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# Ca²⁺ Receptor, Prostate Cancer, and Bone Metastases

## Abstract

While bony metastases of prostate cancer are often osteoblastic, excessive bone resorption also occurs in the sites of metastases, which contributes to skeletal complications (e.g., pain, fractures). This research evaluates whether prostate cancer cells express the extracellular calcium (Ca²⁺)-sensing receptor (CaSR) and whether the CaSR in bony metastases of prostate cancer participates in a vicious cycle involving CaSR-mediated secretion of the bone-resorbing cytokine, parathyroid hormone-related protein (PTHrP). The secreted PTHrP would promote further bone resorption, thereby increasing Ca²⁺ locally and stimulating further PTHrP release. The project entails four tasks—namely showing that: (1) prostate cancer cells express the CaSR, (2) the CaSR mediates high Ca²⁺-induced stimulation of PTHrP secretion, (3) the CaSR transactivates the epidermal growth factor (EGF) receptor, and (4) CaSR-stimulated PTHrP secretion from prostate cancer cells increases the severity of metastatic bone disease in vivo in mice. We have accomplished tasks 1, 2, and 3 and are still working on developing the stably transfected cell lines needed for the studies in task 4. These results support a role for the CaSR in a vicious cycle that increases the severity of bone resorption in vivo in humans.

## Subject Terms

Ca²⁺-sensing receptor, prostate cancer, bone metastases, PC-3 cells, EGF receptor, transactivation

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INTRODUCTION:
Prostate cancer research has generally emphasized the osteoblastic nature of prostate cancer metastases to bone. However, a wealth of recent data documents the nearly universal presence of excessive bone resorption as well, which participates importantly in the associated bone pain and fractures. The goal of this research is to determine whether prostate cancer cells express the extracellular calcium (Ca$^{2+}$)-sensing receptor (CaSR) and whether the CaSR participates in a vicious cycle promoting excessive bone resorption. This vicious cycle involves CaSR-mediated secretion of the bone-resorbing cytokine, parathyroid hormone-related protein (PTHrP), by prostate cancer metastatic to bone. The secreted PTHrP would produce further bone resorption, which would elevate the local level of Ca$^{2+}$, thereby stimulating further PTHrP release by the prostate cancer cells, and so forth. The scope of the project encompasses four specific aims: (1) to show that prostate cancer cells express the CaSR; (2) to prove that the CaSR mediates high Ca$^{2+}$-evoked stimulation of PTHrP secretion in vitro; (3) to determine whether the CaSR initiates a paracrine pathway producing transactivation of the epidermal growth factor receptor (EGFR), which then produces EGFR-mediated stimulation of MAPK and, in turn, increased PTHrP production; and (4) to document that CaSR-mediated stimulation of PTHrP secretion from prostate cancer cells injected into the femora of nude mice contributes to the severity of metastatic bone disease by knocking out the receptor using a dominant negative CaSR construct.

BODY:
Task 1—To document that prostate cancer cell lines express the CaSR (months 1-18).

We have completed the studies in task 1, which are described in detail in a publication of this work submitted with the previous Annual Report (1). A PDF file of this publication is also appended to the report. The results of these studies are as follows: Reverse transcriptase-polymerase chain reaction (RT-PCR) with intron-spanning primers amplified a product of the expected size, 480 bp, for having been derived from authentic CaSR transcript(s). In addition, Northern analysis, carried out using a CaSR-derived riboprobe and poly(A$^+$) RNA derived from both LnCaP and PC-3 cells, revealed a major transcript of 5.2 kb, which is of the same size as the major transcript in human parathyroid gland (2).

With regard to documenting the presence of CaSR protein, immunocytochemistry with a polyclonal, CaSR-specific antiserum revealed specific staining of both PC-3 and LnCaP cells. Furthermore, western blotting with the same antiserum identified specific immunoreactive bands of 160-170 kDa in PC-3 and LnCaP cells, comparable in size to bands identified in the positive
controls—bovine parathyroid gland and CaSR-transfected human embryonic kidney (HEK293) cells (1).

Thus we have demonstrated that LnCaP as well as PC-3 cells express both CaSR transcript and protein. Note that while we originally proposed studies determining whether prostate cancer specimens removed at the time of prostatectomy expressed CaSR transcript(s) and protein, the contract for our grant expressly forbids the use of human anatomical substances.

Task 2—To show that the CaSR mediates the stimulation of PTHrP secretion from prostate cancer cell lines by high Ca\(^{2+}\) (months 6-24).

To investigate whether the CaSR mediated the stimulatory effect of high Ca\(^{2+}\) on PTHrP secretion from PC-3 cells (1), we utilized polycationic agonists (i.e., neomycin and spermine) known to activate the cloned CaSR (3, 4). The potencies of these two polycations were equal to or more effective than high Ca\(^{2+}\) in stimulating PTHrP secretion from PC-3 cells. We next used a naturally occurring, dominant negative construct of the CaSR (R185Q) to assess the CaSR's role in mediating high Ca\(^{2+}\)-evoked PTHrP secretion. To achieve high efficiency expression of the CaSR in PC-3 cells, we utilized infection with an adenoviral construct expressing the mutated CaSR. Compared to vector-infected cells, cells infected with the dominant negative CaSR showed a substantial reduction in the stimulation of PTHrP secretion by 1.5 and 3.5 mM Ca\(^{2+}\) (1), levels of Ca\(^{2+}\) that could potentially be encountered by bony metastases of prostate cancer near sites of active bone resorption (5).

Task 3—To investigate whether the CaSR transactivates the EGFR in prostate cancer cells (months 6-24).

In addition to the studies accomplished in tasks 1 and 2, we have shown that the CaSR transactivates the EGFR (see PDF file of the published paper appended to this report), thereby completing Task 3. Since the MAP kinase, ERK1/2, is a major signal transduction pathway utilized by the EGFR, we initially documented a delayed phosphorylation of ERK1/2 by Western blotting. Maximal activation was observed at 30 min, and a strong signal persisted at 60 min on Western blots of phospho-ERK1/2. At 120 minutes, in contrast, the signal had nearly dissipated. The phosphorylation of ERK1/2 was dose-dependent with regard to the level of Ca\(^{2+}\) employed; the strongest signal was observed with 7.5 mM Ca\(^{2+}\), while signals of intermediate intensity were observed at 1.5 and 3.0 mM Ca\(^{2+}\).

In order to document that high Ca\(^{2+}\)-evoked activation of ERK was CaSR-mediated, we examined the effects of the known polycationic CaSR agonist, spermine, and of a selective CaSR activator, NPS R-467, on phospho-ERK1/2. Incubation of PC-3 cells with 100 \(\mu\)M spermine for 30 min increased the level of phospho-ERK1/2. Moreover, NPS R-467 produced a much greater increase in phospho-ERK1/2 than did its less potent stereoisomer, NPS S-467. Since NPS R-467 is 10
to 100 fold more potent than NPS S-467 in activating the CaSR, our results indicate that high Ca$^{2+}$-induced ERK phosphorylation is mediated by the CaSR.

Next, we examined the effects of various inhibitors and neutralizing antibodies to assess the involvement of transactivation of the EGFR in CaSR-mediated activation of ERK1/2. AG1478, an EGFR kinase inhibitor, and PD98059, a MEK1 inhibitor, inhibited most of the high Ca$^{2+}$-evoked ERK phosphorylation. GM6001, a pan matrix metalloproteinase (MMP) inhibitor, and antibodies against the EGFR and HB-EGF (heparin-bound EGF) also reduced ERK phosphorylation, consistent with the model of transactivation shown on page 7 of this report. In contrast, AG1296, an inhibitor of the platelet-derived growth factor receptor kinase, had no effect on ERK phosphorylation. These results provide indirect evidence that activation of the CaSR transactivates the EGFR, but not the PDGFR, at least in part through activation of MMP(s).

We next directly measured the effect of high Ca$^{2+}$ on the extent of phosphorylation of the EGFR. Phosphorylation of the EGFR was assessed using Western analysis with a monoclonal anti-phosphotyrosine antibody following immunoprecipitation of cell lysates with a rabbit polyclonal anti-EGFR antibody. The EGFR was phosphorylated to some extent even under basal (0.5 mM Ca$^{2+}$) conditions; following 10 min incubation in medium with 7.5 mM Ca$^{2+}$, however, the phosphorylation of the EGFR increased and was sustained for at least 30 min.

