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

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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\(^{2+}\)]\(_{o}\)) stimulate parathyroid hormone-related protein (PTHrP) secretion from normal and malignant cells, potentially acting via the [Ca\(^{2+}\)]\(_{o}\)-sensing receptor (CaR). Because prostate cancers produce PTHrP, if high [Ca\(^{2+}\)]\(_{o}\) stimulates PTHrP secretion via the CaR, this could initiate a mechanism whereby osteolysis caused by bony 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\(^{2+}\)]\(_{o}\), neomycin, and spermine each concentration dependently stimulated PTHrP secretion from PC-3 cells, as measured by immunoradiometric assay, with maximal, 3.2-, 3.2-, and 4.2-fold increases, respectively. In addition, adenovirus-mediated infection of PC-3 cells with a dominant negative CaR construct attenuated high [Ca\(^{2+}\)]\(_{o}\)-evoked PTHrP secretion, further supporting the CaR’s mediatory role in this process. Finally, pretreating PC-3 cells with transforming growth factor (TGF)-\(\beta\)\(_1\) augmented both basal and high [Ca\(^{2+}\)]\(_{o}\)-stimulated PTHrP secretion. Thus, in PTHrP-secreting prostate cancers metastatic to bone, the CaR could initiate a vicious cycle, whereby PTHrP-induced bone resorption releases [Ca\(^{2+}\)]\(_{o}\) and TGF-\(\beta\) stored within bone, 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 of skeletal metastases and their complications are important goals of prostate cancer research.

Recent studies have shown that parathyroid hormone (PTH)-related protein (PTHrP) is a central mediator of malignancy-associated hypercalcemia and osteolysis. In addition to causing most cases of humoral hypercalcemia of malignancy, where skeletal metastases are absent, PTHrP, originally isolated from renal, lung, and breast cancers (7, 37, 39), is the biological mediator in ~70% of cases of malignant osteolysis with or without hypercalcemia, particularly that caused by common epithelial cancers [i.e., breast (16)]. Although prostate cancers metastatic to bone generally cause osteoblastic lesions, substantial increases in 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.

The extracellular calcium ([Ca\(^{2+}\)]\(_{o}\)-sensing receptor (CaR) is a G protein-coupled cell surface receptor that is a central element in [Ca\(^{2+}\)]\(_{o}\) homeostasis (6). In
parathyroid cells, high [Ca\(^{2+}\)]\(_{o}\), by activating the CaR, inhibits PTH secretion and parathyroid cellular proliferation (6), whereas in the kidney, stimulating the receptor reduces renal tubular Ca\(^{2+}\) reabsorption (20). Physiological proof of the CaR's key roles in Ca\(^{2+}\) 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 calcitonin from C cells (12, 14) and of ACTH from AtT-20 cells (11). Furthermore, several studies have shown that high Ca\(^{2+}\)\(_{o}\) 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\(^{2+}\)\(_{o}\)-evoked PTHrP release from both normal and malignant cells. In the case of PTHrP-secreting prostate cancers metastatic to bone, this CaR-mediated action could create an inappropriate "feed-forward" stimulation of PTHrP secretion, causing release of Ca\(^{2+}\)\(_{o}\) from bone that would stimulate further PTHrP secretion and promote worsening bone resorption. Moreover, interrupting high Ca\(^{2+}\)\(_{o}\)-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\(^{2+}\)\(_{o}\)-induced PTHrP secretion from PC-3 cells. Furthermore, transforming growth factor (TGF)-\(\beta\)_1, stimulates PTHrP secretion from PC-3 cells synergistically with high Ca\(^{2+}\)\(_{o}\), suggesting that release of this growth factor, along with calcium, during PTHrP-induced bone resorption could contribute to a feed-forward mechanism in which PTHrP-mediated osteolysis associated with prostate cancers metastatic to bone 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 \(\mu\)g/ml streptomycin. The cells were grown at 37°C in a humidified 5% CO\(_2\) atmosphere and were passaged every 5–7 days with the use of either 0.25% 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 \(\mu\)g of poly(A\(^{+}\)) RNA obtained using oligo-dT cellulose chromatography of total RNA (8). Poly(A\(^{+}\))-enriched RNA samples were denatured and electrophoresed in 2.2 M formaldehyde-1% agarose gels along with a 0.24- to 9.5-kb DNA ladder (GIBCO-BRL) and transferred overnight to nylon membranes (Duralon; Stratagene, La Jolla, CA). A \(^{32}\)P-labeled riboprobe corresponding to nucleotides 1745–2230 of the human parathyroid CaR cDNA was synthesized with the MAXIscript T\(_3\) kit (Pharmacia Biotech, Piscataway, NJ) with the use of T\(_3\) RNA polymerase and [\(^{32}\)P]UTP. Nylon membranes were then prehybridized, hybridized overnight with the labeled cRNA probe (2 \(\times\) 10\(^{6}\) cpn/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 \(\mu\)g) 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, 1.8 MgCl\(_2\), and 0.2 dNTP and 0.4 \(\mu\)M forward primer, 0.4 \(\mu\)M reverse primer, and 1 \(\mu\)L ELongase enzyme mix (a Taq/Pyrrocosus species GB-D DNA polymerase mixture; Gibco-BRL). Human parathyroid CaR sense primer 5'-CGGGGCCACCCATGACGCTTGA-3' and antisense primer 5'--GCTCTAGAGTTAACCGGATCCAAA-GGCTC-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 QIAquick 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 (ABI377; Applied Biosystems, Foster City, CA) as previously described (35).

