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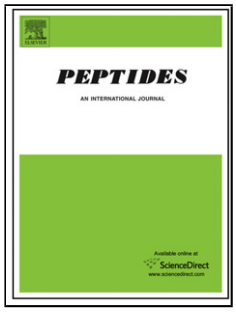

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PACAP and VIP signalling in chondrogenesis and osteogenesis

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Main findings presented in this Manuscript are as follows:

- Elements of VIP and PACAP signalling are present in cartilage and bone cells.
- Exogenous PACAP exerts a positive effect on *in vitro* cartilage and bone formation.
- PACAP plays a chondroprotective role under oxidative stress.

Abstract

Skeletal development is a complex process regulated by multifactorial signalling 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 signalling 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- or 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, however the source of the peptides is not obvious. Crosstalk and collateral connections of the discussed signalling 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.

Keywords

PKA; CREB; hedgehog; BMP; Runx2

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

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 regulating mechanisms and signalling 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 remodelling 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].

1. 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 area of central nervous system [7]. VIP consists of 28 aminoacids and is produced by a variety of cells and tissues in addition to neuronal cells. Among others, specific cells of the intestinal system can produce VIP 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 later 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 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 if 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]. The so called “canonic “signalling activation 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 signalling 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 signalling 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 signalling pathways such as TGFβ [23], BMP [24], Hedgehog [25] and Notch signalisation [26]. Moreover, the general

protective and regenerative effects of PACAP originate from its antiapoptotic function [27] and its ability to decrease inflammatory reactions [28].

2. 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 signalling pathways of proper cartilage development are still under investigation since plenty of the molecular signalling 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 signalling 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 was published in both mature and differentiating chondrocytes which includes BMP pathway connections [33]. Finally, we have shown that the activation of signalling 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 signalling pathways are cAMP or Ca^{2+} dependent it could be a question of interest whether PACAP/VIP neuropeptides have any signalisation 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 have been 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 signalling 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 signalling cascades play essential role in chondrogenesis [32,35,41]. It has been published that PKA phosphorylates CREB and Sox9 transcription factors [32], the latter one being 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 via regulating Ca^{2+} dependent phosphatases such as 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 signalling pathways and connection between PP2B activity and PACAP signalling was proved [17] (Fig 1.), similarly to 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 alteration 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 signalling 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 behaviour 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 signalling with developmental pathways connected to various morphogens, as well as certain compensatory mechanisms of PACAP signalling cascades. For instance MAPK and Wnt signalling 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 signalling activation plays a crucial role in chondrogenesis [51] and exerts modulatory function in osteoarthritis [52] Recently, crosstalk of G protein coupled receptors and Notch signalling has been reported in bacterial LPS induced macrophages [53]. SHH pathway is

another essential positive chondroregulatory pathway [54] and it can be inhibited by PACAP activation [55].

Recently we have demonstrated a chondro-protective effect of PACAP in chondrogenic cell cultures where the administration of the neuropeptide compensated the harmful effects of oxidative stress. It has been shown that PACAP can prevent the harmful effects of cerebral ischemia or oxidative stress induced apoptosis in the central nervous system [56]. PACAP deficient mice showed higher sensitivity to injury during retinal ischemic conditions, axonal lesion, intestinal inflammation or oxidative stress of the kidneys [57]. The presence of PACAP/VIP had preventing role in rheumatoid arthritis [58,59], and cardioprotective effects of these peptides have also been demonstrated [60]. In the light of these data, the cartilage protecting effect of PACAP was predictable; however the exploration of the molecular background of this phenomenon has only started yet. In chicken chondrogenic cells, the addition of PACAP 1-38 during oxidative stress prevented the inhibition of cartilage matrix production by free oxygen radicals and the increased activity of PKA seemed to take part in this compensatory effect [17]. The addition of the neuropeptide also exerted effect on matrix metalloproteinase (MMP) expression in chondrogenic cell cultures in the presence of reactive oxygen species (our unpublished data). Similar results have been published in alveolar cells where both VIP and PACAP were able to decrease the expression of certain MMPs and reduced the activation and expression of caspase3 [61]. VIP and its receptors are expressed in synovial fibroblasts [62] and it enables the release of inflammatory factors either by these cells or immunocompetent cells residing in the surrounding synovial tissues [63]. Finally, PACAP has been shown to have modulatory effects on inflammatory processes of rheumatoid arthritis [64]. These data all strongly suggest that PACAP is a promising future therapeutic agent in inflammatory and degenerative joint diseases [65].

