Calcium-Sensing Receptor Promotes Breast Cancer by Stimulating Intracrine Actions of Parathyroid Hormone–Related Protein

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Abstract

Parathyroid hormone–related protein (PTHrP) contributes to the development and metastatic progression of breast cancer by promoting hypercalcemia, tumor growth, and osteolytic bone metastases, but it is not known how PTHrP is upregulated in breast tumors. Here we report a central role in this process for the calcium-sensing receptor, CaSR, which enables cellular responses to changes in extracellular calcium, through studies of CaSR–PTHrP interactions in the MMTV-PymT transgenic mouse model of breast cancer and in human breast cancer cells. CaSR activation stimulated PTHrP production by breast cancer cells in vitro and in vivo. Tissue-specific disruption of the casr gene in mammary epithelial cells in MMTV-PymT mice reduced tumor PTHrP expression and inhibited tumor cell proliferation and tumor outgrowth. CaSR signaling promoted the proliferation of human breast cancer cell lines and tumor cells cultured from MMTV-PyMT mice. Further, CaSR activation inhibited cell death triggered by high extracellular concentrations of calcium. The actions of the CaSR appeared to be mediated by nuclear actions of PTHrP that decreased p27Kip1 levels and prevented nuclear accumulation of the proapoptotic factor apoptosis inducing factor. Taken together, our findings suggest that CaSR–PTHrP interactions might be a promising target for the development of therapeutic agents to limit tumor cell growth in bone metastases and in other microenvironments in which elevated calcium and/or PTHrP levels contribute to breast cancer progression. Cancer Res; 76(18); 5348–60. ©2016 AACR.

Introduction

The calcium-sensing receptor (CaSR) is a G protein–coupled receptor (GPCR) that signals in response to extracellular calcium and other organic and inorganic cations (1, 2). It is expressed in the parathyroid glands and kidneys, and regulates parathyroid hormone (PTH) production and renal calcium handling to maintain circulating calcium levels within a narrow range (2). The CaSR is also expressed in other tissues, where it modulates cell proliferation and differentiation and is also known as the production of parathyroid hormone (PTH) and renal calcium handling to maintain circulating calcium levels within a narrow range (2). The CaSR is expressed in other tissues, where it modulates cell proliferation and differentiation and is also known as the production of parathyroid hormone (PTH) and renal calcium handling to maintain circulating calcium levels within a narrow range (2).

The CaSR is expressed on lactating mammary epithelial cells and coordinates the flow of calcium into milk. Stimulating the CaSR on normal breast cells inhibits PTHrP production, which circulates to mobilize skeletal calcium stores for milk production (3–5). The CaSR is also expressed in breast cancer cells where it stimulates, rather than inhibits, PTHrP secretion (6, 7). Whether the CaSR regulates breast cancer growth remains uncertain since prior studies have reported that it can stimulate, inhibit, or have no effect on breast cancer cell proliferation (3, 8). To date, no studies have attempted to manipulate the CaSR in breast cancer models in vivo (8).

PTHrP (gene symbol, PTHLH) is secreted by cancer cells and causes humoral hypercalcemia of malignancy by activating the type 1 PTH/PTHrP receptor (PTH1R) in kidney and bone (9). PTHrP also contains canonical nuclear localization sequences and appears to traffic in and out of the nucleus to affect cellular function independent of the PTH1R (10). PTHrP is required for embryonic development of the breast and, during lactation, it is secreted from mammary cells to regulate maternal and neonatal calcium and bone metabolism (9). PTHrP also affects breast cancer. Tumor production of PTHrP can contribute to the expansion of osteolytic bone metastases by stimulating osteoclast activity (11). The actions of PTHrP in primary tumors are less clear. Studies in breast cancer cell lines, genetically altered mice, and clinical series have reported conflicting results regarding its effects on cell turnover, tumor growth, or clinical outcome (9, 11–18). Nevertheless, several GWAS studies have implicated the PTHLH gene as a breast cancer susceptibility locus, suggesting that alterations in PTHrP expression or signaling contribute to breast cancer initiation and/or progression (19–21).

We examined interactions between the CaSR and PTHrP in rodent and human breast tumors, in breast cancer cell lines and in a transgenic model of breast cancer. Our results confirm that...
CasR signaling stimulates PTHrP production in vitro and in vivo. We also demonstrate that the CasR stimulates the growth of mammary tumors in MMTV-PyMT mice. Finally, we demonstrate that activation of the CasR promotes proliferation and inhibits apoptosis in breast cancer cells, in part, through nuclear actions of PTHrP.