Immunocytochemistry. A CaR-specific polyclonal antiserum (4637) was generously provided by Drs. Forrest Fuller and Karen Krapcho 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 immunocytochemistry and Western blot analysis as described in the following paragraphs. The specificity of the antiserum for the CaR is documented in RESULTS 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 4637 antiserum (5 \(\mu\)g/ml in blocking solution). Negative controls were carried out by incubating cells treated in an otherwise identical manner with the same concentration of 4637 antiserum that had been preabsorbed with 10 \(\mu\)g/ml of the FF-7 peptide. The cells were then washed, incubated with peroxidase-conjugated goat anti-rabbit IgG (1:100; 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 EGTA, 1 mM EDTA, 0.25 M sucrose, 1% Triton X-100, 1 mM dithiothreitol, 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 though a 22-gauge needle 10 times. Nuclei and other cellular debris were removed by low-speed centrifugation (1,000 rpm) followed 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 (0.275 ml/well). Six hours later, 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× SDS-Laemmli gel loading buffer containing 100 mM dithiothreitol, incubated at 37°C for 15 min, and resolved electrophoretically on linear 3–10% 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 antiserum 4637 at 1 μg/ml with or without preincubation with 2 μg/ml of the FF-7 peptide in blocking solution with 1% dry milk. The blots were subsequently washed, incubated with a 1:2,000 dilution of horseradish peroxidase-coupled goat anti-rabbit IgG in blocking solution, and washed five times again, 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 [Ca^2+]o) for 2 h. Additional calcium was then added to the wells as needed to achieve the final concentrations indicated in RESULTS, and the cells were incubated overnight. At the end of the incubation, conditioned medium was collected and subjected to PTHrP assay as described in PTHrP secretion studies. The data were normalized to the amount of protein in each well. Experiments were carried out using triplicate wells for each level of [Ca^2+]o.

**PTHrP secretion studies.** For studies on the effects of various CaR agonists on PTHrP secretion, PC-3 cells were seeded in 96-well plates (5,000 cells/well) in 0.15 ml of medium A (RPMI-1640 supplemented with 10% FCS and 100 U/ml 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 [calcium-free DMEM (GIBCO-BRL) supplemented with 4 mM l-glutamine, 2% FCS, 100 U/ml penicillin-100 μg/ml streptomycin, and 0.5 mM CaCl_2]. Medium B alone or medium B supplemented with either additional CaCl_2 (to final concentrations of 1, 3, 5, 7.5, or 10 mM) or the polycationic CaR agonists neomycin (100 or 300 μM) or spermine (2 mM) was then added to each well (0.275 ml/well). Six 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 U/ml penicillin-100 μg/ml streptomycin, and 0.5 mM CaCl_2) containing 0, 0.2, 1, or 5 ng/ml TGF-β1 was added to each well. Twenty-four hours later, this “pretreatment” medium was removed from each well, the cells were rinsed once with 0.15 ml/well 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 (0.275 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.

