Effects of calcium-sensing receptor on the secretion of parathyroid hormone-related peptide and its impact on humoral hypercalcemia of malignancy

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Chattopadhyay, Naibedya. Effects of calcium-sensing receptor on the secretion of parathyroid hormone-related peptide and its impact on humoral hypercalcemia of malignancy, Am J Physiol Endocrinol Metab 290: E761–E770, 2006; doi:10.1152/ajpendo.00350.2005.—The extracellular calcium-sensing receptor (CaR) plays a key role in the defense against hypercalcemia by “sensing” extracellular calcium (Ca$^{2+}$) levels in the parathyroid and kidney, the key organs maintaining systemic calcium homeostasis. However, CaR function can be aberrant in certain pathophysiological states, e.g., in some types of cancers known to produce humoral hypercalcemia of malignancy (HHM) in humans and animal models in which high Ca$^{2+}$, via the CaR, produces a homeostatically inappropriate stimulation of parathyroid hormone-related peptide (PTHrP) secretion from these tumors. Increased levels of PTHrP set a cycle in motion whereby elevated systemic levels of Ca$^{2+}$ resulting from its increased bone-resorptive and positive renal calcium-reabsorbing effects give rise to hypercalcemia, which in turn begets worsening hypercalcemia by stimulating further release of PTHrP by the cancer cells. I review the relationship between CaR activation and PTHrP release in normal and tumor cells giving rise to HHM and/or malignant osteolysis and the actions of the receptor on key cellular events such as proliferation, angiogenesis, and apoptosis of cancer cells that will favor tumor growth and osseous metastasis. I also illustrate diverse signaling mechanisms underlying CaR-stimulated PTHrP secretion and other cellular events in tumor cells. Finally, I raise several necessary questions to demonstrate the roles of the receptor in promoting tumors and metastases that will enable consideration of the CaR as a potential antagonizing/neutralizing target for the treatment of HHM.

G protein-coupled receptor; osteolysis; metastasis; cytokine; receptor tyrosine kinase

HUMORAL HYPERCALCEMIA OF MALIGNANCY AND THE ROLE OF PTHrP

HYPERCALCEMIA IS USUALLY DEFINED as a state in which serum calcium concentrations are greater than 12 mg/dl, corrected for serum albumin concentration. Hypercalcemia frequently arises from primary hyperparathyroidism or in the context of various malignancies including, most frequently, breast, prostate, lung, and renal carcinomas (for review see Ref. 41). Many factors, including vascular endothelial growth factor (VEGF) and interleukin-8 and -11, have been implicated in promoting hypercalcemia of malignancy (41). However, parathyroid hormone-related peptide (PTHrP) has now been shown to be the major pathogenic factor (31, 98–100). This is because the tumor burden and bone lesions have been shown to be decreased significantly by treatment with PTHrP-neutralizing antibody in mice inoculated with a human breast cancer cell line [MDA-MB-231 (33)] or lung squamous cell carcinoma-derived cells [HARA (44)]. When produced in excess by extraskeletal tumor cells, i.e., in humoral hypercalcemia of malignancy (HHM), PTHrP spills into the systemic circulation and acts on the same PTH receptor that mediates the extracellular calcium (Ca$^{2+}$) elevating actions of PTH on bone and kidney. The resultant hypercalcemia can rapidly become severe and life threatening.

HHM is one of the most frequent paraneoplastic syndromes. Circulating levels of PTHrP are elevated in 80% of the patients with HHM (98). Up to two-thirds of HHM patients have bone metastases, in agreement with the “seed and soil” theory of metastases proposed by Paget (77), as bone seems to provide a fertile environment for the growth of tumor cells and enhances the production of PTHrP.

Bone metastases can be osteolytic, osteoblastic, or mixed (21). Because of their capacity to stimulate osteoclastic bone resorption, tumor cells (particularly those from breast tumors) may enrich the bone microenvironment with growth factors that favor tumor growth and metastasis (for review see Ref. 47). Mineralized bone matrix is a unique storage site of immobilized growth factors such as transforming growth factor (TGF)-β, insulin-like growth factor (IGF) I and II, fibroblast growth factor (FGF)-1 and -2, and platelet-derived growth factors (PDGF) within bone matrix. TGF-β plays a major role in metastasis through its local release in active form during osteoclastic resorption and then the stimulation of PTHrP production by tumor cells, thus expanding the cycle between the tumor cells and bone cells (48).
Tumor metastasis to bone can also be osteoblastic, giving rise to the net formation of disorganized new bone, as frequently observed in prostate cancer (52). Endothelin-1 (ET-1), which is secreted by tumor cells, appears to be a key factor in stimulating bone formation (120). A selective antagonist of ET-1 blocks prostate cancer metastasis and is undergoing clinical trials in men with advanced metastatic prostate cancer (for reviews see Refs. 32 and 74).