Materials and Methods

Cell culture

MCF-7 and BT474 cells were obtained from ATCC in 2011 and MDA-MB-231.1833 cells were a gift of the Massague Lab in 2013. Cells were maintained in DMEM (Gibco-life Technologies) containing 10% FBS and pen/strep (Gibco-life Technologies). All cells were routinely screened for mycoplasma contamination at least every 6 months and remained negative during the course of these studies. Experimental results were stable across different batches of cryopreserved cells. Stable cell lines expressing shRNA directed against CASR, PTHLH, and PTH1R were generated by transducing cells with commercially prepared lentiviruses: control (sc-108080), Casr (sc-44373-V), PTHrP (sc-39695-V), and PTH1R (sc-39695) from Santa Cruz Biotechnology. Mammalian cell tumors were purchased from Jackson Laboratories on a FVB background.

Cell proliferation and apoptosis

Cell proliferation was measured by assessing BrdUrd incorporation (Cell proliferation ELISA Kit 11647229001; Roche). Apoptosis was measured by TUNEL assay (In Situ Cell Death Detection Kit 11684817910; Roche).

RNA extraction and real-time RT-PCR

RNA was isolated using TRIzol (Invitrogen). Quantitative RT-PCR was performed with the SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen) using a Step One Plus Real-Time PCR System (Applied Biosystems) and the following TaqMan primer sets: PTHLH, Hs00174969_m1; CASR, Hs01047795_m1; Pthlh, Mm00436057_m1; Casr, Mm00443375_m1. Human HPRT1 (4326321E) and mouse Actb (4352341E) were used as reference genes (Invitrogen). Relative mRNA expression was determined using the Step One Software v2.2.2 (Applied Biosystems).

Biochemical measurements

Serum calcium concentrations were measured using the QuantiChrom Calcium Assay Kit (DICA-300, BioAssay Systems) according to manufacturer’s instructions. Plasma PTHrP was measured as previously described (4) using an immunoradiometric assay (DSL-8100; Beckman Coulter), in which we substituted custom and rabbit anti-PTHrP (1–36) antibody as capture antibody. This assay has a sensitivity of 0.5 pM/L.

Cell proliferation and apoptosis

Cell proliferation was measured by assessing BrdUrd incorporation (Cell proliferation ELISA Kit 11647229001; Roche). Apoptosis was measured by TUNEL assay (In Situ Cell Death Detection Kit 11684817910; Roche).

CasR conditional knockout mice

Floxed Casr (Casrlox/lox) mice (4) were backcrossed onto an FVB background for five generations and bred to MMTV-PyMT mice. MMTV-Cre (line D) and MMTV-PyMT mice were purchased from Jackson Laboratories on a FVB background. MMTV-Cre, Casrlox/lox, MMTV-PyMT and control Casrlox/lox, MMTV-PyMT mice were assessed for tumor incidence, latency, and metastases as detailed in Supplementary Methods.

Breast cancer tissue microarray

YTMA49 contains 652 primary breast cancer specimens retrospectively obtained from 1953 to 1983. Cases were evenly divided between lymph node-positive and -negative, with a median follow-up of 8.9 years (23, 24). Details of staining, automated image acquisition, and analysis using the semiautomated AQUA system have also been described (23) and are detailed further in Supplementary Methods. Histospots were excluded from analysis if the tumor mask represented less than 5% of the area. Analyses of CasR staining were based on 377 histospots with adequate tumor representation and staining.

Immunofluorescence and immunoblotting

Cells were grown on coverslips, fixed in 4% paraformaldehyde for 25 minutes, permeabilized with 0.2% Triton X100 for 15 minutes, and stained for immunofluorescence using standard techniques. Whole-cell lysates were prepared using standard methods and cytoplasmic and nuclear proteins were extracted using Thermo Scientific Pierce NE-PER Nuclear and Cytoplasmic Extraction Kit (78833, Pierce Technology). Protein samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane by wet Western blot transfer (Bio-Rad). Membranes were incubated with primary antibodies overnight at 4°C and staining was analyzed using an infrared imaging system (LI-COR). Primary antibodies included those against: HA-Tag (2367 or 3724), PARP (9542), p27Kip1 (3686 or 3698), CDK2 (2546), HPSTO (4872), AIF (5318), and Caspase-3 (9665) from Cell Signaling; and PTH1R (sc-12722) from Santa Cruz. All experiments were performed at least three times and representative blots are shown in the figures.

Kinase assay

Cdk2 kinase activity was measured by assessing phosphorylation of histone H1 by immunoprecipitated cdk2 using standard techniques. Immunoprecipitates were resuspended in 50 μL of kinase reaction buffer containing 50 μmol/L ATP and 2 μg of Histone H1 (382150) from EMD Millipore and incubated for 30 minutes at 30°C. The samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane and assessed with phospho-histone H1 (05-1324) from EMD Millipore. Further details are provided in Supplementary Methods. All assays were performed at least three times and representative blots are shown in the figures.