**Detection of CaR mRNA in LnCaP and PC-3 cells by Northern analysis and RT-PCR.** Northern blot analysis was performed essentially as described previously (35). Northern blot analysis and RT-PCR. Northern analysis and RT-PCR 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.

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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 antiserum (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 antiserum with the specific peptide (FF-7) against which it was raised (Fig. 2C 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 (26), 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 antiserum 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 HEK-CaR 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 160–170-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 antiserum 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 antiserum for the CaR by comparing the pattern of CaR-immunoreactive bands recognized by antiserum 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+}]_o (0.5, 1, 3, 5, 7.5, or 10 mM), neomycin (100 or 300 μM in 0.5 mM [Ca^{2+}]_o), or spermine (2 mM in 0.5 mM [Ca^{2+}]_o), and PTHrP in the conditioned medium was determined by IRMA. PC-3 cells produce a readily measurable amount of PTHrP at 0.5 mM [Ca^{2+}]_o. Higher levels of [Ca^{2+}]_o stimulated PTHrP secretion in a dose-dependent manner (Fig. 4A). At 1, 3, and 5 mM [Ca^{2+}]_o, PTHrP secretion was increased 1.2-, 1.5-, and 1.8-fold, respectively, compared with that observed at

![Fig. 1. A: Northern blot analysis of extracellular Ca^{2+} concentration ([Ca^{2+}]_o)-sensing 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 1) and PC-3 prostate cancer cell lines (lane 2), as described in MATERIALS AND METHODS, using a human CaR-specific riboprobe. 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 480-bp amplified fragment is indicative of a product arising from authentic CaR-derived transcript(s). 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).](http://ajpendo.physiology.org/)

![Fig. 2. Expression of CaR protein as assessed by immunocytochemistry using CaR-specific polyclonal antiserum 4637 in PC-3 and LnCaP cells. Immunocytochemistry, carried out using anti-CaR antiserum 4637 as described in MATERIALS AND METHODS, revealed readily apparent immunostaining of both cell lines. LnCaP cells (A) and PC-3 cells (B), which was eliminated by preincubating the CaR antiserum with the peptide FF-7 against which it was raised (C: LnCaP cells; D: PC-3 cells) (×400).](http://ajpendo.physiology.org/)
0.5 mM [Ca\textsuperscript{2+}]\textsubscript{o}, [Ca\textsuperscript{2+}]\textsubscript{o} at 7.5 and 10 mM evoked more substantial increases in PTHrP secretion (3.0- and 3.2-fold, respectively). The polycationic CaR agonists neomycin and spermine also elicited robust secretory responses: 100 and 300 μM neomycin increased PTHrP secretion 3.4- and 3.6-fold, respectively, relative to that at 0.5 mM [Ca\textsuperscript{2+}]\textsubscript{o}, whereas 2 mM spermine induced a 4.6-fold increase in secretion.

Because TGF-β stimulates PTHrP secretion from some cancer cell lines [e.g., the MDA-MB-231 breast cancer cell line], we investigated the effect of pretreatment with TGF-β1 on PTHrP secretion in the presence of high levels of extracellular calcium. Cells were pretreated overnight with 0.2, 1, or 5 ng/ml of TGF-β1, and then incubated for 6 h with the indicated levels of Ca\textsuperscript{2+}. The results showed that PTHrP secretion was statistically significantly stimulated at 7.5 mM Ca\textsuperscript{2+} (**P, 0.01 vs. 0.5 mM Ca\textsuperscript{2+} alone, n = 3) and with all concentrations of TGF-β1 (**P, 0.01 vs. no TGF-β1, n = 3) relative to the respective basal values at 0.5 mM Ca\textsuperscript{2+}. Essentially identical results were observed in another experiment carried out using the identical experimental protocol.