Mixed osteoclastic/osteoblastic metastasis can occur in both breast and prostate cancers. However, few studies have been done on the combined actions of osteoclastic and osteoblastic factors on bone, and therefore the overall response of bone at the metastatic site remains unpredictable.

Bone is the depot for 99% of the body’s calcium, and one of the direct effects of increased bone resorption during HHM is an increase in Ca\(^2+\) released during the resorptive process into the bony microenvironment that could modulate the normal remodeling process as well as metastatic osteolysis. The levels of Ca\(^2+\) in the vicinity of resorbing osteoclasts are manyfold higher than the level of systemic Ca\(^2+\) (i.e., ranging from 8 to 40 mM) (97). These large changes in local Ca\(^2+\) could be “sensed” by the cancer cells as well as nearby cells, such as osteoblasts, osteoclasts, and monocytes. Earlier reports suggested that high Ca\(^2+\) stimulates PTHrP production in both normal (10, 58) and malignant cells (71, 87). However, an extracellular calcium-sensing receptor (CaR) could enable Ca\(^2+\) to contribute directly to this vicious cycle by stimulating the production of PTHrP by the cancer cells instead of being a mere by-product of these malignancies (12). Under these circumstances, the CaR could serve as a central element in a physiologically inappropriate “feed-forward” mechanism, whereby malignant osteolysis begets further osteolysis (Fig. 1). CaR-stimulated secretion of PTHrP could offer a selective advantage by contributing to tumor cell survival and growth and/or skeletal complications (e.g., osteolysis), as PTHrP has been shown to promote growth and survival of tumors that secrete it (95, 96).

**EXTRACELLULAR CALCIUM-SENSING RECEPTOR**

The CaR is a key mediator of direct actions of Ca\(^2+\) on parathyroid and kidney and regulates homeostatic responses that restore Ca\(^2+\) to its normal levels (12, 13, 85). The CaR has the “signature” G protein-coupled receptor (GPCR) topology, a seven-membrane-spanning, “serpentine” domain, as well as a large extracellular ligand-binding domain and an intracellular COOH-terminal domain. Activation of the CaR by high Ca\(^2+\) results in suppression of PTH release (13, 18). Therefore, in the parathyroid gland, stimulus-secretion coupling is manifested by an increase in Ca\(^2+\), with attendant suppression of hormone secretion, in contrast to the stimulatory effect of increases in Ca\(^2+\); observed in many secretory processes. CaR activation in the parathyroid gland is a key event in the maintenance of systemic Ca\(^2+\) homeostasis. The essential role of the CaR in the regulation of systemic Ca\(^2+\) homeostasis has been established in two ways. 1) In humans, activating and inactivating mutations of the CaR produce hypo- or hypercalcemic conditions, respectively. The former condition is termed autosomal dominant hypocalcemia (81) and the latter familial hypocalciuric hypercalcemia (80). The condition resulting from homozygous inactivating mutations is known as neonatal severe hyperparathyroidism (80). 2) Mice in which both copies of the CaR gene have been disrupted exhibit severe hypercalcemia and hyperparathyroidism (39). Conversely, a recent report identified a mouse model (the nuf mouse) for the human condition resulting from an activating CaR mutation that displays reduced PTH associated with hypocalcemia and hyper-phosphatemia (42). Updated information on the growing list of CaR mutations in these human disorders can be obtained from www.csrdb.mcgill.ca.

Activation of the CaR occurs by two distinct mechanisms. Factors that interact with the extracellular domain of the CaR, leading to stimulation of phospholipases C and A\(_2\) (PLC and PLA\(_2\)), have been designated type I calcimimetics. Ca\(^2+\) is the prototype for this class of agents, but other di-, tri-, and polyvalent cations, such as spermine and spermidine, can also activate the CaR in vitro (83). In contrast, type II calcimimetics potentiate the effect of Ca\(^2+\) by functioning as allosteric activators of the CaR (76). These calcimimetic compounds interact with membrane-spanning portions of the receptor [although the recently identified type II calcimimetic agents, aromatic amino acids, interact with the extracellular domain (23, 72)] and enhance CaR signaling by inducing conformational changes in the protein (for review see Ref. 43). Type II calcimimetics inhibit PTH secretion across a wide range of Ca\(^2+\) concentrations. One of these compounds is currently in clinical use for the treatment of primary and secondary hyperparathyroidism (30, 78). High-throughput screening using the cloned CaR has also identified CaR antagonists, so-called calcilytics (75). These agents, in contrast to calcimimetics, stimulate PTH release.