Statistical analysis

Analyses were performed with Prism 6.0 (GraphPad Software). Error bars represent SEM except in Fig. 3E, where they represent SD. Significance for all comparisons between two conditions were calculated using paired t-tests and significance for multiple comparisons were performed using one-way ANOVA with Turkey post test corrections. Significance for Kaplan–Meier analyses were calculated using the log-rank test and correlations between CasR and PTHrP were calculated using Pearson’s correlation coefficient.
Results

CaSR and PTHrP expression in breast cancers

We examined CaSR and PTHrP gene expression in mammary tumors in a microarray study of rats treated with the chemical carcinogen, N-Nitroso-N-methylurea (NMU; ref. 25). CaSR and PTHrP mRNA levels were significantly higher in tumors as compared to normal mammary tissue, although the increase for PTHrP was more striking than that for CaSR (Fig. 1A and B). In addition, there was a significant positive correlation between CaSR and PTHrP mRNA levels in the tumors (Fig. 1C). We also examined CASR and PTHHL gene expression in 204 human breast tumors (26) and again found a positive correlation between their mRNA levels in breast cancers (Fig. 1D). Next, we examined CaSR protein expression using the semi-automated immunofluorescence AQUA platform in a tissue microarray consisting of 652 breast tumors with a median clinical follow up of 8.9 years (YTMA49; refs. 23, 24). In YTMA49, CaSR levels correlated inversely with pathologic progesterone receptor positive status and positively with node-positive status. CaSR levels above the median value were associated with significantly shorter survival than those below the median (Fig. 1E), although when considered as a continuous variable, they did not significantly predict survival. We have also stained YTMA49 for PTHrP (manuscript in preparation) and as shown in Fig. 1F, there was a positive correlation between CaSR and PTHrP expression in this cohort. Together, these data document direct correlations between CaSR and PTHrP mRNA and protein expression in breast cancers.

CaSR activation stimulates PTHrP production by breast cancer cell lines

Next, we examined CaSR and PTHrP expression in three human breast cancer cell lines: MCF7, MDA MB231.1833, and BT474 cells. Figure 2A and B show baseline mRNA levels for each transcript in the cell lines cultured in growth media. Cells were also serum-starved for 24-hours and then treated with 0.1 or 2.5 mmol/L calcium in serum-free media for 16 hours. In each cell line, increasing the extracellular calcium concentration stimulated PTHHL mRNA expression (Fig. 2C). MDA-MB-231.1833 cells and BT474 cells had higher baseline levels of CaSR and PTHrP expression and a more robust PTHrP response to calcium, so we used these cell lines for subsequent studies. Figure 2D and G demonstrate dose-dependent increases in PTHHL mRNA levels in both cell lines in response to extracellular calcium or gadolinium, which also activates the CaSR. Exposing the cells to increasing doses of extracellular calcium also increased intracellular cAMP levels, and treatment with the PKA inhibitor, H89, blocked the increase in PTHHL mRNA in response to calcium (Fig. 2E–I; ref. 6). Increased PTHHL expression was mediated by CaSR activation because it could be inhibited by treating the cells with a selective CaSR antagonist, NPS2143 (Fig. 2J and L) or by knocking down CaSR expression using shRNA (Fig. 2K and M). These data confirm that, in breast cancer cells, CaSR activation stimulates cAMP/PKA signaling, which, in turn, increases PTHHL gene expression (6).

The CaSR regulates PTHrP production and tumor growth in MMTV-PyMT mice

To test whether the CaSR regulates PTHrP expression and/or tumor growth in vivo, we generated MMTV-Cre; CaSRlox/lox, MMTV-PyMT (CaSR-CKO-PyMT) mice to ablate the CaSR gene in mammary tumor cells. MMTV-PyMT mice recapitulate the progression from hyperplasia to ductal carcinoma in situ to invasive ductal carcinomas, and invasive tumors often loose estrogen receptor expression (27). These mice develop palpable mammary carcinomas within 60–70 days (27) and a previous study had demonstrated that PTHrP promotes tumor growth in this model (15). Although the MMTV promoter is expressed...
Figure 2.

