The role of calcium-sensing receptor and signalling pathways in the pathophysiology in two in vitro models of malignant hypercalcemia: The rat rice H-500 leydig testis cancer and prostate cancer (PC-3) cells. Expression and regulation of pituitary tumor transforming gene in leydig testis cancer and astrocyte and astrocytoma cells

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INTRODUCTION

Calcium is a crucial biological cation. The body has a fine tuned system to regulate the level of calcium in the extracellular fluid. The system regulating extracellular calcium is termed the calcium homeostatic system. The calcium homeostatic system involves several hormones and organs. The hormones involved comprise, amongst others, the parathyroid hormone, vitamin D and calcitonin. The organs involved are mainly the parathyroid glands, kidneys, skeleton and intestine. Calcium is very important extracellularly as well as intracellularly. In the cell Ca^{2+} serves as an intracellular messenger that participates in muscle contraction, neurotransmission, and enzyme activity (53). Outside the cell calcium probably plays just as important roles for the organism as it does inside the cell. The extracellular calcium ion is important as the cation participates in blood clotting, contributes to maintaining the membrane potential across the cell membrane, functions as a reservoir of calcium e.g. during every heart beat, and controls the release of hormones crucial for calcium homeostasis. Thus calcium controls a plethora of essential cellular functions, ranging from release of hormone and muscle contraction to gene expression. These functions in turn control cell growth, proliferation and cell death.

One of the key players in extracellular calcium homeostasis is the calcium-sensing receptor (CaR). The two major functions of this receptor in calcium homeostasis are to inhibit PTH release from the parathyroid gland and to inhibit renal reabsorption of calcium. Besides being expressed in the chief cells of the parathyroid gland and along the nephron of the kidney, the CaR has also been found to be functionally expressed in tissues not related to calcium homeostasis, including some cancer cells. The loss of regulation of growth is a key factor in the development of cancer. The CaR has been implicated by circumstantial evidence as well as proven to be involved in the progression of cancer disease in vitro (218). But much remain to be learned about the function of the CaR expressed in many cancer cells. Likewise the identification of many intracellular signalling pathways has provided a novel opportunity to understand the molecular mechanism behind the receptor's function in cancer cells. One such novel molecule is the pituitary tumor transforming gene initial found in pituitary adenomas and subsequently expressed in the fetal liver as well as in the testis. Furthermore studies have shown a correlation between the expression of pituitary tumor transforming gene and degree of malignancy in the e.g. colon, and increasing evidence points toward a role of the protein in the genesis of cancer. The pituitary tumor transforming gene is a key player in the anaphase of cell division, and no previous studies have investigated the regulation of this protein by the CaR.

The aim of this thesis was:

1. To identify functions of the calcium-sensing receptor in a primary culture of Leydig testis cancer cells, a model of humoral hypercalcemia of malignancy,
2. To identify the signaling pathways used by the calcium-sensing receptor in exerting its biological functions in an in vitro model of humoral hypercalcemia of malignancy,
3. To identify a novel-signaling pathway using a CaR-expressing prostate cancer cell line associated with malignant hypercalcemia.
4. To investigate whether the up-regulation of the oncogene pituitary tumor transforming gene by the calcium-sensing receptor in our model of humoral hypercalcemia of malignancy was a general phenomenon also seen in other types of cancer with a functional CaR that are not involved in malignant hypercalcemia using a CaR expressing astrocytoma cell line.
5. Lastly, to investigate other regulators of the oncogene pituitary tumor transforming gene in the CaR-expressing astrocytoma cell lines.

Initially I will describe the models, materials, and methods used. Then briefly describe calcium homeostasis with a focus on the CaR. Hereafter I will describe the syndrome of malignant hypercalcemia and in particular humoral hypercalcemia of malignancy with a focus on the role of the CaR. To give a background on the field of pituitary tumor transforming gene, I will describe the pituitary tumor transforming gene as marker of malignancy and its possible roles in the development and progression of malignant disease. Lastly I will present our data in the context of the literature and finally summarize and propose future directions and perspectives.

CELL MODELS USED IN THIS THESIS

HUMORAL HYPERCALCEMIA OF MALIGNANCY

Before the existence of the syndrome of humoral hypercalcemia of malignancy was recognized, it was reported that in inbred aged Fisher rats testicular neoplasms of Leydig cell origin develop spontaneously (123). The tumors were capable of being passaged by serial subcutaneous transplantations. The tumors grew rapidly but did not metastasize. The rat Leydig cell cancer was discovered to cause hypercalcemia with hypophosphatemia, hypercalcuria, and hyperphosphaturia in rats (199, 211, 212). Later Stewart et al. found that in patients with malignant hypercalcemia two groups could be identified: one group with solid tumors but no bone metastases and a

second much larger group of patients with metastases to bone (245). The hypercalcemic state of the first group of patients was termed humoral hypercalcemia of malignancy, indicating that some humoral factor secreted by the solid tumor would activate bone resorption and lead to hypercalcemia. Sica and co-workers found that the rat Leydig hypercalcemia tumor did not metastasize to bone and that the hypercalcemia could be reversed by removal of the tumor from the rats. This study verified that the rat Leydig cell cancer was a suitable model of the humoral hypercalcemia occurring in patients with solid tumors (238). The tumors were investigated by light and electron microscopy and verified to be of Leydig cell origin (212). No sex hormone production was found in the tumor. In vivo, human chorionic gonadotropin caused an acute rise in serum calcium in 3 to 5 hours in tumor-bearing hypercalcemic rats and the binding of chorionic gonadotropin was competed by luteinizing hormone. This indicates that an active chorionic gonadotropin/luteinizing hormone receptor is functionally expressed on the H-500 cells. Hereafter studies found that the mediator of this hypercalcemia induced cAMP-production by adenylate cyclase as seen in PTH-driven hypercalcemia, and that this was mediated through the PTH receptor (120, 217, 242). In 1987 PTHrP was discovered (249). This peptide (or peptides as there are three isoforms) resembles PTH and activates the PTHR1 similar to PTH. Using a two side immunoradiometric assay, it was shown soon thereafter that indeed PTHrP is the humoral factor produced by the H-500 Leydig tumor (74) and that neutralizing the peptide could abolish the hypercalcemia in Fisher rats transplanted with the H-500 tumor (103). Furthermore EGF has been shown to induce PTHrP release whereas testorone, dexamethasone and 1.25 vitamin D_3 downregulate PTHrP in H-500 cells (154). Thus the Rice H-500 Leydig testis cancer cells seem to be a valid, well-established model of humoral hypercalcemia. The H-500 cells differ from the normal Leydig cells as they are not polygonal, and most human Leydig cell tumors do not have central necrosis in contrast to the H-500 cell tumor. In human PTHrP has been reported to be expressed in the Leydig cell, although its function is unknown (9). All the studies, investigating the H-500 cells, used serial transplantations of the tumor. Simultaneously, with the in vivo characterization of the H-500 cells, the cells were also characterized in vitro. In primary cell culture the cells produce PTHrP and grow like cancer cells for up to 10 cell passages (154, 206).

HYPERCALCEMIA OF MALIGNANCY DUE TO METASTASES TO THE BONE

In the paper by Stewart and co-workers that defined HHM, the larger group of patient with malignant hypercalcemia were the patients with metastases to bone (245). The cancers that metastasize to bone are among the most frequent cancers, namely kidney, lung, breast, and prostate cancers (90). Prostate cancers, that metastasize to bone, generally form mixed osteolytic and osteosclerotic lesions. Substantial increases in bone resorption occur in this setting as assessed by biochemical markers such as pyridinoline and deoxypyridinoline (56, 119, 251). Interestingly, the markers of bone resorption may be lower in those with skeletal metastases of breast cancer than in patients with metastatic prostate cancer, indicating that more bone degradation is taking place in the latter (56). The PC-3 cell line was derived from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian (127). The PC-3 cell line induced hypercalcemia in the human subject from which it originated as well as in animal models when inoculated subcutaneously (127). Furthermore, it was shown the PC-3 cells express a functionally active calcium sensing-receptor and that high calcium through the CaR induced PTHrP release in these cells when grown in vitro (223). With this in mind, we chose the PC-3 cell line as a well-established model system to investigate new signaling pathways for the CaR in malignant cells with metastases to bone.

MALIGNANT CELLS EXPRESSING THE CaR THAT ARE NOT INVOLVED IN HYPERCALCEMIA OF MALIGNANCY

The last cell model used in our studies is the astrocytoma cell line U-87. The U-87 cell line is derived from a malignant gliomas grade III from a 44 year old Caucasian female (198). The astrocytoma cells do not induce systemic hypercalcemia neither by a humoral factor nor by metastases to bone. In the literature there are two astrocytoma cell lines that have been reported to express a functional CaR, the U373 and the U-87 cell lines (43, 44). Thus, the astrocytoma represents a model of cancer with no effect on systemic calcium and only local changes in calcium along with the normal changes in systemic calcium that will potentially stimulate the CaR expressed on these cells. The U-87 astrocytoma cells were chosen to investigate the effects of CaR stimulation on the PTTG oncogene due to its robust CaR-induced PTHrP release and ion channel regulation (40, 44). To validate the findings on the levels of PTTG expression in the U-87 cell line, we used human primary tumor samples that had been collected at surgery in the Brigham and Women's Hospitals surgical department as well as primary astrocytes, and the T98G and U-343 astrocytoma cell lines. Before we used the tumor to investigate mRNA expression levels, another part of the same tumor had undergone histological classification by the department of pathology.

MATERIALS AND METHODS

MATERIALS

Antibodies: Polyclonal antisera against phosphorylated and non-phosphorylated ERK1/2, p38 MAPK, SEK1, AKT (ser473) and ATF-2 kinases were purchased from New England Biolabs (Beverly, MA). A rabbit polyclonal antiserum against iNOS was purchased from BD Transductions Laboratories (Lexington, KY). Neutralizing antibodies against EGFR and HB-EGF were obtained from R&D Systems (Minneapolis, MN). Polyclonal antisera to EGFR and PTTG (M-16) and a mouse monoclonal antibody against phosphotyrosine PY99 were purchased from Santa Cruz Biotech (Santa Cruz, CA).

Inhibitors and activators: Selective inhibitors of p38 MAPK (SB203580), MEK1 (PD98059), JNK (SP600125), PI3K (LY294002), EGFR kinase (AG1478), PDGFR kinase (AG1296), pan MMPs (GM6001), and PKC (GF109203X) were all obtained from Calbiochem-Novabiochem (San Diego, CA). The PKC activator, phorbol 12-myristate 13-acetate (PMA), was purchased from Biomol (Plymouth Meeting, PA). The choice of dose of the individual inhibitors was based on data from the literature, previous experience from the laboratory and from pilot studies on dose response curves of the individual inhibitors (15, 64, 68, 125, 136, 142, 156, 183, 185, 268, 279). Most of the signalling molecules modulated by the chemical inhibitors were also investigated by western blotting or their upstream or downstream signaling molecules in order to validate whether calcium would activate the signalling molecules that were blocked pharmacologically, providing some support for the specificity of the inhibitors.

Other: The enhanced chemiluminescence kit, Supersignal, was purchased from Pierce (Rockford, IL). Protease inhibitors were obtained from Boehringer Mannheim (Mannheim, Germany). TGFα, EGF, 17-β-estradiol, BMP-2, TGFβ, TNFα, TRAIL, TTNP3, I3CRA, Ciglitazon, and PGJ2 were obtained from Calbiochem (La Jolla, CA, USA). All cell culture reagents to H-500, PC-3, and U-87 cells were purchased from GIBCO-BRL (Grand Island, NY) with the exception of FBS, which was obtained from Gemini Bio-Products (Calabasas, CA). NPS R-467 and NPS S-467 were donated by NPS Pharmaceuticals, Inc. (Salt Lake City, UT). Other reagents were from Sigma Chemical (St. Louis, MO).

METHODS

Protocol for H-500 in-vitro cell culture

The Rice H-500 rat Leydig cell tumor was obtained from the National Cancer Institute-Frederick Cancer Research and Development Center DCT Tumor Repository (Frederick, MD). Male Fischer 344 rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 200

to 220 g (10 weeks of age) were used for all experiments. A fragment of the H-500 tumor was implanted subcutaneously in each rat, and the tumor was allowed to grow for 8 to 14 days, with the size of the tumor determining when the tumor was removed; the average tumor removed was around 0.5-1 cm in radius. The encapsulated tumor was cut open, and tumor pieces were excised with a minimum of other tissues from the animal, rinsed several times with cell culture medium (see below), minced into smaller pieces, and dispersed by repeated pipetting and several passages through a 22-gauge needle. Dispersed H-500 cells were subsequently plated 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_2 atmosphere. Cells were passaged every 3 to 5 days using 0.05% trypsin-0.53 mM EDTA and used for experimentation within the first 10 passages. After the 10 passages the response to calcium at times would be either less robust or absent, indicating that CaR expression was downregulated or that the H-500 cells had undergone more substantial phenotypical changes. No contamination of host cells was identified as assessed by the presence of homogeneous morphology under light microscopy. Rats were handled in accordance with local institutional guidelines. In one instance an animal died, and in two cases the tumor ulcerated through the skin. No secondary tumors were detected in the animals.

Protocol for PC-3 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_2 atmosphere. Cells were passaged every 4-5 days using 0.05% trypsin-0.53 mM EDTA.

Protocol for human primary astrocytes and U-87 cell culture

Human primary astrocytes were purchased from Clonetics-Biowhittaker (Walkersville, MD), and U-87, T98G, and U-343 astrocytoma cells from the American Type Culture Collection (Manassas, VA); they were maintained in monolayer culture in DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin-100 μg/ml streptomycin and grown at 37°C in a humidified 5% CO_2 atmosphere. Primary astrocytes were derived from a fetal source. The primary astrocyte cultures are more than 90% pure as certified by the company based on assessment of the expression of glial fibrillary acidic protein.

Specimen selection and tissue samples

Nine glioma samples were collected at the time of surgery in patients who underwent craniotomy for glioma resection with human study approval. All operations were performed by Dr. Peter Black at the Brigham and Women's Hospital in Boston. A senior neuropathologist at the hospital evaluated all specimens and classified them in accordance with World Health Organization standard criteria. At the time of surgery, all tissue specimens were immediately snap frozen and stored in liquid nitrogen for RNA isolation.

