting mitogenesis, survival and neurite outgrowth on immature rat cerebellar granule cells (Vaudry et al., 1999), and to stimulate male primordial germ cell proliferation in early development (Pesce et al., 1996).

PACAP RECEPTORS

Three receptors for PACP have been cloned in mammals: VIPR1 and VIPR2 which present a similar affinity for VIP and PACAP, and the specific PAC1 receptor (the official symbol Adcyap1r1). When bound to their ligands, PACAP receptors initiate a complex network of signaling pathways that include the phospholipase C/protein kinase C (PKC)/calcium and adenylate cyclase/protein kinase A (PKA) pathways. The predominant receptor for PACAP – Adcyap1r1 preponderantly couples to membrane Gαs protein, which results in a rapid cAMP production, which in turn activates PKA (Miyata et al., 1989). In both humans and rats Adcyap1r1 has nine subtypes resulting from alternative splicing, and six of these subtypes are distinguished from each other by the absence or presence of two cassettes named Hip and Hop which are located at the end of the 3rd intracellular loop of Adcyap1r1 (Dickson and Finlayson, 2009). They are named Adcyap1r1-null (neither hip nor hop), Adcyap1r1-Hop1, Adcyap1r1-Hop2, Adcyap1r1-Hip, and Adcyap1r1-Hiphop1 and Adcyap1r1-Hiphop2. Beyond these six variants, other subtypes were also discovered, including Adcyap1r1-Vs, Adcyap1r1-TM4 and Adcyap1r1-3a (Chatterjee et al., 1996; Pantaloni et al., 1996; Daniel et al., 2000). Adcyap1r1 variants express not only different affinity for PACAP but may also mediate different signaling pathways in various cell types (McCulloch et al., 2001; Alexandre et al., 2002; Niewiadomski et al., 2002; Dickson and Finlayson, 2009). Adcyap1r1 variants that differ from the null receptor in the amino-terminal extracellular domain have also been identified (Pantaloni et al., 1996). N-terminal extracellular domain of the Adcyap1r1 receptor is a major binding site for the central and C-terminal helical segments of PACAP (Cao et al., 1995; Bourgault et al., 2008). Adcyap1r1 is specific for binding to PACAP but not VIP because different sequence regions (4–13 and 24–28) between PACAP27 and VIP are Adcyap1r1 selective sites (Schafer et al., 1999; Onoue et al., 2001). The rat Adcyap1r1 gene spans 40 kb with 15 exons (Chatterjee et al., 1997), whereas the human gene is located in the region p15 of chromosome 7 (Brabet et al., 1996). The mouse Adcyap1r1 gene spans more than 50 kb and is divided into 18 exons (Aino et al., 1995). The proximal promoter region has no apparent TATA box but contains a CCAAT box and two potential Sp1-binding sites that act as transcriptional activators (Skak and Michelsen, 1999).

PACAP (ADCYAP1) GENE STRUCTURE AND REGULATION OF EXPRESSION

The PACAP genes from different species were cloned soon after polypeptide isolation in 1989 (Montero et al., 2000). The PACAP gene has five exons and four introns, and the introns are bounded by the consensus splicing sequences 5'-GT and 3'-AG. PACAP38 and PACAP27 are encoded by exon 5, whereas exon 4 encodes PRP (PACAP-related peptide), and exon 1 is untranslated (Hannibal et al., 1995). The p11 region of chromosome 18 is a site where human PACAP gene is located, whereas the rat PACAP gene has been mapped to 9q37. (Hosoya et al., 1992). In the PACAP gene, two CRE (cAMP-response-like

element) and growth hormone trans-activator factor-1 response elements, a GATA box, and a C-rich domain with GC boxes are conserved across different species (White et al., 2000). Additionally, the 5'-flanking region contains two neural-restrictive silencer-like elements 1 and 2, which might be involved in neuron-specific PACAP gene expression. (Sugawara et al., 2004; Lee et al., 2006).