CASR (A) and PTHLH (B) mRNA levels in breast cancer cell lines (relative to baseline levels in MCF7 cells). C, PTHLH expression in response to extracellular calcium. D, response of PTHLH mRNA to increasing calcium or gadolinium (100 μmol/L) in BT474 cells. E, cAMP response to increasing levels of calcium in BT474 cells. F, response of PTHLH mRNA to 5 mmol/L calcium/C6H89 in BT474 cells. G, response of PTHLH mRNA to increasing calcium or gadolinium (100 μmol/L) in MDA-MB-231-1833 cells. H, cAMP response to increasing levels of calcium in MDA-MB-231-1833 cells. I, response of PTHLH mRNA to 5 mmol/L calcium/C6H89 in MDA-MB-231-1833 cells. J, response of PTHLH mRNA to calcium/C6PS2143 in BT474 cells. K, response of PTHLH mRNA to calcium in control or CaSRKD BT474 cells. L, response of PTHLH mRNA to calcium/C6PS2143 in MDA-MB-231-1833 cells. M, response of PTHLH mRNA to calcium in control or CaSRKD MDA-MB-231-1833 cells. Bars represent mean ± SEM, n = 3; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
heterogeneously in mammary epithelial cells, using the MMTV-Cre transgene together with the MMTV-PyMT transgene allowed us to disrupt CaSR expression in the same cells expressing the PyMT oncogene.

Ablating the Casr gene did not impair normal mammary development during puberty or pregnancy (Supplementary Fig. S1). As compared to control MMTV-PyMT tumors, mammary tumors from CaSR-CKO-PyMT mice had an 85.6 /C6 5.6% reduction in CaSR mRNA (Fig. 3A). This was associated with a concomitant 48.0 /C6 3.9% reduction in tumor PTHrP mRNA, demonstrating that, in mammary tumors, PTHrP production is regulated by CaSR signaling at physiologic calcium levels (Fig. 3B).

As expected, circulating PTHrP and calcium levels in CaSR-CKO-PyMT mice were unchanged (Supplementary Fig. S1). Reducing CaSR expression did not alter the incidence or latency of tumor formation in MMTV-PyMT mice; 100% of both control and CaSR-CKO-PyMT mice developed tumors, and the time to 50% tumor incidence was identical for both genotypes (Fig. 3C). However, CaSR-CKO-PyMT mice survived longer (Fig. 3D) and had a slower rate of tumor growth and an overall reduction in tumor burden (Fig. 3E and F). There were no differences in the incidence of lung metastases (60% of mice in both groups) or the number of lung metastases per mouse in CaSR-CKO-PyMT mice compared to controls (Fig. 3G). Because ablation of the CaSR

Figure 3.
Csr (A) and Pthlh (B) mRNA levels in MMTV-PyMT (control) or CaSR-CKO-PyMT tumors. n = 5. C, tumor incidence and latency in MMTV-PyMT versus CaSR-CKO-PyMT mice. n = 10. D, survival in MMTV-PyMT versus CaSR-CKO-PyMT mice. n = 10. E, tumor growth (first tumor noted in each mouse) in MMTV-PyMT versus CaSR-CKO-PyMT mice. n = 10. F, tumor weight (sum of all tumors from each mouse) in MMTV-PyMT versus CaSR-CKO-PyMT mice at sacrifice. n = 8. G, lung metastases in MMTV-PyMT versus CaSR-CKO-PyMT mice. n = 10. H, Typical staining for BrdUrd in MMTV-PyMT versus CaSR-CKO-PyMT tumors. I, quantitation of BrdUrd incorporation in MMTV-PyMT versus CaSR-CKO-PyMT tumors. n = 7. J, proliferation in MMTV-PyMT versus CaSR-CKO-PyMT tumor cells cultured ex vivo exposed to different calcium concentrations or gadolinium. N = 3. Bars represent mean ± SEM. Curve in E shows mean ± SD, *, P < 0.05; **, P < 0.01; ***, P < 0.001.
slowed tumor growth, we examined proliferation and apoptosis in tumors by measuring TUNEL staining and BrdUrd incorporation. There was a significant reduction in BrdUrd incorporation in CaSR-CKO-PyMT tumors as compared to controls (Fig. 3H and I). These results reflected a direct effect on tumor cell proliferation because we saw similar effects in tumor cells cultured ex vivo. As shown in Fig. 3J, control PyMT tumor cells, but not CaSR-CKO-PyMT tumor cells, demonstrated increased BrdUrd incorporation in response to increasing doses of extracellular calcium (within and above the physiologic range) or gadolinium. Finally, rates of apoptosis, as assessed by TUNEL staining, were insignificant in both types of tumors in vivo (not shown).