Infecting H-500 cells with CaR constructs in rAAV

High-efficiency gene transfer into H-500 cells was accomplished using a recombinant Adeno-Associated Virus (rAAV)-based method. The CaR sequence with a naturally occurring, dominant negative mutation (R185Q), as well as the same vector containing the cDNA for the beta galactosidase protein (referred to hereafter as BG) were under the control of a cytomegalovirus immediate-early (CMV-IE) promoter element, and packaged as previously described by our collaborator (292). The BG served as the control for non-specific effects of rAAV infection. Cells were seeded (1000 cells/well) in 96-well plates in 0.1 ml of growth medium and cultured overnight. About 1000 virus particles/cell (as optimized by pilot studies) were used to infect each well. Cells were washed once with serum-free RPMI 1640. Virus particles were then added, and the culture was incubated for 90 min in the serum-free medium at 37°C in a cell-culture incubator. Equal volumes of RPMI 1640 containing 20% serum were added to the cells to achieve a final serum concentration of 10%. The cells were then cultured for 48 h, and experiments with low and high calcium concentrations were performed. No studies were done on cell growth or response to calcium besides the PTHrP and mRNA expression measurements with infected H-500 cells.

Measurements of PTHrP in media

PTHrP was measured using a two-site immunoradiometric assay (IRMA) (Nichols Institute Diagnostics, San Juan Capistrano, CA) that detects PTHrP (1-72) with a sensitivity of 0.3 pmol/L. At 3.4 pmol/L our inter- and intra-assay variance were 8.8% and 4.0% respectively. PTHrP assays were initiated immediately after removing the conditioned medium to minimize loss of peptide. PTHrP concentrations were calculated from a standard curve generated by adding recombinant PTHrP(1-86) to the treatment medium (i.e. unconditioned medium). Calcium with or without inhibitors had no effect in the PTHrP assay.

The effects of $Ca^{2+}{}_o$ as well as MAPK and PKC inhibitors on PTHrP release were determined by seeding H-500 or PC-3 cells in 96-well plates (1×10^4 cells/well) in 0.1 ml of growth medium. After 48 h, the growth medium was removed and replaced with 0.1 ml of Ca^{2+}-free DMEM containing 4 mM L-glutamine, 0.2% BSA, 100 U/ml penicillin-100 μg/ml streptomycin, and 0.5 mM $CaCl_2$. Two h later, this medium was removed and replaced with 0.225 ml of the same medium or that supplemented with additional $CaCl_2$ (to a final concentration of 2.5, 5.0, or 7.5 mM) for 6 h. In other experiments the medium was supplemented either with the kinase inhibitors described or with 7.5 mM $CaCl_2$ together with the same inhibitors, in the PC-3 cells the inhibitors were preincubated for 0.5 h before stimulation. In the experiment with ADP the concentrations used were 10^{-7}, 10^{-8}, and 10^{-9} M. Six h later, the conditioned medium was collected for determination of PTHrP release. The 6 h time point was chosen for subsequent experiments because it yielded PTHrP values falling on the linear portion of the PTHrP assay, whereas at 4 and 24 h, PTHrP was at the lower or upper portion of the curve, respectively. The fold increase in PTHrP release at high calcium compared to low calcium did not vary over the first 24 h. Furthermore the 6 hour time point was also chosen since results from our laboratory previously had shown no change on proliferation of the H-500 cells in this time frame as assed by MTT assay a colometric assay (224).

Determination of HGF secretion

To study HGF secretion, U87 cells were grown to 70-75% confluence in complete growth medium in 24-well plates. They were then serum-starved overnight in growth medium minus FBS containing 0.2% BSA along with various concentrations of EGF. Medium samples were cleared by centrifugation, and HGF was measured in this conditioned medium with an ELISA. The ELISA employs a quantitative sandwich, enzyme-linked immunoassay technique, utilizing a monoclonal antibody specific for HGF that is bound to microtiter wells. Assay sensitivity was 125 pg/ml. Data are expressed as picograms per microgram protein.

Western blot analysis

For the determination of PTTG, iNOS, AKT, ATF-2, ERK1/2, p38 MAPK, SEK1, or phosphorylation, monolayers of H-500, PC-3, or U-87 cells were grown on six-well plates. Cells were incubated for 18 h in serum-free, Ca^{2+}-free DMEM containing 4 mM L-glutamine, 0.2% BSA, and 0.5 mM $CaCl_2$. This medium was removed and replaced with the same medium supplemented as described in the papers. At the end of the incubation period, the medium was removed, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing 1 mM sodium vanadate and 25 mM

NaF, and then 100 mL of ice-cold lysis buffer was added [20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium vanadate, 50 mM glycerophosphate, and a cocktail of protease inhibitors]. The protease inhibitors were aprotinin, leupeptin, soybean trypsin inhibitor, pepstatin, and calpain inhibitor (10 mg/ml of each), all from frozen stocks, as well as 100 mg/ml of Pefabloc. The sodium vanadate, NaF, and Pefabloc were freshly prepared on the day of the experiment. The cells were scraped into the lysis buffer, sonicated for 5 sec, and then centrifuged at 6,000 × g for 5 min at 4°C. The supernatants were frozen at –20°C. After thawing, equal amounts of supernatant protein (100 μg) were separated by SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell) and incubated with blocking solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.25% BSA) containing 5% dry milk for at least 1 h at room temperature. AKT, ERK1/2, p38 MAPK, SEK1, and ATF-2 phosphorylation were detected by immunoblotting using an 18-h incubation with 1:1000 dilutions of rabbit polyclonal antisera specific for phospho-AKT, phospho-ERK1/2, phospho-p38 MAPK, phospho-SEK1, or phospho-ATF-2, respectively. For iNOS and PTTG 1:2000 and 1:1000, respectively, dilutions of specific primary rabbit polyclonal antisera was used. Blots were washed for five 15-min periods at room temperature (1% PBS, 1% Triton X-100, and 0.3% dry milk) and then incubated for 1 h with a secondary goat anti-rabbit, peroxidase-linked antiserum (1:2000) in blocking solution. Blots were then washed again (5 × 15 min). Bands were visualized by chemiluminescence according to the manufacturer's protocol (Supersignal, Pierce Chemical). The same membrane was used after stripping (Restore Western Blot Stripping, Pierce) to measure non-phospho AKT, ERK1/2, p38 MAPK, and SEK1. Protein concentrations were measured with the Micro BCA protein kit (Pierce).

Immunoprecipitation

After serum starvation for at least 48 h, PC-3 cells were stimulated with 7.5 mM calcium and 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 mM EGTA, 1% Triton X-100, 0.2 mM sodium vanadate, and protease inhibitors (as described above). The cell lysate was centrifuged at 10,000 × 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 pellets were eluted by boiling for 5 min with 2 × Laemmli sample buffer. Supernatant proteins were separated by 7.0% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with monoclonal anti-phosphotyrosine antibody (PY99). The stripped membrane was then reblotted with EGFR antibody.

DNA synthesis

Two methods for measuring DNA synthesis were used: [^{3}H]thymidine and BrdU uptake. H-500 cells were seeded in 24-well plates. The cells were cultured for 72 h and then serum-starved for 4 h, after which calcium (0.5-7.5 mM) alone or with NPS R-467, NPS S-467, PD98059, SB203580, or LY294002 was added along with [^{3}H]thymidine (1 μl/ml; 50 μCi/ml), and the cells were cultured again for 24 h in 1 ml media. Incorporation of [^{3}H]thymidine in H-500 cells was measured by removing the medium and lysing the cells with 0.5 ml of 10% trichloroacetic acid. The resultant DNA pellet was dissolved in 0.5 ml of 200 mM NaOH. Incorporated radioactivity was measured by counting in a scintillation analyzer on a ^{3}H program (Tri-carb 2900TR, Packard Bioscience, USA). The effect of calcium on [^{3}H]thymidine was verified by using BrdU.

U-87 cells were seeded in 96-well plates and transfection with siRNA was performed using a cocktail consisting of OptiMEM (Invitrogen), siPort lipid, and 20 nmol oligonucleotide that was designed in our laboratory. U-87 cells were pulsed with 5-bromo-2'-deoxyuridine (BrdU) for 4 h, and its incorporation was measured using a kit obtained from Roche Diagnostic (Indianapolis, IN).

TUNEL assay

The TUNEL reaction was performed to detect apoptosis. Cells were plated on coverslips and treated as described for the DNA synthesis assay. The cells were stained with an in situ cell-death detection kit, (Roche Diagnostics, Indianapolis, IN) following the manufacturer's recommendations. Briefly, cells were washed with PBS once and fixed with 4% formalin for 5 min. After fixation, the coverslips were dried and then stored at -80°C. After the cells were washed with PBS, they were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Slides were rinsed twice with PBS and then incubated for 60 minutes at 37°C with terminal deoxynucleotidyl transferase (TdT) enzyme in reaction buffer. The slides were rinsed three times with PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Samples were analyzed by confocal and fluorescence microscopy. TUNEL-positive nuclei were detected by the bright color in condensed or ruptured nuclei. Two researchers blinded to the status of the samples counted the apoptotic cells as well as the total number of cells in eight different fields from two independent experiments. The total number of cells ranged from 26 cells to 105 cells per field.

Northern blot analysis

To study effects on the expression of iNOS, PTHrP, PTTG mRNA, we performed Northern blot analysis as described before (38). Briefly, cellular RNA was isolated (50) using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The RNA recovered was quantitated by spectrophotometry, and aliquots of 20 μg of total RNA from treated cells were loaded on a formaldehyde agarose gel after denaturation. The gel was stained with ethidium bromide to visualize RNA standards and ribosomal RNA, in order to document equal loading of RNA from the various experimental samples. The RNA was then blotted onto nylon membranes (Duralon, Stratagene, La Jolla, CA). Blots were hybridized with a cDNA probe for PTTG, PTHrP, and iNOS and washed under high stringency conditions as described previously (156). An iNOS cDNA probe was prepared by one-step RT-PCR using 2 μg total RNA derived from H-500 cells using the following primers: 5'- TGC TAT TCC CAG CCC AAC AAC -3' (iNOS sense, 120-140), 5'- TTT TGC CTC TTT GAA GGA GCC -3' (iNOS antisense, 486-466). The PCR product was then subcloned into the TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions and sequenced to confirm its homology with the corresponding region of the rat iNOS mRNA (NM_ 012611.1). A full-length human PTHrP cDNA probe (1.7 kb) was generously provided by Dr. E. Schipani, Massachusetts General Hospital, Boston, MA and a rat PTTG probe was donated by professor Shlomo Melmed, Cedars-Sinai Research Institute, Los Angeles, CA. Equal loading was also confirmed by reprobing the membranes with GAPDH cDNA. Specific radioactive signals were analyzed on a Molecular Dynamics, Inc. PhosphorImager (Sunnyvale, CA) with the ImageQuant program.

Reverse Transcriptase-PCR

One-step RT-PCR (kit from Qiagen, Santa Clarita, CA) was used for determining the presence of: 1 PTTG transcript(s) using a pair of primers that would yield a 352-bp product spanning nucleotides 165 to 517 of the human PTTG cDNA (NM_004219). Primer sequences are; 1) sense – 5'-AGT TTC AAC ACC ACG TTT TGG C -3'; 2) antisense – 5'-GCT TTT CAA GCT CTC TCT CCT CG -3'; 2 PTHR1 transcript(s) using a pair of primers that would yield a 411-bp product spanning from nucleotides 181-592 of the rat PTHR1

cDNA (NM_020073). Primer sequences are 1) sense, 5'-CAG ATT TTC CTG CTG CAC CG-3'; 2) antisense, 5'-TGA ACT TGA GGC ACT CGC TGT-3'. In brief, we used the following procedure for RT-PCR: 2 µg total RNA was mixed with a master cocktail containing RT-PCR buffer, sense and antisense PTTG primers, dNTPs, RNase inhibitor, and an enzyme mixture containing reverse transcriptase (Omniscript and Sensiscript) and HotStart Taq DNA polymerase at the concentrations recommended by the manufacturer (Qiagen) to a final volume of 50 µl. The temperature-cycle protocol was as follows: 30 min at 50°C for RT reaction, followed by denaturation and activation of HotStart DNA polymerase for 15 min at 95°C, and PCR amplification (30 sec at 94°C, 30 sec at 58°C, and 1 min at 72°C for 40 cycles). A final extension for 10 min at 72°C was performed after the end of 40 cycles. In order to eliminate amplification from contaminating genomic DNA, we omitted RT as a negative control for the RT-PCR reaction for each sample. RT-PCR products were fractionated on 1.5% agarose gels. The presence of a 352-bp or 411-bp amplified product, respectively, was indicative of a positive PCR reaction arising from the presence of a PTTG- or PTHrP-related sequence within the cDNA.

Quantitative real-time PCR

To amplify human pituitary tumor transforming gene (PTTG1) (NM_004219), human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (J_02642), rat PTTG (NM_022391), rat vascular endothelial growth factor (VEGF) (NM_031836), rat inducible nitric oxide synthase (iNOS) (NM_012611.1) and rat GAPDH (M_17701) cDNA, sense and antisense oligonucleotide primers were designed based on the published cDNA sequences using the Primer Express ver. 2.0.0 (Applied Biosystems, Foster City, CA). Oligonucleotides were obtained from Genosys (Woodlands, TX). The sequences of the primers were as follows: 5'- CGG CTG TTA AGA CCT GCA ATA ATC -3' (human PTTG sense, 18-41), 5'- TTC AGC CCA TCC TTA GCA ACC -3' (human PTTG antisense, 119-99), 5'-TTC AAT GGC ACA GTC AAG GC-3' (human GAPDH sense), 5'-TCA CCC CAT TTG ATG TTA GCG -3' (human GAPDH antisense), 5'- ATG ACC CTG GCG TGA AGA TTT -3' (rat PTTG sense, 127-147), 5'- AAG CAG CAA CAG AGA CCA GAG C -3' (rat PTTG antisense, 227-206), 5'- AGC CTT GTT CAG AGC GGA GAA-3' (rat VEGF sense 500-520), 5'- TAA CTC AAG CTG CCT CGC CTT-3' (rat VEGF antisense 606-586), 5'-GAT TCA GTG GT CCA ACC TGC A-3' (rat iNOS sense, 621-641), 5'- CGA CCT GAT GTT GCC ACT GTT-3' (rat iNOS antisense, 738-718), 5'-TTC AAT GGC ACA GTC AAG GC-3' (rat GAPDH sense), and 5'-TCA CCC CAT TTG ATG TTA GCG-3' (rat GAPDH antisense). cDNA was synthesized with the Omniscript RT Kit (Qiagen, Valencia, CA) using 2 µg total RNA in a 20 µl reaction volume. For real-time PCR, the cDNA was amplified using an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The dsDNA-specific dye SYBR Green I incorporated into the PCR reaction buffer QuantiTech™ SYBR PCR (Qiagen, Valencia, CA) to allow for quantitative detection of the PCR product in a 25-µl reaction volume. The temperature profile of the reaction was 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. An internal housekeeping gene control, GAPDH, was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the RT. The size of the PCR product was first verified on a 1.5% agarose gel, followed by melting curve analysis. Inter-sample differences <0.5 cycles were accepted for calculating the average crossing point (Cp).

