Chapter 49
Examination of PACAP During Lactation

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Abstract Breast milk is a complex biological fluid that contains nutritional components and non-nutritive bioactive factors promoting survival and healthy development of the newborn. PACAP has important functions, as a bioactive factor, in reproductive and developmental processes. Shortly after its discovery, PACAP and its receptors were identified in normal and cancerous mammary gland samples. The present review summarizes data obtained in breast milk during different periods of lactation by radioimmunoassay. Our group showed, for the first time, that PACAP is present in the human milk at levels 5- to 20-fold higher than in the respective plasma samples. PACAP-like immunoreactivity (LI) is higher in colostrum compared to transitional and mature human milk samples. PACAP level seems stable until the 10th month of lactation and there after, a significant increase can be observed between samples obtained in the interval 11th–17th months of breastfeeding. The presence of PACAP can also be confirmed in milk and plasma samples from the most commonly used ruminant domestic animals (cow, sheep and goat), pasteurized cow milk and infant formula samples. Similarly to the human results, the concentration of PACAP in the milk whey is almost ten times higher than in the plasma of the respective animals, while pasteurized cow milk and infant formula samples contain PACAP-LI levels comparable to human milk samples. The exact function of PACAP in the milk is not known at the moment. We hypothesize that PACAP (1) is essential for the growth and development of the

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newborn, (2) may be required for the development of the immune system and immunological microenvironment of the gastrointestinal tract, and (3) could be important in the growth and function of the mammary gland.

Keywords PACAP-like immunoreactivity • Milk • Lactation • Mammary gland

Based on the recommendation of the World Health Organization the exclusive breastfeeding is the normative standard for infant feeding for the first 6 months of life [1, 2]. Human milk is a complex biological fluid [3, 4], it contains nutritional components and non-nutritive bioactive factors that promote survival and healthy development of newborn [5]. The nutritional components of human milk vary depending on maternal diet and the period of lactation. The mean macronutrient composition of the mature milk is approximately 0.9–1.2 g/dl for protein, 3.2–3.6 g/dl for fat, and 6.7–7.8 g/dl for lactose. Many micronutrients are also present in the milk including vitamins A, B1, B2, B6, B12, D and iodine [5]. The definition of the bioactive components of the nutrition is "the elements that affect biological processes or substrates and hence have an impact on body function or condition and ultimately health" [6]. The bioactive factors in the human milk originate from different sources: (1) secreted by the mammary epithelium, (2) produced by cells carried within the milk, (3) drawn from maternal serum and transported across the mammary epithelium [5]. Several bioactive factors have been described in the milk, including different hormones (adrenal, gonadal, gastrointestinal, pituitary, hypothalamic), growth factors, prostaglandins, immunoglobulins, cytokines, chemokines, and different neuropeptides [3, 5]. Before the isolation of pituitary adenylate cyclase activating polypeptide (PACAP) Werner et al. [7] described the presence of vasoactive intestinal peptide (VIP), the neuropeptide structurally the closest to PACAP, in milk samples.

Shortly after the discovery of PACAP [8, 9], Skakkebaek et al. [10] investigated the occurrence and distribution of PACAP immunoreactivity in the mammary gland of lactating and non-lactating rats by radioimmunoassay and immunohistochemistry. They showed PACAP-immunopositive nerve fibers associated with blood vessels and smooth muscle surrounding the lactiferous duct of the nipple. PACAP-immunoreactive fibers were present in the subepidermal connective tissue of the nipple, in the mammary parenchyma and around secretory alveoli. Although there was no significant change in PACAP-immunohistochemical level during pregnancy and lactation, the concentration of PACAP38-immunoreactivity was elevated in the extract of mammary gland during lactation [10]. Double immunostaining demonstrated that PACAP-immunoreactive fibers were co-localized with VIP and calcitonin gene-related peptide (CGRP) originated from the neurons of sensory ganglia indicating the role of PACAP in the transport of suckling stimuli centrally [10]. The presence of both PACAP mRNA and PACAP immunoreactivity was also demonstrated in human normal mammary gland samples and breast carcinoma [11]. Normal, peritumoral and tumoral mammary gland samples expressed both

preproPACAP mRNA and protein, the levels of which increased from normal to tumoral breast tissue. Immunohistochemistry showed PACAP-immunoreactivity both in normal and tumoral tissue in the alveolar epithelial cells, but not in the connective tissue. In tumoral samples duct-like structures of some invasive tumors expressed very strong PACAP-immunoreactivity supporting the important role of this peptide not only in physiological conditions but also in tumorigenesis [11–13].

All three PACAP receptors (PAC1, VPAC1, and VPAC2) were identified in normal and cancerous human mammary glands [13–18]. Both mRNA and protein of VPAC1 and VPAC2 receptors, as well as different isoforms (null, hip/hop) of PAC1 receptors were identified in normal, peritumoral, and tumoral breast tissue samples. In normal tissue immunoreactivity to all three PACAP receptors were located in the ductal and glandular epithelial cells. On the other hand, tumoral tissue expressed stronger immunopositivity compared to normal tissue samples. There were no immunopositive signals in the connective tissue stroma of the mammary gland [14]. Zhang et al. [19] demonstrated that the analogs of VPAC1, VPAC2, and PAC1 receptors are potent, have biological activity and it is suitable of further evaluation for accurate PET imaging of benign and malignant lesions of human breast cancers.

