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PACAP and VIP signaling in chondrogenesis and osteogenesis

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ABSTRACT

Skeletal development is a complex process regulated by multifactorial signaling cascades that govern proper tissue specific cell differentiation and matrix production. The influence of certain regulatory peptides on cartilage or bone development can be predicted but are not widely studied. In this review, we aimed to assemble and overview those signaling pathways which are modulated by PACAP and VIP neuropeptides and are involved in cartilage and bone formation. We discuss recent experimental data suggesting broad spectrum functions of these neuropeptides in osteogenic and chondrogenic differentiation, including the canonical downstream targets of PACAP and VIP receptors, PKA or MAPK pathways, which are key regulators of chondro- and osteogenesis. Recent experimental data support the hypothesis that PACAP is a positive regulator of chondrogenesis, while VIP has been reported playing an important role in the inflammatory reactions of surrounding joint tissues. Regulatory function of PACAP and VIP in bone development has also been proved, although the source of the peptides is not obvious. Crosstalk and collateral connections of the discussed signaling mechanisms make the system complicated and may obscure the pure effects of VIP and PACAP. Chondro-protective properties of PACAP during oxidative stress observed in our experiments indicate a possible therapeutic application of this neuropeptide.

Development of skeletal elements is influenced by several regulatory peptides, which may derive from the evolving tissue or the surrounding nerve terminals. Production of proper long bone architecture requires a cartilage template and involves time and growth factor dependent activation of precisely defined regulatory mechanisms and signaling cascade systems [1]. Hyaline cartilage is an avascular and aneural tissue [2] with a uniquely organized extracellular matrix. Parallel with the bone formation, vessels and nerves penetrate the cartilage template and release various regulatory factors, which can be responsible for the remodeling of cartilage and initiation of bone matrix production by osteoblasts. During the last decade several theories have emerged regarding the regulation of the formation of these tissues by different autocrine and paracrine mechanisms, with presumed involvement of various regulatory peptides [3–6].

Pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP)

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) are neurohormones and members of the VIP–secretin–GHRH–glucagon superfamily. Originally, both of these short neuropeptides were demonstrated predominantly released in specific areas of the central nervous system [7]. VIP consists of 28 amino acids and is produced by a variety of cells and tissues in addition to neuronal cells, such as specific cells of the intestinal system along with some immune and endocrine cells. Among its diverse physiological effects, VIP has important functions in neuronal development and both in innate and acquired immunity [8].

PACAP was originally isolated from ovine hypothalamus extracts and two bioactive forms were identified: a shorter, 27 amino acid (PACAP 27) and a longer 38 amino acid (PACAP38) form [9]. The N-terminal region of the polypeptide is evolutionary

Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; ECM, extracellular matrix; HH, Hedgehog; IHH, Indian Hedgehog; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; PAC1, pituitary adenylate cyclase-activating polypeptide type I receptor; PACAP, pituitary adenylate cyclase polypeptide; PKA, protein kinase A; PKC, protein kinase C; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; PTHrP, parathyroid hormone related peptide; Runx2, Runt-related transcription factor 2; SHH, Sonic Hedgehog; TGFβ, transforming growth factor-β; VIP, vasoactive intestinal peptide; VPAC, vasoactive intestinal peptide receptor.

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conserved and shows a high homology with that of VIP [7]. PACAP is a pleiotropic neuropeptide with various effects in the central nervous system, including trophic effects during neuronal development and protective effects in neuronal regeneration. This protective effect is one of its most promising features for therapeutic use, even considering the short half-life *in vivo* [10,11]. In the last decade, increasing amount of evidence has emerged regarding the important roles of PACAP in peripheral organs such as uterus [12], ovary [13], testis [14], moreover its presence has been proved in human milk [15]. Nonetheless, only sporadic data exist about its function in skeletal elements [16–18].

PACAP and VIP can be ligands of three main receptors; PAC1, VPAC1 and VPAC2. PACAP binds to PAC1 with the highest affinity, while the latter two attract PACAP and VIP with equal affinity [19]. All of the three receptors are well-characterized G protein coupled receptors, the activation of which induces elevation of intracellular cAMP levels activating protein kinase A (PKA) [7]. This so called "canonic" signaling may lead to the nuclear translocation of CREB transcription factor and consequent activation of the expression of various genes. PACAP binding is also able to control the MAPK pathways, such as ERK and p38 kinases [7]. The versatility of PACAP/VIP receptor induced signal transduction indicates its multifactorial regulation, implying a vast array of signaling connections. This includes, for example, activation of IP_3 receptors inducing the release of Ca^{2+} from endoplasmic reticulum (ER) [20]. The elevation of ic. Ca^{2+} concentration activates various Ca^{2+} dependent signaling molecules such as classical PKCs, MAPK [21] or protein phosphatases like PP2B [22]. The diversity of the developmental function is also hallmarked by the fact that PACAP receptor activation may crosstalk with other signaling pathways such as TGFβ [23], BMP [24], Hedgehog [25] and Notch signalization [26]. Moreover, the general protective and regenerative effects of PACAP originate from its antiapoptotic function [27] and its ability to decrease inflammatory reactions [28].