Microarray

Total ribonucleic acid (RNA) was quantified by measuring ultraviolet (UV) absorption ratio at 260/280 nm and checked for quality using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Preparation of the biotin-labeled complementary RNA (cRNA) target was performed using the BioRobot 9604 (Qiagen, Valencia, CA, USA) and a PTC-225 DNA Engine Tetrad™ Cycler (MJ Research, Inc., Boston, MA, USA). Single-stranded cDNA was prepared from 2 µg of total RNA using a T7-(dT) 24-oligonucleotide primer and Superscript™ II RNaseH-reverse transcriptase (200 U/µl). Included in this reaction was a mixture of six bacterial RNAs of known concentration for use as positive controls (2.5 pg/ml of araB/entF, 8.33 pg/ml of fixB/gnd and 25 pg/ml of hisB/leuB). Double-stranded cDNA was then generated with *E. coli* DNA polymerase I (10 U/µl) and RNase H (2 U/µl). After purification using a Qiagen QIAquick purification kit, the double-stranded cDNA served as a template to prepare biotin-labeled cRNA via in vitro transcription (IVT), performed in the presence of biotinylated nucleotides. The labeled-cRNA transcripts were purified using RNEasy columns (Qiagen) and assessed for quantity and quality using the same methods described above. The biotin-labeled cRNA was then randomly fragmented by incubating 2 µg of the sample in the presence of magnesium for 20 min at 94°C. This resulted in fragmented target with a size range between 100 and 200 bases.

Hybridization and Scanning. The biotinylated cRNA target was hybridized to two ADME-Rat Expression Bioarrays (Motorola Life Sciences). For each array, 2 µg of the fragmented target cRNA was added to 260 µl of hybridization buffer, denatured, and then injected into hybridization chambers, sealed, and incubated for 18 h at 37°C while shaking at 300 rpm. Each array was rinsed in a stringent 46°C wash in 0.75× TNT solution for 60 min, followed by Streptavidin labeling for 30 min (RT), then the 0.75× TNT and 0.05% TNT in series. The slides were spin-dried in an Eppendorf 5810R centrifuge (2000 rpm for 3 min) (swinging bucket rotor). Processed arrays were scanned using an Axon GenePix Scanner, and array images were acquired and analyzed using CodeLink™ Expression Analysis Software.

Motorola cDNA chip data analysis. The "normalized intensity" probe data generated by the CodeLink Expression Analysis Software (Amersham) were exported into Microsoft Excel. The data were then separated into two classes: high dose and low dose. The Excel function TTEST (low dose data, high dose data, 2, 2) was applied to each gene probe. Probes with p-values greater than the PVALTHRESH of 0.05 were eliminated. The Excel function AVERAGE (high dose data)/ AVERAGE (low dose data) was next applied. Probes with ratios between the RATIO-THRESH of 2.0 and 1/RATIO-THRESH of 0.5 were removed. The remaining probes were candidates for significantly changed mRNAs. These genes demonstrated acceptable p-values and exhibited at least a two-fold change between the high- and low-dose calcium treatments.

Messenger RNA silencing

U-87 cells were plated in 96-well plate with 60-70% confluency. Twenty four hours after plating, cells were transfected with either negative control or two different PTTG mRNA silencing oligonucleotides, purchased from Ambion (Austin, TX). For human PTTG RNA silencing (siRNA), we first tested two different siRNA oligonucleotide sequences designated as PTTG1.1 and PTTG1.2. The sense and antisense sequences used were: PTTG1.1, 5'-GAU CUC AAG UUU CAA CAC Ctt-3' (sense) and 5'-GGU GUU GAA ACU UGA GAU Ctc-3' (antisense); PTTG1.2, 5'-GUC UGU AA A GAC CAA GG GAtt-3' (sense) and 5'-UCC CUU GGU CUU UAC AGA Ctt-3' (antisense). For a negative control, we used the following oligonucleotides (Ambion): 5'-AGU ACU GCU UAC GAU ACG Gtt-3' (sense) and 5'-CCG UAU CGU AAG CAG UAC Utt-3' (antisense). Chemically synthesized annealed oligonucleotide of the above mentioned PTTG siRNA sequences were used. The 100-nm final concentration of siRNA sequences were used for transfecting the U87 cells. Efficacy of silencing was determined by real-time PCR of human PTTG gene 48 h post transfection, and PTTG1.2 siRNA sequence was found to have 70-80% efficiency in reducing human PTTG mRNA, compared with negative control, whereas PTTG (1.1) was less than 50% efficient. Therefore, we used PTTG (1.2) to study the role of PTTG in the growth of U87 cells.

Measurement of Ca^{2+}_i by fluorimetry in cell populations

Coverslips with H-500 cells were loaded for 2 h at room temperature with Fura-2/AM in 20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 1.25 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 0.1% bovine serum albumin (BSA), and 0.1% dextrose and then washed once with a bath solution (20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% dextrose, and 0.1% BSA) at 37°C for 20 min. The coverslips were then placed diagonally in a thermostatted quartz cuvette containing the bath solution using a modification of the technique employed previously in this laboratory (12). In the experiment with agonists for other G-protein-coupled receptors, angiotensin II was used at 10^{-8} M, ADP at 10^{-8} M, thrombin peptide agonist at 10^{-5} M, and carbachol at 10^{-4} M. The angiotensin II, ADP, thrombin peptide agonist, and carbachol were added into the bath solution. Excitation monochrometers were centered at 340 and 380 nm, and emission light was collected at 510 ± 40 nm through a wide-band emission filter. The 340/380-excitation ratio of emitted light was used to estimate Ca^{2+}_i as described previously (12).

Biological NO imaging by DAF-FM Diacetate

H-500 Cells were plated on coverslips in a 6-well plate. After 72 h at 70-80% confluency, the cells were challenged with 0.5 mM or 7.5 mM calcium for 18 h in serum-free, Ca^{2+}-free DMEM containing 4 mM L-glutamine and 0.2% BSA. The cells were then washed twice with RPMI 1640 media without phenol red. The cells were then loaded for 1 h with 1 ml RPMI 1640 (without phenol red) containing 10 µM DAF-FM diacetate (4-amino-5-methylamino- 2',7'-difluorofluorescein diacetate) (Molecular Probe, Eugene, OR), 0.1% pluronic acid, and 1 mM probenecid at 37°C in a 5% CO_2 incubator with no light. Finally, the coverslips were washed twice in RPMI 1640 media without phenol red.

Direct visualization of NO production. The coverslips incubated in 37°C RPMI 1640 media without phenol red were placed horizontally under the microscope lens. Photomicrographs with the fluorescent NO indicator were acquired with a laser scanning confocal microscope (LSCM, Leica TCS-NT, Heidelberg, Germany) equipped with an argon-krypton laser at an excitation wavelength of 488 nm and a band-pass filter for 500-550 nm (250). Simultaneous visualization of cell morphology by differential interference contrast (DIC) microscopy was performed to confirm equal cell numbers on the coverslips. The fluorescence images were obtained as a 1024 × 1024 pixel frame. All other settings, including scanning speed, pinhole diameter, and voltage gain, remained the same for all experiments. The images were stored on magneto-optical storage devices.

Measurement of NO by fluorometry in cell populations. The coverslips were placed diagonally in a thermostatted quartz cuvettes containing 37°C RPMI 1640 medium without phenol red. Excitation monochrometers were centered at 490 nm, and emission light was collected at 520 ± 40 nm through a wide-band emission filter.

Statistics

The data are presented as the mean ± SEM of the indicated number of experiments equal to N. Data were analyzed by one-way ANOVA followed by Fisher's PLSD test or by Dunnett's multiple comparison test or Student's *t-test* when appropriate. A P value of <0.05 was considered to indicate a statistically significant difference unlikely due to chance.

CALCIUM HOMEOSTASIS

Extracellular calcium is delicately regulated in the blood, and the handling of calcium that keeps it in equilibrium is, as mentioned above, termed the calcium homeostatic system. This system regulating calcium homeostasis involves several hormones and organs. The hormones involved comprise, amongst others, the parathyroid hormone, vitamin D and calcitonin. The organs are mainly the parathyroid glands, kidneys, skeleton and intestine. In the body most of the calcium (around 99%) is stored in the bone. Thus the extracellular ionized calcium concentration constitutes a minor fraction of total body calcium. Of the extracellular calcium approximately 46% is protein bound (mainly to albumin), around 7% is bound to small ions, (e.g., phosphate) and the remaining 47% is ionized. Thus less than 1% of the calcium in the body is present in an ionic form in the extracellular compartment, and it is this small fraction of the calcium that is believed to be the biologically active species. The extracellular concentration of ionic calcium (Ca^{2+}_o) in the blood is kept constant, with extremely small changes (1.1-1.35 mmol/l) (254), and minute-to-minute calcium homeostasis is maintained through the rapid actions of parathyroid hormone (PTH) (77). PTH release is regulated through minute changes in the extracellular calcium concentration that are sensed by the calcium-sensing receptor (CaR) (24), although phosphate and $1.25(OH)_2$ Vitamin D_3 also play a non-redundant role by incompletely understood mechanisms of feedback. Calcium is not only very important extracellularly but maybe even more so intracellularly. Here Ca^{2+} serves as an intracellular messenger that participates in muscle contraction, neurotransmission, enzyme and hormone secretion as well as several other key functions, e.g., blood clotting (53). Thus intracellular calcium controls and is a part of a plethora of essential cellular functions, ranging from release of hormone and muscle contraction to gene expression. These functions in turn can control cell growth, proliferation and cell death. The reservoir for intracellular calcium is extracellular calcium, and the intracellular calcium is therefore dependent on that in the extracellular compartment. Outside the cell calcium probably plays just as important roles for the organism as inside the cell. The extracellular calcium ion is important as the cation participates in neuromuscular activity, secretion, signal transduction, blood clotting, and the membrane potential across the cell membrane; it also functions as a reservoir of calcium that is used, for example, in every heart beat, and it controls the release of hormones crucial for calcium homeostasis. The many roles of the divalent cation, calcium, in the physiology of the body stress the importance of a very tight regulation of the extracellular calcium concentration and availability.

HORMONE SYSTEMS AND RECEPTORS INVOLVED IN EXTRACELLULAR CALCIUM HOMEOSTASIS

PTH and PTH receptor

As mentioned above PTH, calcitonin, and $1,25(OH)_2$ vitamin D_3 are the three major Ca^{2+}_o-regulating hormones, the so-called "calciotropic hormones" (260, 262). The overall action of PTH on calcium homeostasis is to increase the level of Ca^{2+}_o. Early studies showed an inverse sigmoidal relation between Ca^{2+}_o and PTH (21, 86, 228). This inverse sigmoidal relation suggests a negative feedback from Ca^{2+}_o to the parathyroid gland and subsequentially the release of PTH. The molecular mechanism for this feedback, the CaR, was indeed discovered in 1993 (24). PTH release is mainly inhibited by high calcium, low phosphorus and low $1,25(OH)_2$ vitamin D_3. Parathyroid hormone (PTH) is an 84 amino acid polypeptide. Its mRNA, in addition to encoding the mature peptide, also encodes a 'pre' or signal sequence of 25 amino acids and a basic 'pro' peptide of 6 amino acids. The PTH gene has been mapped to chromosome 11 (176). PTH exerts its actions through the PTH receptor (PTHR1), which like the receptors for peptide hormones such as secretin, vasoactive intestinal peptide, glucagon and calcitonin belongs to class B of the seven transmembrane receptors, also termed G -protein-coupled receptors (139). PTHR1 has an extracellular amino terminal of about 185 amino acids, seven transmembrane spanning helixes (approximately 290 amino acids), and an intracellular domain, the carboxy-terminal cytoplasmic tail, of 110 amino acids (160). The PTH peptide is thought to act on the PTHR1 mainly in kidney and bone, although the receptor has been found in many organs not involved in calcium homeostasis, such as rat aorta, heart, bladder, stomach, ileum, and pregnant uterus. Furthermore, knock out mice with no PTHR1 die intrauterine and studies have

suggested an association with abrupt cardiomyocyte death, suggesting that this receptor is important for the normal intrauterine development of the heart (201). The PTH peptide shares significant amino acid homology with parathyroid hormone related peptide (PTHrP), especially in the first 13 residues. PTHrP exists in 3 isoforms in humans: 139,141, and 173 amino acids. PTHrP is frequently expressed in the same cells that express the PTH receptor, or in cells immediately adjacent to them. This spatial proximity of PTHrP to the receptor, together with the fact that little if any PTHrP circulates under normal physiological conditions, have suggested that PTHrP acts as a paracrine or autocrine factor. PTHrP has several important physiological roles encompassing promotion of calcium mobilization from bone during lactation (278) transport of maternal calcium across the placental membrane (141), and the regulation of chondrocyte growth and differentiation in the growth plates of developing long bones (144).

