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INVITED REVIEW

Calcium-sensing receptor in cancer: good cop or bad cop?

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Abstract The extracellular calcium-sensing receptor (CaR) is a versatile 'sensor' for di- and polycationic molecules in the body. CaR plays a key role in the defense against hypercalcemia by "sensing" extracellular calcium levels in the parathyroid and kidney, the key organs maintaining systemic calcium homeostasis. Although mutation of CaR gene has so far not been associated with any malignancy, aberrant functions of CaR have implications in malignant progression. One situation is loss of CaR expression, resulting in loss of growth suppressing effects of elevated extracellular Ca^{2+} by CaR, reported in parathyroid adenoma and in colon carcinoma. Another situation is activation of CaR, resulting in increased production of parathyroid hormone-related peptide (PTHrP), a primary causal factor in hypercalcemia of malignancy and a contributor to metastatic processes involving bone. CaR signaling and effects have been studied in several cancers including ovarian cancers, gastrinomas, and gliomas in addition to comparatively detailed studies in breast, prostate, and colon cancers. Studies on H-500 rat Leydig cells, a xenotransplantable model of humoral hypercalcemia of malignancy has shed much light on the mechanisms of CaR-induced cancer cell growth and survival. Pharmacological agonists and antagonists of CaR hold therapeutic promise depending on whether activation of CaR is required such as in case of colon cancer or inactivating the receptor is required as in the case of breast- and prostate tumors.

Keywords Hypercalcemia of malignancy · Epidermal growth factor receptor · Parathyroid hormone-related protein · Wnt signaling · Metastases

Introduction

Calcium is required for all living cells to maintain their normal structure and function [1, 2]. Calcium signaling occupies a pre-eminent position in the signal transduction system of the cell by virtue of its participation in a variety of physiological functions, biological events associated with cell proliferation and apoptosis [3, 4] as well as cell differentiation [5]. Calcium signaling is also an important feature of cell adhesion and motility [6, 7]. Certainly all these events are integral to normal cell functions, and disruptions in calcium signaling are well-established features in the genesis and progression of cancer [8]. Role of calcium as a second messenger has traditionally received more attention, as a multitude of growth factors and cytokines signal through this ubiquitous molecule [9]. However, the role of calcium as first messenger in either the cause or prevention of cancer has been under active investigation for the previous decade [10].

CaR: the apparatus for Ca^{2+} to act as first messenger

First cloned from the parathyroid, calcium-sensing receptor (CaR) was characterized as a G-protein coupled receptor

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(GPCR) [11]. Upon its activation by its physiological ligand, Ca^{2+}, CaR acutely inhibits secretion of parathyroid hormone (PTH) [12]. Nonredundant role of the CaR in the maintenance of systemic calcium homeostasis has been proved by the generation of CaR-*null* mouse, which exhibits unregulated hyperparathyroidism with hypercalcemia due to the loss of feedback control of parathyroid cells by high Ca^{2+} [13]. Homozygous inactivating mutations of the CaR in humans, known as neonatal severe hyperparathyroidism, closely resemble the condition in CaR-*null* mouse [14]. Unlike homozygous inactivating mutations of CaR, parathyroid function is affected only mildly in heterozygous inactivating mutations in both humans and mice ($CaR^{\pm}$ genotype) [15]. A more pronounced renal defect in calcium metabolism is observed in familial hypocalciuric hypercalcemia, which is characterized by an inappropriate elevation in calcium reabsorption from the tubules. In contrast to inactivating mutations, activating mutations of the CaR gene in humans result in reduced PTH and associated hypocalcemia and hyperphosphatemia [16]. Essential role of CaR in calcium homeostasis makes it an important drug target and offers an attractive means to control hormonal disorders related to calcium homeostasis by resetting body's calciostat by modulating Ca^{2+}-sensing apparatus to restore normocalcemia.

CaR and cancer

GPCRs are known to regulate cellular motility, growth and differentiation, and gene transcription, the factors central to the biology of cancer. GPCRs represent a rich source of drug targets in several therapeutic regimens [17]. A recent examination of publicly available gene expression data identified a variety of types of GPCRs, including protease activated receptor and receptors for various chemokines, adenosine 2B, neuropeptide, metabotropic glutamate, and CaR that are overexpressed in diverse types of cancer tissues [18]. Therefore, CaR's role in cancer biology and its possible role in therapeutics are discussed. Figure 1 describes various roles of CaR in different types of cancer cells.

CaR and PT adenoma

The CaR, located on the surface of parathyroid chief cells, is critical in mediating the effect of extracellular calcium [Ca^{2+}_o] on PTH secretion. It is well known that patients with primary hyperparathyroidism require a greater concentration of Ca^{2+} to inhibit PTH secretion [19, 20]. To date, no mutations in CaR gene have been reported in sporadic parathyroid adenomas [21]. However, reductions in CaR expression have been described both in parathyroid adenomas and in hyperplastic parathyroid tissue [21, 22]. No correlation was seen between CaR mRNA in the adenoma and preoperative serum calcium, PTH, or weight of the adenoma [23]. Alterations in CaR expression may contribute to disturbances in cell cycle regulation and to monoclonal cell growth in parathyroid tissue [24].

The level of expression of one splice variant of CaR has been reported to be diminished in human parathyroid adenomas [25]. Authors identified and characterized the promoter regions of human CaR [25]. Expression of CaR mRNA produced by one of the two promoters of CaR gene was found to be specifically reduced in parathyroid adenomas. A lower expression of CaR mRNA and CaR protein has also been reported in parathyroid adenomas and hyperplastic glands from patients with uremic hyperparathyroidism [26, 27].