Knocking down CaSR or PTHrP expression inhibits proliferation in breast cancer cells

The effects of ablating the CaSR on the growth of mammary tumors in MMTV-PyMT mice mirrored those previously described for ablating PTHrP expression (15). Therefore, we examined proliferation in BT474 and MDA-MB-231.1833 cells stably transfected with shRNAs to knockdown either CaSR (CaSRKD; Fig. 4A and D) or PTHrP (PTHrPKD; Fig. 4B and E) expression. As assessed by BrdUrd incorporation, treatment of control cells (transfected with a nonspecific, control shRNA) with increasing doses of calcium or gadolinium stimulated proliferation in a dose responsive fashion (249 ± 19% over baseline for BT474 cells and...
225 ± 6% over baseline for MDA-MB-231.1833 cells at 5 mmol/L calcium; Fig. 4C and F). The CaSR antagonist, NPS2143, decreased BrdUrd incorporation in response to extracellular calcium or gadolinium (Fig. 4G and J) and knocking down CaSR expression reduced proliferation at baseline and blunted the increase in proliferation in response to calcium or gadolinium (Fig. 3H and K). Knocking down PTHrP expression reproduced the effects of knocking down CaSR expression on proliferation (Fig. 3I and L).

In vascular smooth muscle cells, PTHrP has been shown to regulate cell proliferation at the G1–S checkpoint by altering p27kip1 levels and CDK2 activity (28, 29). Therefore, we examined p27Kip1 and CDK2 in response to high calcium in CaSRKD and PTHrPKD cells. In control BT474 and MDA-MB-231.1833 cells, activation of the CaSR with 5 mmol/L calcium was associated with increased p27Kip1 levels and decreased CDK2 activity (Fig. 5A–C, D–F).

Bars represent mean ± SEM. For L, n = 5; all others, n = 3. **, P < 0.01; ***, P < 0.001.
a clear decrease in \( p27^{kip1} \) levels (Fig. 5A–F). By contrast, control cells treated with NPS2143, CaSRKD cells and PTHrPKD cells all demonstrated either a blunting of the reduction in \( p27^{kip1} \) levels (MDA-MB-231 cells) or an actual increase in \( p27^{kip1} \) levels (BT474 cells) when treated with 5 \( \text{mM} \) calcium (Fig. 5A–F). We also examined CDK2 levels by immunoblotting and CDK2 activity by assessment of histone H1 phosphorylation by immunoprecipitated CDK2. As shown in Supplementary Fig. S2, neither treatment with NPS2143 nor knockdown of the CaSR or PTHrP affected total levels of CDK2. However, inhibition of the CaSR or PTHrP did reduce baseline levels of CDK2 activity and prevented the usual increase in CDK2 activity seen in response to increased extracellular calcium (Fig. 5G–I). These data suggest that activation of the CaSR promotes proliferation in breast cancer cells in vitro, in part, by stimulating PTHrP production, which, in turn, decreases expression of the cell-cycle inhibitor, \( p27^{kip1} \). To determine whether this was also true in PyMT tumors in vivo, we examined \( p27^{kip1} \) in extracts of control PyMT tumors and CaSR-CKO-PyMT tumors. As shown in Fig. 5K and L, \( p27^{kip1} \) levels were significantly higher in tumors lacking the CaSR, correlating with reduced PTHrP expression and decreased proliferation.

**Knocking down CaSR or PTHrP expression sensitizes breast cancer cells to calcium-induced cell death**

Although ablation of the CaSR did not affect apoptosis in PyMT tumors in vitro, both the CaSR and PTHrP have been shown to regulate cell death in other settings (1, 2, 9). Therefore, we exposed control and CaSR-CKO PyMT tumor cells to increased extracellular calcium \( \text{ex vivo} \). When exposed to 5 \( \text{mM} \) calcium for 40 hours, tumor cells from CaSR-CKO-PyMT mice underwent apoptosis whereas cells from control PyMT tumors did not (Fig. 6A and B). However, control tumor cells underwent apoptosis when treated with NPS2143 at 5 \( \text{mM} \) calcium (Fig. 6A and B). Likewise, control BT474 and MDA-MB-231.1833 cells remained viable when exposed to 5 \( \text{mM} \) calcium for 40 hours (Fig. 6C and D and Supplementary Fig. S3). In stark contrast, when treated with NPS2143, 28 ± 2.4% of control BT474 cells and 38 ± 3.8% of control MDA-MB-231.1833 cells became TUNEL positive (Fig. 6C and D and Supplementary Fig. S3). Similar results were noted for both BT474 and MDA-MB-231.1833 CaSRKD and PTHrPKD cells (Fig. 6C and D and Supplementary Fig. S3).