Vitamin D and Vitamin D receptor

The overall effect of vitamin D on calcium homeostasis is to increase the level of Ca^{2+}_o; its actions are slower than the rapid effects of PTH. Vitamin D is not a vitamin as such, since a vitamin is generally defined as a compound that the body is not able to produce. In the skin 7-dehydrocholesterol is converted to previtamin D_3 by ultraviolet radiation (113). Previtamin D_3 is subsequently spontaneously converted to vitamin D_3, cholecalciferol. Vitamin D from the diet is in two forms cholecalciferol and ergocalciferol. Vitamin D is hydroxylated in the liver to 25(OH)-(ergo- or) cholecalciferol. There is little evidence that the active forms of ergocalciferol and cholecalciferol differ in their mode of action, however, more is known about vitamin D_3. Lastly 25(OH)-cholecalciferol is hydroxylated in the proximal tubular cells of the kidney to the active metabolite, $1,25(OH)_2$-cholecalciferol ($1,25(OH)_2$ vitamin D_3), by the enzyme 1-alpha-hydroxylase (also termed CYP27B1) (148) and the less potent metabolite $24,25(OH)_2$-cholecalciferol by the 24-hydroxylase enzyme (also termed CYP24) (271). The 1-alpha-hydroxylase is the enzyme that will determines the rate of production of $1,25(OH)_2$ vitamin D_3. 1-alpha-hydroxylase is upregulated by several factors, the most important are: high serum parathyroid hormone (PTH), hypocalcemia, hypophosphatemia and low 1,25 vitamin D_3. $1,25(OH)_2$ vitamin D exerts its effects on calcium homeostasis through the vitamin D receptor (VDR). The VDR is a sterol receptor that is mainly found in the nucleus of the cell. The receptor is widely expressed throughout the cells of the body, but the effects of the VDR on calcium homeostasis are mainly in the epithelial cells of the intestine and kidney. This was demonstrated in the VDR knock out mice. Bone formation and mineralisation were found by two groups to be severely impaired in VDR knockout mice (150, 309). However, when the mutant mice were maintained on a rescue diet (calcium/ phosphorous/ lactose enriched) they had a normal development, and the impaired bone formation and mineralization were rescued (8). As the nuclear receptor is expressed in most cells, including many that are not involved calcium homeostasis, $1,25(OH)_2$ vitamin D has been found to have other physiological roles that are less well understood, including cytokine production, cell growth and differentiation and secretion of hormones (51).

Calcitonin and calcitonin receptor

The third and last calciotropic hormone is the calcitonin (CT). The overall effect of CT in calcium homeostasis is to reduce the level of Ca^{2+}_o. The parafollicular cells (C cells) of the thyroid gland produce and release the hormone. CT is a 32 amino acid peptide with an N-terminal disulfide bridge and a C-terminal prolineamide residue. CT receptors are seven transmembrane receptors in superfamily B, and share homology with PTHR1 (151). In the case of the human CT receptors at least five splice variants have been described (145). In pharmacological doses CT reduces bone resorption and renal reabsorption of calcium (267), but the significance of these actions in the normal calcium homeostasis is still debated. Data that CT may not be redundant in calcium homeostasis came from studies in mice. CT knockout mice exhibited no identifiable developmental defects at birth, and had normal baseline calcium values. However, the knockout mice responded more to exogenous human PTH as evidenced by a greater increase in serum Ca^{2+} and urine deoxypyridinoline crosslinks, an effect that could be prevented by CT (111). Surprisingly the mice had higher trabecular bone volume. These findings support a hypothesis that CT inhibits bone resorption in the face of an acute hypercalcemic challenge but may also imply that calcitonin is important in regulation of normal bone formation. As described above there is a positive relationship between Ca^{2+}_o and the release CT. This positive feedback loop of calcium on CT secretion is most likely mediated by the CaR (82, 165).

CALCIUM AND CALCIUM-SENSING RECEPTOR (CaR)

It was early described that extracelullar calcium in the blood is kept nearly constant. The coefficient of variation of the serum ionized calcium concentration about its mean value is 2% or less in normal people (186). Furthermore, the inverse sigmoidal relationship between PTH and Ca^{2+}_o was recognized in the 1980-1990s, and Dr. Schwarz and Dr. Brown were among the international group that delineated the physiologic nature of the sigmoidal relationship (228-230, 232, 262). The release of PTH by the parathyroid glands is regulated by a seven transmembrane receptor (7TM), the calcium-sensing receptor (CaR, abbreviated by some as CaSR). The CaR was cloned more than a decade ago by expression cloning by a group of researchers led by Dr. Brown (24). The CaR is extremely sensitive to changes in Ca^{2+}_o, so that decreases in Ca^{2+}_o will, in matter of minutes, result in the release of PTH, an effect mediated by the CaR (24, 25, 86, 231, 233). This is illustrated by the Hill coefficient of around 3 for the CaR response to calcium in a heterologous system of cells transiently transfected with wild type CaR (12). The level of extracellular calcium at which Ca^{2+}_o is maintained is called "set point". The set point from the perspective of the parathyroid gland is defined as the calcium concentration causing half-maximal inhibition of PTH secretion. The CaR needs to be so sensitive to Ca^{2+}_o deviations because life-threatening states may develop if extracellular calcium levels change from normal to either hypo- or hypercalcemic levels, and a rapid response of the CaR is crucial since rapid changes in Ca^{2+}_o are much more dangerous than changes that develop gradually. The CaR functions as a "thermostat" detecting Ca^{2+}_o rather than temperature and the CaR act as a "calciostat", deciding the rate of PTH synthesis and secretion by the parathyroid glands. The rapidity of the secretory responses of PTH and CT to perturbations in extracellular calcium restores Ca^{2+}_o to its normal level within minutes to hours. The CaR is now known to be expressed not only in the calciotropic hormone-secreting organs (parathyroid gland and C-cells of the thyroid glands), but also in tissues that regulate the extracellular calcium concentration by translocating calcium ions into or out of the bodily fluids: kidney and, at lower levels, bone and intestinal cells (254). The kidney plays a major role in calcium homeostasis. In the kidney, the CaR is widely expressed along nearly the entire nephron. The cellular localization and apparent function(s) of the CaR seem to depend upon the site of the nephron in which it is expressed (286). The overall effect is to inhibit reabsorption of calcium from the ultrafiltrated fluid. In the gut and bone, the expression and function of the receptor is still somewhat controversial (for review see Tfelt-Hansen et al. 2005) (254). In the literature there is an on-going search for more regulators of the minute-to-minute regulation of calcium homeostasis. This has been shown to be needed as rats with no PTH or CT due to thyroparathyroidectomy still recover from hypocalcemia induced by calcitonin (282). And in rats $1.25(OH)_2$ vitamin D_3 only participates in the long term regulation of serum calcium (281). Seen in this light the presence of the CaR in these four organs – all major players in Ca^{2+}_o homeostasis-enables Ca^{2+}_o to act, in effect, as a fourth calcio-

tropic hormone, or as a "first messenger". Increases in the extracellular ionized calcium concentration stimulate the CaR to lower Ca^{2+}_o by promoting CT secretion, increasing urinary calcium excretion and inhibiting PTH secretion. The missing calcitropic factor indeed may be extracellular calcium, but other factor have been suggested such as fibroblast growth factor-23 (236) although this cytokine seems to have much slower actions than needed in a minute-to-minute regulation of calcium.

DISEASE WITH MUTATIONS IN THE CaR

The nonredundant physiological importance of the calcium-sensing receptor in humans was proven by the discovery of diseases caused by mutations in the receptor that lead to either loss-of-function or gain-of-function of the protein (105). Heterozygous (i.e., the mutation is present in only one allele and the other allele carries wild type CaR) gain-of-function mutations cause autosomal dominant hypoparathyroidism (ADH). Most patients have asymptomatic hypocalcemia with relative or absolute hypercalciuria. Heterozygous loss-of-function mutations give rise to familial hypocalciuric hypercalcemia – FHH, also termed familial benign hypocalciuric hypercalcemia (FBHH) by somein which most patients have an asymptomatic form of hypercalcemia with relative or absolute hypocalciuria. The homozygous or compound heterozygous variant of inactivating CaR mutations, on the other hand, produce neonatal severe primary hyperparathyroidism (NSHPT), a severe, sometimes deadly disease if left untreated. The mouse models with heterozygous or homozygous inactivation of the CaR gene have phenotypes comparable to the respective human conditions (108). Thus one way in which the mutated receptors may exert their effects on calcium homeostasis is through a reduced level of expression of wild type receptors, as in the heterozygous knock out mice.

Clinically FHH is mostly a benign state of hypercalcemia with few or no symptoms. The diagnosis of FHH is made in a patient with a family history of mild to moderate hypercalcemia averaging approximately 2.75 mM (total calcium), with an inappropriately low rate of urinary calcium excretion. The best distinction between FHH and other forms of hypercalcemia is often made by determining the ratio of calcium clearance to creatinine clearance (Ca/Cr). A value below 0.01 is found in about 80% of cases of FHH, while a similar proportion of cases of primary hyperparathyroidism caused by hyperplasia or parathyroid adenoma have values higher than this (23). While benign and asymptomatic hypercalcemia is typical of most FHH families, two families have been found to have calcium concentrations that average 3.13 and 3.35 mM, causing a neonatal severe hyperparathyroid-like state in some affected infants (11, 12). Due to the lack of symptoms in most cases, patients with FHH are often undiagnosed until a routine blood sample shows an unexpectedly high serum calcium level or family screening is done due to the birth of a child with NSHPT (163). Patients with FHH usually have normal PTH levels, despite the hypercalcemia, although in some cases the PTH levels are elevated (32, 101).

Neonatal severe primary hyperparathyroidism is clinically overt in most cases within the first half year. The infant has, as indicated by the name, severe, symptomatic hypercalcemia driven by PTH, as well as bony changes characteristic of hyperparathyroidism. Infants with NSHPT may have hypotonia, polyuria, dehydration, and failure to thrive. The mass of the parathyroid glands in NSHPT is often increased dramatically. Pathological investigation reveals chief cell hyperplasia. The hallmark of the disease is the associated hyperparathyroid bone disease. This bone disorder often leads to multiple low energy fractures (65, 87). Fractures in the ribs can produce a "flail chest" syndrome that secondarily causes respiratory difficulties. Biochemical analysis reveals hypercalcemia, hyperparathyroidism, and relative hypocalcuria (55). In the literature, levels of serum total calcium are reported to be moderately elevated (e.g., 3-3.25 mM) to as high as 7.7 mM in the most severe cases (23). PTH levels are often observed to be 10-fold higher than the upper limit of normal. Early diagnosis is crucial, as untreated NSHPT can be a severe neurodevelopmental disease that may be lethal when not treated surgically with total parathyroidectomy (55). Lately a wider clinical spectrum for the condition has become apparent; and the growing availability of genetic testing of the CaR gene has documented that some infants have milder hyperparathyroidism and a distinctively less severe clinical presentation and natural history than that just described. In these patients with the calcimimetics (CaR agonists, reviewed later) in theory might represent a means of lowering the serum calcium concentration and determining whether the patient derived any symptomatic benefit, thereby potentially providing a long term, effective medical therapy.

Patients with autosomal dominant hypoparathyroidism have an inherited form of hypocalcemia and are often clinically asymptomatic, similar to most patients with FHH. As stated above the genetic background is a gain of function mutation of the CaR. A few patients may develop seizures, neuromuscular irritability and calcification of the basal ganglia. A paraclinical landmark of the disease is the mild to moderate hypocalcemia, with serum PTH levels that are inappropriately within the lower half of the normal range or frankly subnormal (as opposed to normals who respond vigorously to the hypocalcemia) (197). They often have relative or absolute hypercalciuria, with normal or elevated levels of urinary calcium excretion, respectively, despite the low serum calcium. Their renal calcium excretion has been found to be higher than that of patients with typical hypoparathyroidism in some studies, and, analogous to the diagnosis of FHH, a high urinary Ca/Cr ratio (e.g., the ratio of the calcium to creatinine concentrations in the urine) may be better than hypocalcemia alone for differentiating ADH from primary hypoparathyroidism. However, not all studies showed this difference in the level of urinary calcium excretion between idiopathic hypoparathyroidism and ADH (182, 298). It is important to prevent renal complications such as nephrocalcinosis, nephrolithiasis, and renal impairment. These renal complications are often iatrogenic and caused primarily by the hypercalciuria where the clinician has tried to correct the low serum calcium with calcium and vitamin D treatment. Therapy with calcium supplements and vitamin D metabolites should be administered only to patients with symptomatic ADH, and the aim should be to increase the serum calcium concentration only to a level that will make the patient asymptomatic (189). The development of the calcilytic class of drugs (a CaR antagonist, reviewed later) may, in theory at least, provide an optimal treatment of symptomatic ADH patients by "resetting" the parathyroid and kidney to maintain a more nearly normal serum calcium concentration without hypercalciuria.

IS CaR THE ONLY CALCIUM SENSING MECHANISM?

Recently an orphan receptor, GPRC6A, in the superfamily C of the 7TM's was deorphanized and was found to resemble the CaR in its amino acid homology and several of its pharmacological properties (287). GPRC6A, like the CaR, is sensitive towards certain L-amino acids, but unlike the CaR, GPRC6A is a sensor of basic amino acids (288). The CaR senses aromatic amino acids most effectively (58). It was subsequently shown that this receptor is also sensitive towards extracellular calcium (albeit at high concentrations) and calcimimetics (194), allosteric activators of the CaR, which may implicate GPRC6A as a second calcium-sensing receptor (CaSR2). Another possible mechanism underlying some calcium-induced effects, e.g. in osteoblasts, is the calcium-binding intracellular protein, calcyclin (273). Lastly it should be noted that calcium could also have effects through ion channels such as L-type calcium channels, this is indeed the case in calcium regulated release of calcitonin by C-cell of the thyroid in vitro (165). Although data that the CaR is crucial for the calcitonin release is quite solid the L-type calcium channel may play an additional role (72), the data suggest that the L-type calcium channel is a calcium sensing mechanism involved in the calcium homeostasis.