Similar decreases in CaR protein expression in parathyroid tumors have been reported in another study [28]. This study also showed a significant correlation between apparent CaR protein expression and PTH-Ca^{2+} set-point

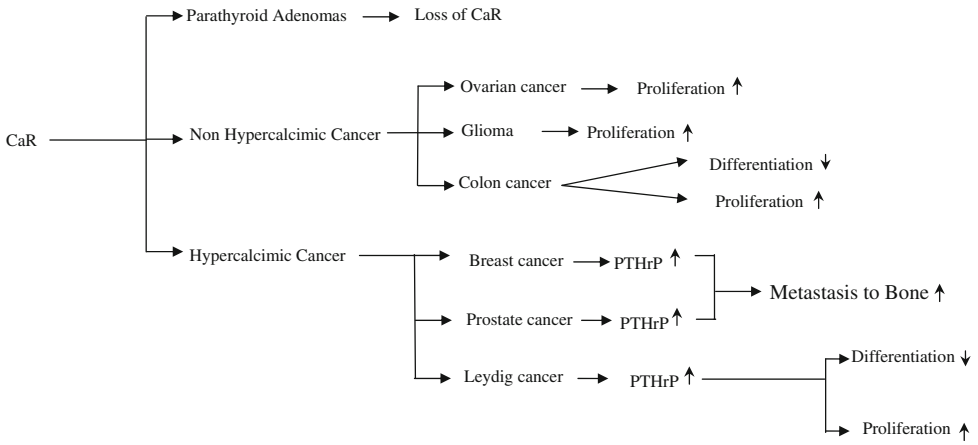

Fig. 1 Summary of various functions of CaR in different types of cancer cells

abnormality. Apparent lack of expression of CaR was associated with a more severe abnormality in the control of Ca^{2+}-regulated PTH release [28]. Therefore, reduced receptor content might have an important role in pathogenesis of PHPT. CaR and caveolin-1 colocalize in parathyroid cells, and reduced levels of caveolin-1 also correlate with reduced suppressibility of PTH secretion by high Ca^{2+} in parathyroid adenomas [29].

It is suggested that abnormality of the signaling pathways of CaR or of the secretory machinery is perhaps involved in pathogenesis of primary hyperparathyroidism. Cytosolic Ca^{2+} [Ca^{2+}_i] rather than absolute value of [Ca^{2+}_o] appears to be the main regulator of secretion from parathyroid adenoma cells. In primary hyperparathyroidism, a defective Ca^{2+}-sensing mechanism could result in the maintenance of low [Ca^{2+}_i] by the parathyroid cells under the condition of hypercalcemia, leading to uninhibited PTH secretion [30]. Alterations in the expression levels of the CaR as well as its coupling to signaling apparatus could also represent defective Ca^{2+} sensing in parathyroid adenoma [31].

CaR's role in parathyroid adenomas is further emphasized by the therapeutic use of calcimimetics, the allosteric modulators that increase the sensitivity of CaR to [Ca^{2+}_o], for primary hyperparathyroidism. Calcimimetics normalizes serum calcium and lowers PTH concentrations in patients with primary hyperparathyroidism [32, 33].

CaR and hypercalcemia of malignancy

By far, the most common causes of hypercalcemia are primary hyperparathyroidism and malignancy [34]. Humoral hypercalcemia of malignancy (HHM) is a syndrome seen in breast, prostate, lung, testicle, and kidney cancer [35]. Hypercalcemia of malignancy, one of the more common paraneoplastic syndromes, arises from coexisting primary hyperparathyroidism or humoral factor secreted by tumors that act systemically on target organs of bone, kidney, and intestine to disrupt normal calcium homeostasis. Up to 30% of patients with cancer may develop hypercalcemia during the course of their disease [36].

Frequent bone metastases seen in HHM patients can be explained by the 'seed and soil' hypothesis proposed by Paget [37]. Bone is not simply a depot of calcium but also a rich store of immobilized growth factors that are released during bone resorption. Bone metastases can be osteoblastic, osteolytic, or mixed [38]. Osteoblastic metastases are commonly associated with prostate cancer and much frequently with breast cancer. Endothelin-1 (ET-1) is a potent osteoblast-stimulatory factor that is involved in osteoblastic bone metastases through its activation of the ET A receptor (ETAR) [39, 40]. Histological analysis of osteolytic bone metastases indicates that bone destruction is mediated by osteoclast rather than directly by tumor cells. Bone-derived transforming growth factor (TGF-β) plays an integral role in promoting development and progression of osteolytic bone metastases by inducing tumor production of parathyroid hormone-related protein (PTHrP), a known stimulator of osteoclastic bone resorption [41]. This is typically described as a vicious cycle between tumor cell and bone (Fig. 2). Mixed osteolytic–osteoblastic metastases are characteristic of both breast and prostate cancers [34]. The effects of combined expression of osteolytic and osteoblastic factors on bone have not been studied, so the net response of bone at the metastatic site is unpredictable. Osteolytic factors such as PTHrP and IL-11 act on osteoblasts to increase expression of RANKL [42].

CaR and Leydig cancer cells

Rice H-500 rat Leydig cell tumor is a widely used model of HHM. H-500 cells have been used extensively to study the role of CaR in PTHrP secretion [43–47]. This tumor is a xenotransplantable model of HHM but does not display skeletal metastases [44], making it resembles cancers arising from squamous cells and kidney epithelial cells [48, 49]. 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 [48]. That PTHrP is required for the growth of this tumor was elegantly shown in a study in which antisense PTHrP-transfected H-500 cells elicited reduced cell growth and tumor volume and near-normal levels of serum calcium [50]. Treatment of the primary cultures of H-500 cells obtained from those tumors when treated with elevated levels of [Ca^{2+}_o] resulted in a concentration-dependent increase in PTHrP release [44]. Changes in [Ca^{2+}_i] concentration as a result of increasing [Ca^{2+}_o] did not modify PTHrP release, because the calcium ionophore ionomycin, which elevates cytosolic free calcium and dissipates extra/intracellular calcium gradient, or [Ca^{2+}_i] chelators, such as BAPTA AM, failed to abolish [Ca^{2+}_o] induced stimulation of PTHrP release in H-500 cells [51]. Transfection of H-500 cells with a dominant negative CaR, R185Q, attenuated Ca^{2+}-stimulated PTHrP release [52, 53]. A similar approach was taken to demonstrate that CaR mediates the effect of high [Ca^{2+}_o] in the stimulation of PTHrP secretion in PC-3, a human prostate cancer cell line [54]. Stimulation of PTHrP release due to CaR activation involves de novo synthesis of PTHrP mRNA [44], as the pan-RNA polymerase inhibitor actinomycin D inhibits the effect of [Ca^{2+}_o] on expression of PTHrP mRNA and protein release in H-500 cells as well as in CaR transfected HEK-293 cells (HEKCaR) [53, 55].