We have previously demonstrated that high Ca$^{2+}$ stimulates PTHrP secretion from PC-3 cells (1). This action of Ca$^{2+}$ is at least partially mediated by the CaSR, since hormonal secretion is reduced by transfecting the cells with a dominant negative CaSR, and known CaSR agonists, e.g., neomycin and gadolinium, promote PTHrP secretion (1). Thus, we wondered if the CaSR might stimulate PTHrP secretion through transactivation of the EGFR.

High Ca$^{2+}$ dose-dependently stimulated PTHrP secretion by PC-3 cells. This stimulation was inhibited by 20 μM PD98059 and by 0.7 μM AG1478. In contrast, 1 μM AG1296 had no effect on PTHrP secretion. When the cells were preincubated with anti-HB-EGF antibody for 30 min, 5 μg/ml of the antibody significantly inhibited PTHrP secretion (by 42%) even under basal conditions (0.5 mM Ca$^{2+}$). At 7.5 mM Ca$^{2+}$, the anti-HB-EGF antibody likewise produced a dose-dependent inhibition of PTHrP secretion. The anti-EGFR antibody gave similar results (data not shown). Preincubation with 10 μM GM6001 also reduced PTHrP secretion by 40% at 0.5 mM Ca$^{2+}$, and by about 50% at 3.0 and 7.5 mM Ca$^{2+}$. These findings indicate that EGF and HB-EGF activate the EGFR even under basal conditions and that high Ca$^{2+}$-induced PTHrP secretion is reduced by blockade of the CaSR-EGFR-ERK pathway. The former result is consistent with the presence of phosphorylated EGFR at 0.5 mM Ca$^{2+}$ even following serum starvation. These results are consistent with model shown below.
in which activation of the CaSR stimulates transactivation of the EGFR by activating a currently unidentified matrix metalloproteinase. The latter then cleaves heparin-bound EGF from its precursor, and the soluble HB-EGF activates the EGFR, thereby stimulating the activity of ERK1/2, likely by a ras and raf-dependent mechanism. The activated ERK1/2 then stimulates PTHrP secretion, which could participate in the feed-forward mechanism of enhanced bone resorption described above.

Task 4—To show that knocking out the CaSR reduces the severity of bone resorption in the femora of nude mice injected with PC-3 cells (months 6-36).

We continued during months 24-36 the development of PC-3 cells stably transfected with a dominant negative CaSR or with the corresponding vector. We have transfected PC-3 cells with a standard mammalian expression vector (pcDNA3) and subjected the transfected cells to selection with hygromycin. To date we have not yet been successful in obtaining individual, stably transfected PC-3 clones, in part because the cells grow very slowly at low density. While we have been able to select cells transfected with the dominant negative CaSR that grow in the presence of hygromycin, on immunocytochemistry only about 20% were positive for the CaSR. During the remaining few months of the grant, we will continue to develop individual clones of stably transfected with the dominant negative CaSR so as to avoid
the apparent heterogeneity in our studies to date. In addition to using the pcDNA3 vector, we will also try infecting the cells with the rAAV vector noted above and selecting for stably transfected cells.

KEY RESEARCH ACCOMPLISHMENTS:

• Documented the presence of CaSR transcripts in PC-3 and LnCaP cells as assessed by RT-PCR and Northern analysis.
• Demonstrated the presence of CaSR protein in PC-3 and LnCaP cells as assessed by immunocytochemistry and Western analysis.
• Shown that polycationic CaSR agonists stimulate PTHrP secretion from PC-3 cells, consistent with the CaSR’s involvement in mediating high Ca\(^{2+}\)-evoked PTHrP secretion.
• Documented reduction of high Ca\(^{2+}\)-stimulated PTHrP secretion from PC-3 cells by infection of the cells with a dominant CaSR construct, supporting the CaSR’s medatory role.
• Shown that high Ca\(^{2+}\) and EGF stimulate ERK1/2 in PC-3 cells; Furthermore, the polycationic CaSR agonist, spermine, and the potent calcimimetic, NPS R-467, increase ERK1/2 in PC-3 cells to a greater extent than the less potent calcimimetic, NPS S-467, consistent with the medatory role of the CaSR in this action.
• Demonstrated that an inhibitor of the EGF receptor kinase, a matrix metalloproteinase inhibitor, as well as antibodies against the EGFR and HB-EGF reduce high Ca\(^{2+}\)-evoked ERK activation, consistent with the involvement of CaSR-mediated transactivation of the EGFR, via matrix metalloproteinase-induced release of soluble EGF, in ERK1/2 activation.
• Shown that high Ca\(^{2+}\)-stimulated PTHrP secretion is reduced by the EGFR inhibitor, the matrix metalloproteinase inhibitor, and the antibodies to the EGFR and HB-EGF, providing further evidence that the CaSR transactivates the EGFR.
• Documented that high calcium stimulates a time dependent increase in the tyrosine phosphorylation of the EGFR, providing direct evidence for CaSR-mediated transactivation of the CaSR.

REPORTABLE OUTCOMES:
CONCLUSIONS:

Our results support the major underling hypotheses driving this research, namely that the CaSR mediates high Ca\textsuperscript{2+}-stimulated PTHrP secretion from PC-3 cells and could provide the basis for a "feed-forward" mechanism in vivo that would serve to aggravate the skeletal complications of prostate cancer metastatic to bone. The importance of this research lies in the implication that the CaSR could serve as a therapeutic target for CaSR antagonists that could diminish the severity of the skeletal complications of prostate cancer. Furthermore, it is possible that expression of the CaSR in other cancers that metastasize to bone (e.g., breast cancer) could serve as the mediator of a similar "feed-forward" mechanism and thereby provide the basis for a novel therapy of cancers other than prostate cancer.

REFERENCES:


Ca\(^{2+}\)-sensing receptor expression and PTHrP secretion in PC-3 human prostate cancer cells

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Sander, Jennifer L., Naibeda Chattopadhyay, Olga Kifor, Toru Yamaguchi, and Edward M. Brown. Ca\(^{2+}\)-sensing receptor expression and PTHrP secretion in PC-3 human prostate cancer cells. Am J Physiol Endocrinol Metab 281:E1267–E1274, 2001.—Prostate cancer metastasizes frequently to bone. Elevated extracellular calcium concentrations ([Ca\(^{2+}\)]) stimulate parathyroid hormone-related protein (PTHrP) secretion from normal and malignant cells, potentially acting via the [Ca\(^{2+}\)]\(_{\text{in}}\)-sensing receptor (CaR). Because prostate cancers produce PTHrP, if high [Ca\(^{2+}\)] stimulates PTHrP secretion via the CaR, this could initiate a mechanism whereby osteolysis caused by bone metastases of prostate cancer promotes further bone resorption. We investigated whether the prostate cancer cell lines LnCaP and PC-3 express the CaR and whether polycationic CaR agonists stimulate PTHrP release. Both PC-3 and LnCaP prostate cancer cell lines expressed bona fide CaR transcripts by Northern analysis and RT-PCR and CaR protein by immunocytochemistry and Western analysis. The polycationic CaR agonists [Ca\(^{2+}\)]\(_{\text{in}}\)-sensing receptor (C\(\text{aR}\))-, neomycin, and spermine each concentration maximally stimulated PTHrP secretion from PC-3 cells, as measured by immunoradiometric assay, with maximal, 3.2-, 3.6-, and 4.2-fold increases, respectively. In addition, adenovirus-mediated infection of PC-3 cells with a dominant negative CaR construct attenuated high [Ca\(^{2+}\)]-evoked PTHrP secretion, further supporting the CaR's mediatory role in this process. Finally, pretreating PC-3 cells with transforming growth factor (TGF)-β, augmented both basal and high [Ca\(^{2+}\)]-stimulated PTHrP secretion. Thus, in PTHrP-secreting prostate cancers metastatic to bone, the CaR could initiate a vicious cycle, whereby PTHrP-stimulated bone resorption releases [Ca\(^{2+}\)]\(_{\text{in}}\)-sensing receptor, further increasing PTHrP release and osteolysis.