In a set of earlier studies, our research group examined the changes of PACAP38-like immunoreactivity (LI) in the serum and milk samples of different species during pregnancy and lactation. First we investigated the concentration of PACAP38 in human plasma of healthy male and female volunteers, pregnant women and lactating women having 1- to 6-month-old babies using radioimmunoassay (RIA) analysis [20, 21]. We found relatively small interindividual differences among healthy volunteers (both sexes, age between 20 and 40 years), there were no significant differences between PACAP38 level of females with different age or hormone cycle. However, in the second and third trimester of pregnancy and during lactation a significant elevation could be observed compared to the earlier gestational period and nonpregnant healthy volunteers [20, 21]. We detected 5- to 20-fold higher concentration of PACAP38 in the milk whey compared to the respective plasma samples [20]. Similarly to our results numerous studies measured higher concentration of bioactive factors in the milk than in the plasma. Several hormones such as estrogen, gonadotropin releasing hormone (GnRH), thyrotropin releasing hormone, VIP, somatostatin, bombesin, neurotensin, oxytocin, prolactin, and different growth factors such as insulin-like, epidermal, transforming growth factors are present in higher concentrations in the milk than in the plasma [3].

It is well known that the composition of milk is changing during lactation based on the requirements of the newborns [5, 22]. Therefore, we next investigated the differences in PACAP38-LI in human milk samples from different periods of lactation. We collected colostrum samples at the beginning of lactation (1–3 days), transitional milk at fourth day of lactation, and mature milk samples every month during the whole period of lactation (1–17 months). Similarly to earlier results showing higher levels of different bioactive factors and nutrients in the colostrum we found significantly higher PACAP38-LI in the colostrum samples compared to transitional and mature milk samples [23]. Higher levels of hormones, growth factors, oligosaccharides,

cytokines, and immune regulating factors in the colostrum have important role in the development of the immune system and protective effects in allergic and inflammatory disorders [24–26]. The first 6 months of lactation have special importance for the development of the newborn, therefore, the WHO recommends exclusive breastfeeding in the first half year of life. We did not find significant alterations in the level of PACAP38-LI during the first 6 months of lactation. We measured a stable PACAP38-LI level until the tenth month and after a significant increase was observed in the samples from 11th to 17th months of lactation [23]. The composition of the milk also depends on the time that passes between the successive sucklings. At the beginning of the suckling the foremilk has lower fat content and later the hindmilk contains more fat and lactose. Several bioactive factors have different concentration in the foremilk compared to the hindmilk. Endothelin-1, ghrelin and cholesterol have higher level, and triglyceride, leptin, retinol have lower level in the foremilk compared to hindmilk [27, 28]. However, we did not find significant difference in PACAP38-LI between foremilk and hindmilk samples similarly to atrial natriuretic peptide and granulocyte colony-stimulating factor [29–31].

Investigation of bioactive factors in the milk of domestic animals has important nutritional value and agricultural significance [32]. Numerous studies investigated different growth factors, hormones in the milk of pig, cow, sheep and goats [33–38]. Therefore, we aimed to compare the PACAP38 level of milk and plasma samples from the most commonly used ruminant domestic animals: cow, sheep and goat. Similarly to our earlier human results the concentration of PACAP in the milk whey was almost ten times higher than in the plasma of the respective animals, and the PACAP38-LI did not change during the 3-month-period of lactation [39]. Fresh cow milk had similar PACAP content as human samples [31]. We also examined PACAP38-LI in the homogenates of sheep udder biopsies with RIA. Localization of PAC1 receptor was also investigated in the lactating and non-lactating sheep mammary gland samples with immunohistochemistry compared to non-lactating human mammary gland samples. In the non-lactating human and sheep samples, a very weak PAC1 receptor immunopositivity was detected in the glandular epithelial cells [23]. In contrast, significantly increased PAC1 receptor expression was detected in lactating sheep mammary gland epithelial cells compared to non-lactating samples, while the surrounding connective tissue remained unstained in all samples [23, 39]. RIA examination of the homogenates of lactating mammary gland samples from sheep showed significantly higher PACAP38-LI 7 days after delivery compared with samples from postpartum 30th days [39].