**ROLE OF CaR IN CELLULAR EVENTS UNRELATED TO SYSTEMIC CALCIUM HOMEOSTASIS**

The discovery of the CaR a decade ago initiated a reevaluation of the known effects of Ca\(^2+\) on various cellular pro-

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**Invited Review**

E762  CALCIUM RECEPTOR AND PTHrP SECRETION

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**Fig. 1.** Schematic illustration of the feedforward cycle between systemic calcium levels in humoral hypercalcemia of malignancy (HHM) and the bone microenvironment with metastases of breast or prostate cancer cells to the skeleton. In local osteolysis, owing to PTH-related protein (PTHrP) released from bony metastases, high local levels of Ca\(^2+\) acting via the calcium-sensing receptor (CaR), along with TGF-β (or other growth factors) acting via its receptors on tumor cells, evoke further PTHrP release. PTHrP binds with PTHR1 on osteoblasts (OB) and stimulates secretion of receptor activator of NF-κB ligand (RANKL), which binds with its receptor RANK on preosteoclasts (pre-OC) to induce osteoclastic bone resorption, producing more osteolysis. TGFβ, transforming growth factor-β; BMP, bone-morphogenetic proteins; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor.
cesses beyond the realms of systemic calcium homeostasis that might be mediated by direct effects of Ca\(^{2+}\) rather than by indirect actions mediated by changes in Ca\(^{2+}\). The CaR is known to regulate cellular processes that are important determinants of cell fate under both normal and pathological conditions, such as proliferation, chemotaxis, apoptosis, and differentiation (13).

The effects of CaR activation have been shown to be either pro- or antiproliferative, depending on the cell types studied. For example, CaR activation inhibits proliferation of parathyroid cells in vivo (113, 118). In vitro, the CaR inhibits proliferation of colonic crypt cells (49–51) and keratinocytes (6, 55) but stimulates the proliferation of cells of the osteoblastic lineage (19, 115), rat-1 fibroblasts (69), and ovarian surface cells (40, 70). The CaR has also been shown to mediate the actions of Ca\(^{2+}\) in promoting the differentiation of a number of cell types, including colonic epithelial cells (49), osteoblasts (24), and keratinocytes (55). Differentiation-specific genes that are upregulated by the CaR in these cells include E-cadherin in colonic epithelial cells (16), osteocalcin in osteoblasts (24), and loricrin and profilaggrin in keratinocytes (55). In addition, a role for the CaR in protecting against apoptosis was found in AT-3 rat prostate carcinoma cells, c-myc-overexpressing rat-1A fibroblasts, and CaR-transfected HEK 293 cells (62). Therefore, CaR activation by locally high levels of Ca\(^{2+}\) at sites of osteolytic metastases could exert proproliferative and antiapoptotic effects and/or alter the cellular phenotype, thereby rendering the cancer cells metastasizing to the skeleton resistant to chemotherapy and/or radiation.

**CaR AND PTHrP INTERACTION UNDER PHYSIOLOGICAL CONDITIONS**

High Ca\(^{2+}\), via the CaR, suppresses PTH secretion and serum PTH levels. Unlike PTH, serum levels of PTHrP are normally very low, although PTHrP is widely synthesized in normal tissues, implying that PTHrP has important physiological functions. Both the extensive distribution of the PTH/PTHrP receptor in nonclassical PTH target tissues and the synthesis of PTHrP by these tissues suggest an autocrine/paracrine mode of action. By these mechanisms, PTHrP has been shown to regulate vascular smooth muscle relaxation, chondrocyte growth and differentiation, branching morphogenesis of mammary gland, and fetus-directed transport of maternal calcium across the placental membrane (for review see Ref. 29). In many of these tissues, PTHrP regulates cell growth and apoptosis via an intracrine mode by virtue of a putative nuclear localization sequence (residues 61–94), which implied nuclear import of PTHrP (for review see Ref. 22). Subsequent studies revealed that phosphorylation of PTHrP at Thr\(^{45}\) by the cyclin-dependent kinases p33cdk2 and p34cdc2 regulates its nuclear import (61) with the participation of the saturable transport receptor importin-\(\beta\) (60).