The sensitivity of the Adcyap1 gene to PACAP stimulation was observed in both in vitro (Suzuki et al., 1994; Yamamoto et al., 1998) and in vivo studies (Radleff-Schlimme et al., 1998). Also GnRH activates Adcyap1 expression and this effect is mediated via the PKA, PKC and MAPK pathways acting on CRE/AP-1 sites in the proximal part of the Adcyap1 promoter (Grafer et al., 2009). Moreover, estrogen was reported to stimulate the PACAP level in the ventromedial nucleus and arcuate nucleus (Apostolakis et al., 2004), whereas progesterone alone induced Adcyap1 and Adcyap1r1 mRNA expression in the medial basal hypothalamus of ovariectomized rats (Ha et al., 2000). The importance of gonadal steroids in the regulation of pituitary PACAP is further supported by an increase in both PACAP mRNA and protein levels found in late proestrus, and a decline observed after gonadectomy (Koves et al., 2003; Szabo et al., 2004; Moore et al., 2005).

PACAP AND FOLLISTATIN CONNECTIONS

A specific mechanism which selectively regulates FSHβ gene expression and protein synthesis involves activin and follistatin (Fst) from the pituitary as well as inhibin from the gonads (Harrison et al., 2005). In this mechanism, follistatin binds to activin to form a biologically inactive complex that counteracts activin signaling and blocks its stimulatory effects on FSHβ expression. Follistatin is precisely regulated and its rapid half-life enables an abrupt start or termination of its physiological effects (Kogure et al., 1996). PACAP was shown both to induce an increase in the follistatin mRNA levels in primary pituitary cell cultures and to activate the Fst promoter by stimulating cAMP/PKA signaling (Winters et al., 1997; Lariviere et al., 2008). PACAP readily increased FSHβ mRNA levels in follistatin-deficient LβT2 cells as well as the transcription of a reporter gene placed under the control of the FSHβ gene promoter, whereas both these effects were abolished by co-expression of follistatin. Furthermore, quantitative in situ hybridization coupled to immunostaining revealed that follistatin expression in both gonadorophs and FS cells is increased by PACAP (Fujii et al., 2002). Also in gonadotrope-derived αT3-1 line transiently transfected with a rat follistatin promoter-luciferase reporter, PACAP was shown to stimulate follistatin gene transcription, and this effect required cAMP-dependent protein kinase A pathway activity (Winters et al., 1997). Two alternatively spliced mRNAs are derived from the Fst gene, including follistatin-288 having no exon 6 sequence and the greater activin-neutralizing activity (Sidis et al., 2006), and PACAP is believed to have its greatest effect on the production of follistatin-288. Altogether, PACAP induction of Fst expression promotes suppression of FSHβ mRNA and may regulate many of the downstream targets of activin signaling. Also the results obtained from the in vivo study, showing that the level of Fst mRNA falls sharply at birth with a decrease in PACAP, further support the idea that PACAP is a major regulator of follistatin protein expression (Moore et al., 2009). Moreover, the decrease in the pituitary Fst-288 at birth is accompanied by a substantial increase in FSHβ and GnRHR mRNA levels, which suggests that the high level of PACAP in the embryonic anterior pituitary facilitates the early appearance of gonadotropin alpha subunit and delays the ontogeny of FSHβ as compared with LHβ.

The nitric oxide pathway represents another example of GnRH and PACAP interaction occurring at the level of intracellular signaling. Nitric oxide synthase type I (NOS1) is synthesized in gonadotropes and folliculostellate cells (Ceccatelli et al., 1993). PACAP-induced NOS1 stimulates the action of GnRH upon nitric-oxide-dependent cGMP production (Garrel et al., 1998, 2002). A possible functional link between PACAP and NOS1 activity is based on NOS1 involvement in activin gene expression inhibition (Shafiee-Kermani et al., 2007) as well as on NO stimulatory impact exerted on follistatin expression (Pisconti et al., 2006). Altogether, NO might participate in PACP regulation of follistatin and FSH activity in pituitary cells.