3. VIP and PACAP in osteogenic signalling cascades

Similarly to chondrogenic differentiation, proper osteogenesis requires high spatial and temporary organization supported by complex bone specific developing mechanisms and signalling. Development of this skeletal tissue involves differentiation of osteoblasts from osteoprogenitors. It is followed by an initial deposition of a bone specific organic ECM abundant in collagen type I completed with certain bone specific matrix components such as osteocalcin or osteonectin. This osteoid undergoes calcification then meaning deposition of calcium hydroxyapatite crystals in the bone matrix with active contribution of osteoblasts. Differentiation of osteoblast is regulated by three main signalling cascades such as BMP, WNT and Hedgehog cascades [66-68]. BMPR activation subsequently induces the phosphorylation of Smad1/5 and with the help of Smad4 the complex is translocated into the nuclei of osteogenic cells and initiates expression of bone specific genes such as the transcription factor osterix, alkaline phosphatase (ALP) or collagen type I [69,70]. The expression of BMPs is regulated by CREB transcription factor activated via PKA signalling pathways [70]. On the other hand a well balanced expression of hedgehog signalling elements governed by another bone specific transcription factor, Runx2 is also essential for proper long bone formation [71]. Runx2 can be directly phosphorylated by PKA [72] and subsequently activates the expression of bone specific signalling elements or ECM components. This complex signalisation involves broad spectrum crosstalk opportunities with the PACAP/VIP signalisation, further highlighting the significance of neuropeptide signalling in bone formation and regeneration.

During endochondral ossification, after the invasion of vessels and nerves into the cartilage template osteoprogenitor cells start to migrate into the diaphysis of the developing long bone and differentiate into osteoblasts. This process can also be regulated by neuropeptides [73]. During the elongation of long bones PACAP positive nerve fibers penetrate the bone matrix

[37]. VIP positive sympathetic nerve endings were also identified releasing these neuropeptides [74]. As an interesting observation, receptor composition and effects of VIP exhibited differences in cells of bones developed in different ways (i.e. membraneous or endochondral). Moreover, the direct communication of sympathetic nerve fibers with osteoblasts showed an embryonic origin dependent response and signalisation, suggesting that the innervation of periosteum by peptidergic fibers plays important function both in bone regeneration and formation [75]. The role of PACAP and VIP in osteogenesis was further supported by the observations where MC3T3 E1 mouse calvaria derived osteoblast cell line [76] and UMR-106 cells isolated from rat osteosarcoma [16] were shown both expressing the receptors for these neuropeptides. Accumulation of cAMP in osteoblasts is proved to be as a result of combined activation of PACAP and VIP and regulates diverse signalling pathways influencing osteoblast differentiation. In line with this, presence of certain neuropeptides was shown to be elevated after bone fracture, indicating their importance in successful regeneration [77]. A recent report demonstrated release of various neuropeptides from periosteal nerve endings resulting in enhancement of intercellular communication and increased metabolic activity of osteoblasts [78]. As it was described above, osteogenic transformation, bone matrix production and mineralization are regulated by multiple signalling cascades [79], where the activation of MAPK and PKA plays essential roles. Runx2 is one of the key transcription factors which governs osteoblast differentiation [80] and it is regulated by PKA signalling pathways [81]. We have demonstrated that the administration of PACAP into the medium of UMR-106 cell line enhanced the nuclear translocation of Runx2 and increased expression of collagen type I, ALP and osterix genes was observed (Fig. 2.). Interestingly, the phosphorylation of CREB by PKA was not remarkably increased after PACAP addition in this ostesarcoma derived cell line [16] (Fig 2.). BMP signalling pathway is another fundamental regulator of osteogenesis and crosstalk with Runx2 has been reported

[83]. Moreover, the TGFβ/BMP pathways are activated by PACAP or VIP [24]. Indeed, the administration of PACAP increased the expression of BMPs in UMR-106 cells and expression of BMPR1, one of its major receptors, became also elevated. As a consequence of BMPR activation, a pronounced elevation of the nuclear presence of Smad1 transcription factor was detected under the effect of PACAP administration [16] (Fig 2.). VIP can also be regulated by TGFβ/BMP signalling pathways as Smads may activate VIP expression [85] suggesting a complex reciprocal signalling with numerous compensatory escape routes during bone development [16].