Under physiological conditions, PTHrP levels are increased during pregnancy and lactation (for review see Ref. 111). Several organs, including the placenta, amnion, decidua, umbilical cord, fetal parathyroid, and breast, probably contribute to the rise in PTHrP in the maternal circulation during pregnancy. In human placenta, CaR expression has been shown in the cell layers facing the maternal circulation, i.e., the cytotrophoblasts, as well as the transporting cells, the syncytiotrophoblasts (8, 9). Elevated Ca\(^{2+}\) in JEG-3 cytotrophoblasts stimulated secretion of PTHrP (37), and PTHrP is known to promote transplacental calcium transport. Indeed, participation of the CaR in transplacental calcium transport has been elegantly shown in a mouse model in which the measurement of \(^{45}\)Ca transport across the placenta revealed ~60% reduction in the CaR\(^{-/-}\) fetuses compared with that in their wild-type counterparts (56, 57). Because the reduction of placental calcium transport in CaR\(^{-/-}\);PTHrP\(^{-/-}\) fetuses was not greater than that observed in CaR\(^{-/-}\);PTHrP\(^{+/+}\) fetuses, it appears that the CaR is required for the normal control of calcium transport across the placenta.

The lactating breast is a rich source of PTHrP, and lactation appears to be the only physiological state in which PTHrP acts as an endocrine hormone by entering the systemic circulation, increasing the rate of bone resorption and mobilization of skeletal calcium in the milk (111). CaR is expressed in the epithelial ducts of the normal human breast (20). In mouse, the mammary gland has been shown to become a calcium-“sensing” organ during lactation by robustly upregulating CaR expression (111). CaR resides in the basolateral side of the luminal epithelial cells of the mammary ducts, and CaR activation decreases PTHrP secretion (112). In this respect, the lactating breast resembles the parathyroid gland and appears to act to provide an optimal level of calcium for transport into milk.

**REGULATION OF PTHrP SECRETION BY THE CaR IN NONMALIGNANT CELLS**

Under normal conditions, PTHrP is produced locally. Therefore, the regulation of its production by the CaR would require local activation of the receptor rather than systemic changes in Ca\(^{2+}\) concentrations. An emerging body of data suggests that, depending on the composition of the local extracellular milieu, the CaR can sense variations in extracellular levels of Ca\(^{2+}\), other di- and multivalent cations (Mg\(^{2+}\) and polyamines), ionic strength (Na\(^{+}\)), pH, and L-amino acids. In addition, changes in Ca\(^{2+}\) that are dependent on cellular and physiological activity take place in almost all tissues and organs (for review see Ref. 11). Therefore, expression of the CaR by a given tissue could result in the regulation of PTHrP by either high Ca\(^{2+}\) or other physiological CaR agonists. For example, human primary astrocytes have been shown to express CaR and type I CaR agonists such as neomycin and gadolinium in addition to elevated levels of Ca\(^{2+}\)-stimulated PTHrP release from these cells (17). Key evidence was obtained from a heterologous system demonstrating that elevated Ca\(^{2+}\)-induced PTHrP secretion is indeed mediated by CaR (66). For this purpose, HEK 293 cells with no endogenous CaR expression were stably transfected with human parathyroid CaR (referred to hereinafter as HEKCaR) and NPS R-467, a phenylalkylamine derivative that is a positive allosteric modulator of the CaR, increased PTHrP secretion but S-467, its less active enantiomer, did not. CaR activation by the calcimimetic compound (NPS R-467) also stimulated PTHrP secretion in growth plate chondrocytes, which express the CaR (15). Furthermore, because the CaR is a member of the GPCR family that uses G\(_{q/11}\) for coupling to its major signaling pathway polyphosphoinositol/phospholipase c (PI/PLC) (53), it was asked whether PTHrP release could be induced by activation of other GPCRs coupled to
Gq/11. ADP, which activates an endogenous purinoreceptor coupled to Gq/11, had no effect on PTHrP secretion from HEKCaR cells, indicating that stimulation of PTHrP release is specific to the CaR (66). These studies provide the basis for investigating the role of the CaR in cancer cells producing PTHrP and giving rise to HHM.