PACAP *IN VITRO* AND *IN VIVO* REGULATES GONADOTROPH FUNCTION

The importance of PACAP for regulation of the pituitary function is underscored by the fact that at least one of the PACAP receptors is present in each of the anterior pituitary endocrine cell types and in pituitary folliculostellate (FS) cells (Rawling and Hezareh, 1996). In the rat pituitary as well as in gonadotrope αT3-1 cells both null and hop variants of Adcyap1r1 are predominantly expressed (Vertongen et al., 1995; Rawlings et al., 1995). In this line, PACAP activated cAMP production and, independently, increased intracellular calcium concentration by stimulating inositol phosphate (IP) turnover through phospholipase C (Rawlings et al., 1993; Vigh et al., 1993). Also in a more mature gonadotrope line LβT2, in which Adcyap1r1is expressed at a much lower level than in αT3-1 cells (Fowkes et al., 2003), PACAP38 was shown to promote cAMP/PKA pathway (Winters et al., 2007). Interestingly, an interaction between GnRH and PACAP signaling was reported both in LβT2 and αT3-1 cells in which significant inhibition of PACAP38-induced cAMP production required GnRH-induced PKC activity (McArdle et al., 1994; Lariviere et al., 2008). In rat anterior pituitary culture cAMP inductors or agonists, such as forskolin or 8-Br-cAMP, effectively reproduced PACAP-induced time-course profiles, while intracellular effects of PACAP were abolished either by the protein kinase A inhibitor H89 or by decreasing the extracellular calcium concentration with ethylene glycol tetraacetic acid (Hart et al., 1992; Garrel et al., 2002).

It is now established that GnRH and PACAP signaling evidently interact in gonadotropes. Indeed, GnRH was shown to stimulate PACAP (Grafer et al., 2009) and its receptor (Kanasaki et al., 2011) expression in gonadotropes as well as in folliculostellate cells. In turn, also PACAP acting via cAMP/PKA pathway induction increased the level of the GnRH receptor (Pincas et al., 2001; Kanasaki et al., 2009), which implies that both GnRH and PACAP modulate their activity by changing their receptor levels. Moreover, the expression of both GnRH-R and Adcyap1r1 depends on their specific ligand patterns of pulsatile stimulation: the Adcyap1r1 cellular level is higher under lower frequencies of PACAP pulses (Kanasaki et al., 2009; Purwana et al., 2010), whereas the number of cell-surface GnRH receptors is increased at more frequent GnRH pulses (Kaiser et al., 1997).

Apart from the essential role of GnRH exerted on gonadotropin synthesis and release, the gathered evidence shows that also PACAP is an important regulator of gonadotropin secretion and subunit gene expression (Counis et al., 2007). Although PACAP stimulates the release of LH as well as free alpha gonadotropin subunit from rat pituitary cell culture (Culler and Paschall 1991; Hart et al., 1992; Perrin et al., 1993) and these effects are related to concomitant cytosolic calcium increase (Canny et al., 1992), PACAP releasing efficiency is modest compared with that of GnRH and stimulated LH secretion desensitizes rapidly in rat pituitary cells (Tsuji et al., 1995). Besides directly affecting LH secretion, PACAP was shown to augment the response to GnRH (Culler and Paschall, 1991) as well as to increase the GnRH receptor level via a cAMP-dependent mechanism (Pincas et al., 2001; Kanasaki et al., 2009). PACAP also affects gonadotropin activity at each gonadotropin subunit gene level. The enhancement of the alpha subunit mRNA level in αT3-1 cells occurred via stimulation of gene transcription and required PACAP-induced cAMP/PKA pathway activation (Attardi and Winters, 1998). In LβT2 cells, PACAP was reported to activate LHβ promoter (Ferris et al., 2007), which resulted partly from an increase in early growth response protein expression (Horton et al., 2004), whereas in primary pituitary culture it lengthened LHβ mRNA transcripts (Tsuji et al., 1994). Recently a significant increase in LHβ and FSHβ subunit promoter activities has been observed in LβT2 cells transfected with Adcyap1r1 (Purwana et al., 2011). Elevated concentrations of PACAP or increasing densities of its receptor were shown to strengthen the action of PACAP on gonadotropin subunit gene expression (Purwana et al., 2011), whereas simultaneous treatment with GnRH and PACAP increased LHβ and FSHβ gene promoter activity to a degree that far exceeded the response to either hormone alone (Purwana et al., 2010). In the αT3-1 line PACAP stimulated GnRH-R mRNA expression via PKA activation, whereas in LβT2 cells this gene activation resulted from the up-regulatory impact on the expression of CREB and SF1 transcription factors (Pincas et al., 2001). Also in the GnRH-producing line GT1-7 transfected

with Adcyap1r1, increased GnRH receptor gene expression was reported after PACAP stimulation (Kanasaki et al., 2013). In the case of the FSHβ gene, PACAP involvement appears to be dependent on the experimental model used: in the rat primary pituitary culture PACAP decreased FSHβ transcript (Tsujii and Winters, 1995), whereas in LβT2 cells this effect was not observed (Kanasaki et al., 2009).