Parathyroid hormone-related protein; ion-sensing receptor; osteolysis; prostate cancer; LnCaP cells; skeletal metastases

Prostate cancer is a common cancer and the second leading cause of cancer death in men (4). A substantial percentage of elderly men have microscopic prostate cancers, but these small lesions usually remain localized to the prostate and never come to clinical attention. Nevertheless, skeletal complications of prostate cancer are a difficult clinical problem, causing disabling pain and other complications such as fractures (10). Radiation, hormonal manipulations, and/or chemotherapy offer palliation but, unfortunately, little hope of cure for skeletal metastases of prostate cancer. Therefore, further understanding of the biology of prostate cancer metastatic to bone and the development of improved therapies for skeletal metastases and their complications are important goals of prostate cancer research.

Recent studies have shown that parathyroid hormone (PTH)-related protein (PTHrP), a central mediator of malignancy-associated hypercalcemia and osteolysis, increases bone resorption also occur in this setting, as assessed by biochemical markers (10, 24, 40). Indeed, markers of bone resorption can be higher in patients with metastatic prostate cancer than in those with skeletal metastases of breast cancer (10). Prostate cancers often express more PTHrP than normal prostate epithelial cells (1, 25), suggesting that PTHrP could contribute to the increased bone resorption (10) in patients with prostate cancer metastatic to bone (1, 25, 38). PTHrP secreted by prostate cancer cells could then activate osteoclasts and potentially contribute to skeletal invasiveness, bone pain, and/or pathological fractures. Therefore, further understanding of the factors regulating the production and secretion of PTHrP by prostate cancer cells could elucidate the mechanisms underlying the excessive bone resorption associated with this tumor and potentially provide clues to novel therapeutic strategies.

Orthocel calcium (Ca\(^{2+}\))-sensing receptor (CaR) is a G protein-coupled cell surface receptor that is a central element in [Ca\(^{2+}\)]\(_{\text{in}}\) homeostasis (6).
parathyroid cells, high [Ca\textsuperscript{2+}] byactivating the CaR, inhibits PTH secretion and parathyroid cellular proliferation (6), whereas in the kidney, stimulating the receptor reduces renal tubular Ca\textsuperscript{2+} reabsorption (20). The biological proof of the CaR's key roles in [Ca\textsuperscript{2+}]\textsubscript{homeostasis} has come from the identification of hyper- and hypocalcemic disorders caused by inactivating or activating CaR mutations (5), respectively, and from mice with targeted disruption of the CaR gene (23).

In addition to inhibiting PTH release from parathyroid cells, the CaR stimulates the secretion of calcitin from C cells (12, 14) and of ACTH from AP-20 cells (21). Furthermore, several studies have shown that high [Ca\textsuperscript{2+}], can stimulate PTHrP release from normal keratinocytes (22), normal cervical epithelial cells (28), oral squamous cancer cells (31), and JEG-3 cells (21), suggesting that the CaR could be the mediator of high [Ca\textsuperscript{2+}]-evoked PTHrP release from both normal and malignant cells. In the case of PTHrP-secreting prostates tumors metastatic to bone, this CaR-mediated action could create an inappropriate “feed-forward” stimulation of PTHrP secretion, causing release of Ca\textsuperscript{2+} from bone that would stimulate further PTHrP secretion and promote worsening bone resorption. Moreover, interrupting high [Ca\textsuperscript{2+}]-evoked CaR-mediated PTHrP secretion from prostate cancer cells [e.g., with a CaR antagonist (15)] could potentially be of substantial clinical benefit in this setting. The goals of the present study, therefore, were to determine whether two commonly employed prostate cancer cell lines, LnCaP and PC-3, express the CaR, and, if so, whether this receptor participates in the regulation of PTHrP secretion. Our results suggest that the CaR is expressed in and likely mediates high [Ca\textsuperscript{2+}]-induced PTHrP secretion from PC-3 cells. Furthermore, transforming growth factor (TGF)-β1, stimulates PTHrP secretion from PC-3 cells synergistically with high [Ca\textsuperscript{2+}], 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 cancer metastatic to bone begets worsening osteolysis.

MATERIALS AND METHODS

Cell culture. The LnCaP and PC-3 human prostate cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 100 U/ml penicillin-100 μg/ml streptomycin. The cells were grown at 37°C in a humidified 5% CO\textsubscript{2} atmosphere and were passaged every 5–7 days with the use of either 0.2% trypsin-0.53 mM EDTA (LnCaP cells) or 0.05% trypsin-0.53 mM EDTA (PC-3 cells). All cell culture reagents were purchased from Gibco-BRL (Grand Island, NY), with the exception of FCS, which was obtained from Gemini Bio-Products (Calabasas, CA).

Northern blotting. Total RNA was prepared using TRIzol reagent (GIBCO-BRL). Northern blot analysis was performed on 7.5 μg of poly(A)\textsuperscript{+} enriched RNA samples that were denatured and electrophoresed in 2.2 M formaldehyde-1% agarose gels along with a 0.24- to 8.5-kb RNA ladder (GIBCO-BRL) and transferrred overnight to nylon membranes (Duralon; Stratagene, La Jolla, CA). A 32P-labeled riboprobe corresponding to nucleotides 1745–2230 of the human parathyroid CaR cDNA was synthesized with the MAXiScript T7 kit (Pharmacia Biotech, Piscataway, NJ) with the use of T7 RNA polymerase and [32P]UTP. Nylon membranes were then prehybridized, hybridized overnight with the labeled cRNA probe (2 x 10\textsuperscript{6} cpm/ml), and washed at high stringency for 30 min as described previously (35). Membranes were sealed in plastic bags and exposed to a Phosphorimager screen. The screens were analyzed on a Molecular Dynamics Phosphorimager (Sunnyvale, CA) with the ImageQuant program.

RT-PCR. Total RNA (3–5 μg) was used for the synthesis of first-strand cDNA (cDNA synthesis kit, Gibco-BRL). The resultant first-strand cDNA was used for PCR, which was performed in a buffer containing (in mM): 20 Tris-HCl, pH 8.4, 50 KCl, 1.5 MgCl\textsubscript{2}, and 0.2 dNTP and 0.4 μM forward primer, 0.4 μM reverse primer, and 1 μl E.coli ONGCASE enzyme mix (a Taq/Hymenice species GBD-D RNA polymerase mixture; GIBCO-BRL). Human parathyroid CaR sense primer 5'-CCGCGGATCTTAAACGCTACGGCATCTAA-3' and antisense primer 5'-GGCTCTAGATGGACGATCCCAAAGGGCTTC-3', which are intron spanning, were used for the reactions. To perform "hot start" PCR, the enzyme mixture was added during the initial 3-min denaturation and was followed by 35 cycles of amplification (30-s denaturation at 94°C, 30-s annealing at 47°C, and 1-min extension at 72°C). The reaction was completed with an additional 10-min incubation at 72°C to allow completion of extension. PCR products were fractionated on 1.5% agarose gels. PCR products in the reaction mixture were purified using the Qiagen PCR purification kit (Qiagen, Santa Clarita, CA) and were subjected to bidirectional sequencing by employing the same primer pairs used for PCR by means of an automated sequencer (AB377, Applied Biosystems, Foster City, CA) as previously described (35).

Immunocytochemistry. A CaR-specific polyclonal antiserum (4377) was generously provided by Drs. Forrest Fuller and Karen Kruppa of NPS Pharmaceuticals. This antiserum was raised against a peptide corresponding to amino acids 345–359 of the bovine CaR, which is identical to the corresponding peptide in the human CaR and resides within the predicted amino-terminal extracellular domain of the CaR. The antiserum was subjected to further purification by means of an affinity column conjugated with the FF-7 peptide (27), and the affinity-purified antiserum was used for immunochemistry and Western blot analysis as described in the following paragraph. The specificity of the antiserum for the CaR is documented in the present study by the use of suitable positive and negative controls.

For immunocytochemistry, prostate cancer cells were grown on glass coverslips (27), fixed for 5 min with 4% formaldehyde, and then treated for 10 min with peroxidase blocking reagent (DAKO, Carpenteria, CA) to inhibit endogenous peroxidases. After washing with PBS, the cells were blocked for 30 min with PBS containing 1% BSA. The cells were then incubated overnight at 4°C with the 4377 antisem (5 μg/ml in blocking solution). Negative controls were carried out by incubating cells treated in an otherwise identical manner with the same concentration of 4377 antiserum that had been preabsorbed with 10 μg/ml of the FF-7 peptide. The cells were then washed, incubated with peroxidase-conjugated goat anti-rabbit IgG (1:1000; Sigma Chemical, St. Louis, MO) and washed again, and the color reaction was developed using the DAKO AEC substrate system (DAKO) as
before (27). The cells were observed by light microscopy and photographed at ×400 magnification.