THE CALCIUM-SENSING RECEPTOR AND ITS SIGNALING APPARATUS

CLONING AND STRUCTURE

The CaR was discovered, by an expression cloning technique, in *Xenopus laevis* oocytes, using a bovine cDNA library. A single clone of 5.3-kb showing the same pharmacological properties as the Ca^{2+}_o-sensing apparatus was found in a cDNA library prepared from mRNA extracted from bovine parathyroid glands (24). Soon afterward, the nucleic acid hybridization technique was used to clone the CaR in humans (6, 81), rats (210), mice (115), and rabbits (31). The nucleotide sequences of these receptors are approximately 85% identical to the original bovine parathyroid CaR, while the amino acid sequences are more than 90% identical (using http://www.ncbi.nlm.nih.gov/BLAST/), implying that functionally important mutations in the receptor are discarded through evolution. The CaR belongs to family C II of the superfamily of the seven transmebrane receptors (26). The 7TMs constitute the largest group of cell surface membrane receptors, and they carry significant importance in clinical medicine, as approximately 50% of the current prescription drugs target the 7TMs. The human CaR is 1078 amino acid residues long and, like all 7TMs, has three structural domains (Figure 1): a hydrophilic N-terminal end of 612 residues that is an unusually large extracellular domain (ECD) characteristic of the family C receptors of the 7TMs; a hydrophobic transmembrane domain (TMD) of 250 amino acids, and an intracellular domain (ICD) 216 amino acids long, which is the hydrophilic C-terminal end of the protein. The receptor is modified by N-linked glycosylation, which is important in promoting the cell membrane expression of the receptor. The cell surface CaR consists primarily of a dimer linked by covalent disulfide bonds involving two cysteine residues in each monomer (e.g., cys129 and cys131) (13, 69, 124). The location of the binding sites for Ca^{2+}_o in the CaR is, at present, somewhat of a conundrum. The ECD is thought to contain one or more binding sites for Ca^{2+}_o in the CaR, but a mutated CaR expressed without the ECD also responds to Ca^{2+}_o and other polyvalent cations, implying that the TMD also senses Ca^{2+}_o (116, 209).

INTRACELLULAR SIGNALING APPARATUS OF THE CaR

There are five protein kinase C (PKC) (Figure 1) and two protein kinase A (PKA) phosphorylation sites in the CaR (14). Phosphorylation of these PKC sites inhibits activation of phospholipase C (PLC) by the CaR, a major downstream mediator of the receptor's biological responses; this phosphorylation thereby acts as a negative feedback system, since PKC is also activated by CaR and is often situated downstream of PLC. The ICD directly or indirectly binds the scaffolding proteins filamin-A, beta arrestin and caveolin (10, 107, 135, 195). All three also bind to signaling partners activated by the CaR. This binding to filamin-A was recently found to inhibit the degradation of the CaR (312).

The CaR, like other 7TMs, acts through G-proteins. The first step following activation of the receptor is the release of a guanosine diphosphate (GDP) bound to the G-protein complex of $G\alpha\beta\gamma$. Subsequently, the $G\alpha\beta\gamma$ complex is bound to the ligand-bound 7TM and activated, which releases the complex from the receptor. Then GTP binds to $G\alpha$ in the $G\alpha\beta\gamma$ complex, and the activated $G\alpha$ is dissociated from $G\beta\gamma$. There are three main groups of $G\alpha$ subunits: 1) $G\alpha_s$ which activates adenylate cyclase (AC) to produce cyclic adenosine monophosphate (cAMP) from ATP; 2) $G\alpha_i$, which inhibits AC and inward calcium channels and activates outward potassium channels; and 3) $G\alpha_q$, which activates PLC and thereby the production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). Before the CaR was cloned, it was recognized that activation of parathyroid cells with high calcium induced PLC activity and thereby IP_3 production and also inhibited hormone-dependent cAMP production, suggesting that the CaR activated $G\alpha_q$ as well as $G\alpha_i$ (45, 97). Counter intuitively, two studies have suggested that the CaR is linked to the $G\alpha_s$ signaling pathway in growth hormone- and ACTH-producing producing pituitary adenoma cells respectively (66, 219), although this has not been confirmed by others, it might be due to stimulation of a calcium stimulated adenylate cyclase not through $G\alpha_s$.

A wide range of intracellular signaling pathways of the CaR have been recognized using a heterologous system comprising human embryonic kidney (HEK) cells with or without stably transfected CaR (HEK-CaR) (Figure 2). Many of these intracellular signaling

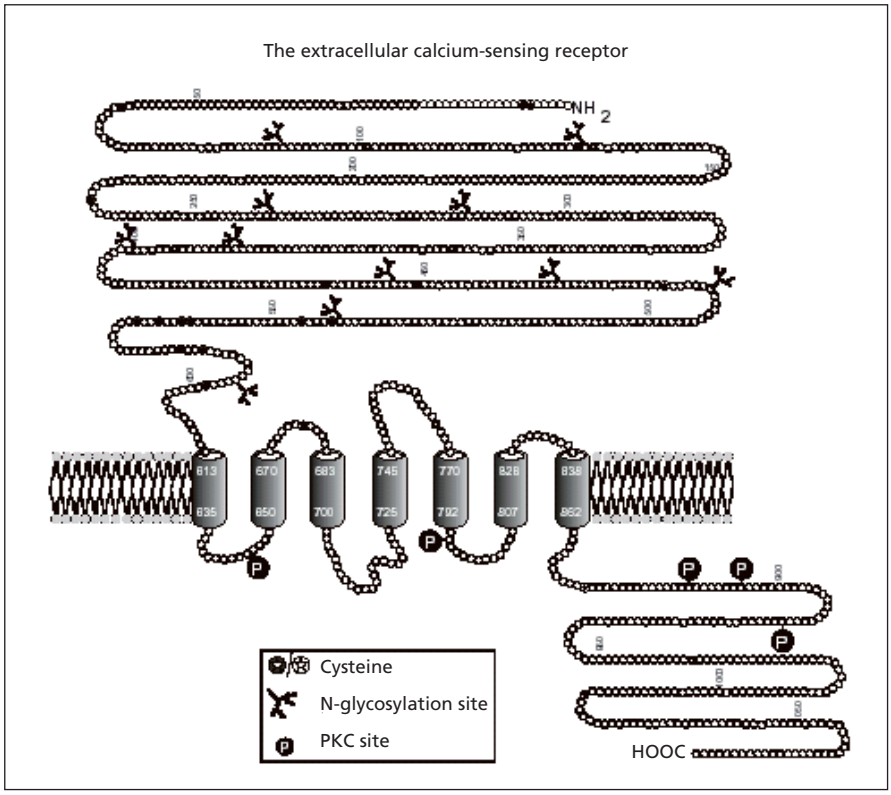

Figure 1. Schematic representation of the principal predicted topological features of the extracellular Ca^{2+}-sensing receptor cloned from human parathyroid gland. The extracellular domain contains approximately 600 amino acids, and the transmembrane and intracellular domains each contain approximately 200 amino acids. Also illustrated are the protein kinase C (PKC) phosphorylation sites, N-glycosylation sites, and cysteines (260).

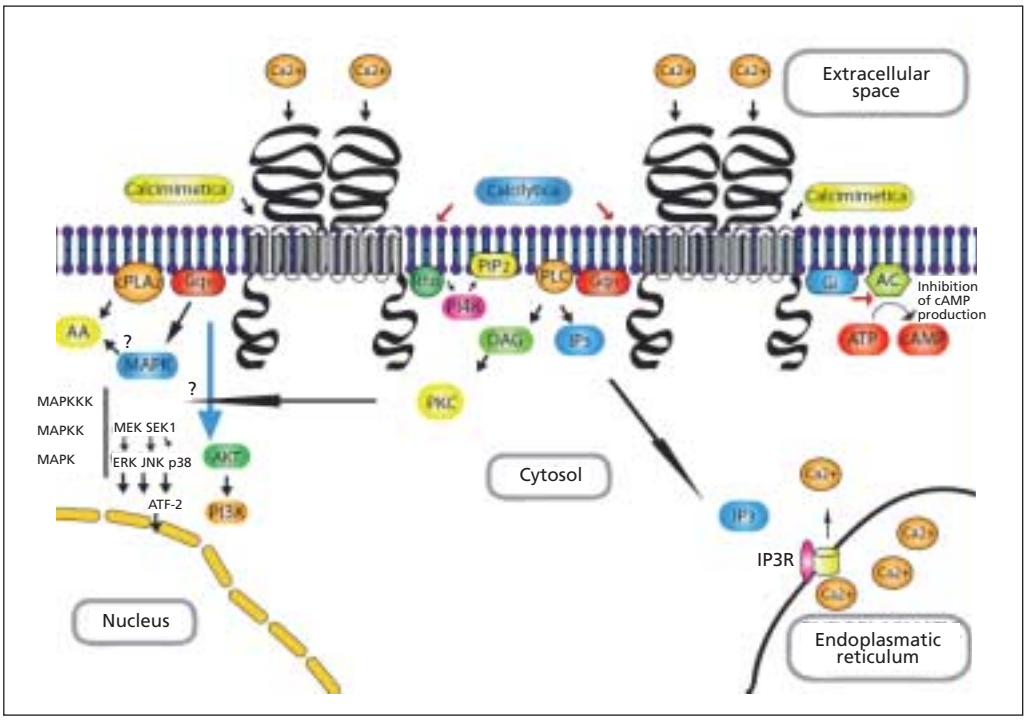

Figure 2. Signaling pathways activated by CaR. CaR is activated by Ca^{2+}_o, calcimimetics and numerous other agents. Please refer to text for detailed information. Black and blue arrow signifies stimulation and red arrow signifies inhibition. Abbreviations: Arachidonic acid (AA), Adenylate cyclase (AC), protein kinase B (AKT), activating transcription factor-2 (ATF-2), adenosine trisphosphate (ATP), cyclic adenosine monophosphate (cAMP), extracellular regulated kinase (ERK), alpha subunit of I and q subtype of the heterotrimeric G proteins (Gi and Gq_{11}), inositol-1,4,5-triphosphate (IP_3), Jun amino terminal kinase (JNK), mitogen-activated protein kinase (MAPK), MAPK kinase (MEK), p38 MAPK (p38) phosphatidylinositol 4-kinase (PI4K), phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), phosphatidylinositol-4,5-biphosphate (PIP_2), and stress-activated protein kinase ERK kinase 1 (SEK1).

pathways have also been shown to be active in CaR-mediated signaling in other cells naturally expressing the CaR. In parathyroid cells and HEK-CaR cells, the CaR activates phospholipase (PL) A_2, C, and D (134). PLC produces IP3, which then activates the IP3 receptor in the endoplasmic reticulum, resulting in release of calcium from intracellular stores, thereby producing spikes in the cytosolic free calcium concentration (Ca^{2+}_i). The elevation in Ca^{2+}_i is sustained by the opening of calcium-dependent calcium channels on the cell membrane. The first step in the production of inositol lipids is the activation of phosphoinositol 4-kinase, which converts PI to PI_4P. The CaR has been shown to activate phosphoinositol 4-kinase through $G\alpha_q$ in parallel to activation of PLC in HEK-CaR cells (117). Mitogen-activated protein kinases (MAPKs) are also activated by phosphorylation of their respective upstream kinases. MAPK is an important intracellular signaling pathway that often acts through the nucleus, e.g., in the regulation of the cell cycle, but it can also regulate events close to the cell membrane, such as release of peptides. Kifor et al. have shown that the CaR in HEK-CaR cells and parathyroid cells activates MAPKs. More specifically, the CaR activated extracellular signal regulated kinase (ERK1/2, also termed the p42/44 MAPK), which, in turn, phosphorylated and activated PLA_2 (136). Soon after that finding, Handlogten et al. showed that the CaR promotes ERK1/2 phosphorylation in HEK-CaR cells; as a control, they used HEK cells stably transfected with a dominant-negative CaR (Arg796Trp) (93). They also showed that the CaR activates PLA_2 through $G\alpha_q$, PLC, calmodulin, and calmodulin-dependent kinase but not through Gas or ERK1/2 in HEK-CaR cells. The difference in the results of these two studies regarding the role of ERK1/2 in PLA_2 activation remains to be clarified. In cells expressing the CaR at a lower level than in parathyroid cells and HEK-CaR cells, MAPK and phosphatidylinositol 3-kinase (PI3K), a classical prosurvival pathway, have both been found to be activated by the CaR. The role of the CaR and these pathways in testicular Leydig cell cancer, prostate cancer, breast tissue, and astrocytoma cells will be discussed in detail later.

AGONISTS OF THE CALCIUM-SENSING RECEPTOR

The CaR is a promiscuous receptor with many ligands. CaR agonists are divided into type I and type II. Type I are direct agonists, whereas type II are allosteric modulators, i.e., they require the presence of calcium to activate the CaR; the type II modulators left shift the calcium dose-response curve. The type I ligands are all poly-charged cations, both inorganic and organic. Inorganic di- and trivalent cations have been tested for their potency on the CaR, and they rank as follows: $La^{3+}>Gd^{3+}>Be^{2+}>Ca^{2+}=Ba^{2+}>Sr^{2+}>Mg^{2+}$ (81, 177). The best known type I organic polycationic CaR agonists are neomycin, spermine, and amyloid β-peptides (204, 305, 306). Neomycin, gadolinium, and spemine are often used to show that an effect of Ca^{2+}_o is likely to be mediated through the CaR. Slight changes in Ca^{2+}_o (micromolar) regulate CaR activity; most likely due to a lower affinity of the CaR for Ca^{2+}_o than of other receptors for their ligands where the EC_{50} are much lower, e.g. EC_{50} for angiotensin II receptor type I is around 10^{-8} M (94). Furthermore, the affinity towards calcium can be substantially decreased by changing serine residues at position 147 and 170 in the ECD to alanine (20). A CaR-S170A mutant showed no significant response to Ca^{2+}_o even at 50 mM. And CaR-S147A showed an impaired function as compared with wild-type CaR both with respect to the potency of Ca^{2+} (4-fold increase in EC_{50}) and maximal response (79% of wild-type response). The low affinity of the CaR, together with the steepness of the relationship between Ca^{2+}_o and CaR activity, makes the CaR a perfect "calciostat" to inform the cell of the exact concentration of Ca^{2+}_o in the immediate vicinity of the cell membrane. The Hill coefficient, a measure of how well the receptor responds to small changes in agonist, is 3 to 4 in HEK-CaR cells (12). In dispersed parathyroid cells in vitro, the CaR is even more sensitive to Ca^{2+}_o: PTH secretion is maximal at 0.75 mM and minimal just below 2 mM ionized Ca^{2+}_o (204). HEK-CaR cells and other cells expressing the CaR at lower levels than the parathyroid chief cells, such as primary testicular Leydig cancer cells, have higher EC_{50} values (~3-4 mM) (12, 224).