To characterize the nature of the cell death caused by inhibiting the CaSR and PTHrP, we assessed caspase 3 activation. Interestingly, we observed no cleaved caspase 3 in BT474 CaSRKD or BT474 PTHrPKD cells despite the fact that they were TUNEL positive (Fig. 6E). Therefore, we examined nuclear translocation of apoptosis inducing factor (AIF). In response to DNA damage or cellular calcium stress, AIF is cleaved by calpain, released from mitochondria and enters the nucleus, where it can trigger caspase-dependent chromatin condensation and DNA fragmentation (30, 31). Treatment of control BT474 or MDA-MB-231.1833 cells with 5 \( \text{mM} \) calcium resulted in a slight increase of AIF in the nucleus (Fig. 6F and Supplementary Fig. S3). Although NPS2143 had little effect on AIF at low or physiologic extracellular calcium, it markedly increased levels of nuclear AIF at 5 \( \text{mM} \) calcium (Fig. 6F and G and Supplementary Fig. S3). Likewise, there was little difference in nuclear AIF levels between cell lines when control, CaSRKD and PTHrPKD cells were cultured in 0.05 or 1 \( \text{mM} \) calcium. However, when exposed to 5 \( \text{mM} \) calcium, significantly more AIF accumulated in the nucleus of the knockdown cell lines compared to controls (Fig. 6H and I and Supplementary Fig. S3). We also observed a marked increase in nuclear AIF expression in control PyMT tumor cells treated with NPS2143 at 5 \( \text{mM} \) calcium or in CaSR-CKO-PyMT cells exposed to 5 \( \text{mM} \) calcium (Supplementary Fig. S4). To determine the functional consequences of nuclear AIF, we treated control, CaSRKD, and PTHrPKD cells with the AIF inhibitor, N-phenylmaleimide. As shown in Fig. 6J and K and Supplementary Fig. S5, N-phenylmaleimide had no effect on apoptosis in control cells but significantly reduced the percentage of TUNEL-positive CaSRKD and PTHrPKD cells after they were treated with 5 \( \text{mM} \) calcium. These data suggest that activation of the CaSR-PTHrP axis allows breast cancer cells to resist apoptosis by inhibiting nuclear accumulation of AIF, which otherwise can be triggered by high extracellular calcium levels.

**Nuclear PTHrP mediates the actions of the CaSR**

To determine whether secreted PTHrP acts on the PTH1R to mediate the actions of the CaSR, we treated BT474 and MDA-MB-231.1833 CaSRKD and PTHrPKD cells with 10 or 100 \( \text{mM} \) soluble PTHrP(1–86), which failed to stimulate BrdUrd incorporation at baseline or after exposure to 5 \( \text{mM} \) calcium (Fig. 7A and B and Supplementary Fig. S5). Similarly, adding PTHrP to the media did not prevent apoptosis in CaSRKD or PTHrPKD cells treated with 5 \( \text{mM} \) calcium (Fig. 7C and Supplementary Fig. S5). Stable knockdown of PTH1R expression in BT474 cells reduced receptor expression by 89.3 ± 4.50% (Fig. 7D), which failed to alter proliferation or cell death in PTH1R KD cells exposed to high extracellular calcium and gadolinium (Fig. 7E and F). The inability of soluble PTHrP to rescue proliferation and/or cell death in CaSRKD or PTHrPKD cells, together with the failure of PTH1R KD cells to recapitulate the phenotype of CaSRKD and PTHrPKD cells, suggest that neither secreted PTHrP nor the PTH1R mediate the CaSR's actions in breast cancer cells.

We next examined whether PTHrP might act in an intracrine fashion by utilizing adenoviruses to express either wild-type PTHrP (WT-PTHrP) or mutant PTHrP with disrupted nuclear localization sequences (ΔNLS-PTHrP; refs. 28, 29). As shown in Fig. 7G, both wild-type and mutant PTHrP constructs included an HA-tag that allowed us to ensure that we expressed equivalent amounts of WT-PTHrP or ΔNLS-PTHrP, 5 mmol/L calcium decreased p27Kip1 levels but in cells transduced with ΔNLS-PTHrP, 5 mmol/L calcium increased BrdUrd incorporation in CaSRKD cells at low calcium concentrations but expression of ΔNLS-PTHrP did not. As noted previously, 5 \( \text{mM} \) calcium increased BrdUrd incorporation in control BT474 cells but this response was blunted in BT474 CaSRKD cells (Fig. 7I). Expression of WT-PTHrP in CaSRKD cells significantly increased BrdUrd incorporation partly back to the level of control cells at 5 \( \text{mM} \) calcium, whereas the expression of ΔNLS-PTHrP failed to increase proliferation (Fig. 7I). As before, 5 \( \text{mM} \) calcium reduced \( p27^{kip1} \) levels in control cells, but increased \( p27^{kip1} \) levels in CaSRKD cells. In CaSRKD cells transduced with WT-PTHrP, treatment with 5 \( \text{mM} \) calcium decreased \( p27^{kip1} \) levels but in cells transduced with ΔNLS-PTHrP, 5 \( \text{mM} \) calcium increased \( p27^{kip1} \) levels (Fig. 7I and Supplementary Fig. S5). These data suggest that PTHrP mediates the proliferative effects of CaSR activation in these cells through an intracrine/nuclear pathway.