Western Blotting. For Western blotting, confluent monolayers of LnCaP and PC-3 cells in 6-well plates were rinsed with ice-cold PBS and scraped on ice into lysis buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.25% v/v sucrose, 1% Triton X-100, 1 mM diethiothreitol, and a cocktail of protease inhibitors (10 μg/ml each of aprotinin, leupeptin, and calpain inhibitor, as well as 100 μg/ml of Pefabloc) (26). The cells were then passed through a 22-gauge needle 10 times. Nuclei and other cellular debris were removed by low-speed centrifugation (1,500 g for 10 min), and the resultant total cellular lysate in the supernatant was used either directly for SDS-PAGE or stored at −80°C. Bone parathyroid cells, CaR-transfected HEK-293 cells (designated HEKCaR), or nontransfected HEK-293 cells, included as positive (parathyroid and HEKCaR) and negative controls (nontransfected HEK-293 cells), were harvested according to the same protocol.

Immunoblot analyses were performed essentially as described before (26, 27). Aliquots of 20–40 μg of protein were mixed with an equal volume of 2 x SDS-sample loading buffer containing 100 mM diethiothreitol, incubated at 37°C for 15 min, and resolved electrophoretically on linear 4–15% gradient gels. The separated proteins were then transferred to nitrocellulose blots (Schleicher & Schuell, Keene, NH) and incubated with blocking solution (PBS with 0.25% Triton X-100 and 5% dry milk) for 1 h at room temperature. The blots were incubated overnight at 4°C with affinity-purified anti-CaR polyclonal antibody R5 (26) or an antibody against PTHrP (generously provided by M. Yasumura, Juntendo University, Tokyo, Japan). The blots were then incubated with a 1:2,000 dilution of peroxidase-conjugated goat anti-rabbit IgG and washed five times, and protein bands were detected using an enhanced chemiluminescence (ECL) system (Renaissance Kit, Du Pont-NEN).

Adenoviral infection of dominant negative CaR into PC-3 cells. Confluent PC-3 cells were scraped, dispersed by repeated pipetting, and then seeded in 24-well plates (≈2.5 × 10^4 cells/well). Approximately 10,000 infective particles containing dominant negative CaR (R185Q) or empty vector as a negative control were added to each well at the time the cells were seeded in growth medium. The cells were then cultured for 48 h, washed with PBS, and then incubated with DMEM containing 0.2% BSA and 0.5 mM CaCl_2 for 2 h. Additional calcium was then added to the wells as needed to achieve the final concentrations indicated in the results, and the cells were incubated overnight. At the end of the incubation, conditioned medium was collected and subjected to PTHrP assays as described in PTHrP secretion studies. The data were normalized to the amount of protein in each well. Experiments were carried out using triplicates wells for each level of Ca^2+.

PTHRP secretion studies. For studies on the effects of various CaR agonists on PTHrP secretion, PC-3 cells were seeded in 24-well plates (5,000 cells/well) in 0.15 ml of medium A (RPMI-1640 supplemented with 10% FCS and 100 μM penicillin-100 μg/ml streptomycin). After 72 h, medium A was carefully removed, and the subconfluent cells in each well were rinsed once with 0.15 ml of medium B (Iscove's modified DMEM [GIBCO-BRL] supplemented with 4 mM L-glutamine, 2% FCS, 100 μM penicillin-100 μg/ml streptomycin, and 0.5 mM CaCl_2). Medium B alone or medium B supplemented with additional CaCl_2 to final concentrations of 1, 3, 5, 7.5, or 10 mM was added to each well. Twenty-four hours later, "pretreatment" medium was removed from each well, the cells were rinsed once with 0.15 ml medium B, and then medium B alone, or medium B supplemented with additional CaCl_2 (to final concentrations of 3, 5, 7.5, or 10 mM) was added to each well (0.25 ml/well). Six to twenty-four hours later, the conditioned medium was removed for determination of PTHrP content. Triplicate incubations were performed for each treatment, and each experiment was carried out at least twice.

For studies on the effects of pretreatment with TGF-β1 on PTHrP secretion, PC-3 cells were seeded as described earlier. After 48 h, medium A was carefully removed from each well, and 0.15 ml of medium C (calcium-free DMEM supplemented with 4 mM L-glutamine, 0.2% BSA, 100 μM penicillin-100 μg/ml streptomycin, and 0.5 mM CaCl_2) containing 0.2, 1, or 3 μg/ml TGF-β1 was added to each well. Twenty-four hours later, "pretreatment" medium was removed from each well, the cells were rinsed once with 0.15 ml medium B (0.25 ml/well), and then medium B alone, or medium B supplemented with additional CaCl_2 (to final concentrations of 3, 5, 7.5, or 10 mM) was added to each well (0.25 ml/well). Six to twenty-four hours later, the conditioned medium was removed for determination of PTHrP content. Triplicate incubations were performed for each treatment, and each experiment was carried out at least twice.

PTHRP was measured in PC-3 cell-conditioned medium by means of a two-site immunoradiometric assay (IEMA; Nichols Institute Diagnostics, San Juan Capistrano, CA) that detects PTHrP-(1-72) and has a sensitivity of 0.3 pmol/l (35). PTHrP assays were initiated immediately after removal of the conditioned medium from the cell cultures to minimize the loss of PTHrP that occurs with freezing-thawing or other manipulations. PTHrP concentrations were calculated from a standard curve generated by adding recombinant PTHrP-(1-86) to the treatment medium employed in this study (i.e., unconditioned medium B). CaCl_2 and the additional polycationic CaR agonists (neomycin, spermine) employed in these experiments had no effects in the PTHrP assay when added in the absence of PC-3 cell-conditioned medium.

To ensure that the CaR agonists employed in the PTHrP studies had no significant effects on cell number or viability over the 6-h treatment period, we employed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (19), in which only viable cells convert water-soluble MTT to insoluble formazan crystals, as described previously (35).

Statistical analyses. A minimum of two independent PTHrP secretion experiments were performed for each of the PTHrP secretion studies described earlier. Results are presented as means ± SE for three determinations. Data were analyzed by analysis of variance followed by Fisher's protected least significant difference test. For all statistical tests, a P value < 0.05 was considered to indicate a statistically significant result.

RESULTS

Detection of CaR mRNA in LnCaP and PC-3 cells by Northern analysis and RT-PCR. Northern blot analysis carried out using a CaR-specific riboprobe on poly(A)+ RNA isolated from LnCaP and PC-3 cells revealed a major transcript of ~5.2 kb (Fig. 1A). This transcript is similar in size to a major CaR transcript in human parathyroid gland (13). RT-PCR performed with intron-spanning primers specific for the human CaR amplified a product of the expected size, 480 bp, for a CaR-derived product in both LnCaP (Fig. 1B, lane 2) and PC-3 cells (Fig. 1B, lane 3). DNA sequence analysis of the PCR products revealed >99% sequence identity with the corresponding region of the human CaR.
Car and PTHrP Release in Prostate Cancer

**A**

- 5.2 Kb

**B**

Mol. wt.  
500 bp  
300 bp

Fig. 1. Northern blot analysis of intracellular Ca\(^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)) and receptor (CaR) transcripts in the PC-3 and LnCaP prostate cancer cell lines. Northern analysis was performed on poly(A)^+ RNA isolated from the LnCaP (lane 2) and PC-3 prostate cancer cell lines (lane 3), as described in Materials and Methods. B: expression of CaR transcripts as assessed by RT-PCR using CaR-specific primers in PC-3 and LnCaP cells. RT-PCR was performed on cDNA prepared from the same sample of RNA extracted from LnCaP cells (lane 2) or PC-3 cells (lane 3), as described in Materials and Methods, using an intron-spanning primer pair specific for the human CaR. A 481-bp amplified fragment is indicative of a product arising from authentic CaR-derived transcripts. Lane 1 shows a DNA ladder for size comparison. No such product was apparent when cDNA was replaced with water or the reverse transcriptase was omitted from the RT reactions (not shown).

parathyroid CaR CDNA (not shown). These results indicate that the PCR products derived from both PC-3 and LnCaP cells were amplified from authentic CaR transcript(s).