As mentioned earlier, type II agonists are allosteric modulators of the CaR, i.e., they potentiate the effect of Ca^{2+}_o on the CaR, and comprise two groups: small molecular drugs and amino acids. Drugs targeting the CaR as type II agonists are termed "calcimimetics" (178). NPS R-467 and R-568 and AMG 073 have all been used in experimental studies and clinical trials and lately in the treatment of uremic secondary hyperparathyroidism (27, 85, 153, 188, 237). AMG 073 is the drug of choice in the clinic because NPS R-467 and NPS R-568 are degraded by a cytochrome P-450 enzyme, CYP2D6 (Amgen, unpublished data). Five to seven percent of the population expresses CYP2D6 as an isoenzyme with reduced enzymatic activity. The calcimimetic binds to the TMD and enhances the affinity of the

CaR for calcium. Some L-amino acids have been found to be type II agonists of the CaR, whereas the respective D-amino acids are several-fold less potent in stimulating the receptor (59, 60). Thus the CaR's capacity to respond to both extracellular calcium and L-amino acids may enable it to act as a receptor that senses nutrients in the gut, for example. Most likely, the amino acids bind to the CaR in the ECD (315). This may be of pharmacological relevance, as the calcimimetics NPS R-467 and L-phenylalanine have synergistic effects on the CaR (314).

CELLULAR PROCESSES REGULATED BY THE CaR IN TISSUE NOT INVOLVED IN CALCIUM HOMEOSTASIS

Soon after the CaR was cloned, it was described in various cell types not considered to be a part of the calcium homeostatic mechanism. Such cell types include astrocytes (40), neurons (221), lens epithelial cells (42), pituitary cells (66), breast ductal cells (46), testis (169), keratinocytes (180), chondrocytes (37), human β-cells of the pancreas (243), bone marrow cells (115), adipocytes (52), monocytes (296), vascular smooth muscle cells from aorta (240), cardiomyocytes (257, 280), endothelial cells (289), and fibroblasts (167). While the physiologic roles of the CaR in these cells that do not participate in mineral ion homeostasis still remain to be fully defined, studies of the functions of the CaR in these diverse cell types have widened our knowledge of the range of regulatory functions of the CaR. These CaR-modulated processes include: (1) *regulation of proliferation* of colonic and ovarian surface epithelial cells (109, 166), testis Leydig cell cancer cells (255), fibroblasts (167), cardiomyocytes (257), and keratinocytes (17); (2) *differentiation* of keratinocytes (272), goblet cells (17), and mammary epithelial cells (46); (3) *apoptosis* of testicular Leydig cell cancer cells (255), fibroblasts, HEK-293 cells stably transfected with the CaR, and prostate cancer cells (152); (5) *chemotaxis* of preosteoblastic cells (294) and macrophages (296); (6) *secretion* of peptides, e.g., CT (71, 72, 82), PTH, PTHrP (156, 259), ACTH (66), gastrin (29), insulin (243, 244), and growth hormone (219); (7) *ion channel/transporter activity*, e.g., aquaporin-2 water channels (226, 227), non-selective cation channels (43, 306), voltage-dependent Ca^{2+} channels, calcium-activated potassium (K^+) and other K^+ channels (295, 304, 307); (8) *gene expression*, of the pituitary tumor-transforming gene (261); (9) *extracellular cell to cell calcium measurements*, two reports have nicely showed stimulation of cells with hormones may cause readily measurable changes in $Ca^{2+}{}_o$ (33, 110) (caused by rise in the cytosolic calcium concentration and subsequent pumping of calcium into the extracellular space), which may be so potent that in may be sensed by the CaR on adjacent cells (110); (10) *Homing of stem cells*, hematopoietic stem cells from CaR knock out mice have been reported to be lacking in the endosteal niche (2). As seen in the extended list of CaR functions above, the CaR has been found not only in normal tissues but also in many cancers, including astrocytoma, breast cancer, colon cancer, prostate cancer, ovarian surface epithelial cell cancer, and testicular cancer (218, 254). This knowledge may be important as extracellular calcium has been found to induce changes in the cell cycle and secretion of growth factors, two crucial features of most forms of cancer (254).

The widespread expression of the CaR throughout the body implies expanded biological roles that are unrelated to its major role in Ca^{2+} homeostasis; these "non-homeostatic" roles, however, are not well defined. From the data available, the CaR emerges as a multimodal sensor that integrates signals from various metabolic inputs (26). Depending on the composition of the local extracellular milieu, the CaR can act as a sensor for extracellular Ca^{2+}, ionic strength, pH, amino acids and positively charged molecules (60, 202, 203). This exceptional flexibility appears to place the CaR in positions of responsibility for maintaining homeostasis not only at the physiological level but also under pathophysiological conditions.

Several provocative, new lines of evidence indicate interesting (and sometimes unanticipated) roles for the CaR in tumor growth, progression, and/or metastasis and therefore underscore the need to reevaluate this receptor in the context of cancer biology. Later in this thesis I will describe how the CaR fulfills several aspects of Paget's "seed and soil" hypothesis (184), which posits that that tumor (the "seeds") will metastasize to locations that provide a fertile "soil" in which those particular tumor cells can grow. Indeed, under the changing microenvironments necessary for the initial growth and differentiation of cancer cells, with the subsequent modulation of cancer cell invasion and site-permissive metastasis, the CaR appears to be well poised as an effector of tumor cell behavior in the context of these parameters.

PTTG AS A MARKER OF MALIGNANCY

The CaR is involved in the regulation of cell division as indicated above. A newly discovered protein identified as having an important role in cell division is the pituitary tumor transforming gene (PTTG) (317). PTTG, also termed securin, is a pituitary tumor-specific oncogene, as it is expressed in pituitary tumors but at very low level in normal pituitary cells. The oncogene is currently the best available marker of pituitary adenomas (258). Available data increasingly implicates PTTG overexpression in the malignancy grades in thyroid, colon and astroglial cancers. Furthermore, there is an increasing body of evidence suggesting that PTTG and its binding protein participate in the genesis of some cancers (258).

In the cell cycle equal separation of sister chromatids is a key step for chromosomal segregation. Sister chromatid separation occurs once in the cell cycle in somatic cells during a brief mitotic step called anaphase. Anaphase is triggered by a protease called separase, which is normally kept inactive by an associated inhibitor: the PTTG (174, 175, 317). When PTTG is inactivated by ubiquitination by the anaphase-promoting complex or cyclosome (95, 192, 310), this leads to separase activation, loss of chromosomal cohesion, and the onset of anaphase (174, 175). Loss of chromosomal cohesion allows segregation of sister chromatids toward opposite spindle poles during anaphase and thereby the creation of cells with equal numbers of chromosomes. Under aberrant conditions, when sister chromatids are pulled to the same pole, aneuploid daughter cells are produced.

In the field of neurohormonal malignancies the cloning of PTTG has been a promising discovery, since it was soon after revealed that about 90% of all pituitary adenomas overexpress this gene compared with normal pituitary, which expresses very little PTTG. Several reports have shown that, although PTTG was upregulated in all histological subtypes, its expression is highest in ACTH-secreting and nonfunctioning pituitary tumors (164, 222, 313). Therefore PTTG and possibly FGF-2 and FGF-R-1 are currently the best available markers of pituitary tumors. The high level of expression of PTTG in all pituitary adenoma subtypes suggests that the protein is involved in the genesis of pituitary tumors. That this is likely the case was implied by a mouse model where the overexpression of PTTG in the pituitary gland induced in focal pituitary hyperplasia and adenoma formation (1).

PTTG has been shown to induce proliferation and malignant transformation in vitro and to promote tumor formation in nude athymic mice implanted with NIH-3T3 fibroblasts that overexpress PTTG (191). NIH-3T3 fibroblasts overexpressing PTTG were also shown to induce angiogenesis through the production of basic fibroblast growth factor (bFGF) (121). PTTG is thought to work in collaboration with a PTTG-binding protein necessary for the translocation of PTTG from the cytoplasm to the nucleus (47). PTTG has been found to be expressed in many malignant tissues but not in the corresponding nonmalignant tissue. The normal tissues that express PTTG mRNA include the testis, thymus, and fetal liver, as assessed by northern analysis (283). Although expression of PTTG is limited to a few tissues, PTTG knock-out mice have been found to have testicular hypoplasia and to display diabetes due to impaired pancreatic b-cell development (284, 285). PTTG is abundantly expressed in the testis, and its expression is stage-specific-being highest in spermatocytes and spermatids during the rat spermatogenic cycle (190).

ADENOMAS EXPRESSING THE CALCIUM-SENSING-RECEPTOR

CaR EXPRESSION IN PRIMARY HYPERPARATHYROIDISM

The expression of the CaR in the body is highest in the chief cells of the parathyroid glands. The role of the CaR in parathyroid neoplasia has, therefore, been found to be extensive. Adenomas are the most common cause of primary hyperparathyroidism (HPT). In primary HPT, the calcium set point of the pathologic parathyroid gland(s) is shifted to the right, as in familial hypocalciuric hypercalcemia, but the alteration in Ca^{2+}_o-regulated PTH release is not due to loss-of-function mutations in the CaR gene (114). All human studies (70, 84, 131, 137, 303) but one (80) have found that the amount of the CaR protein expressed on the cell surface and/or the amount of its mRNA are downregulated in adenomas from patients with primary HPT.

In a mouse model of primary HPT it was found that the parathyroid glands proliferated and the CaR was downregulated before there was HPT biochemically (159). Whether this observation is an association or CaR downregulation is playing a part in the proliferation of the glands is discussed below.

CaR EXPRESSION IN SECONDARY HYPERPARATHYROIDISM

In secondary hyperparathyroidism the etiology of the disease is not the parathyroid gland but may be due to renal insufficiency or lack of vitamin D. In hyperplastic parathyroid glands from patients with secondary HPT (e.g., in patients with renal failure), as for primary HPT, the amount of the CaR protein expressed on the cell surface and the amount of its mRNA are downregulated (84, 162, 276). In a human study, the parathyroid glands of 56 subjects, 52 from uremic patients with secondary HPT and 4 from normal subjects, were studied for CaR content. This study showed an association of decreased CaR protein with enhanced proliferation of parathyroid cells in secondary HPT by immunochemical techniques (302). This observation suggests that the CaR may participate in the genesis of the hyperplasia. To study this in detail the field has used animal models.

In a rat study using a model of secondary HPT, 5/6 nephrectomized rats received a high-phosphate diet to produce uremic secondary HPT; downregulation of the CaR was found at both the RNA and protein levels, suggesting an association between the decrease in CaR expression and parathyroid proliferation as seen in humans (22). Ritter et al. (213), however, showed that parathyroid cellular hyperplasia precedes the downregulation of the CaR in the rat 5/6 nephrectomized model of secondary HPT. The same group found that feeding a low-phosphate diet to the rats with secondary HPT reversed the downregulation of the CaR and the secondary HPT, but again, this was preceded by normalization of PTH and parathyroid-gland growth (214). Lewin et al. (149) performed isogenic kidney transplantation in rats with uremic secondary HPT and found that the PTH levels normalized rapidly despite the continued downregulation of both the CaR and the vitamin D receptor in the same rat model. This study suggests that normalization of levels of the CaR and vitamin D receptor are not necessary to reestablish the normal set point of the parathyroid glands. In contrast to these findings, increased parathyroid mass and manifest hyperplasia were reported in CaR knock-out mice, a finding that favors the involvement of the CaR in the inhibition of parathyroid cellular proliferation (108). Recently, a Japanese report showed that the calcimimetic NPS R-568 reversed the downregulation in CaR expression on the parathyroid glands in the rat model of uremic secondary HPT (170). The cause-and-effect relationships of the CaR, parathyroid hyperplasia, and altered set point in the pathophysiology of primary and secondary HPT, however, require further study. In the parathyroid glands, there are two mRNA transcripts of the CaR; they arise from two different promoters (48). The transcripts differ in the reading of exon 1, and a study showed that one transcript (type 1A) is downregulated in parathyroid adenomas. The significance of the splice variants in parathyroid gland adenomas and other forms of neoplasia is presently unknown, but could be a part of the explanation of the conflicting data.

CaR EXPRESSION IN NON-PARATHYROID ADENOMAS

The CaR has been shown to be expressed in human gastrinomas, with the levels of expression varying in the eight samples investigated by quantitative PCR (83). Gastrinomas are notorious for being difficult to diagnose, and a widely used diagnostic method is to infuse calcium intravenously and study its effect on the gastrin levels. Itami et al. incubated CaR-expressing gastrinoma cells in vitro with calcium and observed an enhanced release of gastrin that could not be obliterated by the calcium channel blocker nifedipine (122). Also favoring a role for the CaR in the calcium-induced release of gastrin in gastrinomas is the finding of a small Japanese study of healthy humans that the calcimimetic KNR568 increased serum gastrin levels transiently (118). In a clinical study investigating the effects of oral calcium and peptone (amino acids, type II CaR agonists) in women with absorptive hypercalciuria and in normal controls, Bevilacqua et al. found that the effects of calcium and peptone on gastrin release were increased in the hypercalciuric group, indicating the presence of more-responsive CaR in these patients than normals (16). Taken together, these data strongly suggest that CaR is the mediator of the calcium-induced gastrin release. In a related tumor, the insulinoma, the CaR is likewise reported to be expressed (132, 140). Normal human β-cells of the pancreas also express the CaR and respond to an increase in Ca^{2+}_o by decreasing insulin release in response to low and high glucose levels in one study (243) and to increase insulin release in other studies (88). Two studies found that the responses to an increase in Ca^{2+}_o were opposite in insulinomas and normal β-cells, i.e., an increase in Ca^{2+}_o leads to an increase in insulin release in insulinomas (133, 291). The effect of Ca^{2+}_o seems to be mediated through the CaR, as nifedipine did not change the action of Ca^{2+}_o (140). In a study on human pituitary adenomas the CaR has been reported to expressed in nonfunctioning and growth hormone adenomas (219). The type I agonists of the CaR induced increase in cAMP, and in growth hormone producing adenomas calcium augmented the effect of growth hormone releasing hormone on growth hormone release from the adenoma cells. These data shows that the CaR is active in at least three endocrine adenomas. Whether a functional CaR on these adenomas is important in the development and progression of the diseases is unknown. It could be speculated from the data on pituitary adenomas that the CaR somehow modulates the effect of growth hormone releasing hormone on growth hormone release; this could have clinical implications in the diagnosis and perhaps treatment of the diseases. Whether the effect of a CaR-specific agonist, such as AMG 073, on gastrin, insulin, and growth hormone release will be useful in clinical tests for adenomas remains to be tested. CaR expression has also been found on premalignant adenomas, e.g. from the colon; the literature on these adenomas will be described in the section on malignant tumors below.