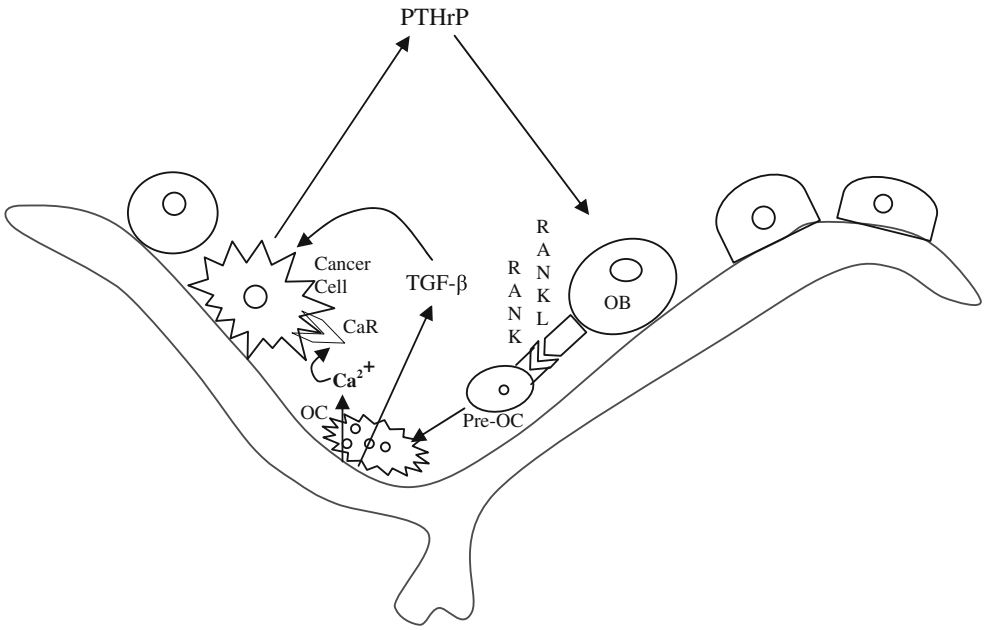

Fig. 2 Schematic illustration of the 'vicious' cycle between systemic calcium levels in HHM and the bone microenvironment with skeletal metastases of cancer cells. In local osteolysis, due to increased secretion of PTHrP from bony metastases, high levels of ambient [$Ca^{2+}{}_o$], acting via the CaR, 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 osteoclasts (OC) to induce osteoclastic bone resorption, producing more osteolysis. BMP, bone-morphogenetic proteins; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor

Taken together, these studies in HHM models suggest a mediatory role of CaR in PTHrP secretion.

Just as activation of CaR stimulates PTHrP release, it also stimulates proliferation and protects cells from apoptosis [34]. 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 [$Ca^{2+}{}_o$] increases uptake of [^{3}H] thymidine (a measure of proliferation) in H-500 cells as well as by using allosteric modulator of the CaR, NPS R-467 [46]. Effect of CaR on the proliferation of H-500 cells appears to be direct and not through an autocrine mechanism of increased PTHrP release by CaR activation, since PTH-(7–34) peptide, an antagonist of PTHR1, had no effect on [$Ca^{2+}{}_o$] induced proliferation [46]. CaR activation results in upregulation of a proliferative and angiogenic oncogene, pituitary tumor-transforming gene (PTTG) [56, 57], in H-500 cells [58]. PTTG protein is highly expressed by various tumors [59] and may be a critical element in 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 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 classical PI-3-kinase/Akt survival pathway [46]. Therefore, high Ca^{2+} produced, CaR-mediated stimulation of proliferation as well as resistance to apoptosis may be complementary mechanisms by which tumors that cause HHM are resistant to anti-cancer chemotherapy.

Angiogenesis, or the development of new blood vessels, 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 PTTG [58]. CaR-dependent upregulation of PTTG may provide a mechanism for enhanced new vessel formation via the production of the recognized angiogenic factor basic-FGF [60]. High [$Ca^{2+}{}_o$] also regulates a second potent angiogenic factor, VEGF, and stimulates inducible nitric oxide (NO) synthase and attendant NO production in H-500 cells [61]. NO promotes neovascularization in xenograft tumors, which not only enhances tumor growth but also increases invasiveness and metastatic ability [62, 63].

CaR and breast cancer

Strong CaR immunostaining has been detected along the epithelial cells of the ducts in normal and fibrocystic breast tissue [64], suggesting that the CaR could serve important functions in normal and pathophysiological conditions of

the mammary gland. Growth and differentiation of human mammary epithelial cells are responsive to changes in calcium concentration in vitro [65, 66]. [Ca^{2+}_o] appears to have biphasic action on mammary epithelial cells; stimulating differentiation at [Ca^{2+}_o] concentration of ~0.5 mM but proliferation is induced at very low [Ca^{2+}_o] concentrations (40–50 μM) [65]. In contrast, mouse mammary epithelial cells exhibited optimal growth at a relatively high [Ca^{2+}_o] concentration (0.8 mM) [67]. In addition, growth inhibitory effect of 1,25 $(OH)_2$ vitamin D on breast cancer lines is abrogated by high [Ca^{2+}_o], indicating that the effects of Ca^{2+} on mammary epithelial cells are species- and state-specific [68]. A specific mechanism that may regulate the effects of Ca^{2+} in human mammary cells has come to light with the observation of CaR expression in normal and malignant human breast tissues [64, 69]. Although the mediatory role of CaR on proliferation of human mammary tumor has not been assessed directly, CaR elicits "estrogen-like" effects in MCF-7 human breast cancer cells by enhancing transcriptional activity of estrogen receptor (ER) [70]. The concentration of [Ca^{2+}_o] required in this study for such effect was very high (15–20 mM). However, since ER-positive breast tumors exhibit higher osteotropism and that local [Ca^{2+}_o] near the bone resorption pits are ~40 mM, thus giving rise to a situation of very high levels of [Ca^{2+}_o] being encountered by breast cancer cells metastasizing to bones [70]. In this regard, buffering of [Ca^{2+}_i] becomes crucial for avoiding the toxicity of large (micromolar) sustained increases in [Ca^{2+}_i] [71]. CaR activation in MCF-7 cells by a polyvalent cation agonist, neomycin results in spatial interaction of this receptor with calbindin-D_{28K}) [72]. However, the functional implication of this interaction in MCF-7 cells is currently unknown.

