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Calcium Sensing by the Mammary Gland

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Calcium is an important nutrient that is secreted into milk in quantities that put a considerable stress upon maternal calcium homeostasis. Here we summarize the evidence that two important entities, the extracellular calcium-sensing receptor (CaR) and parathyroid hormone-related protein (PTHrP) are involved in a feedback loop that regulates calcium fluxes to the mammary gland. The CaR may also play a role in regulating milk secretion, and may regulate the proliferation of normal and neoplastic mammary epithelial cells. Finally, the relationship between the CaR and PTHrP in breast cancer cells may promote the formation of osteolytic bone metastases.

KEY WORDS: calcium-sensing receptor; lactation; calcium metabolism; bone, PTHrP.

INTRODUCTION

The primary function of the mammary gland is the transfer of nutrients, including calcium, from the lactating mother to the newborn offspring. Milk is the only source of calcium for the neonate's rapidly developing skeleton, and so the mammary gland must drive large amounts of calcium against a strong concentration gradient. This presents challenges to calcium metabolism at the level of the whole organism and at the level of the mammary epithelium. First, the movement of large quantities of calcium from the bloodstream into milk stresses the mechanisms that maintain systemic calcium homeostasis. Second, in order to be efficient in the task of calcium transport, the mammary gland should be able to match the transport of calcium across the mammary epithelium to the supply of calcium. Herein we present evidence that two molecules, the calcium-sensing receptor (CaR) and parathyroid hormone-related protein (PTHrP), allow the mammary gland to participate in the regulation of systemic calcium pools. We also summarize the evidence that the CaR regulates the transport of calcium into milk. Finally, we discuss the evidence that calcium may regulate mammary epithelial cell proliferation and/or the metastasis of breast cancer cells to bone.

THE CALCIUM-SENSING RECEPTOR

The circulating calcium concentration in terrestrial organisms remains relatively constant, regardless of fluctuations in calcium intake. Such stability relies on cooperation between several organs, principally the parathyroid glands, the kidneys, the skeleton, and the gut *(4)*. The ability of parathyroid cells to sense very small changes in the circulating calcium ion concentration, and to adjust parathyroid hormone (PTH) secretion appropriately, is central to the maintenance of calcium homeostasis *(4)*. For example, a decrease in calcium concentration leads to increased secretion of PTH from the parathyroid which, in turn, acts on the kidney, skeleton, and gut to raise the calcium concentration back to baseline. Conversely, an elevation in the plasma calcium ion

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Abbreviations used. BLG, β-lactoglobulin; CaR, calcium-sensing receptor; DCT, distal convoluted tubule; GPCR, G-protein-coupled receptor; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PMCA, plasma membrane calcium-ATPase; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein.

concentration decreases PTH secretion, which leads to the excretion of excess calcium via the kidney and a decrease in the circulating calcium concentration back to baseline *(4)*.

In 1993, the protein that allows the parathyroid cells to sense changes in the concentration of calcium was discovered *(5)*. The CaR is a seven transmembrane-spanning G-protein coupled receptor (GPCR), belonging to the GPCR family C, which also includes the metabotropic glutamate receptors, the $GABA_B$ receptors, and the vomeronasal receptors (putative pheromone receptors) *(4)*. The mature CaR has a molecular weight between 150–160 kDa, and contains a large glycosylated extracellular domain with multiple calcium binding sites *(4)*. The CaR appears to function as a homodimer *(6)*, and mutations interfering with its dimerization disrupt its function *(7)*. In addition to calcium, the receptor can be activated, although with less potency, by other polycations, including magnesium, gadolinium or aminoglycoside antibiotics such as neomycin *(4)*. A series of pharmacologic activators of the calcium receptor have also been synthesized. These compounds have been termed calcimimetics and are not true agonists of the receptor, but rather allosteric activators that sensitize the receptor to low concentrations of calcium *(8–10)*. In contrast, calcilytic agents are pharmacological compounds that inactivate the CaR *(9,10)*. The signaling pathways stimulated by the CaR remain incompletely defined *(4,11)*, but it is known that the CaR can signal through both $G_{q\alpha}$ and G_i and activates PLC, PLA_2, and PLD in parathyroid cells and HEK293 cells transfected with the CaR *(12)*. Signaling is dependent on the cell type, and in various cells it activates the MAPK, ERK1 through a G_i-dependent pathway *(13)*, and MAPK family members such as JNK by transactivation of focal adhesion kinase, Src, or a receptor tyrosine kinase (EGFR) *(14,15)*.