Fig. 3. A and B: expression of CaR protein as assessed by Western blot analysis using CaR-specific polyclonal antiserum 4637 in PC-3 and LnCaP cells. Western blot analyses of CaR proteins in whole cell lysates isolated from PC-3 or LnCaP prostate cancer cells, or from CaR-transfected HEK-293 (HEKCaR) cells as a positive control, were carried out as described in MATERIALS AND METHODS. Each protein sample, 20 μg for HEKCaR cells (right lanes) and 40 μg for LnCaP and PC-3 cells (left and middle lanes, respectively), was subjected to SDS-PAGE. A: CaR-specific antiserum 4637 was used as described in MATERIALS AND METHODS to identify expression of CaR protein in the resultant blots as indicated in the figure. B: results observed when the antiserum was preabsorbed with the peptide against which it was raised. C and D: Western blots of proteins in crude membrane preparations from bovine parathyroid (lane 1), HEKCaR (lane 2), and nontransfected HEK-293 cells (lane 3) using anti-CaR antiserum 4637. C: crude plasma membrane proteins were prepared, SDS-PAGE was carried out, and Western blotting was performed as described in MATERIALS AND METHODS. D: results observed when the antiserum was preabsorbed with the peptide against which it was raised. The Western blots shown in A-D are representative of ≥2 such blots for each cell type.

0.5 mM [Ca\textsuperscript{2+}]\textsubscript{o}, [Ca\textsuperscript{2+}]\textsubscript{o} at 7.5 and 10 mM evoked more substantial increases in PTHrP secretion (3.0- and 3.2-fold, respectively). The polycationic CaR agonists neomycin and spermine also elicited robust secretory responses: 100 and 300 μM neomycin increased PTHrP secretion 3.4- and 3.6-fold, respectively, relative to that at 0.5 mM [Ca\textsuperscript{2+}]\textsubscript{o}, whereas 2 mM spermine induced a 4.6-fold increase in secretion.

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cancer cell line (35)], we examined the possibility that there might be an interaction between TGF-β and 
[Ca2+]o 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 
[Ca2+]o) and high 
[Ca2+]o-stimulated PTHrP secretion was observed (Fig. 4B). Neither 
[Ca2+]o, 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 
[Ca2+]o-evoked PTHrP secretion, we examined the effect of adenovirus-mediated infection of PC-3 cells with a dominant negative CaR construct (2) on 
[Ca2+]o-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 
[Ca2+]o and attenuates the response observed at 10 mM 
[Ca2+]o 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 substantially 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 
Ca2+-activated K+ channels (9).

[Ca2+]o and the polycationic CaR agonists neomycin and spermine each stimulated PTHrP secretion from LnCaP and PC-3 cells in a dose-dependent manner (Fig. 3A), with maximal stimulation occurring at 7.5–10 mM 
[Ca2+]o. The levels of 
[Ca2+]o in the vicinity of resorbing osteoclasts are thought to be many times higher than the level of systemic 
[Ca2+]o (i.e., as high as 8–40 mM) (36). Therefore, in the bony microenvironment, metastatic prostate cancer cells will likely encounter levels of 
[Ca2+]o at least as high as those used in the present studies. Our results are consistent with those in other cell types exhibiting high 
[Ca2+]o-evoked PTHrP secretion, including normal keratinocytes (22), normal cervical epithelial cells (28), oral squamous cancer cells (31), JEG-3 cells (21), and H-500 rat Leydig cells, a model of humoral hypercalcemia of malignancy (34). The molecular mechanism underlying 
[Ca2+]o-stimulated PTHrP secretion in these cell types, however, is not clear. Our data suggest that the CaR is the likely mediator of this effect in PC-3 cells, because the receptor is clearly expressed in this cell line and PTHrP secretion is stimulated not only by elevated levels of 
[Ca2+]o but also by the polycationic CaR agonists neomycin and spermine. Furthermore, adenovirus-mediated infection of the PC-3 cells with a dominant negative CaR (R185Q) (2) attenuated and right-shifted high 
[Ca2+]o-stimulated PTHrP secretion, providing additional strong evidence for mediation of this action by the CaR. Others have successfully utilized transfection of CaR-expressing cells with a different dominant negative CaR construct (R795W) to document the CaR's involvement in other biological responses (30).