MALIGNANT TISSUES EXPRESSING THE CALCIUM-SENSING-RECEPTOR

In the last part of this thesis I will describe the role of the CaR in malignant tissues as well as that of pituitary tumor transforming gene with a special focus on the data obtained in my studies. It is epidemiologically debated whether or not dietary calcium has a preventive effect on colon cancer (147). Investigating the expression of the CaR in biopsies from human colonic mucosa and in cancerous lesions, Sheinin et al. found expression of CaR at the mRNA and protein levels in both normal and malignant tissue (235). In normal tissue, the CaR was expressed primarily in chromogranin A-positive enteroendocrine cells, whereas in the biopsies of adenocarcinomas, the CaR was expressed primarily in the differentiated areas. Two groups have shown that expression of the CaR tended to be downregulated with increasing degree of malignancy of the colonic adenocarcinoma (34, 129). Furthermore, two other studies investigating

the effects of polymorphisms in the ICD of the CaR showed a possible clinical significance of the CaR in colon adenocarcinomas. Both studies found that polymorphisms in the CaR were associated with the risk of advanced adenoma (193, 241). The presence of the CaR has also been established in both the large and small intestines of rats (39, 76). Overall, the presence of the CaR in the colon seems indisputable, as seven different human epithelium/adenocarcinoma cell-derived cell lines have been shown to express the CaR (34, 76, 130). Kallay et al. found that decreasing $Ca^{2+}{}_o$ from a normal level to subphysiologic levels increased the in vitro proliferation of Caco-2 cells, a human colon adenocarcinoma cell line, suggesting that normal stimulation of the CaR has antiproliferative effects on the adenocarcinoma. They also found that low calcium induced activation of the transcription factor c-Myc through PKC (128) and that the effects of calcium could be mimicked by the type I agonist gadolinium, again pointing toward the CaR as the mediator of the antiproliferative response to calcium. Chakrabarty et al. showed that, in adenocarcinoma cell lines in vitro, stimulation of the CaR promotes expression of E-cadherin and suppression of β-catenin/T cell factor; events that are both thought to inhibit the growth of the tumor cells (34). In a subsequent study, they saw that the epithelial cells acquire CaR expression as they differentiate and migrate towards the apex of the crypt (35). $Ca^{2+}{}_o$, as well as $1,25(OH)_2$ vitamin D, stimulated the CaR promoter. Thus, the CaR seems to be present in the differentiated epithelial cells, and stimulating the cells with calcium upregulates the CaR. Therefore, the role of the receptor in colon cancer seems to be as a tumor suppressor in colon adenocarcinoma, and loss of CaR expression or polymorphisms in the ICD of the CaR correlate with a severe prognosis.

The CaR has been found to be functionally expressed in human astrocytes and astrocytoma cell lines (40, 43, 44). Ubiquitously expressed in the CNS, astrocytes are support cells for neurons, acting as spacers that organize the spatial distribution of the neurons. Changes in $Ca^{2+}{}_o$ in the CNS are observed when secretory granules are delivered into the synaptic cleft, during seizures, and as a result of cell death due to ischemia or cancer (300). In the astrocytoma cell lines, the CaR has been shown to regulate the cell cycle, peptide release, and two ion channels (43, 44, 308). In the human U373 astrocytoma cell line, stimulation of the CaR induced proliferation and activation of a nonselective cation channel (43), whereas in the human U-87 astrocytoma cell line, activation of the CaR led to the release of parathyroid hormone-related peptide (PTHrP) and activation of a midi-type outward potassium channel (44, 308); both cell lines are high-grade astrocytoma cell lines. Thus, the functions of the CaR observed in astrocytoma cells depend on the properties of individual clones of the malignancy, and this points to a complex etiology of the disease.

McNeil et al. found expression of a functional CaR in ovarian surface cell cancer (167). The authors showed that increasing extracellular calcium produced a marked proliferative response in normal ovarian surface epithelial cells and that the signaling pathways involved in the CaR-mediated response were the MAPK and src pathways (109). In support of this we found that the CaR also signals a proliferative response to calcium through MAPK in primary cultured rat Rice H-500 testicular Leydig cancer cells (255), and these data will be reviewed in the subsequent chapter.

REGULATION OF PTHrP RELEASE BY THE CALCIUM-SENSING-RECEPTOR IN MALIGNANT IN VITRO MODELS

PTHrP was discovered as a hypercalcemia-inducing systemic factor in humoral hypercalcemia of malignancy (HHM) as described earlier (89) and was later shown to be the humoral factor in approximately 80% of HHM cases. Other humoral factors causing HHM are $1,25(OH)_2$ vitamin D_3, tumor necrosis factor á, interleukin (IL) 1, and IL-6 (Figure 3). Many important functions of PTHrP other than its actions in normal physiology have since been described, e.g., it participates in cell-cycle events, such as differentiation, proliferation, and apoptosis. PTHrP is crucial for bone development and important in the regulation of smooth muscle cell contraction (248). In the breast and placenta, PTHrP participates in regulating local calcium homeostasis. These effects of PTHrP are reported to be systemic, paracrine, autocrine, and, recently, intracrine. The N-termini of the three isoforms of human PTHrP are structurally similar to that of PTH; therefore, PTHrP acts on the PTH receptor 1 (PTHR), the same receptor by which PTH exerts its effects as described earlier. Of note, PTH is presently, along with perhaps strontium ranelate, the only efficient anabolic drug in the treatment of osteoporosis. $Ca^{2+}{}_o$ has been found to regulate PTHrP release in keratinocytes (104), cervical epithelial cells (143), oral squamous cancer cells (168), JEG-3 cells (102), breast duct cells (278), astrocytoma cells (40), breast cancer cells (225), testis tumor cells (215), and prostate cancer cells (223). In astrocytoma (40), meningioma (40), breast cancer (225, 278), prostate cancer (223, 301), and testicular cancer (259), the CaR probably mediates the effect of $Ca^{2+}{}_o$ on PTHrP release. These studies will be the focus of the last part of this chapter, in addition to the discussion of astrocytoma and meningiomas reviewed above.

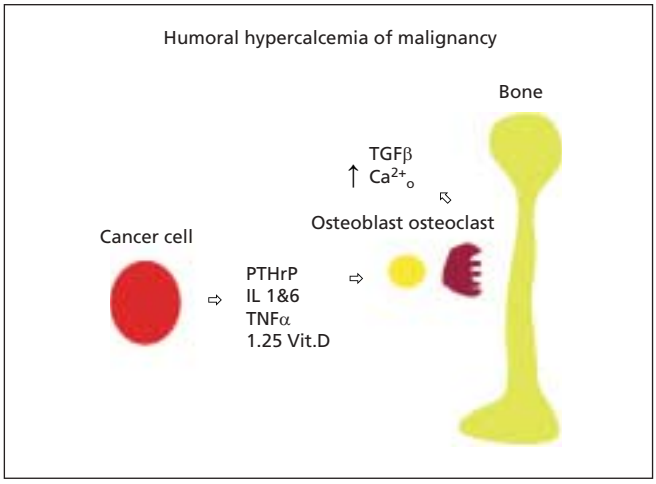

Figure 3. Schematic drawing of the pathophysiology of humoral hypercalcemia of malignancy (HHM). A tumor produces a systemic hypercalcemia-elevating hormone or cytokine that, in turn, activates bone degradation. The degradation of calcified bone matrix is the proximate cause of the hypercalcemia (253). Abbreviations: extracellular calcium ($Ca^{2+}{}_o$), interleukin (IL), parathyroid hormone-related peptide (PTHrP), Tumor necrosis factor alpha (TNFα), and 1.25 dihydroxy vitamin D (1.25 Vit.D).

CaR AND PTHrP IN PROSTATE CANCER

Prostate cancer is known to metastasize frequently to bone. PTHrP has been described as a central mediator of osteolysis and growth of bone metastases (90). Sanders et al. reported the expression of CaR mRNA and protein in two human prostate cancer cell lines, PC-3 and LnCaP (223). The PC-3 cells responded to stimulation with $Ca^{2+}{}_o$ and type I agonists with a dose-dependent response, approximately two-fold increase in PTHrP release. To further prove that the CaR is involved in the $Ca^{2+}{}_o$-evoked PTHrP release, they infected the PC-3 cells with the dominant-negative Arg185Gln CaR in a recombinant adeno-associated viral (rAAV) vector. The infected cells showed a blunted response to $Ca^{2+}{}_o$ as compared with that of cells infected with empty vector. This result strongly supports the claim that the CaR is the mediator of the $Ca^{2+}{}_o$-induced PTHrP release in PC-3 cells. In the extracellular bone matrix, there are embedded growth factors (90). One of these growth factors, transforming growth factor β (TGFβ), was found to act additively with $Ca^{2+}{}_o$ on the release of PTHrP by the PC-3 cells. This leads to a potentially destructive cycle, with $Ca^{2+}{}_o$ and TGFβ as stimulators of PTHrP release, owing to the release of these two substances from the resorbed bone. Of note, the concentration of $Ca^{2+}{}_o$ in the bone microenvironment in HHM can reach much higher levels than those of systemic

Ca^{2+}_o. In fact, the levels of Ca^{2+}_o in the immediate vicinity of resorbing osteoclasts can reach concentrations as high as 8 to 40 mM (239). We found in a later study that, in same PC-3 cell line, MAPKs are involved in the CaR-mediated PTHrP release (301). Serendipitously, we found that the CaR activates ERK1/2 through a triple membrane passing signaling pathway (TMPS) in the PC-3 cells. 7TMs use the epidermal growth factor receptor (EGFR) or other tyrosine kinase receptors as an intermediate signaling molecule in a process termed TMPS (290). A brief description of TMPS; the 7TMs activate intracellular signaling molecules, e.g., src or reactive oxygen species (274), which, in turn, activate a membrane-bound metalloprotease (200, 299) to cleave membrane-bound heparin-binding EGF (HB-EGF) or TGFα. The released HB-EGF then activates its receptor (the EGFR), which subsequently phosphorylates its own intracellular domain and activates downstream proteins such as MAPKs (155). ERK1/2 is maximally activated by the CaR at 30 minutes in PC-3 cells. These studies used NPS R-467 to show that the CaR activated the ERK1/2 pathway. Another non-trivial approach could have been the infection with a virus containing a dominant negative CaR. This method has been used in later studies on the phosphorylation of p38 MAPK (308). Inhibiting the Ca^{2+}-induced phosphorylation of ERK1/2 by inhibiting metalloproteinases (GM6001), neutralizing the extracellular domain of the EGF and the EGFR with antibodies, or inhibiting the EGFR with a specific inhibitor (AG1478) all showed that the CaR utilizes the EGFR as a downstream signaling molecule in the activation of ERK1/2. The platelet-derived growth factor receptor (PDGFR) inhibitor, AG1296, had no effect on Ca^{2+}_o-induced ERK1/2 phosphorylation, showing that the CaR specifically used the EGFR in the TMPS mechanism. The inhibitors of MMP and EGFR described above had the same effect on Ca^{2+}_o-induced PTHrP release; furthermore, the potency of the MEK1/2 inhibitor, PD98059, on PTHrP release induced by Ca^{2+}_o was the same as that of the EGFR inhibitor (AG1478). These results suggest that CaR-mediated PTHrP release occurs through the EGFR and ERK1/2 signalling pathways in PC-3 cells. Similar results showing that the CaR uses EGFR as a mediator in high Ca^{2+}_o-induced ERK1/2 phosphorylation were also observed in H-500 and HEK-CaR cells as well, suggesting that this may be a widely used pathway by the CaR (157, 264). The participation of the EGFR pathway in CaR signaling makes the EGFR a promising drug target for treatment of prostate cancer, since targeting the CaR itself could jeopardize calcium homeostasis. The PC-3 cells are a model of malignant hypercalcemia as described initially. Another choice could have been the breast cancer cell line MDA-MB-231. They have also been shown to induce malignant hypercalcemia due to bone metastases with a functionally active CaR, Calcium induces PTHrP release from these cells (225). The PC-3 cells were chosen since the evidence for CaR-mediated PTHrP release was stronger, as the calcium induced PTHrP release in PC-3 cells could be abolished by the dominant negative CaR described above, whereas involvement of the CaR in MDA-MB-231 cells was suggested by effects of type I agonist of the CaR. To my knowledge it was never investigated whether the level of CaR protein in prostate tumors is correlated to malignancy and or bone metastasis. This could be interesting because an increased expression in higher malignancy grades would be suggestive of CaR as an active player in the progression of the diseases.

CaR AND PTHrP IN BREAST CANCER

Breast tissue is active in calcium homeostasis during pregnancy and lactation and must be able to concentrate the calcium in milk; these observations have led to studies investigating a possible role for the CaR in breast tissue. Cheng et al. found CaR expression in normal, fibrocystic, and ductal carcinoma mammary epithelial cells (46), and Sanders et al. found the CaR in the human breast cancer cell lines MDA-MB231 and MCF-7 (225). Stimulation of MCF-7 cells with calcium and neomycin induced Ca^{2+}_i spikes, as measured by Fura-2 (187). Confocal microscopic studies showed that the CaR and calbindin-D-28k co-localized when the CaR was stimulated, suggesting that calbindin-D-28k, which binds calcium, is recruited to the CaR. Stimulating the MDA-MB231 cells with calcium and the type I CaR agonist neomycin induced release of PTHrP, with a maximal, two-fold increase (225). In normal breast duct epithelial cells, in contrast, stimulation of the CaR leads to decreased PTHrP release (278). Highly speculatively this could suggest that a switch in the function of the CaR takes place during the malignant transformation. Also, stimulation of the human MCF-7 breast cancer cell line with 100 μM of the type II agonist NPS R-568 or calcium decreased the estrogen receptor α protein, whereas both CaR ligands upregulated transcription of the receptor. The estrogen receptor a is thought to play an important role in the function and growth of breast cancer (126), as estrogen receptor positive breast tumors display a greater tendency to grow in the bone and this finding could enhance the progression of the disease. Of note in this study, the effect of the calcium ionophore A23187 was opposite to that of calcium, indicating that calcium did not act in a nonspecific manner as a result of its influx through calcium channels. Although the doses of NPS R-568 were very high in this study, these data indicate that the CaR, in addition to its effect on PTHrP release, modulates the important estrogen receptor a in breast cancer. Breast cancer and prostate cancer are known to metastasize to bone, and the metastases are often mixed osteolytic and osteosclerotic lesions (90). Tumors that stimulate new bone formation, through a complex stimulatory effect by growth factors and other agents on the osteoblast, are thought to result in osteosclerotic metastases. This new bone formation may indeed cause hypocalcemia although this is not common. Breast cancer cells metastatic to bone will be exposed to a locally high level of Ca^{2+}_o, which, through stimulation of the CaR, can, in turn, induce two promalignant features, PTHrP release and estrogen receptor α regulation. Thus, the stimulation of PTHrP release by the CaR constitutes a vicious cycle in bone metastases of the breast and prostate cancer cell lines. Growth factors in addition to PTHrP that can induce such vicious cycles are endothelin-1, vascular endothelial growth factor, IL-8, and IL-11 (49). Bone itself stimulates the tumor by releasing insulin-like growth factors and TGF-β as described above. Secreted factors transmit the interactions between tumor and bone and provide novel therapeutic targets to interrupt the vicious cycle of bone metastases. Investigation of the effects of the CaR on the effects and/or production of endothelin-1, vascular endothelial growth factor, IL-8, and IL-11 might further clarify the role of the CaR in these malignant cycles.