In addition to parathyroid cells, the CaR is expressed on a wide variety of other cell types. These include several types of renal epithelial cells, osteoclasts, osteoblasts, osteocytes, chondrocytes, cytotrophoblasts, neurons, breast epithelial cells, and keratinocytes *(4)*. The distal convoluted tubule is the major site of regulation of calcium reabsorption in the nephron *(16)*. In the DCT, calcium reabsorption is an active, transcellular process involving either a sodium–calcium exchange mechanism or a plasma membrane calcium ATPase (PMCA) *(16)*. In addition to being regulated by PTH, there is evidence that calcium suppresses its own reabsorption through the CaR on the basolateral surface of DCT cells *(16)*. Not all of the sites where the CaR is expressed are involved in the regulation of calcium metabolism, however, it has been shown that the CaR regulates a variety of cellular processes in addition to parathyroid hormone secretion. For example, in renal tubules and in the intestines the CaR has been shown to regulate sodium and water transport *(4)*. CaR signaling has also been shown to participate in the regulation of the proliferation and/or differentiation of intestinal epithelial cells, parathyroid cells, and keratinocytes *(17)*. Finally, as will be discussed in more detail below, in several cell types the CaR has been shown to regulate the secretion of parathyroid hormone-related protein (PTHrP) rather than PTH *(18–23)*.

THE CaR AND CALCIUM METABOLISM DURING LACTATION

During lactation, large amounts of calcium are transferred into milk, placing nursing mothers under calcemic stress. Concurrent with lactation, bone turnover increases and bone mass decreases, presumably to free skeletal calcium for milk production *(24,25)*. Bone loss during lactation is rapid and completely reversible upon weaning *(24–27)*. In the mouse, for instance, between day 18 of gestation and day 12 of lactation, bone mineral density decreases by 16–21% *(28)*. Just 1 week after forced weaning done in the experiment cited on day 12 of lactation, maternal bone density is already substantially recovered *(28)*. Although both bone formation and bone resorption are increased during lactation, bone resorption must increase more, since there is a net loss of bone. The classic calciotrophic hormones, PTH and 1,25-dihydroxyvitamin D, are not necessary for bone loss during lactation *(24,29–31)*. However, PTHrP, normally thought of as a local paracrine or autocrine/intracrine factor *(32)*, is elevated in the circulation of lactating women *(33–37)* and mice *(28)*. PTHrP can act on bone cells to increase bone resorption, and epidemiologic studies have suggested that increased levels of circulating PTHrP are involved in bone loss during lactation *(35)*. Furthermore, it had been suggested that the lactating mammary gland was the source of the circulating PTHrP *(38)*. We recently used the cre-lox system to address these problems directly *(39)*. In mice with one null PTHrP allele, one floxed PTHrP allele ($PTHrP^{lox/-}$)

and the β-lactoglobulin-Cre transgene (BLG-Cre), expression of PTHrP in the mammary gland was abolished (Fig. 1A). Lactating PTHrP$^{lox/-}$/BLG-Cre mice, in addition to having no PTHrP RNA in the mammary gland and no PTHrP in milk, had reduced levels of circulating PTHrP (Fig. 1B), compared to lactating littermate controls (PTHrP$^{lox/-}$) *(39)*. Bone turnover and bone loss (Fig. 1C) were also reduced in the lactating PTHrP$^{lox/-}$/BLG-Cre mice *(39)*. Therefore, the mammary gland is an endocrine organ during lactation and releases PTHrP into the circulation to promote maternal bone loss.

There is strong evidence implicating the CaR and PTHrP in a feedback loop that allows the mammary gland to regulate systemic calcium metabolism in lactating animals. This control is possible due to the presence of the CaR on mammary epithelial cells *(3)*. The CaR is expressed at low levels in the mammary gland of virgin mice (Fig. 2). At the onset of lactation, CaR expression in the mammary gland increases dramatically, and then returns to baseline virgin levels after the initiation of involution *(3)*. By immunofluorescence, the CaR appears to be located basally, ideally positioned to detect the level of systemic calcium (Fig. 2). In several other cell types, including human breast cancer cells *(40)*, the CaR regulates secretion of PTHrP *(18,19,21–23)*. In primary cultures of mouse mammary epithelial cells, we found that stimulation of the CaR by extracellular calcium or the calcimimetic, NPS-R467, caused a dose-dependent decrease in PTHrP secretion (Fig. 3A) *(3)*. We investigated the function of the CaR in the mammary gland during lactation *in vivo* by placing lactating mice on a calcium-restricted diet to lower their systemic calcium levels, and then specifically stimulating the CaR by infusing the calcimimetic compound NPS-R467. Mice on the low-calcium diet had significantly lower plasma calcium concentrations; therefore CaR signaling in the mammary gland was reduced. Consistent with our *in vitro* data in primary cultures of normal mouse mammary epithelial cells, calcium-restricted mice had increased mammary gland PTHrP RNA expression and higher concentrations of PTHrP in milk. Furthermore, treatment with the calcimimetic, NPS-R467 returned the PTHrP RNA and protein expression to normal levels (Fig. 3B), indicating that the effect of calcium on PTHrP production by the mammary gland *in vivo* is mediated by the CaR *(3)*.