Detection of CaR protein in LnCaP and PC-3 cells by immunocytochemistry and Western analysis. Immunocytochemistry with an anti-CaR antisemur (4637) revealed moderate CaR staining in both LnCaP (Fig. 2A) and PC-3 (Fig. 2B) prostate cancer cells. Staining was eliminated by preincubating the CaR antisemur with the specific peptide (FF7) against which it was raised (Fig. 2, C and D). Considerable intracellular CaR immunoreactivity could be observed in these cells, as in breast cancer (35) and bone cells (43, 44), which express considerably less CaR protein than do parathyroid cells (29), where the CaR displays a predominantly rim-like pattern of cell surface expression. Western blot analyses of proteins isolated from total cellular lysates of LnCaP or PC-3 cells by use of the 4637 antisemur were compared with those obtained using protein preparations from HEKCaR and bovine parathyroid cells as positive controls and nontransfected HEK-293 cells as a negative control (Fig. 3, A and C). Although the level of CaR protein expression in HEKCaR cells was much higher than the level in LnCaP and PC-3 cells (Fig. 3A), the immunoreactive bands in the two prostate cancer cell lines of ~160–170 kDa are comparable in size to those of bands present in the positive controls (Fig. 3, A and C). The specificity of these 160- to 170-kDa CaR-immunoreactive bands in proteins from the prostate cancer cell lines was confirmed by the marked reductions in their intensities after preabsorption of the antisemur with the peptide against which it was raised, although nonspecific bands at lower molecular masses were not abolished by the preabsorption procedure (Fig. 3B).

Figure 3, C and D further documents the specificity of this antisemur for the CaR by comparing the pattern of CaR-immunoreactive bands recognized by antisemur 4637 in proteins prepared from HEKCaR cells, bovine parathyroid cells, and nontransfected HEK-293 cells. There are similar patterns of bands in HEKCaR and parathyroid cells, corresponding to various glycosylated and nonglycosylated forms of CaR monomers and dimers (3, 42), but no CaR-specific immunoreactivity in nontransfected HEK-293 cells, which do not express the CaR endogenously. Figure 3C also shows more clearly the sizes of the immunoreactive bands in HEKCaR cells than does the overexposed lane showing these bands in Fig. 3A.

Effect of CaR agonists, TGF-β1, and dominant-negative CaR on PTHrP secretion. To determine whether CaR agonists modulate PTHrP secretion from PC-3 cells, the cells were treated with varying levels of [Ca\(^{2+}\)]_i (0.5, 1, 3, 5, 7.5, or 10 mM), neomycin (100 or 300 mM [Ca\(^{2+}\)]_i), or spermine (2 mM in 0.5 mM [Ca\(^{2+}\)_i]), and PTHrP in the conditioned medium was determined by RIA. PC-3 cells produce a readily measurable amount of PTHrP at 0.5 mM [Ca\(^{2+}\)]_i. Higher levels of [Ca\(^{2+}\)]_i stimulated PTHrP secretion in a dose-dependent manner (Fig. 4A). At 1, 3, and 5 mM [Ca\(^{2+}\)]_i, PTHrP secretion was increased 1.2-, 1.5-, and 1.8-fold, respectively, compared with that observed at

**Fig. 2.** Expression of CaR protein as assessed by immunocytochemistry using CaR-specific antibody antisemur 4637 in PC-3 and LnCaP cells. Immunocytochemistry, carried out using anti-CaR antisemur 4637 as described in Materials and Methods, revealed readily apparent immuno-staining of both cell lines, LnCaP cells (A) and PC-3 cells (B), which was eliminated by preincubating the CaR antisemur with the peptide FF7 against which it was raised (C: LnCaP cells; D: PC-3 cells) (4637).
4,6-fold increase in secretion. Results were reported in another experiment carried out using the secretion 34- and 36-fold, respectively, relative to that of neomycin and spermine also caused substantial increases in PTHrP secretion (2.0- and 3.2-fold, respectively). The polycationic CaR agonist, neomycin (Neo) and spermine (Sper), increased secretion of parathyroid hormone-related peptide (PTHrP) from PC-3 cells. Cells were treated with 6 mM CaR agonists, and the conditioned media were removed for determination of PTHrP released during the incubation, as described in MATERIALS AND METHODS. There was no significant difference in the MTT colorimetric assay for cells treated with different concentrations of CaR agonists (not shown), suggesting that there was no difference in the levels of PTHrP secretion of the same antigenic response. A: Carboxy-terminal or PTHrP in the conditioned medium was detected by immunoradiometric assay as in A. MTT values for cell number or viability, and results for PTHrP secretion were normalized to the MTT value for that well. Results observed when the neomycin increased PTHrP secretion from identical experimental conditions for NEK3-22 and Spermine (Sper) on secretion of PTHrP released during the incubation, as described in MATERIALS AND METHODS. There was no significant difference in the MTT colorimetric assay for cells treated with different concentrations of CaR agonists (not shown), suggesting that there was no difference in the levels of PTHrP secretion at 37.5 mM [Ca\(^{2+}\)], as well as in the presence of neomycin or spermine (P < 0.01; n = 3) relative to that observed at 0.5 mM [Ca\(^{2+}\)]. Essentially identical results were observed in another experiment carried out using the identical experimental protocol. B: Effect of pretreatment with transforming growth factor (TGF-β) on high [Ca\(^{2+}\)]-stimulated PTHrP secretion from PC-3 cells. Cells were pretreated overnight with 0.5, 1, or 5 ng/mL of TGF-β, as described in MATERIALS AND METHODS, and then incubated for 6 h with the indicated levels of [Ca\(^{2+}\)]. PTHrP secretion was then determined by immunoradiometric assay as in A. The results for PTHrP secretion were normalized to the MTT value for that well. Results observed when the neomycin increased PTHrP secretion from identical experimental conditions for NEK3-22 and Spermine (Sper) on secretion of PTHrP released during the incubation, as described in MATERIALS AND METHODS. There was no significant difference in the MTT colorimetric assay for cells treated with different concentrations of CaR agonists (not shown), suggesting that there was no difference in the levels of PTHrP secretion at 37.5 mM [Ca\(^{2+}\)], as well as in the presence of neomycin or spermine (P < 0.01; n = 3) relative to that observed at 0.5 mM [Ca\(^{2+}\)]. Essentially identical results were observed in another experiment carried out using the identical experimental protocol. B: Effect of pretreatment with transforming growth factor (TGF-β) on high [Ca\(^{2+}\)]-stimulated PTHrP secretion from PC-3 cells. Cells were pretreated overnight with 0.5, 1, or 5 ng/mL of TGF-β, as described in MATERIALS AND METHODS, and then incubated for 6 h with the indicated levels of [Ca\(^{2+}\)]. PTHrP secretion was then determined by immunoradiometric assay as in A. The results for PTHrP secretion were normalized to the MTT value for that well. Results observed when the neomycin increased PTHrP secretion from identical experimental conditions for NEK3-22 and Spermine (Sper) on secretion of PTHrP released during the incubation, as described in MATERIALS AND METHODS. There was no significant difference in the MTT colorimetric assay for cells treated with different concentrations of CaR agonists (not shown), suggesting that there was no difference in the levels of PTHrP secretion at 37.5 mM [Ca\(^{2+}\)], as well as in the presence of neomycin or spermine (P < 0.01; n = 3) relative to that observed at 0.5 mM [Ca\(^{2+}\)]. Essentially identical results were observed in another experiment carried out using the identical experimental protocol.

![Diagram](https://example.com/diagram.png)
cancer cell line (35), we examined the possibility that there might be an interaction between TGF-β and [Ca\(^{2+}\)]\(_0\) on PTHrP secretion in PC-3 cells. When PC-3 cells were pretreated for 24 h with TGF-β1, a substantial dose-dependent increase in both basal (i.e., at 0.5 mM [Ca\(^{2+}\)]\(_0\)) and high [Ca\(^{2+}\)]\(_0\)-stimulated PTHrP secretion was observed (Fig. 4B). Neither [Ca\(^{2+}\)]\(_0\) neomycin, nor TGF-β1 had any significant effect on the MTT values obtained from the PC-3 cells in this study, and the results of the MTT assay were employed to normalize the PTHrP released in each well.

