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Review

The complexity of parathyroid hormone-related protein signalling

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Abstract. Our understanding of the mode of action of parathyroid hormone-related protein (PTHrP) has changed profoundly during the last decade. Most PTHrP activities are mediated by membrane receptors through autocrine/paracrine pathways. However, both endogenous and exogenous PTHrP also appear to have intracrine effects through translocation into the nucleus. The present review proposes unconventional PTHrP signalling, based on novel clues. First, PTHrP binding to its membrane receptor triggers internalization of the whole complex, mediated by beta-arrestin. There is growing evidence that the receptor and arrestin are the effectors of biological responses, rather than the ligand (or in addition to the ligand). Second, the existence of putative PTHrP targets within the cytoplasm is beginning to be supported. Recent findings of interactions between a COOH-terminus of PTHrP and beta-arrestin and between the PTHrP receptor and 14-3-3 proteins represent the starting point for identification of intracellular partners of both the hormone and its receptor.

Key words. PTHrP; PTHrP receptor; arrestin; intracrine; proliferation; apoptosis.

Introduction

Parathyroid hormone-related protein (PTHrP) was originally described as the factor responsible for the hypercalcemic paraneoplastic syndrome called humoral hypercalcemia of malignancy [1].

PTHrP has limited homology to PTH in its N-terminal region and can bind the same receptor as PTH with similar biological effects. In fact, PTHrP, like PTH, is a calciotropic hormone. Both PTHrP and PTHrP receptor are expressed in bone cells and chondrocytes [2]. Their expression has been evaluated in a variety of species at different ages and by using different techniques. Although the results are somewhat conflicting, it is clear that there is a temporal and spatial relationship between expression of PTHrP and its receptor during osteogenesis. Therefore, a paracrine-autocrine role for PTHrP in bone development has been suggested and confirmed by the fact that targeted disruption of the PTHrP gene in mice results in skeletal dysplasia [3, 4].

However, PTHrP has a much broader spectrum of effects. It is also a smooth muscle relaxant, a developmental factor and a controller of cell growth and cell death in almost every target cell [5]. PTHrP, although present in the serum, acts prevalently in an autocrine/paracrine manner and is widely distributed in most if not all tissues in the body [6, 7].

PTHrP should be described as a polyhormone. In fact, a family of peptides arises from alternative splicing of the primary transcript. In addition, by alternative posttranslational cleavage sites, N-terminal, mid-region and C-terminal peptides are generated, with distinct physiological functions and probably distinct receptors on the cell surface [8–10].

However, only the type I PTHrP receptor (PTH1R), which binds both PTHrP 1-36 and PTH 1-34, has been

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cloned to date [11, 12]. PTH1R is a class II G-protein-coupled receptor (GPCR) that couples with the adenylate cyclase-protein kinase A (AC-PKA) and phospholipase C-protein kinase C (PLC-PKC) pathways [12]. The distribution and functional properties of PTH1R have been extensively described elsewhere [13]. Little is known about the receptors for other PTHrP peptides since no direct molecular evidence for them has been reported to date. Their existence has been inferred from the fact that certain mid-region and C-terminal PTHrP species have biological effects that neither PTH nor N-terminal PTHrP possess, for example in placenta [14] and bone [15].

On the other hand, the recently identified receptor for PTH (PTH2R) appears to be relatively unresponsive to PTHrP fragments [16]. Unlike PTH1R, which has a widespread tissue distribution, PTH2R is more tissue specific, being prevalently expressed in brain and pancreas.

In this paper, we will review the most recent advances in the mode of action of PTHrP, distinct from the classical transduction cascades, including the following aspects: (i) updated reappraisal of PTHrP as an intracrine hormone; (ii) emergent evidence for nuclear localization of PTHrP peptides lacking a classical nuclear localization sequence (NLS); (iii) emergent evidence for novel roles of PTHrP in the cytoplasm suggested by the interaction of a C-terminal PTHrP peptide and beta-arrestin; (iv) PTH1R protein and beta-arrestin contributions in mediating cellular responses to the hormone.