THE CaR AND REGULATION OF CALCIUM TRANSPORT INTO MILK

In addition to regulating PTHrP production in the lactating mammary gland, CaR regulates calcium transport into milk *(3)*. Lactating mice on a low-calcium diet had decreased milk calcium contents, which were partially corrected by calcimimetic infusion *(3)*. Furthermore, when primary mammary epithelial cells were grown on matrigel with lactogenic hormones, the accumulation of ^{45}Ca in the luminal fraction was greater with increasing concentrations of calcium in the media or with calcimimetic treatment *(3)*. The effect of the CaR on calcium transport is not related to its effect on PTHrP production, since the same results were obtained in primary cultures of cells derived from mice with a conditional deletion of the PTHrP gene in their mammary epithelial cells (the PTHrP$^{lox/-}$/BLG-Cre mice). These findings suggest that calcium, acting through the CaR, regulates its own transport into milk, based on its availability in the maternal circulation. It should be noted that the evidence for this regulation is based only on the effect of the calcimimetic, and no genetic evidence has been published at this time. The mechanism by which the CaR affects calcium transport in the mammary gland is not known. Reinhardt *et al.* found that the plasma membrane calcium ATPase type 2 (PMCA2) is necessary for the normal secretion of calcium into milk (Reinhardt, 2004 #1007). In the PMCA2 knockout mice, however, the secretion of milk itself is somewhat impaired (Reinhardt, 2004 #1007), so it is unclear whether PMCA2 affects calcium transport directly. Nonetheless, it is known that the CaR regulates transcellular calcium transport in polarized MDCK cells, a model of the DCT, by altering PMCA activity *(16)*. Therefore, it is tempting to speculate that the CaR also regulates PMCA2 activity in mammary epithelial cells.

Finally, milk from lactating mice on a low calcium diet had higher protein content and osmolality than mice on a normal calcium diet *(3)*. Treatment with the calcimimetic reversed these increases *(3)*, indicating that the CaR may regulate the protein content and osmolality of milk, suggesting an effect on water transport. Such a function is feasible, as the CaR has similar roles in the kidney and the large intestine *(4)*. Although all the evidence from these studies support a role for the CaR in regulating PTHrP production, calcium transport, and perhaps water transport in the mammary gland during lactation, potentially