Despite intensive research on PACAP activity in different experimental models, its role in vivo remains less recognized. PACAP and its receptors are widely expressed in rodents, including their central nervous system, with the highest expression detected in the hypothalamus (Arimura et al., 1991; Ghatei et al., 1993). Since its concentration in hypothalamic portal blood exceeds that of peripheral blood (Dow et al., 1994), PACAP is thought to affect pituitary activity as a hypophysiotropic hormone. In rats, PAPAP increased LH secretion when administered in vivo (Osuga et al., 1992) but it was ineffective when given to ovariectomized ewes (Anderson et al., 1996). In most regions of the rat brain PACAP protein levels increase from low levels at birth to peak levels at 30–60 days of age and they are maintained throughout adulthood (Masuo et al., 1994). PACAP mRNA expression in paraventricular nucleus (PVN) of peripubertal (between 20 and 30 days of age) male rats was shown to be reciprocally related to FSH β and GnRH-R mRNA levels (Moore et al., 2003). Also in females, the levels of PACAP mRNA found in the PVN varied during the rat estrous cycle, with a peak 3h before the proestrous LH/FSH surge (Moore et al., 2005). Moreover, PACAP enhances progesterone-mediated female sexual behavior (Apostolakis et al., 2004, 2005) and may regulate GnRH secretion. When injected subcutaneously (sc) on day 1 of life, PACAP delayed vaginal opening and reduced GnRH immunoreactivity in the preoptic region of 9- and 30-day-old rats (Szabo et al., 2002). The physiological importance of PACAP is underscored by the consequences of knocking-out its gene. In several groups PACAP-deficient mice were found, but most of the pups died at birth or by the second week of life with wasting, ketosis and dyslipidemia (Gray et al., 2001). Because of gonadotropin insufficiency, PACAP-deficient females are subfertile (Isaac and Sherwood, 2008), whereas males are testosterone-deficient (Lacombe et al., 2006). Interestingly, an abrupt decline in the pituitary PACAP content is observed in normal neonate mice as compared with the level maintained in the fetal pituitary (Shintani et al., 2002; Sherwood et al., 2007), and it is suggested that precisely regulated PACAP activity be required for physiological support of gonadal function activation. The gathered evidence indicates that PACAP, apart from its hypophysiotropic activity, is also produced in the pituitary and has a paracrine/autocrine mechanism of action. In rats, high pituitary PACAP mRNA and protein expression was detected as early as embryonic day 19 with an abrupt decrease at birth (Moore et al., 2009), and the PACAP as well as Adycap1r1 mRNA levels were evidently higher within the embryonic pituitary that within the adult rat pituitary (Jaworski and Proctor, 2000).

Early immunoassay detected PACAP in the adult rat pituitary, although at much lower levels than in the hypothalamus, as well as in the human pituitary tissue (Arimura et al., 1991, Ghatei et al., 1993). Immunoreactive PACAP was shown in rat gonadotropes at proestrus (Koves et al., 1998), and laser-capture microdissection demonstrated PACAP mRNA expression in folliculo-stelate cells (Jin et al., 2001). In late proestrus, an increase in PACAP secretion was observed in pituitary primary cell cultures (Koves et al., 2003). Also in vivo, a transient increase in pituitary PACAP expression occurred in female rats during overnight hours, between proestrus and estrus when FSH levels are elevated. Therefore, pituitary PACAP was suggested to participate in termination of the secondary FSH surge during the early hours of estrus (Moore et al., 2005). In male rats pituitary PACAP levels are lower than in females (Heinzlmann et al., 2008) and a significant decline in their pituitary PACAP mRNA between 17 and 21 days of age coincides with a pronounced increase in FSHβ compared with LHβ mRNA (Moore et al., 2003).

CONCLUSIONS

Soon after its isolation, PACAP was suggested to play a key regulatory role in hypothamo-pituitary--gonadal axis activity (McArdle, 1994). Since then, intensive studies have provided data which appear to support that idea. Nevertheless, a fur-

ther experimental effort is required to explore the relevance of PACAP activity to reproduction in humans.

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