PTHrP intracrine pathway

PTHrP is currently considered a member of the family of intracrines [17]. Intracrine is the term used to define peptides that are found in the extracellular space, bind to cell surface receptors with the production of a biological effect and are associated with one or more intracellular organelles not involved in the secretory pathway.

As discussed in details in [17], it has become clear that intracellular peptide action is not rare among hormones. Indeed, a growing number of peptide hormones and growth factors have been shown to operate in the intracellular space after either internalization or retention in the cells that synthesize them.

Although most of the documented biological activity of PTHrP is mediated via a signal transduction cascade initiated at membrane receptors, it has become apparent over the past few years that some of the biological actions of PTHrP are cell surface receptor independent and mediated through an intracrine mechanism based on nuclear localization of the peptide.

The presence of PTHrP inside the nucleus has been demonstrated in a number of cell types. The first report is of Henderson et al. in chondrocytes, where PTHrP was found to delay apoptosis induced by serum depletion [18]. In subsequent years, PTHrP intracrine action has been found to be operative in many other target cells. However, as noted earlier by de Miguel et al. [19], the functional consequences of nuclear localization are quite different in different cell types and are likely cell specific. For example, in vascular smooth muscle (VSM) cells, nuclear entry results in enhanced proliferation, rather than in delayed apoptosis. Moreover, nuclear localization can be diffused or concentrated in the nucleolar compartment depending on the cell type, for example VSM cells [19] vs. chondrocytes [18]. It is still unclear whether the different biological effects are linked to different nuclear distribution. From the two examples mentioned above, it appears that diffuse nuclear distribution is associated with proliferative responses (VMS cells), while nucleolar concentration is associated with delayed apoptosis (chondrocytes). Both endogenous and transfected PTHrP bind messenger RNA (mRNA), and a role of PTHrP in the metabolism of mRNA was suspected early on by Aarts et al. [20]. The same group recently demonstrated that nucleolar PTHrP reduces the synthesis of ribosomal RNA (rRNA) in PTHrP-transfected chondrogenic cells. They hypothesized that downregulation of rRNA synthesis could prevent progression through the cell cycle and apoptosis in an unfavourable environment [21].

In addition to modulating proliferative behaviour, the PTHrP intracrine mechanism has also been reported to enhance interleukin (IL)-8 expression in prostate cancer cells [22], further confirming that the functional effects of nuclear PTHrP entry are likely to be more widespread than has been obvious to date, as already suspected by de Miguel et al. [19].

Furthermore, recent studies suggest that the protein likely covers intracrine roles even more complex than those mentioned above. Indeed, in VSM cells, it stimulates mitogenesis after binding to the nucleus, but inhibits proliferation after binding to surface receptors [23]. This dual and paradoxical behaviour has also been described in other cell types such as MCF-7 breast cancer cells [24].

Several mechanisms have been invoked to explain the presence of PTHrP, a prototypical secretory protein, in the nuclear compartment. They include endocytosis via a PTH1R-independent mechanism [25] and via the PTH1R [26], as well as redirection of a nascent protein from the secretory pathway [27, 28]. This explains how PTHrP can exert opposite effects in the cells expressing it: the protein can be synthesized either in forms destined for extracellular transport or in forms lacking the secretory signal and thus confined to the cell interior.

However, the question is more complicated. In fact, in the case of exogenous PTHrP, we must take into account that the hormone can work either in a paracrine or in an intracrine way. The simultaneous involvement of both extracellular and intracellular mechanisms implies a three-

step process: binding to surface receptors, receptor-dependent endocytosis, and transport to the perinuclear structure.

As far as PTHrP receptor-mediated endocytosis is concerned, it is well known that PTH1R undergoes phosphorylation and rapid internalization after agonist binding. In fact, after stimulation with agonist, GPCRs become phosphorylated by G-protein-coupled kinases (GRKs), and most of them translocate cytosolic arrestin proteins to the cytoplasmic membrane. Binding of arrestin to agonist-activated receptors has at least two consequences: uncoupling of the receptors from G proteins, resulting in termination of agonist-mediated signalling and downregulation, and facilitation of receptor internalization via a classic clathrin-coated pits-mediated pathway [29]. This process requires active conformation of the receptor but does not require participation of second-messenger products of receptor signalling or G protein activation [30, 31].