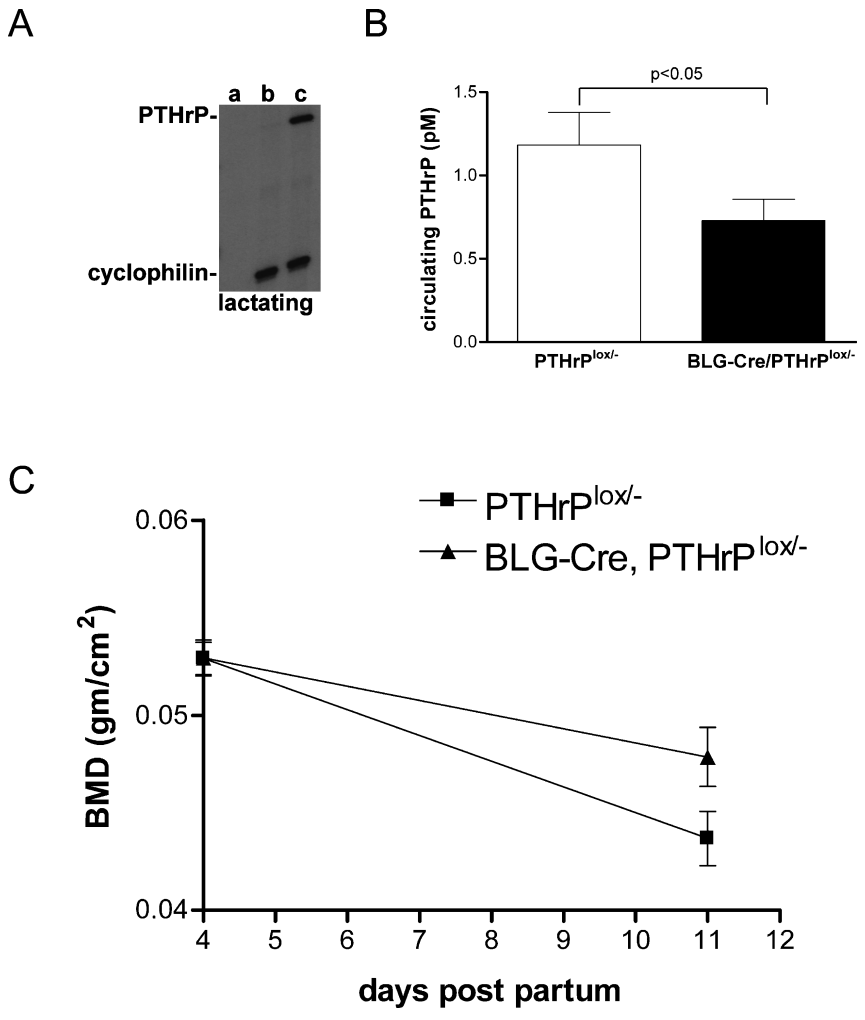

Fig. 1. Cre-mediated deletion of PTHrP from the lactating mammary gland and its effect on maternal bone metabolism. (A) Yeast RNA (a) was a negative control. Ribonuclease protection of PTHrP and cyclophilin in RNA from mammary glands of lactating BLG-Cre/PTHrP$^{lox/-}$ (b) and PTHrP$^{lox/-}$ (c) mice. PTHrP RNA in the BLG-Cre/PTHrP$^{lox/-}$ mammary glands was undetectable. (B) Circulating PTHrP(1–34) levels were significantly lower ($p < 0.05$) during lactation in BLG-Cre/PTHrP$^{lox/-}$ mice (0.73 ± 0.13 pmol/l, $n = 7$) than in PTHrP$^{lox/-}$ controls (1.18 ± 0.19 pmol/l, $n = 3$), indicating that the mammary gland releases PTHrP into the circulation. (C) Bone mineral density was measured *in vivo* at days 4 and 11 of lactation by dual-energy X-ray absorptiometry. At day 4, the vertebral BMD of BLG-Cre/PTHrP$^{lox/-}$ mice ($n = 6$) and controls ($n = 5$) was 0.05294 ± 0.002043 and 0.05290 ± 0.002030 g/cm^2, respectively. By day 11 of lactation, the BMD of the control mice had fallen to 0.04368 ± 0.001386 g/cm^2, while the BMD of the BLG-Cre/PTHrP$^{lox/-}$ mice was 0.04786 ± 0.001512 g/cm^2. Therefore, the BLG-Cre/PTHrP$^{lox/-}$ mice lost significantly less bone mass than controls during 1 week of lactation ($p < 0.01$).

confounding effects of disturbed systemic calcium metabolism caused by the low-calcium diet cannot be absolutely ruled out. Some of the findings have been confirmed in studies of heterozygous CaR knockout mice (L. Ardeshirpour, unpublished observations), but these mice also have abnormal systemic calcium metabolism *(41)*. Ultimately, the conditional deletion of the CaR in the mammary gland by cre-mediated recombination will be necessary to separate the effects of the CaR in other organs from its effects specifically in the mammary gland.