Examining cell death responses produced similar results. As noted in Fig. 7K and Supplementary Fig. S5, transduction with WT-PTHrP almost completely prevented apoptosis in BT474 CaSRKD cells exposed to 5 \( \text{mM} \) calcium for 40 hours. In
Figure 6.
Representative TUNEL staining (A) and quantitation (B) of apoptosis in MMTV-PyMT tumor cells ± NPS2143 and CaSR-CKO-PyMT tumor cells ex vivo. n = 5. C, representative TUNEL staining of indicated BT474 cells at different calcium concentrations. D, quantitation of TUNEL-positive cells at high calcium. n = 5. E, representative immunoblot of caspase 3. F, representative immunoblot showing cytoplasmic and nuclear AIF in BT474 cells treated with NPS2143. G, quantitation of nuclear AIF accumulation in response to NPS2143. n = 3. H, representative immunoblot showing cytoplasmic and nuclear AIF in control and knockdown BT474 cells. I, quantitation of change in nuclear AIF accumulation in knockdown relative to control BT474 cells. n = 3. J, representative TUNEL staining of BT474 cells ± treatment with N-phenylmaleimide. K, quantitation of TUNEL staining in control and knockdown BT474 cells at 5 mmol/L calcium ± N-phenylmaleimide. n = 5. Bars represent mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.
contrast, ΔNLS-PTHrP had little effect on cell death. As before, increased cell death correlated with increased levels of nuclear AIF. CaSRKD cells and CaSRKD cells transduced with ΔNLS-PTHrP demonstrated increased nuclear AIF in response to 5 mmol/L calcium, whereas nuclear AIF levels in CaSRKD cells transduced with WT-PTHrP were no different than in control BT474 cells (Fig. 7L and Supplementary Fig. S5). These data suggest that nuclear PTHrP inhibits nuclear accumulation of AIF and thus prevents cell death in response to high extracellular calcium in vitro.

Discussion

Despite the fact that 75% of breast cancers express the CaSR (32), relatively few studies have examined its functional role in...
breast tumors. Although some studies have reported that the CaSR increases proliferation in breast cancer cells (33–38), others have suggested that it has either no effect on cell proliferation or actually suppresses proliferation and increases apoptosis (39, 40). In this study, we found that activation of the CaSR upregulates PTHrP production, stimulates breast cancer cell proliferation, and protects against apoptosis. Furthermore, we find that the actions of CaSR signaling on cell proliferation and apoptosis in vitro are mediated by PTHrP acting in the nucleus. Reducing CaSR expression in transgenic mammary tumors and in breast cancer cell lines inhibited PTHrP production and slowed tumor cell growth. In the aggregate, these data suggest that activation of the CaSR promotes breast cancer growth, at least in part, through intracrine actions of PTHrP.

Our data are consistent with prior reports that extracellular calcium stimulates PTHrP expression by breast cancer cells in vitro, similar to findings in normal or malignant keratinocytes (6, 7, 41, 42). We also show that ablation of the CaSR in vivo inhibits PTHrP production by mammary tumors from MMTV-PyMT mice. Furthermore, we documented positive correlations between Casr and Pthlh mRNA levels in NMU-generated mammary tumors in rats, and CASR and PTHLH mRNA and/or protein levels in combined cohorts of more than 800 individual breast cancers from human patients. These findings convincingly show that CaSR activation stimulates PTHrP production by breast cancers at physiologic calcium concentrations. By contrast, CaSR activation reduces PTHrP production by normal mammary epithelial cells in culture (4, 6, 43, 44) and conditional ablation of the Case gene in the lactating mammary gland increases PTHrP production (4). Thus, malignant transformation of breast epithelial cells rewrites the relationship between the CaSR and PTHrP such that extracellular calcium, which suppresses PTHrP production in normal cells becomes, instead, a stimulus for PTHrP production by malignant cells. Because PTHrP activates peritumoral osteolysis, this change in PTHrP production may contribute to the pathophysiology of bone metastases. In fact, Mihai and colleagues observed a positive correlation between CaSR expression in primary breast cancers and the presence of bone metastases (32).