One of the mechanisms that could mediate growth and metastasis of breast cancer cells is increased production of PTHrP [44]. Overexpression of PTHrP in MCF-7 results in its increased proliferation through a nuclear/intracrine pathway [73, 74]. In addition, overexpression of PTHrP specifically in the mammary gland shortened tumor latency and increased tumor incidence following exposure to the chemical carcinogen dimethylbenzanthracene [75]. In this case, visceral metastasis was not accompanied by bone metastasis [76]. On the other hand, when mice inoculated with PTHrP secreting MDA-MB-231, bone metastases was significantly attenuated by neutralizing antibody against PTHrP-(1-34) [77]. These data suggest an important role for PTHrP in breast cancer progression and bone metastases.

CaR has been shown to stimulate synthesis and secretion of PTHrP in both MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) human breast cancer cell lines [78]. Activating the CaR by di-or polyvalent cations (type I CaR agonists) but not pharmacological CaR agonists (the calcimimetics or type II CaR agonist), did not alter cellular proliferation and viability of these two cell lines, indicating that stimulation of PTHrP production by the CaR may contribute to breast cancer metastasis [79]. Interestingly, CaR activation in combination with TGF-β increased PTHrP secretion that was more than additive [78]. The mechanism underlying this effect is unclear but might involve TGF-β induced upregulation of CaR expression or its signaling pathways and/or increased expression of PTHrP gene, thereby increasing amount of PTHrP available for secretion in response to elevated levels of [Ca^{2+}_o]. [Ca^{2+}_o] and TGF-β are released from bone matrix during bone resorption induced by PTHrP [78]. Therefore, they are likewise both available to elicit further PTHrP secretion, in effect cooperating to fuel a cycle of tumor-induced bone resorption, begetting further bone resorption in skeletal metastases of breast cancers [78, 80]. Beneficial actions of bisphosphonates on the skeletal complications of metastatic breast cancer and on new metastases [81–86] could result at least in part from reductions in local concentrations of both [Ca^{2+}_o] and TGF-β as a result of decreased bone resorption [80, 87–91].

Although CaR expression as a biomarker of breast cancer may have prognostic limitations [92], it may serve as a drug target for advanced breast cancer, since there are currently few specific therapies that address the primary mediators of osteolytic metastases in breast cancer.

CaR and prostate cancer

The preferential metastasis of prostate cancer cells to bone indicates that bone microenvironment provides a favorable niche [93]. Multiple growth factors in bone microenvironment have been postulated to provide favorable chemotactic- and growth-promoting functions required for tumor localization and expansion in the skeleton [94, 95]. Ca^{2+} is one of the major inorganic factors released as a result of bone remodeling [93]. Due to higher remodeling rates, trabecular bones are metabolically more active than cortical bones. Trabecular regions, such as proximal ends of the long bones, ribs, and vertebral column, happen to be the preferential sites of prostate cancer metastasis [96]. Therefore, CaR could serve as one of the major molecular targets of Ca^{2+} in facilitating the formation and growth of skeletal metastasis of prostate cancer. Expression of CaR in osteoblasts, osteoclasts [97], and its precursor cells and stromal cells lays the groundwork for local cross-talk in event that cancer cells localize in the skeleton [98]. However, CaR could also regulate events guiding the migration of cancer cells to bone.

Several human prostate cancer cell lines have aided in the study of prostate cancer research. PC-3 cells are highly tumorigenic, have xenotransplantable metastatic properties, and are androgen-independent [99, 100]. In contrast to PC-3 cells, LNCaP cells express a functioning androgen receptor (AR) and proliferate in vitro following treatment with androgen [101]. C4-2B is an osteotropic prostate cancer cell line derived from its nonosteotropic parent cell line LNCaP and has an osteoblast-like phenotype including production of alkaline phosphatase, osteocalcin, osteonectin, bone sialoprotein, osteoprotegerin, and production of hydroxyapatite [102]. Two independent groups reported expression of CaR in PC-3 and LnCaP cells. When the expression levels of CaR were compared among these two cell lines, PC-3 had higher levels of CaR mRNA than LNCaP [103, 104] .

Increasing $[Ca^{2+}{}_o]$ levels from 0.5 mM to 2.5 mM for 6 days in culture resulted in a substantial increase in the cellular growth of PC-3 and C4-2B cells but not of LnCaP cells [93, 105]. This observation correlates with the receptor expression data. Stable transfection of short hairpin RNA (shRNA) to downregulate CaR expression in PC-3 cells reduced cell growth both in vitro and in vivo in the craniofacial region of nude mice when these cells were injected in left ventricle [93]. It is interesting to note that silencing CaR expression reduces cell growth of PC-3 cells per se, suggesting a likely constitutive role of the CaR facilitating growth of androgen-independent prostate cancer cells. In PC-3 cells, CaR appears to be coupled with either Gαi or Gαq, as elevated $[Ca^{2+}{}_o]$ or neomycin blocked PGE2-or forskolin-induced cAMP production [93]. However, there has been no molecular or pharmacological confirmation of CaR's role in $[Ca^{2+}{}_o]$-induced production of cAMP. As elevated $[Ca^{2+}{}_o]$ stabilizes cyclin D1 in PC-3 cells and that cyclin D1 is mitogenic, it is possible that CaR could promote PC-3 cell growth by cyclin D1 [93]. However, there is no molecular or pharmacological evidence of modulation of cyclin D1 function by the CaR.