PACAP and VIP may directly activate ERK1/2 e.g. during adipogenesis [86] or in osteoblast cells [87], furthermore CREB phosphorylation is regulated by the MAPK system in MC3T3 cells [88]. Additionally, intracellular Ca^{2+} concentration can be elevated by PACAP [89] or VIP [90], resulting in an activation of classical PKCs and ERK both influencing osteoblast differentiation [91]. Nonetheless, PACAP treatment of UMR-106 cells did not alter the Ca^{2+} concentration of these osteoblast cells, and activation of classical PKCs was not detected, in our experiments [16] (Fig 2.). Ca^{2+} influx can be evoked by PACAP [92] and the presence of PACAP and VIP is able to decrease the Ca^{2+} entry via L- and N-type calcium channels in neurons [93]. It is known that the administration of PACAP affects Ca^{2+} oscillation [94] and alters the Ca^{2+} related vesicular transport of chromaffin cells [95]. Besides this dynamic alteration of intracellular Ca-homeostasis, PACAP also exerts effects on matrix mineralisation. We found that addition of PACAP elevated the deposition of inorganic matrix components in the ECM of UMR-106 cells [16]. Moreover, an altered mineralisation was detected during tooth formation of PACAP deficient mice [96], suggesting a yet unknown connection between PACAP and Ca^{2+} release of osteoblasts, ameloblasts and/or odontoblasts. As a possible mechanism for PACAP induced extracellular Ca^{2+} accumulation during osteogenesis, calcitonin gene-related protein was proved to effect on osteoclast function [97]

and the presence of PACAP decreased the matrix-resorption and consequent Ca-release by these cells [95,96].

Hedgehog signalling is of key importance amongst the regulatory mechanisms of bone and cartilage development [71]. A well defined balance between Indian Hedgehog (IHH) and Parathyroid Hormone Related Peptide (PTHrP) is essential for proper long bone formation, regulation of proliferation and matrix production of osteoblasts via the activation of Runx2 transcription factor [98]. PTHrP directly communicates with PKA signalling inducing the activation of CREB and NFAT factors in osteoblasts [99]. In UMR-106 cells the application of PACAP elevated the expression of PTHrP without altering the IHH expression [16]. Sonic Hedgehog (SHH) pathway is known to be regulated by PACAP signalling [55] and the activation of PKA downregulates the function of Gli1, which consequently decreases the proliferation [25]. In PACAP KO mice, enhanced SHH signalling was detected during tooth development [94]. On the contrary, exogenous administration of PACAP elevated the expression of SHH and a more pronounced nuclear presence of Gli1 was found in rat UMR-106 cells [16]. This contradiction may stem from the osteosarcoma origin of UMR cells, as malignant cells can exhibit alterations of various signalling mechanisms. Although we do not have data about the possible function of VIP in osteogenesis, previous results suggest that multifactorial signalling pathways of these regulatory peptides exert modulatory effect on matrix production and differentiation in bone development [100].

Conclusion

Regulatory pathways of PACAP and VIP form a complex signalling network indicating the communication of a huge variety of signalling cascades accomplishing and supporting the diverse functions of these regulatory peptides. Different compensatory mechanisms can switch on or off upon activation or inactivation of certain signalling cascades in the

interconnected system, which can obscure the physiological function of PACAP and/or VIP during chondrogenesis and osteogenesis. Better understanding of the functions of these neurohormones during skeletal development may help us to find possibilities for their therapeutic application in various skeletal diseases.