REGULATION OF PTHrP SECRETION BY THE CaR IN HHM CELL MODELS

Downregulation of CaR mRNA and protein in adenomatous parathyroid cells with accompanying loss of CaR function is a key factor in the development of hyperparathyroidism (25). This finding suggests that CaR expression could vary in malignancy compared with the respective normal cell/tissue. Therefore, various well-established cancer cell lines were tested for CaR expression and found to express both CaR mRNA and protein. These cell lines were derived from breast cancer [MCF-7, MDA-MB-231 (92)], prostate cancer [PC-3, LnCaP (91)], and H-500 rat Leydig tumor cells (90). Although CaR expression in the ductal cells of breast carcinoma has also been reported (20), there are no reports regarding CaR expression in primary prostate tumors. Analysis of cancer cells indicates that they are likely to be identical to the CaR cloned previously from the parathyroid and kidney. However, the possibility of overexpression of CaR and/or expression of activating forms of CaR mutation in the cancers of HHM type compared with their normal counterparts has not been addressed. Various lines of pharmacological and molecular evidence revealed that elevated Ca\(^{2+}\) upregulates PTHrP synthesis and release via CaR activation in breast cancer [MCF-7 and MDA-MB-231 cell lines (92)], prostate cancer [PC-3 and LnCaP cell lines (91)], and H-500 rat Leydig tumor cells (90). Thus the stimulatory action of the CaR on the secretion of PTHrP from cancer cells causing hypercalcemia and/or osteolysis is in marked contrast to the CaR-mediated inhibition of the release of a calcium-elevating hormone (e.g., PTH) or stimulation of a calcium-lowering hormone (i.e., calcitonin), each a key component of normal mineral ion homeostasis (11, 13, 28).

Altered regulation of PTHrP secretion by the CaR in malignant cells could be the key to the pathogenesis of breast tumors. Consistent with this concept, elevated Ca\(^{2+}\) and a calcimimetic suppressed PTHrP secretion from mammary duct epithelial cells (112), as opposed to the stimulation of PTHrP secretion observed in breast cancer cell lines (92). In addition, high Ca\(^{2+}\), presumably via the CaR, accentuates the stimulatory action of TGF-β on PTHrP secretion in breast cancer cells (92), suggesting that CaR could “inappropriately” synergize actions of other promalignant cytokines during malignancy. Currently, the lack of published studies prevents us from determining whether dysregulation of PTHrP secretion by the CaR also takes place in prostate and other HHM cancer models compared with their normal counterparts.

So far, most studies of the role of the CaR in PTHrP secretion and the pathogenesis of HHM have been on mice H-500 rat Leydig cell tumors (90, 102–106). This tumor is a xenotransplantable model of HHM but does not display skeletal metastases (101), thus resembling cancers arising from squamous cells and kidney epithelial cells (64, 86). H-500 cells secrete PTHrP and produce a rapidly developing, PTHrP-dependent form of hypercalcemia following subcutaneous implantation of a tumor fragment into the groin of male Fisher rats (64). That PTHrP is required for the growth of this tumor was elegantly shown in a study in which antisense PTHrP-transfected H-500 cells showed reduced cell growth and tumor volume and near-normal levels of serum calcium (84). Treatment of primary cultures of H-500 cells obtained from the tumors with elevated levels of Ca\(^{2+}\) resulted in a concentration-dependent increase in PTHrP release (90). Changes in the Ca\(^{2+}\) concentration as a result of increasing Ca\(^{2+}\) did not modify the PTHrP release, because the calcium ionophore ionomycin, which elevates cytosolic free calcium and dissipates the extra/intracellular calcium gradient, or Ca\(^{2+}\) chelators, such as BAPTA AM, failed to abolish Ca\(^{2+}\)-induced stimulation of PTHrP release in H-500 cells (14). These results strongly suggest that a functional CaR mediates the calcium-induced PTHrP release. Unequivocal molecular evidence in support of CaR-mediated upregulation of PTHrP release was demonstrated in H-500 cells infected with a naturally occurring dominant negative Arg\(^{185}\)Gln CaR construct (4, 104), and in these cells high Ca\(^{2+}\)-stimulated PTHrP secretion was markedly attenuated. A similar approach was taken to demonstrate that CaR mediates the effect of high Ca\(^{2+}\) in the stimulation of PTHrP secretion in PC-3, a human prostate cancer cell line (91). Stimulation of PTHrP release due to CaR activation involves de novo synthesis of PTHrP mRNA, as the pan-RNA polymerase inhibitor actinomycin D inhibits the effect of CaR on the expression of PTHrP mRNA and protein release in H-500 cells as well as in HEKCaR (104, 66). Taken together, these studies in HHM models suggest a mediatory role of the CaR in PTHrP secretion.