To provide more definitive evidence that the CaR mediates high [Ca\(^{2+}\)]\(_0\)-evoked PTHrP secretion, we examined the effect of adenovirus-mediated infection of PC-3 cells with a dominant negative CaR construct (2) on [Ca\(^{2+}\)]\(_0\)-stimulated PTHrP secretion. Figure 5 shows that pretreatment of PC-3 cells with an adenoviral vector encoding the dominant negative CaR construct R185Q right-shifts the stimulation of PTHrP secretion by high [Ca\(^{2+}\)]\(_0\), and attenuates the response observed at 10 mM [Ca\(^{2+}\)]\(_0\), relative to the secretory response observed with PC-3 cells infected with a control adenoviral vector.

**DISCUSSION**

The purpose of this study was to determine whether the LnCaP and PC-3 human prostate cancer cell lines express the CaR, and if so, whether CaR agonists modulate PTHrP secretion from them. CaR expression was detected in LnCaP and PC-3 cells by both nucleotide- and protein-based approaches. Northern analysis performed on poly(A)\(^+\) RNA from each of the two cell lines revealed a 5.2-kb CaR transcript (Fig. 1A). This transcript is similar in size to one of the predominant CaR transcripts observed in human parathyroid cells (13). Authentic CaR transcript(s) was also detected by RT-PCR (Fig. 1B), performed using total RNA from LnCaP and PC-3 cells followed by sequence analysis of the PCR products. These two prostate cancer cell lines also express CaR protein as assessed by immunocytochemistry (Fig. 2) and Western blot analysis (Fig. 3) performed using an affinity-purified, anti-CaR antiserum (4637). As assessed by Western analysis, the levels of CaR protein expression in LnCaP and PC-3 cells were lower than in the positive controls, HEKCaR cells and bovine parathyroid cells. They are not dissimilar, however, from those in several other types of cells in which we have shown that the CaR is expressed and modulates various biological responses, such as regulation of Ca\(^{2+}\)-activated K\(^+\) channels (9).

[Ca\(^{2+}\)]\(_0\) and the polycationic CaR agonists neomycin and spermine each stimulated PTHrP secretion from LnCaP and PC-3 cells in a dose-dependent manner (Fig. 5), with maximal stimulation occurring at 7.5–10 mM [Ca\(^{2+}\)]\(_0\). The levels of [Ca\(^{2+}\)]\(_0\) in the vicinity of resorbing osteoclasts are thought to be many times higher than the level of systemic [Ca\(^{2+}\)]\(_0\) (i.e., as high as 6–40 mM) (36). Therefore, in the bony microenvironment, metastatic prostate cancer cells will likely encounter levels of [Ca\(^{2+}\)]\(_0\) at least as high as those used in the present studies. Our results are consistent with those in other cell types exhibiting high [Ca\(^{2+}\)]\(_0\)-evoked PTHrP secretion, including normal keratinocytes (22), normal cervical epithelial cells (28), oral squamous cancer cells (31), JEG-3 cells (21), and H-560 rat Leydig cells, a model of humoral hypercalcemia of malignancy (34). The molecular mechanism underlying [Ca\(^{2+}\)]\(_0\)-stimulated PTHrP secretion, including normal keratinocytes (22), normal cervical epithelial cells (28), oral squamous cancer cells (31), JEG-3 cells (21), and H-560 rat Leydig cells, a model of humoral hypercalcemia of malignancy (34).

In conclusion, our results indicate that CaR expression and function is present in LnCaP and PC-3 cells, and that CaR agonists, such as neomycin and spermine, stimulate PTHrP secretion in these cells.

**Fig. 5.** Attenuation of high [Ca\(^{2+}\)]\(_0\)-stimulated PTHrP secretion from PC-3 cells infected with a dominant negative CaR. Open bars show PTHrP secretion in response to elevated levels of Ca\(^{2+}\) by PC-3 cells infected with the empty adenoviral vector; solid bars show the attenuation of high [Ca\(^{2+}\)]\(_0\)-stimulated PTHrP secretion in the cells infected with the dominant negative CaR. *Significant inhibition of PTHrP secretion from the dominant negative vs. vector-infected cells at the indicated level of Ca\(^{2+}\). Similar results were observed in another experiment carried out using the identical experimental protocol.
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protein: elevated levels in both humoral hypercalcemia of malignancy and hypercalcemia complicating metastatic breast cancer. 


Calcium-sensing receptor activation stimulates parathyroid hormone-related protein secretion in prostate cancer cells: role of epidermal growth factor receptor transactivation

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Abstract

We have previously reported that high extracellular Ca2+ stimulates parathyroid hormone-related protein (PTHrP) release from human prostate and breast cancer cell lines as well as from H-500 rat Leydig cancer cells, an action mediated by the calcium-sensing receptor (CaR). Activating the CaR leads to phosphorylation of mitogen-activated protein kinases (MAPKs) that participate in PTHrP synthesis and secretion. Because the CaR is a G protein-coupled receptor (GPCR), it is likely to transactivate the epidermal growth factor receptor (EGFR) or the platelet-derived growth factor receptor (PDGFR). In this study, we hypothesized that activation of the CaR transactivates the EGFR or PDGFR, and examined whether transactivation affects PTHrP secretion in PC-3 human prostate cancer cells. Using Western analysis, we observed that an increase in extracellular Ca2+ resulted in delayed activation of extracellular signal-regulated kinase (ERK) in PC-3 cells. Pre-incubation with AG1478 (an EGFR kinase inhibitor) or an EGFR neutralizing antibody inhibited the high Ca2+-induced phosphorylation of ERK1/2, GM6001, a pan matrix metalloproteinase (MMP) inhibitor, also partially suppressed the ERK activation, but AG1296 (a PDGFR kinase inhibitor) did not. High extracellular Ca2+ stimulates PTHrP release during a 6-h incubation (1.5- to 2.5- and 3- to 4-fold increases in 3.0 and 7.5 mM Ca2+, respectively). When cells were preincubated with AG1478, GM6001, or an antihuman heparin-binding IGF (IIB-IGF) antibody, PTHrP secretion was significantly inhibited under basal as well as high Ca2+ conditions, while AG1296 had no effect on PTHrP secretion. Taken together, these findings indicate that activation of the CaR transactivates the EGFR, but not the PDGFR, leading to phosphorylation of ERK1/2 and resultant PTHrP secretion, although CaR-EGFR-ERK might not be the only signaling pathway for PTHrP secretion. This transactivation is most likely mediated by activation of MMP and cleavage of proheparin-binding EGF (proHB-EGF) to HB-EGF.

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Keywords: G protein-coupled receptor; Epidermal growth factor receptor (EGFR); Transactivation; Parathyroid hormone-related protein (PTHrP); Prostate cancer

Introduction

Prostate cancer is known as the second most deadly cancer in men in the United States [1]. In most cases, prostate cancer metastasizes to bone, which negatively impacts prognosis [2]. Previous studies demonstrated expression of parathyroid hormone-related protein (PTHrP) in normal and malignant prostate epithelial cells [3,4]. PTHrP, which was originally isolated from renal, lung, and breast cancers in 1987, plays an important role in normal bone formation, development of mammary gland, skin, and teeth, and regulation of the contractility of smooth muscle [5]. Because the amino terminus of PTHrP has structural similarity to PTH, they can act on the same receptor, the type 1 PTH receptor (PTH1R). However, PTHrP acts on cells in a paracrine, autocrine, or intracrine fashion, whereas PTH acts...
in an endocrine manner [6]. In the prostate gland, the physiological role of PTHrP is unknown. However, evidence that there is (1) higher PTHrP expression in prostatic dysplasia (prostate intraepithelial neoplasia) than in normal prostate epithelium and (2) higher PTHrP expression in prostate carcinoma than in benign hyperplasia suggests that there are promalignant or proliferative effects of PTHrP that participate in the pathophysiology of prostate cancer [6–8].

We have previously reported that high concentrations of extracellular calcium (Ca\textsuperscript{2+}) stimulate PTHrP secretion from rat H-500 Leydig cells, human embryonic kidney cells stably transfected with the calcium-sensing receptor (CaR), human breast cancer cell lines, prostate cancer cell lines, and human astrocytes, astrocytomias, and meningiomas, and that this phenomenon is mediated by the CaR expressed on these cells [9–13]. These findings suggest the existence of a vicious cycle that could contribute to the pathophysiology of humoral hypercalcemia of malignancy (HHM) and osteolytic bone metastases. Once PTHrP-producing cancer cells metastasize to bone, for example, locally high levels of Ca\textsuperscript{2+} could stimulate PTHrP secretion further. Excessive production of PTHrP, in turn, would elevate local or systemic levels of Ca\textsuperscript{2+} through the PTH1R expressed on renal tubules and osteoblasts.