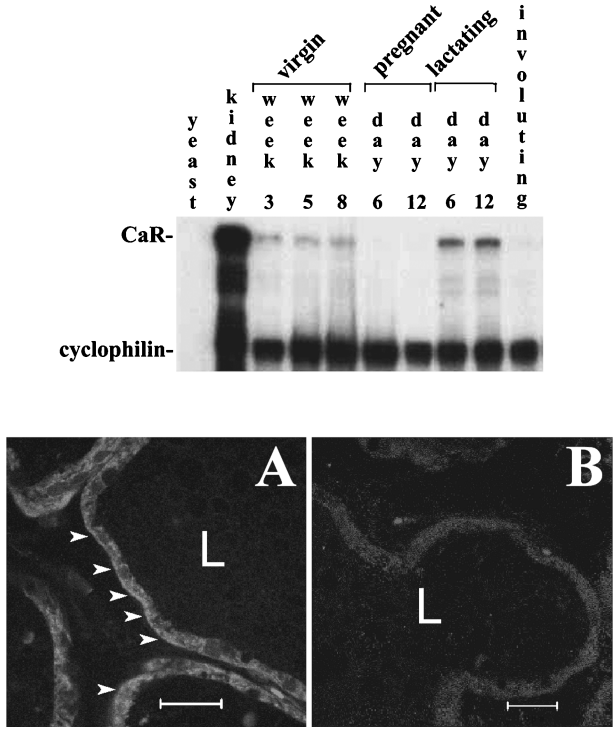

Fig. 2. CaR expression in the mammary gland. *Upper panel.* RNase protection analysis of CaR mRNA levels during mouse mammary gland development. Total RNA (50 μg) prepared from mouse mammary glands harvested at the time-points noted in the figure were assayed. Mouse kidney RNA (5 μg) served as a positive control, and 50 μg of yeast RNA served as a negative control. A cyclophilin probe was used as a loading control. *Lower panel.* Immunofluorescence staining for the CaR in mammary glands of lactating mice. Staining was done with 10 μg/ml anti-CaR antibody (A) or rabbit IgG (B). *Arrowheads* denote the basal area, and "L" denotes the alveolar lumen. The scale bars represents 20 μm.

THE CaR IN BREAST CANCER

Proliferation

In early studies using isolated human mammary epithelial cells, it was found that their growth and differentiation could be altered dramatically by changing the calcium concentration of the media *(1,2)*. Proliferation was favored by low calcium levels, while high calcium levels promoted differentiation. Reducing the calcium concentration from 0.46 to 0.046 mM reduced the population doubling time by 6–12 h *(1)*. In addition to increasing proliferation, low calcium concentrations increased the percentage of cells capable of extended growth by several fold *(1)*. Also, at high calcium concentrations (0.46 mM or higher), the cells showed signs of increased differentiation, including greater cell–cell and cell–substrate attachment and the presence of many lipid-filled vacuoles *(1)*. Although the effects of calcium on mammary epithelial cells in culture have been known for some time, the mechanism by which it acts is unknown. Furthermore, this issue is complicated by the studies of Medina *et al.*, who reported that mouse mammary epithelial cells grew optimally in media with a high calcium concentration (0.8 mM) *(42)*. It remains unknown why human and mouse mammary cells appear to have different sensitivities to calcium *in vitro*. It was also reported that calcium released the growth inhibition of 1,25-dihydroxyvitamin D on the breast cancer cell lines HT-39 and MCF-7 *(43)*, apparently at odds with a growth-inhibiting and differentiation-inducing effect of calcium on breast cells.