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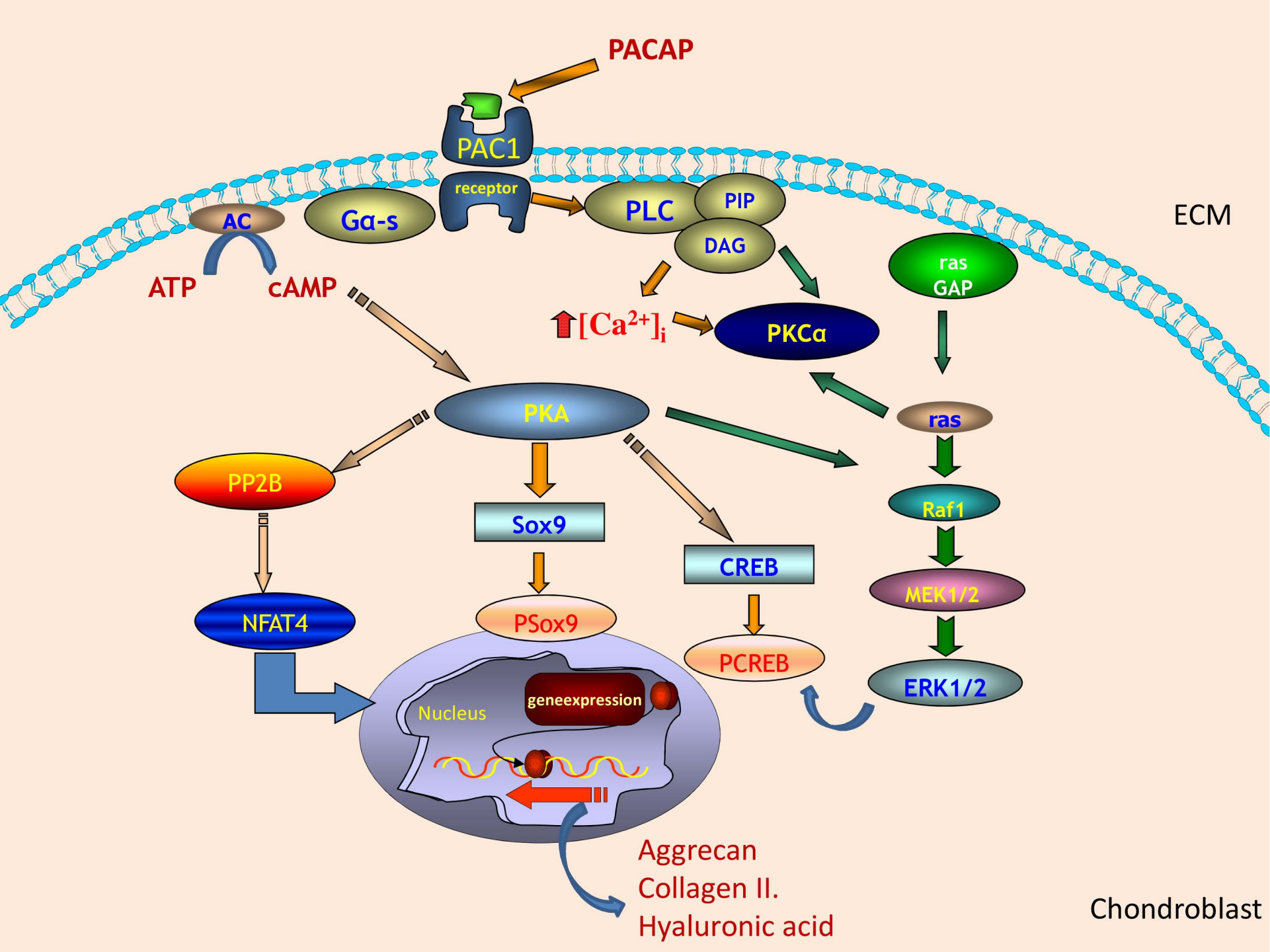
Figure 1. Signalling pathways of PACAP induced chondrogenesis. The increased concentration of cAMP level elevates PKA activity. Phosphorylated form of the downstream targets of PKA such as CREB and Sox9 translocate into the nucleus of chondrogenic cells and induce the gene expression of collagen type II., aggrecan and various GAG such as hyaluronic acid. Activation of PAC1 receptor can also elevate the intracellular Ca^{2+} concentration leading to increased PP2B, PKC or MAPK signalling activity. The elevated expression and nuclear presence of PP2B regulated NFAT4 are also responsible for the augmented matrix production.

Figure 2. Multiple regulation connections' of PACAP signalling pathways in osteogenic differentiation. PACAP binding to its receptors elevates the intracellular cAMP concentration and activates PKA in osteoblast cells. CREB, the canonical downstream target of the kinase is not significantly activated (arrows crossed by red lines) but the nuclear localisation of Runx2 is elevated. Although the cAMP regulated pathway is active the presence of the neuropeptide does not result in a Ca^{2+} concentration increase, subsequently the Ca^{2+} dependent signalling pathways are not activated (arrows crossed by red lines). PACAP also induces the expression of BMPs which may crosstalk via the nuclear activity of Smad1with Runx2 transcription factor. SHH binding to PTCH1 receptor can induce the nuclear translocation of Gli1 transcription factor which is suppressed by the increased activation of PKA.