REGULATION OF OTHER CELLULAR EVENTS BY THE CaR IN HHM CELL MODELS

Just as activation of the CaR stimulates PTHrP release, it also stimulates proliferation and protects cells from apoptosis. Cellular events in the progression of common tumors that metastasize to bone include vigorous cell growth and resistance to cell death in response to apoptotic stimuli. Elevated Ca\(^{2+}\) increases uptake of [\(^{3}H\)]thymidine (a measure of proliferation) in H-500 cells, and the effect is mimicked by the type II calcimimetic NPS R-467 (102). The effect of CaR on the proliferation of H-500 cells was direct and not through an autocrine mechanism of increased PTHrP release by CaR activation, as PTH-(7–34) peptide, an antagonist of the PTHR1, had no effect on Ca\(^{2+}\)-induced proliferation (102). CaR activation results in upregulation of a proliferative and angiogenic oncogene, pituitary tumor-transforming gene (PTTG) (79, 45), in H-500 cells (105). PTTG protein is highly expressed by various tumors (34) and may act as a critical element in the mechanism underlying CaR-stimulated proliferation of H-500 cells. Although PTTG has been shown to exert a mitogenic effect on various cancer cells, it remains to be seen whether the upregulation of PTTG by the CaR is the mechanism underlying CaR-stimulated proliferation of H-500 cells.

The CaR-mediated increase in proliferation of H-500 cells arises in part from protection against apoptosis and involves activation of the classical PI 3-kinase/Akt survival pathway (102). Therefore, high Ca\(^{2+}\)-evoked, CaR-mediated stimula-
tion of proliferation as well as resistance to apoptosis may provide complementary mechanisms by which tumors that cause HHM are resistant to anti-cancer chemotherapy.

Angiogenesis, or new blood vessel development, is one of the hallmarks of unrestricted tumor growth and favors tumor metastasis. Leydig cell tumors exhibit high levels of angiogenesis. In H-500 cells, CaR activation upregulates PTG (105). CaR-dependent upregulation of PTG may provide a mechanism for enhanced new vessel formation via the production of the recognized angiogenic factor basic (b)FGF (48). High Ca\textsuperscript{2+}, also regulates a second potent angiogenic factor, VEGF (105), and stimulates inducible nitric oxide synthase and attendant NO production in H-500 cells (103). NO promotes neovascularization in xenograft tumors, which not only enhances tumor growth but also increases invasiveness and metastatic ability (59, 107). Furthermore, in MCF-7 human breast cancer cells expressing estrogen receptor (ER)\alpha, CaR activation increased transcriptional activity of ER despite downregulation of ER protein (46). Because ER-positive breast tumors show higher osteotropism, transactivation of ER due to CaR activation could contribute to their metastasis to bone.

CaR-MEDIATED SIGNALING IN HHM MODELS

Regulation by PKC/MEK pathway. The postreceptor signaling events following CaR activation are complex and diverse. In bovine parathyroid gland, kidney, and HEK 293 cells stably transfected with human CaR, the CaR activates several mitogen-activated protein kinases (MEK1 and -2, ERK1 and -2, and p38 MAPK), using filamin-A as a scaffold (3, 38, 52, 66, 94). Structure-function studies of the CaR revealed that Thr\textsuperscript{888} in its intracellular domain is readily phosphorylated by PKC, resulting in Ca\textsuperscript{2+}-evoked changes in Ca\textsuperscript{2+} dynamics through the PI/PLC pathway (5). Activators of PKC, such as phorbol 12-myristate 13-acetate, substantially reduce Ca\textsuperscript{2+}-evoked increases in inositol phosphates and Ca\textsuperscript{2+}; in bovine parathyroid cells and HEKCaR (53, 54), suggesting that PKC transduces CaR signaling via the PLC pathway (for review see Ref. 114). In H-500 cells, however, CaR-induced PTHrP release is PLC independent, as high Ca\textsuperscript{2+} did not result in an increase in Ca\textsuperscript{2+}. Failure of the CaR to elicit PLC signaling has also been observed in other cell types that are not involved in calcium homeostasis, including PC-3 prostate carcinoma, MDA-MB-231 breast cancer, and U-87 glioblastoma cells. The origin of the differences in CaR-mediated signaling in different cell types is not yet clear.

Regulation of PTHrP release by PKC has been reported in the NCI-H727 nonsmall lung cancer cell line, alveolar epithelial cells, and human osteosarcoma cell line (10, 35, 88). In some cell systems, PKC participates in the activation of the MEK/ERK cascade (1, 42). The PKC/MEK-1/ERK1/2 pathway represents a classical signaling cascade and has been implicated in the production of PTHrP from MCF-7 breast cancer cells (63). PKC is involved in CaR-induced PTHrP release in H-500 cells, as inactivation of conventional PKC isoforms with bisindolylmaleimide I (GF-109203X) partially attenuated high Ca\textsuperscript{2+}-induced PTHrP release (104, 109). The CaR/MEK/ERK pathway, which is downstream of PKC in many cell systems, also contributes to Ca\textsuperscript{2+}-induced PTHrP release in H-500 and PC-3 prostate cancer cells (104, 117). However, CaR-stimulated ERK activation is apparently independent of PKC in H-500 cells, as Ca\textsuperscript{2+}-induced ERK1/2 phosphorylation was not abolished by GF-109203X (104). This suggests that the CaR activates the PKC and MEK pathways in parallel in H-500 cells. On the other hand, inhibiting the MEK pathway completely abolished high Ca\textsuperscript{2+}-stimulated PTHrP release in HEKCaR cells (66). Therefore, it appears that the coupling of the CaR to PKC varies between malignant (i.e., H-500 and PC-3 cells) and nonmalignant (HEKCaR) cells.