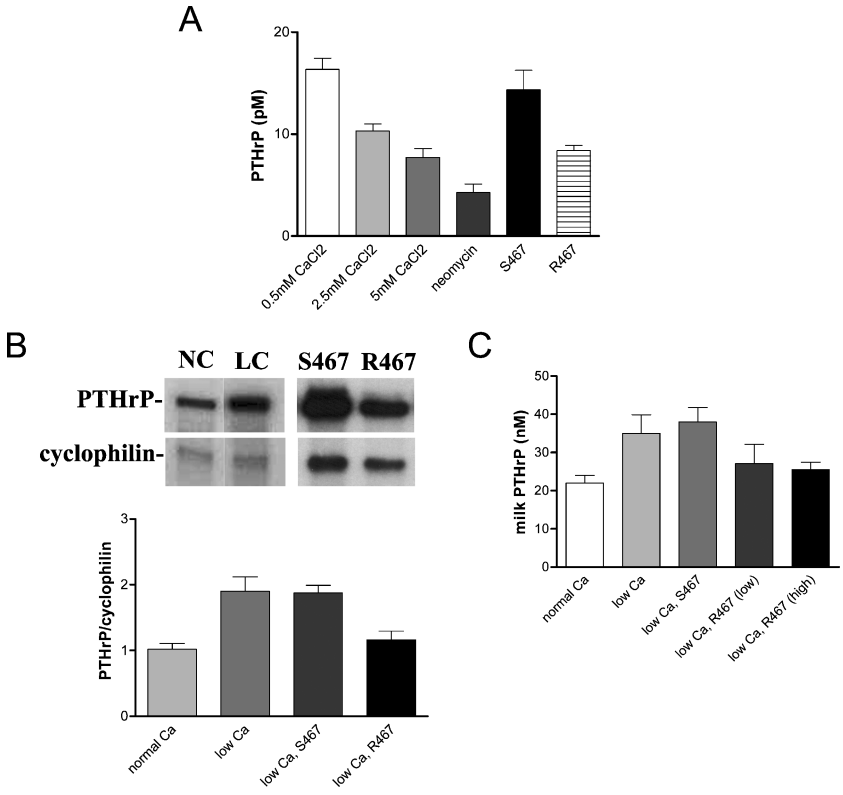

Fig. 3. Regulation of PTHrP production by the CaR in mammary epithelial cells. (A) PTHrP concentrations in conditioned media harvested from cultures of normal mammary epithelial cells exposed to varying concentrations of calcium noted on the graph. Calcium-free DMEM was supplemented with the specified concentration of calcium. Cells were also exposed to 300 μM of neomycin, an agonist of the CaR, and 2.5 μM of the calcimimetic NPS R467 or its less active isomer NPS S467 (as a negative control). Bars represent the mean of three experiments; error bars represent the SEM. (B) PTHrP mRNA levels in mammary glands of lactating mice on either a normal calcium diet (NC), a low calcium diet (LC), a low calcium diet with NPS S467 (control compound) treatment (40 μmol/kg/day, S467), or a low calcium diet with NPS R467 (calcimimetic) treatment (40 μmol/kg/day, R467) as assessed by RNase protection analysis. Total RNA (40 μg) was assayed. The bar graph represents cumulative data from four animals per treatment normalized to cyclophilin. Note that a low calcium diet increases PTHrP mRNA levels in the mammary gland and that treatment with NPS R467, but not NPS S467, prevents this increase. (C) Milk PTHrP concentrations in mice fed either a normal calcium diet (0.6%) or a low calcium diet (0.01%). Mice on a low calcium diet either received nothing (low Ca) or were infused with NPS S467 (low Ca, S467) at 40 μmol/kg/day or NPS R467 at doses of 4 (low Ca, R467 (low)) or 40 μmol/kg/day (low Ca, R467 (high)). Milk PTHrP concentrations were significantly different in the low Ca ($p < 0.05$) and low Ca, S467 ($p < 0.05$) groups as compared to the group on a normal diet. However, milk PTHrP concentrations in the mice treated with NPS R467 were not significantly different from those on a normal calcium diet.

A specific mechanism that may mediate the effects of calcium on human mammary cells arose when it was recently found that normal and malignant human breast tissues *(44)*, as well as the mouse mammary gland *(3)*, express the CaR. Although the effects of the CaR on proliferation in mammary cells has not been studied directly, in MCF-7 and MDA-MB-231 breast cancer cells signaling through the CaR increases the secretion of PTHrP *(40)*. Overexpression of PTHrP has been shown to increase proliferation of MCF-7 cells through a nuclear/intracrine pathway *(45,46)*, however others have shown that

PTHrP in the media inhibits their growth *(47)*. Also, targeted overexpression of PTHrP in the mammary gland shortened tumor latency and increased tumor incidence following exposure to the chemical carcinogen, dimethylbenzanthracene *(48)*. Nonetheless, a significant role for PTHrP in regulating the proliferation of breast cancer cells is still controversial *(49)*, and it is unclear whether the effects of calcium on human mammary epithelial cell growth are a result of CaR action on PTHrP production. Another interesting observation is that dietary calcium appears to have a protective effect against breast cancer *(50–52)*. No study has yet addressed whether this results from increased CaR signaling in mammary cells.

In addition to the evidence outlined above, the expression pattern of the CaR during post-natal mammary gland development in the mouse also suggests that it may affect proliferation of mammary epithelial cells. During pregnancy, the phase of mammary development when massive proliferation results in expansion of the epithelium to fill out the entire mammary fat pad, expression of the CaR disappears (Fig. 2). Thus, low CaR signaling, whether it results from reduced calcium concentration in the media or from decreased expression of the CaR itself, may release growth inhibition and induce proliferation. However, direct evidence supporting this hypothesis is lacking.