Elevated $[Ca^{2+}{}_o]$ also results in increased adherence of PC-3 cells in vitro that is mediated by PI3 K-Akt pathway [46, 93, 106, 107]. Hence, data suggest that $[Ca^{2+}{}_o]$ could play an important role not only in stimulating growth but also in early stages of prostate cancer metastasis to bone. However, it remains to be studied whether CaR activation promotes adherence of prostate cancer cells to collagen, which is the most prominent extracellular matrix protein in bone [93].

PTHrP is one of the major bone metastatic factors. Prostate cancers often express more PTHrP than normal prostate epithelial cells, including tumors with skeletal metastasis [108, 109]. A recent report presented evidence that ectopic expression of PTHrP converted DU-145, a type of noninvasive prostate cancer cells with very little PTHrP expression, into cells that metastasize to bone. Although prostate cancers metastatic to bone generally cause osteoblastic lesions, there are also substantial increases in bone resorption, as assessed by biochemical markers [79, 110, 111]. Indeed, markers of bone resorption can be higher in patients with metastatic prostate cancer than in those with skeletal metastases of breast cancer [79], suggesting that PTHrP contributes to increased bone resorption [79] in these patients with prostate cancer metastatic to bone [108, 109, 112]. Levels of $[Ca^{2+}{}_o]$ were elevated via the CaR stimulated secretion of PTHrP in PC-3 cells, indicating that CaR activation in highly malignant prostate cancer cells not only promotes proliferation and attachment but also stimulates bone metastatic factor, PTHrP. TGF-β stimulates PTHrP secretion from PC-3 cells synergistically with high $[Ca^{2+}{}_o]$, suggesting that release of this growth factor along with calcium during PTHrP-induced bone resorption could contribute to a feed-forward mechanism in which PTHrP-mediated osteolysis associated with prostate cancers metastatic to bone exacerbates osteolysis. CaR stimulates secretion of PTHrP by transactivating epidermal growth factor receptor (EGFR) [47] but not platelet-derived growth factor receptor. Subsequent downstream signaling involves activation of the MEK-Erk cascade.

Signaling mechanism of CaR in HHM cells causing HHM

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. CaR activates several mitogen-activated protein kinases (MEK1 and −2, ERK1 and −2, and p38 MAPK), using filamin-A as a scaffold [55, 113–116]. Structure–function studies of CaR revealed that Thr^{888} in its intracellular domain is readily phosphorylated by PKC, resulting in $[Ca^{2+}{}_o]$ evoked changes in $[Ca^{2+}{}_i]$ dynamics through PI/PLC pathway [117]. Activators of PKC, such as phorbol 12-myristate 13-acetate, substantially reduce $[Ca^{2+}{}_o]$ evoked increases in inositol phosphates and in bovine parathyroid cells and HEKCaR [118, 119], suggesting that PKC transduces CaR signaling via PLC pathway [120]. In H-500 cells, however, CaR-induced PTHrP release is PLC-independent, as high $[Ca^{2+}{}_o]$ does not result in an increase in $[Ca^{2+}{}_i]$. Failure of CaR to elicit PLC signaling has also been observed in cell types that are not involved in calcium homeostasis, including PC-3 prostate carcinoma, MDA-MB-231 breast cancer, and U-87 glioblastoma cells [34]. Origin of the differences in

CaR-mediated signaling between 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 [121–123]. In some cell systems, PKC participates in activation of MEK/ERK cascade [124, 125]. PKC/MEK-1/ERK1/2 pathway represents a classical signaling cascade and has been implicated in production of PTHrP from MCF-7 breast cancer cells [126]. 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^{2+} induced PTHrP release [47, 53, 127]. MEK/ERK pathway, which is downstream of PKC in many cell systems, also contributes to [$Ca^{2+}{}_o$]-induced PTHrP release in H-500 and PC-3 prostate cancer cells [53, 128]. However, CaR-stimulated ERK activation is apparently independent of PKC in H-500 cells, as [$Ca^{2+}{}_o$]-induced ERK1/2 phosphorylation was not abolished by GF-109203X [53]. This suggests that CaR in parallel activates PKC and MEK pathways in H-500 cells. On the other hand, inhibiting MEK pathway completely abolished high [$Ca^{2+}{}_o$]-stimulated PTHrP release in HEKCaR cells [55]. Therefore, it appears that the coupling of CaR to PKC varies between malignant (i.e., H-500 and PC-3 cells) and nonmalignant (HEKCaR) cells.

Activation of MEK/ERK pathway by high [$Ca^{2+}{}_o$] is mediated by the CaR. This was demonstrated in PC-3 cells, in which calcimimetic NPS R-467 stimulated phosphorylation of ERK1/2; however, the less active stereoisomer NPS S-467 did not [47]. In addition, high [$Ca^{2+}{}_o$] induced rapid phosphorylation of ERK1/2 in HEKCaR cells but not in untransfected cells [55]. Although CaR-mediated activation of MEK/ERK pathway requires CaR to interact with the scaffold protein filamin A in HEKCaR cells [114], the molecular requirements for CaR-mediated activation of MEK/ERK in PC-3 and H-500 cells remain unclear.

Transactivation of epidermal growth factor receptor by CaR

After demonstrating that MAP kinase pathways play key roles in CaR-stimulated PTHrP secretion, the next question is how CaR activates MAP kinases. An emerging body of evidence indicates that some GPCRs transactivate receptor tyrosine kinases (RTKs) such as EGFR and PDGF receptor (PDGFR) [129–132]. 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 [131, 133]. These ligands [either heparin-bound (HB)-EGF or TGF-β or PDGF] then secondarily activate EGFR or PDGFR to phosphorylate specific tyrosine residues residing on their own intracellular domains, thereby activating downstream proteins such as MAPKs [132–136]. 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 EGFR and subsequent activation of MAPKs and the ET-1-induced phosphorylation of EGFR in human ovarian carcinoma cells [137].