Among the multiple natural and/or synthetic peptides of the PTH/PTHrP family that bind to PTH1R, not all exhibit the same efficiency in promoting receptor internalization. For example, PTH 1–36 is more potent than 1–28, which in turn is more efficient than 1–14. On the other hand, PTH 3–34 binds to the receptor with high affinity but does not stimulate internalization [31].

Although no pharmacological information exists about analogue PTHrP fragments, they likely possess similar properties. In fact, the first 13 amino acids are homologous to PTH, and the region 14–34 of both peptides shares a similar three-dimensional conformation [7].

Moreover, internalization is neither limited to N-terminal PTHrP peptides, nor linked to the PTH1R. In fact, Aarts et al. have discovered that the biotinylated 87–107 PTHrP peptide can be endocytosed either in the presence or in the absence of the PTH1R. They suggested that a cell surface protein, distinct from the PTH1R, mediates peptide internalization [25].

Nuclear import of PTHrP peptides

The full-length PTHrP molecule contains a bipartite nuclear localization sequence (NLS), which is absent in the 84-amino acid PTH protein. PTHrP NLS is similar to the one found in viral and mammalian transcription factors and composed of multibasic amino acids in the 88–91 and 89–106 region of the peptide [18, 23].

It has been demonstrated that the NLS of PTHrP is necessary to gain access into the nuclear compartment [18]. A subsequent study by Massfelder et al. on VSM cells has also shown that both the bipartite NLSs are involved in nuclear entry [23]. Moreover, the same group has investigated the role of a panel of PTHrP sequences and found that in addition to the well-defined NLS sequence, the C-terminus of PTHrP (amino acids 108–139) is also necessary to stimulate mitogenesis inside the nucleus of transfected A-10 VSM cells [19].

NLS-dependent nuclear protein import can be mediated by several nuclear import receptors (importins). Importin beta is the nuclear import receptor for PTHrP and the region for importin beta binding has been mapped to PTHrP 67–94 [32]. Further studies by Lam et al. have also shown the importance of PTHrP phosphorylation status in its nuclear migration. In fact, importin beta binding is inhibited when PTHrP is phosphorylated at Thr 85 by cyclin-dependent kinases [33]. Up-to-date information about PTHrP trafficking from and to the nucleus can be found in a recent, detailed review [34].

While most of the described PTHrP nuclear effects require an intact 87–107 sequence, nuclear migration of PTHrP peptides lacking the NLS has been reported too. In chondrocytes, the tetrabasic 147–150 motif is required for PTHrP 1–173 nuclear localization [35]. Further, in prostate cancer cells, PTHrP 1–87 has recently been found in the nucleus [22]. Although the nuclear import pathways of these last peptides have not yet been identified, these new findings raise the possibility that many PTHrP fragments, in addition to the ones identified so far, could have intracrine action by association with other nuclear targeting proteins. A number of proteins do not possess an NLS but still have a nuclear mechanism of action. For instance, the transcription factor STAT does not have an NLS but is translocated to the nucleus upon activation by certain cytokines and growth factors [36].

Among the many PTHrP peptides that can be internalized, to date, only the ones containing a NLS sequence have been associated with biological changes. However, if a cell expends energy for internalization, there must be a purpose. Therefore, we can expect that PTHrP fragments that can be internalized, but whose intracellular function is not known, could be imported into the nucleus by yet unknown mechanisms. As already hypothesized by Lam [32] and de Miguel [19], it is also possible that PTHrP could function as a transcription factor and directly interact with DNA. Although direct evidence of PTHrP binding to nuclear factors or to chromatin is missing, this possibility has been deduced on the basis of the ability of PTHrP to accumulate in the nucleus in vitro, in the absence of an intact nuclear envelope [32].

While these intriguing aspects remain speculative, there is emerging evidence that ligand-receptor complex endocytosis is per se a signalling pathway.

Indirect PTHrP signalling mediated by the receptor

In addition to the ligand, this pathway may involve the receptor and/or receptor-associated proteins such as arrestin.