Metastasis

In contrast to its action in normal mouse mammary epithelial cells, stimulation of the CaR has been shown to upregulate PTHrP secretion in MCF-7 and MDA-MB-231 breast cancer cells *(40)*. Because PTHrP acts in bone to increase osteoclastogenesis and increase the activity of osteoclasts that have already formed, it is thought that the upregulation of PTHrP by the CaR in breast cancer cells in response to calcium may be involved in the "vicious cycle" of osteolytic bone metastasis, shown in Fig. 4. In breast cancer cells that colonize bone, the CaR would detect the high calcium concentration in the microenvironment and promote further osteolysis by

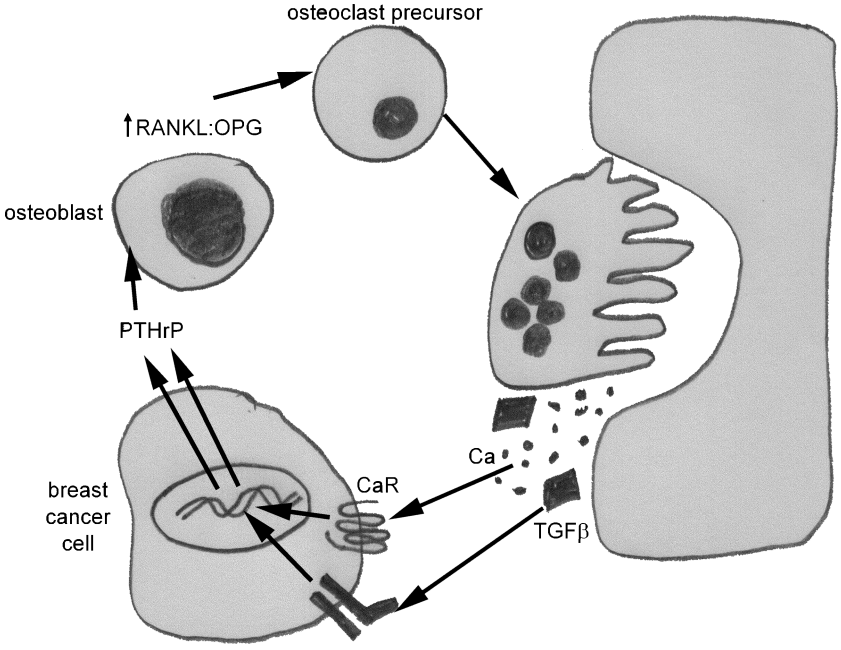

Fig. 4. The vicious cycle of osteolytic bone metastasis. In this feed-forward model of the formation of osteolytic bone metastasis, breast cancer cells producing PTHrP invade bone and stimulate the PTH/PTHrP receptor on osteoblasts, which then increase osteoclastogenesis by increasing the RANKL:OPG ratio. The resulting increase in bone resorption causes an increase in the concentration of free calcium and TGFβ in the bone microenvironment, which then act on receptors on the metastatic breast cancer cells to sustain and augment PTHrP production.