Four different cell systems were used to demonstrate that the CaR transactivates EGFR but not PDGFR. Two of those are models of HHM (i.e., PC-3, human prostate cancer, and H-500 Leydig cell), and other two are rat-1 fibroblast and CaR-transfected HEK 293 cells [43, 47, 128, 138]. High Ca^{2+} was shown to stimulate tyrosine phosphorylation of EGFR in PC-3 and H-500 cells, but role of CaR in the process was not established [43, 47]. Definitive evidence regarding CaR's role in the process was obtained when high [$Ca^{2+}{}_o$] stimulated tyrosine phosphorylation of EGFR in HEKCaR cells but not in nontransfected cells [128]. In addition, inhibition of EGFR by tyrphostin AG-1478 prevented high Ca^{2+}-induced phosphorylation of ERK1/2 and attenuated CaR-stimulated PTHrP release in HEKCaR, PC-3, and H-500 cells. AG-1296, a PDGFR kinase inhibitor, had no effect. These data suggest that CaR not only phosphorylates EGFR but directs EGFR to initiate its downstream signaling events.

In rat-1 fibroblasts, blocking EGFR activation abolishes CaR-stimulated proliferation [138]. Inhibiting MMP by a broadly selective MMP inhibitor (GM-6001) and neutralizing HB EGF with a neutralizing antibody prevented CaR-mediated increases of phospho-ERK and PTHrP release, consistent with a triple-membrane-spanning signaling requirement for transactivation of EGFR by CaR [128, 138]. These data suggest that HB-EGF is the ligand responsible for activating 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 CaR itself, given its central role in calcium homeostasis.

CaR and non-hypercalcemic cancer

Calcium and colon cancer

The epithelium of colon and small intestine is constantly undergoing renewal. Calcium is considered a chemoprotective agent against colon cancer [139, 140]. In vitro studies have demonstrated that maintenance of human colon carcinoma cells in Ca^{2+}-free medium results in increased proliferation of cells that are loosely attached to the

substratum. When $[Ca^{2+}_o]$ (a final concentration of 1.4 mM) is included in the culture medium, cell growth is inhibited, and the cells take on a flattened appearance and behave as a cohesive epithelial unit [141]. In line with these observations, epidemiological studies reveal that high dietary calcium ingestion reduces risks for development of colorectal cancer by inhibiting cell growth and promoting epithelial cell differentiation [142]. CaR expressed on the apical and basolateral membranes of colonic crypt cells [143] may be activated by small changes (<5%) of $[Ca^{2+}_o]$ at millimolar levels as well by other physiological agonists such as spermine and phenylalanine [144]. A significant association was found between CaR diplotypes and advanced colorectal adenoma in three common nonsynonymous single nucleotide polymorphisms (SNPs) in the coding region of intracellular CaR tail [A986S (rs1801725), R990G (rs1042636), and Q1011E (rs1801726)] [145]. However, risks were not specific to any of the three genotypes studied, suggesting that the association between genetic variants in CaR and adenoma risk may depend upon the diplotype patterns studied or upon linked genetic variants upstream or downstream of these three SNPs.

In human colon carcinoma cells lines, elevated levels of $[Ca^{2+}_o]$, presumably by activating the CaR elicited increased expression of a homotypic cell–cell adhesion molecule, E-cadherin, which functions as tumor suppressor in colon carcinoma [146, 147]. Mutation of the adenomatous polyposis coli gene (APC) is regarded as a particularly crucial event in colorectal carcinogenesis [148, 149]. APC protein binds to cytosolic β-catenin, a member of the cell membrane-bound adherens complex. Furthermore, APC mutation leads to nuclear accumulation of β-catenin [150], a feature associated with progression along the adenoma-carcinoma sequence. β-Catenin may exist as part of cell membrane-bound adherens complex with E-cadherin, a physical association that is tightly regulated by tyrosine phosphorylation of β-catenin. The β-catenin-adherens complex is dissociated by tyrosine phosphorylation [151], leading to translocation of β-catenin to the nucleus, where it binds with a member of the T-cell receptor family (TCF) of transcription factors to form a complex that activates transcription of target genes required for cellular proliferation [152]. High levels of $[Ca^{2+}_o]$ and other di- or polyvalent cations have been shown to inhibit β-catenin/TCF4 complex formation in human colon cancer cells. Since downregulation of β-catenin/TCF signaling has been implicated in the promotion of differentiation in colon carcinoma cells, $[Ca^{2+}_o]$ via CaR may act in concert to promote more differentiated and less malignant phenotypes of these cells [153].

c-Myc was the first target gene in the β-catenin signaling pathway to be identified in humans. Kallay et al. [154] have shown that Caco-2 cells respond differentially to changes in Ca^{2+} depending on whether the stimulus is applied to basolateral or luminal surfaces. Reduction of $[Ca^{2+}_o]$ to 0.025 mM on the luminal surface reduced c-myc RNA expression, while a similar change confined to the basolateral surface had no effect [154]. These data correlate well with the observations that c-myc is a major factor regulating cell cycle progression in colonic epithelial cells, where a decreasing levels of $[Ca^{2+}_o]$ in the surface to crypt direction has been proposed [155, 156], and that proliferation of normal intestinal epithelial cells is highest in the base of crypts, where extracellular calcium concentration is lowest [157]. Furthermore, immunohistochemical studies of human colon carcinomas indicated that CaR protein was most highly expressed in well-differentiated regions of the tumor and nearly lacking in poorly differentiated regions [158]. These data suggest that loss of a normal growth inhibitory response to elevated $[Ca^{2+}_o]$, dependent on expression of functional CaR, may contribute to the progression of colon carcinomas.