Activation of the MEK/ERK pathway by high Ca\textsuperscript{2+} is mediated by the CaR. This was demonstrated in PC-3 cells, where the calcimimetic NPS R-467 stimulated phosphorylation of ERK1/2; however, the less active stereoisomer NPS S-467 did not (117). In addition, high Ca\textsuperscript{2+} induced rapid phosphorylation of ERK1/2 in HEKCaR cells but not in untransfected cells (66). Although CaR-mediated activation of the MEK/ERK pathway requires its interaction with the scaffold protein filamin A in HEKCaR cells (38), the molecular requirements for the CaR-mediated activation of MEK/ERK in PC-3 and H-500 cells are currently unclear.

Transactivation of epidermal growth factor receptor by the CaR. After demonstrating that the MAP kinase pathways play key roles in CaR-stimulated PTHrP secretion, the next question is how the CaR activates MAP kinases. An emerging body of evidence indicates that GPCRs are able to transactivate receptor tyrosine kinases (RTKs) such as the endothelial growth factor receptor (EGFR) and PDGF receptor (PDGFR) (27, 65, 82, 93). The initial transactivation process involves stimulation of matrix metalloproteinases (MMPs), which results in the extracellular release of a latent membrane-spanning precursor of a member of the family of ligands known to activate these groups of receptors (82, 89, 116). These ligands [either heparin-bound (HB)-EGF or TGF-\alpha or PDGF] will then secondarily activate the EGFR or PDGFR to phosphorylate specific tyrosine residues residing on their own intracellular domains, thereby activating downstream proteins such as MAPKs (2, 68, 89, 93, 116). This mechanism of GPCR-induced EGFR/PDGFR activation is called “triple-membrane-passing signaling.” Some widely studied examples include the angiotensin II-induced hypertrophy of cardiomyocytes via transactivation of the EGFR and subsequent activation of MAPKs and the ET-1-induced phosphorylation of the EGFR in human ovarian carcinoma cells (for review see ref. 110).

Four different cell systems were used to demonstrate that the CaR transactivates EGFR but not PDGFR. Two of those models are of HHM (i.e., PC-3, human prostate cancer, and H-500 Leydig cell) and the other two are rat-1 fibroblast and CaR-transfected HEK 293 cells (67, 106, 108, 117). High Ca\textsuperscript{2+} was shown to stimulate tyrosine phosphorylation of EGFR in PC-3 and H-500 cells, but the role of CaR in the process was not established (106, 117). Definitive evidence regarding the CaR’s role in the process was obtained when high Ca\textsuperscript{2+} stimulated tyrosine phosphorylation of EGFR in HEKCaR cells but not in nontransfected cells (67). In addition, inhibiting EGFR by the tryphostin AG-1478 prevented high Ca\textsuperscript{2+}-induced phosphorylation of ERK1/2 and attenuated CaR-stimulated PTHrP release in HEKCaR cells (66). Therefore, it appears that the coupling of the CaR to PKC varies between malignant (i.e., H-500 and PC-3 cells) and nonmalignant (HEKCaR) cells.
broadly selective MMP inhibitor, GM-6001, and neutralizing heparin-bound EGF with a neutralizing antibody prevented the CaR-mediated increases of phospho-ERK and PTHrP release, consistent with a triple-membrane-spanning signaling requirement for transactivation of the EGFR by the CaR (67, 117). These data suggest that HB-EGF is the ligand responsible for activating the EGFR following CaR stimulation. The new evidence implicating the participation of EGFR in CaR signaling in cellular models of HHM points to potential new therapeutic targets that need not include the CaR itself, given its central role in calcium homeostasis. Figure 2 is a schematic diagram of the sequence of events following CaR activation that results in EGFR activation.