PACAP
PAC1
receptor
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DAG
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[Ca2+]i
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PKA
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Sox9
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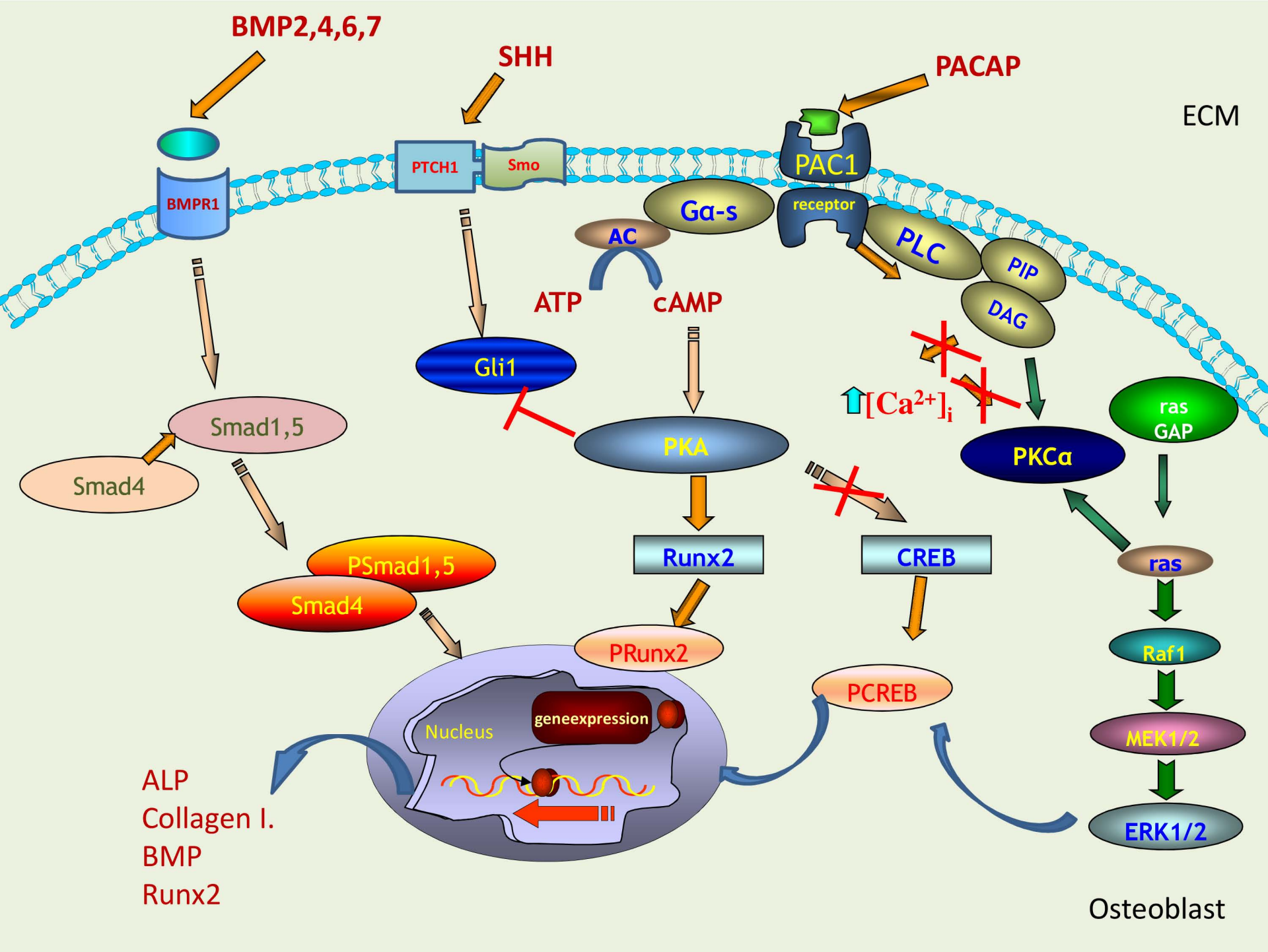
Aggrecan
Collagen II.
Hyaluronic acid
Chondroblast


BMP2,4,6,7
SHH
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Smad4
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PKCα
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GAP
PSmad1,5
Smad4
Runx2
CREB
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Raf1
PRunx2
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MEK1/2
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Collagen I.
BMP
Runx2
Osteoblast