The CaR that was first cloned from bovine parathyroid gland has a central role in the regulation of PTH secretion and calcium metabolism [14,15]. Although the CaR is expressed mainly on parathyroid glands, distal tubules of the kidney, and the thyroid C cells, the receptor has been identified in intestinal epithelial cells, bone cells, several nephron segments other than the distal tubule, and many other tissues and cell lines [16]. The CaR activates MAP kinases (extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), JNK/SAPK) in certain cells, which may mediate some of the known biological actions of the CaR [17–21]. In previous reports, we have demonstrated that the MAP kinase pathways play key roles in CaR-stimulated PTHrP secretion [10,21]. However, it remains unclear how the CaR activates MAP kinases. Because the CaR is a member of the superfamily of G protein-coupled receptors (GPCR), we hypothesized that the CaR activates receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR) and, in turn, MAPKs. Recent evidence suggests that transactivation of the EGFR by GPCRs is mediated by activation of one or more metalloproteinases (MMPs), which cleave proheparin-binding EGF (proHB-EGF) to release HB-EGF [22,23]. This mechanism of GPCR-induced EGFR activation, which has been called the “triple-membrane-passing signaling” model, has been widely accepted [24]. Thus, we wondered if the CaR could also transactivate the EGFR. In this study, we show that the CaR transactivates the EGFR at least in part via metalloproteinase activation, followed by ERK phosphorylation, and that CaR-induced EGFR transactivation stimulates PTHrP secretion in PC-3 human prostate cancer cells.

### Materials and methods

#### Materials

Selective inhibitors of MEK1 (PD98059), EGFR kinase (AG1478), PDGFR kinase (AG1296), and pan MMPs (GM6001) were all obtained from Calbiochem-Novabiochem (San Diego, CA). Neutralizing antibodies against EGFR and HB-EGF were obtained from R&D Systems (Minneapolis, MN). Polyclonal antisera to EGFR and a mouse monoclonal antibody against phosphorylated ERK1/2 and a mouse monoclonal antibody against ERK2 were purchased from Sigma (St. Louis, MO). The enhanced chemiluminescence kit Supersignal was purchased from Pierce (Rockford, IL). Protease inhibitors were obtained from Bio-Rad Laboratories (Hercules, CA). The polyclonal antisera to EGFR and a mouse monoclonal antibody against phospho-EGFR and a mouse monoclonal antibody against phosphotyrosine (Calbiochem-Novabiochem, San Diego, CA) were all obtained from Calbiochem-Novabiochem (San Diego, CA). The polyclonal antisera to EGFR and a mouse monoclonal antibody against phospho-EGFR were purchased from Sigma (St. Louis, MO). The enhanced chemiluminescence kit Supersignal was purchased from Pierce (Rockford, IL).

#### Cell culture

The PC-3 human prostate cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin–100 µg/ml streptomycin and grown at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were passaged every 4–5 days using 0.05% trypsin and 0.5 mM CaCl for 2 h. Cells were then preincubated for 30 min with specific inhibitors or neutralizing antibodies in serum-free medium containing 0.5 mM CaCl<sub>2</sub> for 2 h. Cells were then preincubated for 30 min with specific inhibitors or neutralizing antibodies in serum-free medium containing 0.5 mM CaCl<sub>2</sub> for 2 h. Cells were then preincubated for 30 min with specific inhibitors or neutralizing antibodies in serum-free medium containing 0.5 mM CaCl<sub>2</sub> for 2 h.

#### PTHrP secretion studies

PTHrP secretion from PC-3 cells was determined using the same system as previously described [12]. Briefly, for studies on the effects of the CaR agonist Ca\textsuperscript{2+} and various inhibitors on PTHrP secretion, cells were seeded in 96-well plates (5 × 10<sup>3</sup> cells/well) in 15 µl of growth medium. After 72 h, the growth medium was removed and replaced with 0.15 ml of Ca\textsuperscript{2+}-free DMEM containing 4 mM l-glutamine, 0.2% BSA, 100 U/ml penicillin–100 µg/ml streptomycin, and 0.5 mM CaCl<sub>2</sub> for 2 h. Cells were then preincubated for 30 min with specific inhibitors or neutralizing antibodies in serum-free medium containing 0.5 mM CaCl<sub>2</sub> following which the medium was removed and replaced with 0.275 ml of the same medium or that supplemented with additional CaCl<sub>2</sub> (to a final concentration of 1.5, 3.0, or 7.5 mM) and appropriate inhibitors or neutralizing antibody for 6 h. The conditioned medium was collected to measure PTHrP content. Each experiment was carried out at least three times, and duplicate incubations were performed for each treatment.
Triton X-100, and 0.3% dry milk) and then incubated for five 15-min periods at room temperature (1% PBS, 150 mM NaCl, 1% Triton X-100, and 0.25% BSA) containing signal started to disappear at 60 min. The magnitude of the phosphorylation was detected by immunoblotting using an antibody overnight. After serum starvation for at least 48 h, cells were stimulated with 7.5 mM Ca\(^{2+}\) as described above. At the indicated time points, cells were washed with ice-cold PBS and lysed with immunoprecipitation buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.2 mM sodium vanadate, and protease inhibitors (as described above). The cell lysate was centrifuged at 10,000 \(\times\) g for 10 min. For immunoprecipitation, equal amounts of protein were incubated with polyclonal EGFR antibody overnight, and then incubated with protein A/G agarose beads for a further 1 h at 4°C. Bound immune complexes were washed three times with immunoprecipitation buffer containing protease and phosphatase inhibitors and detergents. The pellet was eluted by boiling for 5 min with 2 \(\times\) Laemmli sample buffer. Supernatant proteins were separated by 7.0% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with monoclonal antiphosphotyrosine antibody (PY99). The stripped membrane was then rebotted with EGFR antibody.

**Statistics**

The data are presented as the mean \(\pm\) SE of the indicated number of experiments. Data were analyzed using one-way ANOVA or Student’s t test. A P value of <0.05 was considered to indicate a statistically significant difference.

**Results**

We have previously reported that ERK activation plays a critical role in high Ca\(^{2+}\)-induced PTHrP secretion, and that high Ca\(^{2+}\) produces a delayed phosphorylation of ERK1/2 in the cell types studied to date [10,21]. In PC-3 cells, we also confirmed a delayed phosphorylation of ERK1/2 by Western blotting (Fig. 1A). Maximal activation was present at 30 min in all three independent experiments, and this signal started to disappear at 60 min. The magnitude of the phosphorylation of ERK1/2 is dependent on the Ca\(^{2+}\) concentration employed, as the strongest signal was observed with 7.5 mM Ca\(^{2+}\), while intermediate signals were observed with 1.5 and 3.0 mM Ca\(^{2+}\) (Fig. 1B).

**CaR activates ERK**

To make sure that the ERK activation is mediated by the CaR, we examined the effects of a known CaR agonist (sperrmine) and of a selective CaR activator (NPS R-467)
Fig. 2. High Ca\(^{2+}\)-induced ERK phosphorylation is mediated by EGFR activation. Cells were serum starved overnight and preincubated with the inhibitors or antibodies indicated. The cells were then treated with 0.5 and 7.5 mM Ca\(^{2+}\) in lanes 1 and 2–3, respectively, for 30 min. Equal amounts of total cellular protein were separated by electrophoresis on 12% polyacrylamide gels, transferred to nitrocellulose membranes, and analyzed by immunoblotting using a polyclonal antibody against phosphorylated ERK1/2 and a monoclonal antibody against ERK2 protein. Experiments were repeated three times, and the data shown are similar to those from the other two experiments.

kinase inhibitor, did not affect the level of ERK phosphorylation. These findings suggest that activation of the CaR results in transactivation of the EGFR, but not of the PDGFR, at least in part through activation of MMP(s).