On the basis of the present study on PC-3 cells and in two breast cancer cell lines (35), our findings have clear implications for the existence of a feed-forward mechanism involving prostate cancer cells metastatic to bone. When prostate and breast, and possibly other, cancers metastasize to the skeleton and induce PTHrP-mediated osteolysis, this will lead to high local levels of 
[Ca2+]o within the bony microenvironment owing to PTHrP-stimulated bone resorption with or without associated systemic hypercalcemia. These high levels of 
[Ca2+]o will elicit further PTHrP secretion from the cancer cells, thereby exacerbating the osteolytic disease. Guise and Mundy (18) have provided strong evi-

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*Fig. 5. Attenuation of high 
[Ca2+]o-stimulated PTHrP secretion from PC-3 cells infected with a dominant negative CaR. Open bars show PTHrP secretion in response to elevated levels of 
Ca2+ by PC-3 cells infected with the empty adenoviral vector; solid bars show the attenuation of high 
[Ca2+]o-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 
[Ca2+]o. Similar results were observed in another experiment carried out using the identical experimental protocol.*
idence for the existence of a similar feed-forward mechanism involving the action of TGF-β released from bone on PTHrP-secreting breast cancer cells. Indeed, we have shown that TGF-β1 increases PTHrP secretion from PC-3 cells and have also demonstrated that TGF-β1 produces at least an additive increase in the stimulation of PTHrP secretion by high [Ca\(^{2+}\)\(_i\)]. The mechanism for this effect is not clear but might involve TGF-β1-induced upregulation of the expression of the CaR or its signaling pathways and/or of the level of expression of the PTHrP gene, thereby increasing the amount of PTHrP available for secretion in response to an elevated level of [Ca\(^{2+}\)\(_i\)]. Because [Ca\(^{2+}\)\(_i\)] and TGF-β are both released from the bone matrix during bone resorption induced by PTHrP, they are both available to elicit further PTHrP secretion. In effect, both could cooperate to generate a vicious cycle of tumor-induced bone resorption begetting further bone resorption in the setting of skeletal metastases of prostate (or breast) cancers. The beneficial actions of bisphosphonates on the skeletal complications of metastatic breast cancer and on the incidence of new metastases (17, 32, 41) could result, at least in part, from reductions in the local concentrations of both [Ca\(^{2+}\)\(_i\)] and TGF-β as a result of decreased bone resorption.

In addition to its potential role in stimulating PTHrP secretion from prostate cancer cells metastatic to bone, the CaR could also impact on tumor progression, osteolysis, and, in some cases, hypercalcemia by modulating the proliferation and/or apoptosis of tumor cells. Recent studies have shown that CaR activation stimulates proliferation in several cell types, including rat-1 fibroblasts (30). In PTHrP-producing tumors, the CaR could potentially increase proliferation directly and/or indirectly by enhancing PTHrP secretion. Indeed, PTHrP has been shown to stimulate the proliferation of H-500 rat Leydig cells in vitro and to increase the rate of tumor growth in vivo when H-500 cells are implanted subcutaneously in rats (33). The CaR also protects some cells against apoptosis, as we have shown recently for AT-3 rat prostate cancer cells and CaR-transfected, but not nontransfected, HEK-293 cells (29). Therefore, high [Ca\(^{2+}\)\(_i\)]-evoked, CaR-mediated stimulation of proliferation and/or inhibition of apoptosis of prostate cancer cells metastatic to bone could clearly contribute to the progression of tumor growth and potentially render the tumor cells resistant to therapy.

In summary, high [Ca\(^{2+}\)\(_i\)]-evoked, CaR-mediated PTHrP secretion could clearly contribute to the excessive bone resorption recently recognized to be an important complication of prostate cancer metastatic to bone. If, as in PC-3 cells, the CaR modulates PTHrP secretion in other prostate cancer cells, then the use of CaR antagonists (15) with some degree of specificity for prostate and other types of cancer cells that metastasize to bone and produce PTHrP and, therefore, osteolysis could potentially offer substantial therapeutic benefits in this setting.

REFERENCES