Colon cancer is a disease of defective Wnt signaling [159, 160]. Wnts are cysteine-rich glycoproteins that interact with coreceptors low-density lipoprotein receptor-related protein (LRP) 5/6 and seven-span transmembrane protein Frizzled. While some Wnts (Wnt1, Wnt3, Wnt3a) signal canonically by stimulating β-catenin signaling by releasing β-catenin from its destruction complex (for example APC), others such as Wnt4, Wnt5a, and Wnt11 signal noncanonically without liberating β-catenin. Increased expression of Wnt5a transcripts have been shown in colon cancer in addition to other malignancies [161–165]. Using HT-29 adenocarcinoma cells, which have wild-type β-catenin and a truncated APC [166, 167], Macleod et al. [168] have shown that high $[Ca^{2+}_o]$ via CaR stimulates wnt5a secretion. CaR-stimulated Wnt5a secretion in HT-29 cells requires truncated APC, because overexpressing wild-type APC in these cells prevents increased secretion of Wnt5a by CaR stimulation [168]. Increased secretion of Wnt5a by CaR activation in turn inhibits β-catenin reporter activity, as demonstrated by abolition of CaR effect on β-catenin reporter activity by using a chelator of Wnt protein, anti-Frizzled-8 antibody [168]. Taken together, the evidence indicates that Wnt5a, likely by autocrine action, activates either the orphan tyrosine kinase Ror2 or an uncharacterized Frizzled receptor to increase transcripts and proteins of E-ubiquitin ligase, seven in absentia homolog 2 (Siah2) [169]. The same study also provides mechanistic insights into the role of CaR in mediating anti-carcinogenic effects of Ca^{2+} supplementation under the condition of APC mutation that is observed in colorectal cancer [170]. Figure 3 is a schematic illustration of the sequence of events following CaR activation that result in wnt5a secretion.

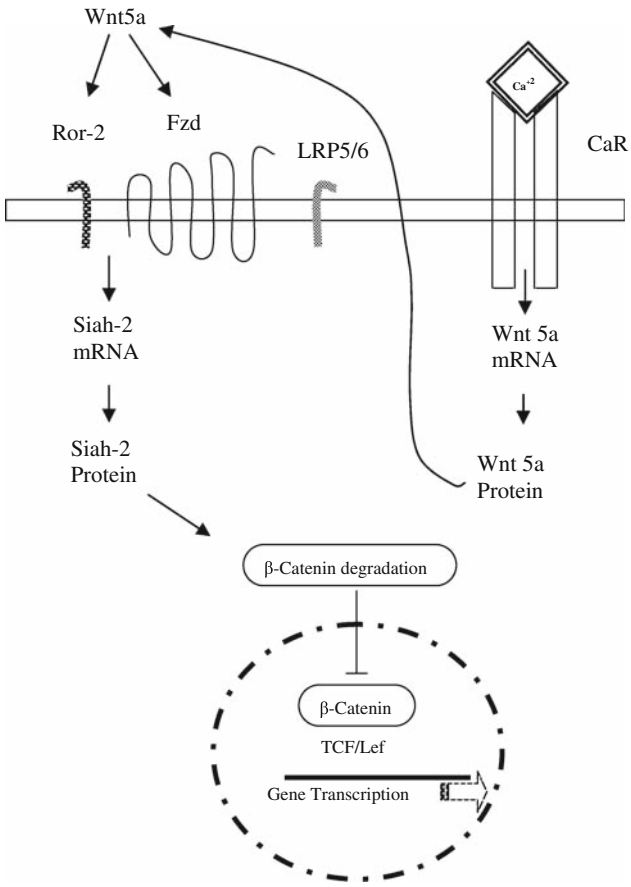

Fig. 3 Effect of CaR activation of a truncated APC colonic epithelial cell. [Ca^{2+}_o] stimulates the CaR in HT-29, APC-truncated adenocarcinoma intestinal cell line resulting in increased synthesis and secretion of Wnt5. Wnt5a by interacting with Ror2 and/or Frizzled (Fzd) increase Siah2. Overexpression of full-length APC prevents CaR-stimulated upregulation of Wnt5a and increases in Siah2 protein. Increase in Siah2 increases β-catenin degradation and its signaling. Modified from Fig. 6 of reference [168]

CaR in insulionams and gastrinomas

CaR has been shown to be expressed in purified rat pancreatic B-cells as well as in human insolinoma cells [171, 172]. Insulin release is known to be dependent on [Ca^{2+}_o] as removal of [Ca^{2+}_o] abrogates sustained secondary phase of glucose-induced insulin release [173]. Elevated levels of [Ca^{2+}_o] itself could also stimulate insulin release without the presence of any secretagogue [174]. On the other hand, in perifusion experiments, elevations in [Ca^{2+}_o] levels produced initial transient increases in insulin secretion, followed by a concentration-dependent and prolonged, but reversible, inhibition of secretion, suggesting a negative insulinotropic action being mediated by the CaR [175]. However, the role of CaR in mediating the effects of elevated [Ca^{2+}_o] is equivocal. In mice, NPS-R467 stimulated glucose-stimulated insulin release but in a beta cell line, the inactive enantiomer NPS-S467 also stimulated insulin release.

A different coupling of the CaR with its signaling apparatus appears to exist in insulioma cells compared with normal B-cells. For example, insulinoma cells are more sensitive in increasing in response to elevated levels of [Ca^{2+}_o] compared with normal B-cells. In addition, increased [Ca^{2+}_i] levels by high [Ca^{2+}_o] was abolished by blocking of PI3 K-Akt pathway. However, it still remains to be understood whether or not CaR is involved in the secretion of insulin from insulinoma as well as growth of these tumors.

Based on the reports that altered calcium levels exert profound effects on the biological effects of gastrinomas, the gastrin secreting tumors, CaR expression was studied in 10 gastrinoma patients with high levels of fasting serum gastrin levels. All gastrinoma tumors exhibited readily detectable levels of CaR mRNA by RT-PCR. Variations in the levels of CaR protein in the tumor samples, however, did not correlated with the aggressiveness growth patterns, metastases to other organs and multiple endocrine neoplasia type 1. Also, the role of CaR in gastrinomas, particularly in the regulation of gastrin release is yet to be documented.