Finally, we examined the extent of phosphorylation of the EGFR using immunoprecipitation. Phosphorylation of the EGFR was assessed using Western analysis with a monoclonal anti-phosphotyrosine antibody following immunoprecipitation of cell lysates with a rabbit polyclonal anti-EGFR antibody. Fig. 3 shows that the EGFR was phosphorylated to some extent even under basal (0.5 mM Ca\(^{2+}\)) conditions; after 10-min incubation in medium with 7.5 mM Ca\(^{2+}\), however, the phosphorylation of the receptor increased and was sustained for at least 30 min.

Next, we examined the effects of various inhibitors and neutralizing antibodies as described below. In Fig. 2, G1478, which is an EGFR kinase inhibitor, and D98059, which is a MEKI inhibitor, inhibited most of the high Ca\(^{2+}\)-induced ERK phosphorylation. GM6001, a matrix metalloproteinase (MMP) inhibitor, and antibodies against the EGFR as well as HB EGF also diminished ERK phosphorylation. However, AG1296, a PDGFR

**Evidence for EGFR transactivation**

When cells were incubated with 100 μM spermine for 30 min, the signal was increased over basal activity (Fig. 1). In addition, a much stronger signal was observed in cells incubated with NPS R-467 than those exposed to the less potent stereoisomer NPS S-467. Because NPS R-467 is 100-fold more potent than NPS S-467 [25], our results indicate that high Ca\(^{2+}\)-induced ERK phosphorylation is mediated by the CaR.
Effect of EGFR and PDGFR inhibitors on PTHrP secretion

We have previously demonstrated that high Ca\(^{2+}\) stimulates PTHrP secretion in PC-3 cells [12]. This action of Ca\(^{2+}\) is at least partly mediated by the CaR, because hormone secretion is attenuated after transfection of the cells with a dominant negative CaR, and known CaR agonists, for example, neomycin and gadolinium, promote PTHrP secretion [12]. Thus, we wondered if the CaR might stimulate PTHrP secretion through transactivation of the EGFR.

High Ca\(^{2+}\) stimulated PTHrP secretion in PC3 cells in a dose-dependent manner (Fig. 4). This stimulation was inhibited by 20 μM PD98059 and by 0.7 μM AG1478. However, 1 μM AG1296 did not affect PTHrP secretion. When the cells were preincubated with anti-HB-EGF antibody for 30 min, 5 μg/ml of the antibody significantly inhibited PTHrP secretion (by 42%) even under basal conditions (0.5 mM Ca\(^{2+}\)) (Fig. 5). At 7.5 mM Ca\(^{2+}\), the anti-HB-EGF antibody suppressed PTHrP secretion in a dose-dependent fashion. The anti-EGFR antibody gave similar results (data not shown). Pre-incubation with 10 μM GM6001 also suppressed PTHrP secretion by 40% at 0.5 mM Ca\(^{2+}\), and by about 50% in 3.0 and 7.5 mM Ca\(^{2+}\) medium (Fig. 6). These findings indicate that EGF and HB-EGF activate the EGFR even under basal conditions and that high Ca\(^{2+}\)-induced PTHrP secretion is suppressed by blockade of the CaR-
EGFR-ERK pathway. The former result seems compatible with the presence of phosphorylated EGFR signal at 0.5 mM Ca\(^{2+}\) even after serum starvation (Fig. 3).

Discussion

EGF has been shown to induce PTHrP secretion in human prostate tissue, mammary epithelial cells, bone, breast, kidney and lung cell lines, keratinocytes, osteosarcoma cells, epithelial cancer cells, and rat Leydig tumor cells [26-32]. This induction has been reported to involve both transcriptional and posttranscriptional mechanisms [32-34]. The likely involvement of the PKC pathway in EGF-induced secretion of PTHrP in cultured mammary epithelial cells [29] was shown by the additive effect of PMA and EGF on the induction of PTHrP mRNA. Multiple GPCR signals converge on the receptor tyrosine kinases (RTKs), particularly the EGFR. Some widely studied examples include the angiotensin II-induced hypertrophy of cardiomyocytes via transactivation of the EGFR and subsequent activation of MAPKs, and the ET-1-induced phosphorylation of the EGFR in human ovarian carcinoma cells.

PTHrP participates in promoting growth in PC-3 cells, which express a functional PTHR1 [6]. Previous work has shown that the level of PTHrP expression is higher in prostate cancer than in normal prostate tissue and that PC-3 cells secrete a significantly higher amount of PTHrP-1-34 than do the DU-145 and LNCaP prostate cancer cell lines [35,36]. PTHrP and the PTHR1 are co-expressed in both primary prostate cancers as well as in bone metastases [37]. In addition, PTHrP seems to influence cell adhesion by enhancing the synthesis of several extracellular matrix proteins and some integrin subunits [38]. These findings suggest that PTHrP may also play a critical role in promoting tumor invasiveness and skeletal metastases through paracrine- and probably intracrine mechanisms [6], although overexpression of PTHrP did not accelerate bone metastasis in a murine breast cancer model [39]. In an in vivo study, neutralizing antibodies to PTHrP or guanine nucleotide analogs, which inhibit PTHrP gene transcription, not only decreased osteoclastic bone resorption but also inhibited the development of metastases to bone by human breast cancer cells [40,41]. Furthermore, the intracrine actions of PTHrP can prevent apoptosis under certain circumstances [42].

In the present study, we showed that high Ca\(^{2+}\) stimulates PTHrP secretion via CaR-mediated activation of ERK in PC3 cells and demonstrated that this activation of ERK is mediated by transactivation of the EGFR but not the PDGFR through activation of MAPKs, followed by cleavage of proHB-EGF to HB-EGF (Fig. 7). The CaR belongs to the superfamily of GPCRs, some of which have previously been shown to be associated with RTKs; for example, the angiotensin II AT1 receptor, bradykinin B2 receptor, vasopressin V1 receptor, cholecystokinin CCK1R, gastrin CCK2R, and bombesin receptors stimulate the ERK cascade via activation of G\(_{q/11}\) followed by transactivation of the EGFR [22,43-49]. Because these peptide receptors can also activate G\(_{q/11}\), there appear to be two pathways activating the MAPK cascade via G proteins. Activation of G\(_{q/11}\) and G\(_{i}\) can stimulate PKC and PI3K, leading to Raf-MEK-ERK activation. Because the CaR is thought to couple to both G\(_{q/11}\) and some isoforms of G\(_{i}\) [14-17], it could activate the dual pathways, PKC and PI3K, as well as transactivate the EGFR. In fact, in our preliminary data, pre-incubation with either PKC or PI3K inhibitors suppressed ERK activation and PTHrP secretion to some extent in PC3 cells. In addition, p38 MAPK and JNK/SAPK inhibitors also partially suppressed high Ca\(^{2+}\)-induced PTHrP secretion (Yano et al., unpublished data). Therefore, PKC, PI3K, and other MAPKs could be activated directly by the CaR or indirectly mediated by EGFR transactivation, and this might explain the partial
Fig. 7. Schema of CaR-induced EGFR transactivation in PC-3 cells. Activation of the CaR stimulates MMPs, following cleavage of proHB-EGF to soluble HB-EGF, which activates the EGFR. Phosphorylation of the EGFR stimulates the Ras-Raf-MEK-ERK pathway, which is involved in regulating PTHrP synthesis or secretion.

inhibition of PTHrP secretion as well as ERK1/2 phosphorylation in the present study.

Transactivation of RTKs has been considered as a mitogenic pathway for GPCRs. Because high Ca\(^{2+}\) stimulates cell proliferation via the CaR in several cell types [50–54], the CaR-EGFR-ERK pathway could be involved in such cases, although other pathways, that is, p38 MAPK or PI3K, might also participate in regulating proliferation [55,56]. When we evaluated cell proliferation 6 h after stimulation with high Ca\(^{2+}\), there was no significant change, suggesting that CaR-induced changes in cell number did not affect the present data. Yet, because high Ca\(^{2+}\) most probably stimulates cell proliferation as well as PTHrP secretion via EGFR transactivation in PTHrP-producing cancer cells, further study is necessary to evaluate any effects of the CaR on cell proliferation and survival.

In conclusion, activation of the CaR transactivates the EGFR, but not the PDGFR, leading to activation of ERK1/2 and resultant PTHrP secretion in PC-3 cells. This transactivation is most likely mediated by CaR-mediated activation of MMP and subsequent cleavage of proHB-EGF to HB-EGF.

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