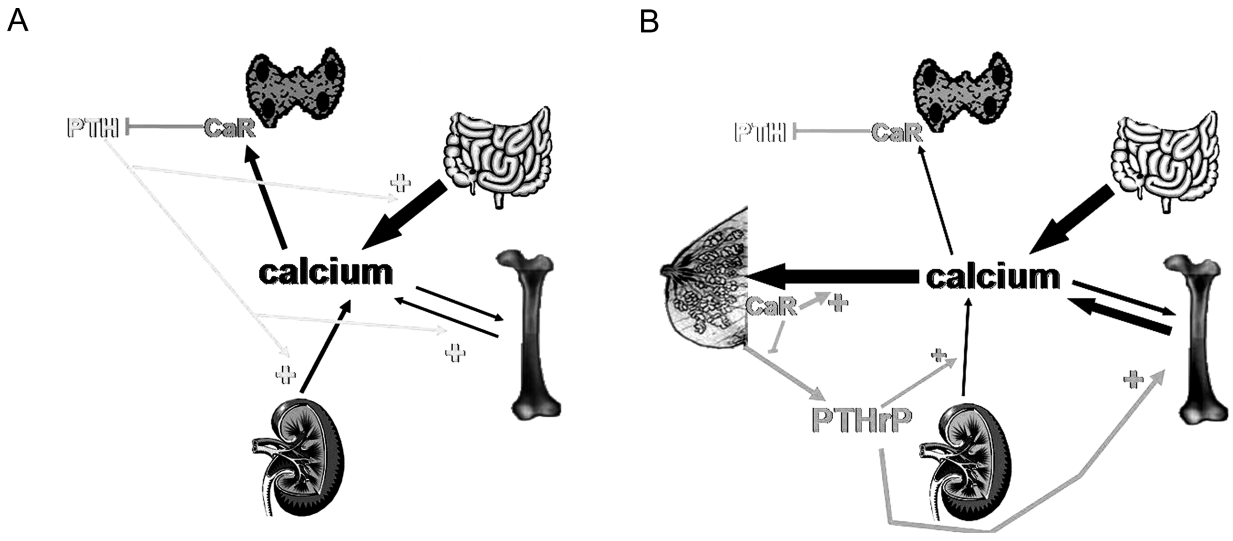

Fig. 5. The mammary gland as an accessory parathyroid gland during lactation. (A) In nulliparous animals, the parathyroid gland is the central regulator of calcium metabolism. The CaR on parathyroid cells senses small changes in circulating calcium levels and adjusts PTH secretion, which then acts on the intestines, bone, and the kidneys to restore the systemic calcium concentration to its original set-point. (B) In lactating animals, we hypothesize that the mammary gland takes over the job of the parathyroid gland, but secretes PTHrP instead of PTH to act on PTH/PTHrP receptors in the intestines, bone, and the kidneys. See the text for full details.

increasing PTHrP production in the cancerous cells. This hypothesis is further supported by the finding that TGF-β potentiates the actions of the CaR on PTHrP production in breast cancer cells *(40)*. Since TGF-β is one of the growth factors released from the bone matrix upon osteolysis, its involvement in such a self-sustaining feed-forward loop seems likely.

Contrary to the findings in MCF-7 and MDA-MB-231 cells, 8701BC human breast cancer cells show the opposite effect of calcium on PTHrP secretion *(53)*. In 8701BC cells, increasing calcium concentrations decreased the production of PTHrP. Whether the CaR's upregulation of PTHrP in MCF-7 or MDA-MB-231 cells *(20)* and downregulation of PTHrP in 8701BC cells *(53)* reflects the derivation of the former from metastatic sites and the latter from a primary, non-metastatic site is an intriguing question. Guise *et al.* have shown that, indeed, MDA-MB-231 clones expressing high levels of PTHrP show increased metastatic potential in bone *(54)*. Interestingly, in normal mouse mammary epithelial cells, as in 8701BC cells, increasing concentrations of calcium also decrease PTHrP production *(3)*. Therefore, it is possible that in the process of malignant progression, changes in the intracellular signaling of the CaR cause its dysregulation of PTHrP production, which may then contribute to the formation of osteolytic metastases.

CONCLUSIONS

A CaR-Mediated Feedback Loop: Is the Mammary Gland an Accessory Parathyroid Gland During Lactation?

During lactation, maternal bone turnover increases dramatically, and is apparently caused largely by low estradiol and elevated PTHrP levels in the circulation *(28,35)*. This scenario results in significant loss of bone mineral density, which is completely and rapidly reversible after weaning *(25)*. We have recently confirmed that the lactating mammary gland is a source of circulating PTHrP during lactation *(39)*, and shown that it promotes maternal bone turnover and bone loss *(28,39)*. This is the only normal situation in which PTHrP acts as an endocrine hormone. Presumably, the skeletal calcium stores are mobilized to ensure an adequate supply of calcium for milk production and/or to protect the lactating mother from severe hypocalcemia, given the pressures resulting from the transfer of large amounts of calcium into milk. Because calcium regulates PTHrP production in the mammary gland through the CaR, we propose the following feedback mechanism (see Fig. 5). The drain of calcium from the maternal circulation into milk would cause a reduction in the systemic calcium concentration. This

loss would reduce CaR stimulation on the basolateral surface of the mammary epithelial cells, causing an increase in their production of PTHrP. More PTHrP production would result in increased PTHrP release from the mammary gland into the circulation, which would promote the mobilization of maternal skeletal calcium by increasing bone turnover. This skeletal calcium would then feed-back on the CaR to downregulate PTHrP production and restore calcium transport into milk. Thus, the mammary gland would serve as an accessory parathyroid gland, using PTHrP instead of PTH, to help maintain maternal calcium homeostasis in the face of the calcemic stress of lactation.

The mammary gland is an "intelligent" calcium-sensing organ. Although not all the details are perfectly clear yet, mammary epithelial cells respond to changes in calcium concentration in several ways. *In vitro* calcium may inhibit the proliferation and cause the differentiation of primary human mammary epithelial cells. In breast cancer cells, calcium regulates PTHrP production through the CaR, and may be involved in the "vicious cycle" of osteolytic bone metastases. The relationship between calcium, the CaR, and PTHrP may be important in maintaining calcium homeostasis during lactation, which would provide the evolutionary pressure to maintain this physiological system despite the disadvantage it poses with regard to its proposed role in osteolytic breast cancer metastasis and the osteotrophism of breast cancer *(40,54,55)*. On the other hand, the CaR could represent a novel therapeutic target which has the potential to make a significant impact on the morbidity and mortality arising from breast cancer's osteolytic metastases.

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