CaR and neoplasm of central nervous system

Glial cells, unlike neurons, have the capacity to undergo cell division postnatally [176]. Such proliferation occurs during reactive gliosis, in response to CNS injury [177] and during malignant transformation of glial cells. Gliomas, a collective term for glial cell-derived tumors, include astrocyte-, oligodendrocyte-, and ependymal cell-derived tumors. Gliomas are the most common primary neoplasm of the central nervous system. They are classified into four clinical grades; grade 4, also referred as glioblastoma multiforme (GBM), is the most aggressive [178, 179]. GBMs can develop de novo (primary) or progress to GBM from lower-grade gliomas (secondary). Low-grade gliomas are generally classified into three major subgroups based on histological characteristics reflecting cellular differentiation lineages: astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas.

Most GBMs express markers for astrocyte differentiation such as glial fibrillary acid protein (GFAP) and S-100β [180, 181]. GBMs show signs of disturbed Ca^{2+} homeostasis and abnormal electrophysiological characteristics [182–186]. Calcification, sometimes combined with cyst formation [187], is a common pathological feature of these tumors [188–190].

CaR expression has been studied in human gliomas. Immunostaining of the tumors revealed that GBMs have

patchy CaR staining (1–30% CaR-positive cells), whereas low-grade and anaplastic astrocytomas have moderate to strong staining, varying from 30 to 100% CaR-positive cells. Since low-grade gliomas such as astrocytomas can progress to GMS, it is surmised that CaR may play some role in the process [191]. CaR immunoreactivity has also been detected in human meningiomas, although its role is unclear.

CaR in the human U373 astrocytoma cell line has been shown to promote proliferation and activation of a nonselective cation channel (NCC) [192]. Whether CaR mediates its mitogenic effect on U373 cells via the activation of NCC is not known. In contrast to U373 cells, U87 astrocytoma cell line does not proliferate in response to high Ca^{2+}; instead, CaR activates a midi-type outward K^+ channel via p38 mitogen-activated protein kinase pathway [193]. Blocking of outward rectifying K^+ channels with 4-aminopyridine inhibits proliferation of U87 cells [194]. It is possible that CaR in these cells modulates a different kind of K^+ channel that does not modulate cellular proliferation. The differences in CaR's functions in these two high-grade astrocytoma cell lines (U373 and U87) indicate the complex heterogeneity of glioma cell biology.

CaR activation results in equal increase in the secretion of PTHrP in normal and malignant astrocytes as well as in menigiomas [191]. Since treatment of U87 cells with antisense PTHrP reduces cell growth and survival, a pathological role of CaR in glioma progression can be suggested due to its stimulatory effects on PTHrP secretion from gliomas. In addition, future studies could address the rate of basal PTHrP secretion in normal human meningoepithelial cells relative to the rate in meningiomas and whether the receptor modulates the growth and/or differentiation of these cell types.

CaR and ovarian cancer

Ovarian cancer is the most lethal female genital malignancy. Malignant ovarian tumors are derived primarily from ovarian surface epithelial (OSE) cells [195], whereas tumors from other ovarian tissues are mostly benign. OSE cells are derived from a mesodermal lineage and represent an extension of the peritoneal mesothelium [196, 197]. This simple cuboidal epithelial layer retains proliferative potential throughout adult life, because the wounding during each ovulation followed by healing requires proliferation. Since the number of ovulatory cycles experienced by a woman is considered to be one of the major risk factors for ovarian cancer, the proliferative potential of OSE cells could contribute to the progression of this malignancy [198, 199]. Therefore, understanding the proliferative pathways activated in normal OSE cells is a prerequisite to identifying possible disruptions of these pathways in ovarian adenocarcinomas.

Some potential mitogens of OSE cells include follicle-stimulating hormone [197], TGF-β, hepatocyte growth factor [200], macrophage-colony stimulating factor, lysophosphatidic acid, and elevated calcium [95, 96]. Elevated $[Ca^{2+}{}_o]$ stimulates proliferation of OSE cells [201], which is dependent upon the presence of functional CaR in these cells. Ovarian cancer cells also express CaR protein [202]. Studies on human and rodent OSE cells revealed Ras-Raf-MKK1 pathway mediating CaR-stimulated proliferation of these cells [201, 203]. SV40-immortalized human OSE cells exhibit a sharp increase in proliferation from 0.8 mM Ca^{2+} to 1.8 mM Ca^{2+} [202], and this proliferative response is dependent on CaR-mediated activation of MAP kinase cascade and PI3 kinase. Thus, CaR appears to mediate the mitogenic effect of elevated $[Ca^{2+}{}_o]$ in OSE cells by promoting cellular survival.

Conclusion and future directions

Current understanding puts CaR as a molecule that can either promote or prevent tumor growth depending on the type of cancer. In the setting of HHM, i.e., in cases of breast and prostate cancers, CaR promotes cancer progression and may favor metastases owing to its stimulatory effect on PTHrP secretion by these tumors. However, final outcomes of the stimulatory effects of CaR in glial cells and gliomas are unclear at this stage. CaR also promotes proliferation of OSE cells that undergo malignant transformation. Interestingly, transactivation of EGFR by CaR has been reported in both normal and malignant cells, which prevents us from concluding that CaR-EGFR communication is a cancer-specific phenomenon. Growth-inhibitory and differentiation-promoting functions of CaR have been suggested in the case of colon carcinoma and PT adenoma. Non-canonical wnt/β catenine signaling elicited by CaR activation in colon carcinoma cells could be part of a cascade specifically used by CaR in these cells. The allosteric modulator of the CaR, cinacalcet (Sensipar®) is used for the management of PT adenoma. However, the feasibility of pharmacological manipulation of CaR by a calcimimetic or calcilytic (CaR antagonist), depending upon the requirement of stimulating or inhibiting CaR function in various cancer cells, remains to be determined.

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