Stimulation of PTHrP production by the CaSR contributes to the growth of breast cancer cells in response to extracellular calcium in vitro. Knocking down either CaSR or PTHrP expression reduced proliferation and increased cell death when BT474 and MDA-MB-231.1833 cells were exposed to increasing concentrations of extracellular calcium. Likewise, whereas targeting the CaSR in MMTV-PyMT mice with normal systemic calcium concentrations did not change the incidence or latency of tumor development, it did significantly reduce the rate of cell proliferation and slowed the overall growth of mammary tumors. CaSR ablation also decreased PTHrP production by tumors and our results are similar to data from Li and colleagues demonstrating that targeting PTHrP expression inhibited the growth of mammary tumors in MMTV-PyMT mice (15). Although there was no significant change in apoptosis rates at physiologic calcium concentrations in vivo, exposure of the CaSR-CKO-PyMT tumor cells to 5 mMol/L calcium ex vivo led to significant cell death. Likewise, using NPS2143 to pharmacologically inhibit CaSR function also killed control MMTV-PyMT cells exposed to elevated extracellular calcium ex vivo. Therefore, inhibiting the CaSR-PTHrP axis appears to convert the response of breast cancer cells to extracellular calcium from supportive of tumor growth to detrimental to tumor growth.

Several observations suggest that the CaSR may activate an intracrine mode of nuclear PTHrP action in breast cancer cells. First, adding PTHrP to the culture media could not rescue the effects of knocking down CaSR or PTHrP expression. Second, knocking down the PTH1R had no effect on the responses to extracellular calcium. Third, re-expressing wild-type PTHrP rescued the effects of knocking down the CaSR but re-expressing mutant PTHrP, lacking the ability to translocate into the nucleus (ANLIS-PTHrP), could not. Although we cannot exclude additional contributions of autocrine/paracrine PTHrP signaling in vivo, our data in vitro are consistent with previous reports that nuclear PTHrP stimulates proliferation and/or inhibits cell death in breast cancer cell lines, chondrocytes, and vascular smooth muscle cells (10, 45). In particular, our data are similar to prior findings that nuclear PTHrP acts to stimulate proliferation of vascular smooth muscle cells by reducing p27kip1 levels and stimulating cdk2 activity (28, 29). Ongoing experiments targeting the PTH1R in MMTV-PyMT tumors will address the relative contribution of autocrine/paracrine versus intracrine PTHrP signaling to tumor growth in vivo.

Activation of the CaSR also protected breast cancer cells from caspase-independent cell death mediated by apoptosis-inducing factor (AIF). AIF is a mitochondrial protein released into the cytoplasm in response to cellular calcium overload or DNA damage and PARP-1 activation (30, 31). From the cytoplasm, AIF can be transported into the nucleus and activate chromatin condensation, DNA fragmentation, and cell death. Knocking down the CaSR or PTHrP augments, the accumulation of nuclear AIF when cells are exposed to high extracellular calcium. Furthermore, pharmacologic inhibition of AIF blocks calcium-induced apoptosis in CasrKD or PthlhpKD cells. Therefore, activation of a CaSR–PTHrP pathway protects these cells from apoptosis by either preventing the release of AIF from mitochondria and/or by preventing its nuclear accumulation. AIF is an important mediator of intracellular calcium-mediated “necroptotic” cell death triggered by excitotoxicity in neurons, and PTHrP has previously been shown to protect hippocampal neurons from excitotoxic cell death (46–48). The CaSR also modulates neuronal cell death in the setting of reperfusion injury, which involves cellular calcium overload as well (46, 49). Although much work will be needed to fully understand how the CaSR and PTHrP regulate AIF in breast cancer, given the examples above, it is interesting to speculate whether interactions between the CaSR, PTHrP, and AIF might have a wider role in regulating cell death stimulated by intracellular calcium.

In summary, we find that the CaSR stimulates proliferative and survival responses in breast cancer cells exposed to increasing doses of extracellular calcium. Both appear to be mediated, in part, by the induction of PTHrP, which acts in the nucleus to stimulate proliferation by suppressing p27kip1 levels and increasing Cdk2 activity, and to prevent apoptosis by inhibiting nuclear accumulation of AIF. As in cells lines in vitro, the CaSR contributes to the secretion of PTHrP and the growth of mammary tumors in MMTV-PyMT mice in vivo. Based on our findings in vitro and the fact that targeting either the CaSR or PTHrP in MMTV-PyMT mice slows tumor growth (15), we suspect that PTHrP may mediate the effects of the CaSR in tumor cells in vivo as well. However, the CaSR could also affect tumor growth independent from changes in PTHrP. These findings may be particularly relevant to bone metastases, where breast cancer cells are likely exposed to high levels of extracellular calcium near active sites of osteoclast-
mediated osteolysis. In that setting, the CaSR may help breast cancer cells grow by triggering PTHrP expression, which might, in turn, act through intracrine pathways to stimulate proliferation and prevent calcium-mediated cell death. This hypothesis needs to be tested in vivo but, if this is the case, then pharmacologic targeting of the CaSR-PTHrP axis may offer new possibilities for breast cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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