Regulation of chondrogenesis focused on VIP and PACAP

As articular cartilage has very poor regeneration capacity, the exploration of new strategies to improve replacement or reconstruction of cartilage is very important. Currently, no effective or curative treatment is available for degenerative cartilage diseases such as osteoarthritis. The signaling pathways of proper cartilage development are still under investigation since plenty of the molecular signaling puzzles have neither been solved nor locked in their adequate positions.

Chondrogenic differentiation is a multistep process involving rapid proliferation and condensation of chondroprogenitor cells. Formation of chondrogenic nodules and cartilage specific extracellular matrix production both are required for proper hyaline cartilage development [29]. Transcription factors of the SoxE family such as Sox5, Sox6 and Sox9 are essential for the induction of mRNA expression of cartilage matrix-specific proteins (*e.g.* COL2A1, aggrecan core protein). Sox9 is one of the pivotal signaling elements of chondrogenesis, therefore, its regulation by reversible phosphorylation can be a key momentum of the proper differentiation cycle. Sox9 promoter is known to be regulated by the CREB that binds to a CRE site upstream of Sox9 [30]. We have demonstrated that Sox9 and CREB transcription factors are phosphorylated by PKA during cartilage formation [31,32]. Moreover, a quite complex regulatory mechanism and synergism between Sox9 function and the cAMP–PKA–CREB pathway has been published in both mature and differentiating chondrocytes which includes BMP pathway connections [33]. Additionally, we have shown that the activation of signaling elements phosphorylated by PKA can be equilibrated by a few Ser/Thr protein phosphatases such as PP2A and PP2B [34,35]. Since the regulation of these cartilage specific signaling pathways are cAMP or Ca^{2+} dependent it could be a question of interest whether PACAP/VIP neuropeptides have any signalization connection with proper hyaline cartilage formation.

Only sporadic data exist on the functions of regulatory peptides in chondrogenesis. Role of various regulatory peptides such as VIP are well known in inflammatory diseases; moreover, VIP is a promising agent in the therapeutic treatment of rheumatoid arthritis [11]. Although the articular cartilage is aneural, the surrounding synovial membrane is rich in nerve endings, which may release VIP into the synovial cavity and subsequently induce anti-inflammatory processes [36]. About the functions of PACAP in the adult joints we still have exiguous knowledge despite the fact that PACAP-positive nerve endings were described in cartilage canals of porcine epiphyseal cartilage more than 15 years ago [37]. Our laboratory was the first to demonstrate that the mRNAs of preproPACAP as well as PAC1, VPAC1 and VPAC2 receptors are expressed in chicken "high density" chondrogenic cell cultures. Furthermore, we have shown the expression of the PAC1 receptor protein in chondroprogenitor cells [17] and increased extracellular matrix synthesis was detected during PACAP administration suggesting the positive effect of this neuropeptide in cartilage development. Our findings suggested the presence of PACAP-related autocrine and/or paracrine effects in cartilage itself, reflecting on a possible new signaling mechanism in the regeneration of hyaline cartilage [38,39]. Although the receptors of VIP were expressed by chondrogenic cells in our experiments, others found that this neuropeptide did not influence the matrix production of chondrocytes and synovial cells [40] suggesting certain tissue specific effects of these neuropeptides. Classical downstream targets of PAC1 receptor activation such as PKA, PKC and MAPK signaling cascades play essential role in chondrogenesis [32,35,41]. It has been published that PKA phosphorylates CREB and Sox9 transcription factors [32] and the latter one is a key regulator of chondrogenesis [42]. PACAP administration into the medium of chondrogenic cell cultures increased the phosphorylation both of Sox9 and CREB, and enhanced matrix production of the differentiating cells was also observed [17] (Fig. 1). PAC1 receptor activation can be responsible for the elevation of intracellular Ca^{2+} concentration and ultimately can regulate the Ca^{2+}dependent protein phosphatase PP2B (also known as calcineurin). This enzyme is one of the positive regulators of *in vitro* chondrogenesis [35,41,43]. Therefore, we investigated the involvement of this Ser/Thr phosphatase in PACAP signaling pathways and connection between PP2B activity and PACAP signaling was proved [17] (Fig. 1). A similar interaction has been described in chromaffin cells [44]. These *in vitro* results indicated that the presence of PACAP is essential for proper cartilage formation, however the phenotype of PACAP KO mice [45] did not show any dramatic macroscopical morphological deformation of skeleton. Although the analysis of the genetically modified animals has not been completed yet, our initial observations suggested alterations in the composition of the cartilage extracellular matrix and in the expression of various signaling molecules in the knee joints of PACAP KO mice (our unpublished data). In the reproductory organ system of these mice, the lack of PACAP gene resulted in reduced fertility and altered mating behavior of females [46], moreover the maturation [47] and the morphology [48] of gonadal cells showed notable differences. The complex phenotypic changes raise the possibility of multiple crosstalk of PACAP signaling with developmental pathways connected to various morphogens, as well as certain compensatory mechanisms of PACAP signaling cascades. For instance MAPK and Wnt signaling both play important roles in the proper cartilage formation and tissue patterning [49] and a PACAP-independent PAC1 receptor activation has been directly linked to the regulation of Wnt/β-catenin pathways [50]. Notch signaling activation plays a crucial role in chondrogenesis