**Involvement of stress-activated kinases.** CaR-stimulated PTHrP release in H-500 and HEKCaR cells also involves a stress-activated kinase, p38 MAPK, which was originally identified as the target of pyrindinylimidazole compounds (SB-203580 and SB-202190) and found to inhibit the production of inflammatory cytokines and cell death following cellular stress (66, 73, 104, 119). High Ca\(^{2+}\) (presumably via the CaR) also stimulates stress-activated protein kinase (SEK-1) in H-500 cells and regulates PTHrP release (104). Phosphorylation activates p38 MAPK, and high Ca\(^{2+}\) phosphorylates p38 MAPK in both H-500 and HEKCaR cells (66, 104). Phosphorylation of p38 MAPK, in turn, phosphorylates ATF-2, which then acts as a transcription factor for various target genes. High Ca\(^{2+}\)-dependent phosphorylation of ATF-2 in H-500 cells (104) could provide a link for the upregulation of PTHrP.

Interestingly, despite its contribution to the release of PTHrP in H-500 cells, the MEK/ERK pathway is not involved in the CaR-stimulated proliferation of H-500 cells, which depends instead on PI 3-kinase and p38 MAPK (102). An ERK-independent, PI 3-kinase-dependent component in the proliferative response mediated by the CaR has been reported in ovarian surface epithelial cells (7). Therefore, the CaR-dependent selection of specific protein kinase-dependent pathways is not only cell type specific but also dependent on the cell function.

**SUMMARY, CONCLUSIONS, AND UNANSWERED QUESTIONS**

The CaR bound to the cell surface mediates direct actions of Ca\(^{2+}\)\(_{o}\) on parathyroid, kidney, and various other cell types. The CaR plays a key role in the maintenance of systemic Ca\(^{2+}\)\(_{o}\) homeostasis by critically regulating PTH secretion. Recently, reports of CaR expression in cancer cells have opened the door to a new understanding of the role of Ca\(^{2+}\)\(_{o}\) as a malignant stimulus by activating the CaR. Hypercalcemia is the most commonly observed metabolic complication of malignancy, and PTHrP has been found to be the most frequent cause of hypercalcemia in malignancy due to its role in bone metastasis. Activation of the CaR has been shown to increase the expression and secretion of PTHrP in various models of HHM. Thus the CaR may play an important role in HHM as a mediator of a malignancy-associated, feedforward loop between the tumor and bone, resulting in osteolysis. In some of these cancer models, the CaR promotes growth and survival of the cancer cells by enhancing cellular proliferation and reducing apoptosis. The CaR accomplishes these functions by activating multiple signaling pathways involving MAPKs and transactivation of EGFR. Thus interrupting CaR-mediated PTHrP secretion from tumors metastatic to bone using a CaR antagonist holds therapeutic potential.

Although substantial progress has been made in understanding the role of CaR in the pathogenesis of HHM during the last five years, many unanswered questions remain. First, there is no systematic study comparing CaR expression and full nucleotide sequence data for normal vs. malignant tissues; hence, it is not known whether upregulation of CaR expression and/or its activation due to activating mutation in the CaR gene accompanies malignant transformation. Second, we do not know what role, if any, the CaR plays in the transformation of normal cells to malignant ones. Noteworthy in this regard is that CaR activation inhibits PTHrP secretion in normal mammary duct cells, whereas it stimulates PTHrP secretion in breast tumor cells. The difference in the coupling of the CaR to its signaling apparatus in normal and corresponding transformed cells could serve as a switch in the genesis of breast and other HHM cancer models. Last, we have yet to determine the relative importance of the CaR in the pathogenesis of HHM in vivo. To that end, antagonizing endogenous CaR function in breast, prostate, and H-500 cells is required before inoculating the cells in the animal models and assessing various parameters of tumor growth such as proliferation and angiogenesis in H-500 cells or metastasis of breast and prostate cancer cells to bone. Moreover, because degradation of extracellular matrix

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**Fig. 2.** Schematic diagram showing transactivation of endothelial growth factor (EGF) receptor (EGFR) by the CaR and subsequent activation of MAP kinases as the mechanism for the regulation of PTHrP release by the CaR. CaR activates one or more matrix metalloproteinases (MMPs), which cleave heparin-bound HB-EGF to release soluble HB-EGF. HB-EGF then binds with EGFR, and the activated EGFR becomes autophosphorylated. This, in turn, sets the Ras/Raf/MEK pathway in motion, finally resulting in increased PTHrP synthesis and secretion. This is called the “triple-membrane-passing signaling” model. PY, tyrosine phosphorylated after activation; YP, mirror image of PY. [Reprinted with permission from Yano et al. (117)]
by MMPs is crucial for malignant tumor growth, invasion, metastasis, and angiogenesis, identifying the MMPs that are activated by the CaR and determining the mechanism of MMP activation could provide important new insights into the role of the CaR in the pathogenesis of HHM. The assessment of CaR as a potential antagonizing/neutralizing target for the treatment of HHM awaits our understanding of these unresolved issues.

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