It was recently reported that beta-arrestin itself contributes to GPCR signalling, acting as a scaffold for extracellular regulated kinase (ERK) activation. However, the beta-arrestin-dependent increase in ERK 1/2 phosphorylation results in an attenuated proliferative response. In fact, the functional role of the receptor-beta-arrestin complex is to control the subcellular localization of activated ERK, which is retained in the cytosol and inhibited in its translocation into the nucleus [37–40].

Furthermore, very recent studies have demonstrated that a truncated form of beta-arrestin (1–36) interacts with the COOH-terminus of PTHrP (122–141). By analogy with the cytoplasmic retention of activated ERK, it has been postulated that the binding of PTHrP with beta-arrestin may retain the hormone in the cytosol, suggesting a role of PTHrP inside the cytoplasm [41].

It is becoming increasingly evident that some of the effects of PTHrP depend on its receptor rather than on the ligand itself. From this point of view, the ligand, independently of its nature, can be considered as merely a way to trigger internalization. Indeed, since most internalized PTHrP peptides probably do not either reach the nucleus or bind to cytoplasmic targets, their role might be to promote receptor endocytosis. This process might have two purposes, to switch the membrane signal off and to trigger an intracellular delayed response mediated by the receptor protein. The nuclear localization of the PTHrP receptor was demonstrated in 2000, and a role for the receptor protein in nuclear events was hypothesized [26]. Very recently, it was demonstrated that internalized PTH1R interacts with 14-3-3 proteins [42], a family of phosphoserine/phosphothreonine-binding molecules that control the function of a wide array of cellular proteins. It has been suggested that one function of 14-3-3 proteins is to support cell survival. This is due in part to an antagonistic effect on pro-apoptotic proteins such as Bad, Bax and apoptosis signal-regulating kinase (ASK1). 14-3-3 binding molecules might be useful to antagonize 14-3-3 anti-apoptotic activity [43]. It has even been proposed that the efficacy of conventional anticancer agents could be enhanced by targeting 14-3-3-ligand interactions [44].

By integrating the most recent findings, we can introduce another level of complexity into the concept of PTHrP signalling. In fact, it is likely that internalization of both PTHrP and its receptor modulates cell proliferation through a second wave of signalling by two opposite and simultaneous actions: a direct nuclear mitogenic effect mediated by the hormone and a cytoplasmic pro-apoptotic activity mediated by the receptor protein.

Since PTHrP binding to PTH1R can result in either mitogenic or antimitogenic responses, depending on the cell type [45], the second wave of signalling may represent a counteracting phenomenon and, at the same time, an amplified, long-lasting membrane effect.

Conclusions

In view of the preceding remarks, it is not surprising that both the old and the new literature report discordant effects of PTHrP in different cell types and/or under varying experimental conditions. As far as PTHrP proliferative effects are concerned, both positive and negative actions have been described depending on the cell type, the cell state or both [45].

However, most of the discrepancies can now be explained. They are linked to different signal transduction pathways (cAMP/PKA and PLC/PKC cascades), different PTHrP fragments (each likely acting through its own receptor) or different modes of action of the peptide (autocrine/paracrine or intracrine).

While recent advances have contributed to a better understanding of PTHrP physiology, the new nuclear functions and the recently identified cytoplasmic partners of PTHrP further extend the potential pathophysiological role of this extraordinary peptide and open exciting areas of investigation. Although it seems clear that the nuclear localization of PTHrP is associated with cellular proliferative and/or anti-apoptotic responses, it has not been explained yet how PTHrP exerts these effects. The clarification of the nuclear and/or cytoplasmic actions of PTHrP would add significantly to our present understanding of this complex protein.

The possible implications for therapeutic purposes are also evident. In particular, since PTHrP nuclear localization in cancer cells that overexpress it is associated with delayed apoptosis and proliferation, strategies to block the nuclear import of certain PTHrP sequences could lead to reduced tumorigenic potential.

Finally, the recent discovery of binding between internalized PTHrP receptors and 14-3-3 proteins might lead to the use in anticancer therapy of selected nontransducing PTHrP fragments with high affinity to the membrane receptor, able to promote receptor endocytosis.

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