When mother's milk is unavailable cow-milk based infant formulas are necessary to use. Therefore, in the next step we aimed to measure the PACAP38-LI in pasteurized cow milk and two basic infant formulas, the hypoantigenic and the non-hypoantigenic infant formula samples by RIA. The infant formula samples contain minerals, vitamins, lactose, fat and some proteins for example parathyroid hormone-like peptide [40], parathyroid hormone-related protein [41], but most of the proteins are hydrolyzed during the preparation process. There are many bioactive factors, which cannot be detected in the infant formula, for example insulin-like growth factors, GnRH, antibodies, and enzymes [3, 41]. In our earlier experiment we showed that the pasteurized cow milk and infant formula samples contain PACAP38-LI at

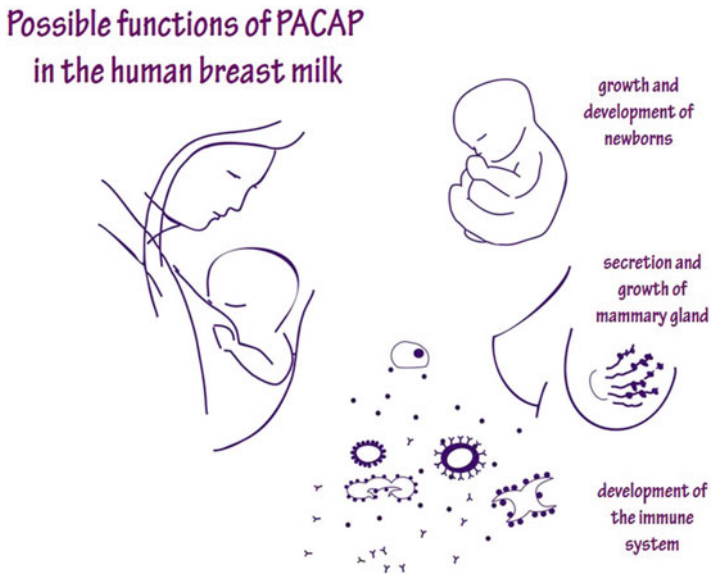

Fig. 49.1 Possible functions of PACAP in the breast milk during lactation

levels comparable to human milk samples. This observation means that because of the low molecular weight PACAP might withstand hydrolysis and the manufacturing processes. Interestingly, we detected higher PACAP level in the hypoantigenic formulas compared to non-hypo antigenic formula samples. These results suggest that PACAP38 could have a carrier not yet known in the milk (such as ceruloplamin) and during the extensive hydrolysis it might be released from the carrier molecule [31].

The exact function of PACAP in the milk is not known at the moment. We hypothesize that PACAP (1) is essential for the growth and development of the newborn; (2) may be required for the development of the immune system and immunological microenvironment of the gastrointestinal tract; (3) could be important in the growth and function of mammary gland (Fig. 49.1).

It is well known that PACAP plays a very important role in the development of the nervous system and other internal organs. PACAP is a potent neurotrophic factor, it plays an important role in neurogenesis, myelinization, neuronal differentiation and migration. A separate chapter of this book summarizes the function of PACAP in neuronal development (Chap. 6. Watanabe et al.). The source of PACAP required for development of the nervous system of the newborn could be partially originating from the milk.

PACAP has well known immunomodulatory effects (Chap. 40. Delgado et al.). We suggest that as other bioactive components of milk, PACAP has a role in protecting newborns against infections and stimulating the development of the immune system and immunological microenvironment of the gastrointestinal tract.

The third possible function of PACAP in the human breast is the regulation of secretion and growth of the mammary gland. Apoptosis plays an important role in the

involution of mammary gland, while during lactation the expression of antiapoptotic factors is elevated by different bioactive factors. PACAP is a potent antiapoptotic factor, and it has influence on cytokines, chemokines, angiogenic factors, and different hormones (estrogen, progesterone, prolactin, and oxytocin) which are also involved in the regulation of lactation [42, 43]. Intravenous PACAP injection increases the prolactin level in human plasma supporting the role of PACAP during lactation [44]. Earlier we examined the effect on the lactogenic hormone-induced terminal differentiation of HC11 mouse mammary epithelial cells. We treated mouse HC11 cells with lactogenic hormones (dexamethasone, insulin, and prolactin) and we measured the β-casein expression to show cell differentiation. We demonstrated that PACAP had no effect on cell differentiation, as PACAP treatment did not influence the β-casein expression. Mouse cytokine and angiogenesis arrays were also used to show the PACAP-induced changes in secreted cytokines, growth and angiogenic factors in differentiated and non-differentiated cells, where PACAP was able to decrease the levels of amphiregulin and epidermal growth factor, which may have physiological implications in the development and progression of mammary gland diseases [45].

The oral bioavailability of PACAP originating from milk is not known at the moment. PACAP has a very short half-life (minutes) in the plasma; exogenous PACAP38 is rapidly degraded in the presence of dipeptidyl peptidase IV [46, 47]. In contrast to the plasma samples PACAP was stable in milk samples, because we could detect PACAP38-LI from milk samples stored in −20 °C without protease inhibitor treatment several days or weeks after the collection. It is known that the mammary gland produces different protease inhibitors which are responsible for the stability of different bioactive proteins and peptides in the milk. In neonates the proteolytic enzymes (including dipeptidyl peptidase) have lower activity and the intestinal epithelium has higher permeability for the macromolecules, and therefore, we suggest that PACAP could be utilized during breastfeeding.

Taken together, the experimental data suggest that PACAP could have an important role during lactation based on its antiapoptotic, immunomodulatory, and neurotrophic effects, but further examinations are necessary to describe the exact functions.

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