PTHrP AND CaR IN HUMORAL HYPERCALCEMIA OF MALIGNANCY

The Rice H-500 rat Leydig cells are a primary cell culture of a rat Leydig cell tumor and represent a xenotransplantable model of HHM used in four of the studies in this thesis. The H-500 model is not the only transplantable model of humoral hypercalcemia of malignancy; additional such tumors include: the PTHrP producing cancer cell lines LC-6 established from human large-cell lung cancer (67), the human lung SCC cell line (RWGT2) from explanted metastatic tumor tissue (91), the C-26 cells that were originally isolated from a chemically induced mouse colon adenocarcinoma (61), and the Walker carcinosarcoma (WCS) 256, a rat mammary carcinoma cell line also producing PTHrP (106). The H-500 cell model was chosen as a model of HHM to investigate possible roles of the CaR, rather than any of the other models mentioned above, since Rizzoli et al. found that challenging Rice H-500 rat Leydig tumor cells with Ca^{2+}_o stimulated the release of a PTH-like bioactivity (215). Two additional studies published at almost the same time showed that the CaR was present on these cells and that type 1 agonists of the CaR such as calcium stimulated PTHrP release (30, 224). In the study by Sanders et al. they found that the CaR was expressed at both the protein and mRNA levels and that stimulation of the H-500 cells with Ca^{2+}_o resulted in a dose-dependent increase in PTHrP release by

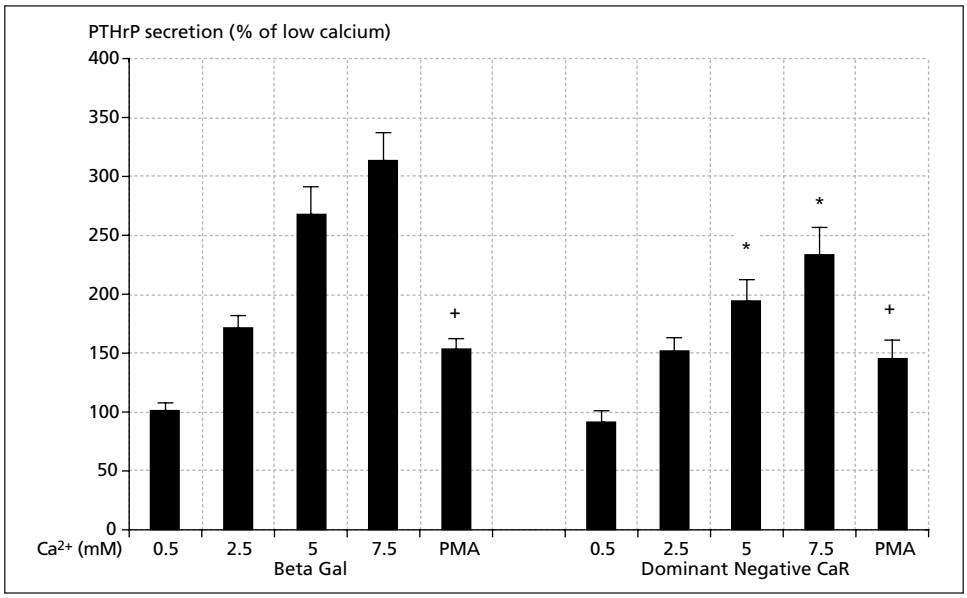

Figure 4. Effect of dominant-negative calcium-sensing receptor (CaR) on high Ca^{2+}_o-stimulated secretion of PTHrP. H-500 cells were infected with either the dominant-negative CaR or the vector expressing the β-galactosidase (BG) gene 48 h before stimulation. The cells were then starved for 2 h in serum-free medium, and the medium was collected after incubation of cells with 0.5, 2.5, 5, or 7.5 mM Ca^{2+}_o or 0.5 mM Ca^{2+}_o with 100 nM PMA in serum-free medium for 6 h. Results represent the percent increase compared with basal PTHrP release and are pooled data from four independent experiments. Ca^{2+}_o-mediated PTHrP release (7.5 mM) was 233±(SE) 24% of basal PTHrP release in cells infected with dominant-negative CaR, whereas it was 313±24% of basal release in cells infected with BG (*P <0.05 vs. BG, +P< 0.05 vs. 0.5 calcium) (259). Beta Gal: β galactosidase, CaR: calcium-sensing receptor, PMA: phorbol 12-myristate 13-acetate.

more than three-fold. The type I agonist neomycin had the same effect as Ca^{2+}_o on PTHrP release (224). Buchs et al. also showed that the presence of the ionophore ionomycin did not block the calcium-induced PTHrP release in H-500 cells (30). These results point toward the CaR functioning as the mediator of the calcium-induced PTHrP release. We later demonstrated that the response to Ca^{2+}_o of H-500 cells infected with a naturally occurring dominant-negative Arg185Gln CaR (11) construct in a rAAV vector (292) was shifted to the right and downward in comparison to the response of cells infected with β-galactosidase, a protein of the same size as the CaR (Figure 4) (259), thereby showing that the Ca^{2+}_o-induced PTHrP release is, at least in part, through the CaR. Therefore, stimulation of the CaR by Ca^{2+}_o in HHM leads to an increase in PTHrP release. The released PTHrP then increases systemic Ca^{2+}_o. This process constitutes a malignant, vicious cycle, and pharmacological blockade of the CaR, its downstream signaling apparatus, or the secreted PTHrP may represent a fruitful therapeutic approach. The post-receptor mechanism by which the CaR stimulates PTHrP release seems to be through a de novo synthesized transcript, as the pan polymerase inhibitor, actinomycin D, inhibits the effect of Ca^{2+}_o on the expression of PTHrP mRNA as well as on protein release. It was shown subsequently that the effect of the CaR on PTHrP release seems to be through PKC, MAPK, and PI3K, as the effects of 7.5 mM Ca^{2+} on PTHrP release were blocked by inhibiting PKC and the three MAPKs-MEK1/2 (upstream of ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK)-and by inhibiting protein kinase B (AKT) (255). The stimulation of the MAPKs by Ca^{2+}_o was independent of PKC, since the phosphorylation of ERK1/2, p38 MAPK, and stress-activated protein kinase ERK kinase 1 (SEK1) (the kinase upstream of JNK) by Ca^{2+}_o could not be blocked by the pan PKC inhibitor GF-109203X, despite the ability of the PKC-activator, phorbol 12-myristate 13-acetate, to phosphorylate ERK1/2 (Figure 5). This may be explained by the finding that H-500 cells loaded with the calcium-sensitive dye, fura 2, did not exhibit Ca^{2+}_i spikes in response to high Ca^{2+}_o, which suggests either that the CaR is expressed at too low a level on the cell membrane to produce Ca^{2+}_i spikes (30) or that the CaR mediates its effects on MAPK in a PLC-independent pathway in H-500 cells. The same phenomenon, that CaR activation does not lead to Ca^{2+}_i spikes, was also reported in intact gastric mucosal cells that expressed a functionally active CaR (33). Filamin-A and beta-arrestin binds to the CaR (107, 195) and have been shown to interact with MAPKs (161), providing a possible explanations for the PLC-independent activation of MAPKs downstream of the CaR. In support of this hypothesis other receptors also coupled to the Gαq like angiotensin II receptor type I have been shown to activate MAPKs independent of Gαq through the scaffolding protein β-arrestin (5). In H-500 cells, the phosphorylation of the ERK1/2 was delayed, and ERK1/2 activation reached a maximum at 30 to 60 minutes and was sustained up to 2 hours before declining (Figure 5). In dispersed parathyroid and HEK-CaR cells, the phosphorylation of ERK1/2 by high Ca^{2+}_o was maximal at 10 to 30 minutes (136). This difference in the kinetics of MAPK activation may be explained by the fact that prolonged activation of ERK1/2 by EGF, in contrast to brief activation by PDGF, leads to translocation of phosphorylated ERK1/2 to the nucleus and to proliferation in Swiss 3T3 fibroblasts (172). As described later in this thesis, stimulating the CaR in H-500 cells indeed enhanced proliferation and protected the cells from apoptosis through the p38 MAPK and PI3K pathways but, surprisingly, not through the ERK1/2 pathway (255). The overall message from the studies investigating the possible role of the CaR as a mediator of calcium-induced PTHrP release by prostate, breast, and testis cancer is that the CaR induces PTHrP release from the tumor cells and that the effect of the CaR appears to be most pronounced in the H-500 cells, as the fold increase in PTHrP was the highest, at least in the models tested. One could speculate that this is due to primary culture versus cell line as the H-500 cells represent the former and the PC-3 and MDA-MB-231 the latter. In the nonmalignant murine Leydig TM3 cell line, indication of a cell membrane bound calcium sensing mechanism has been found (4), but this study did not test whether the CaR was present. Furthermore, the presence of the CaR in human testis or testis cancer has to my knowledge not been investigated. This could be highly interesting as the CaR might have the same function in the Leydig cells as in the GH producing pituitary adenoma, that is modulating the regulatory hormone effect on the Leydig cell; this hypothesis is of course highly speculative.

INDUCTION OF NITRIC OXIDE SYNTHASE AND NITRIC OXIDE PRODUCTION BY CALCIUM-SENSING RECEPTOR IN A MODEL OF HUMORAL HYPERCALCEMIA OF MALIGNANCY

Nitric oxide (NO) is a small hydrophobic molecule with chemical properties that allow it to serve as both an intra- and extracellular messenger. In malignant tissues, the mutagenic effect of chronically high levels of NO is thought to play a role in carcinogenesis. NO also participates in the proliferation, apoptosis, and angiogenesis of cancer cells (146). NO is produced by three isoforms of the enzyme NO synthase (NOS). These are neuronal (n), inducible (i), and endothelial (e) NOS (also termed I, II, and III, respectively) (7). These enzymes converts L-arginine to the free radical NO and citrulline in the presence of several cofactors. iNOS is classically upregulated in inflammation by interferon-γ, tumor necrosis factor α, endotoxin,

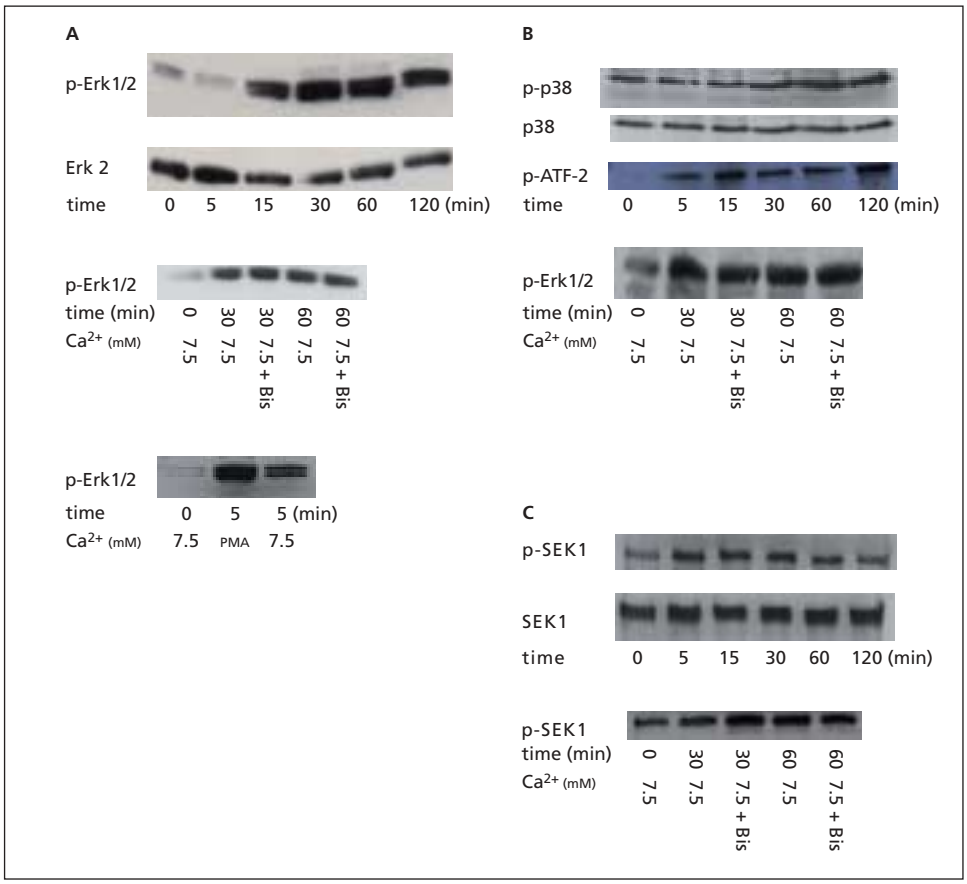

Figure 5. Time course of the phosphorylation of ERK1/2, p38 MAPK, SEK1, and ATF-2 by high Ca^{2+}_o. A) ERK1/2 is maximally phosphorylated at 30 to 60 min in response to high Ca^{2+}_o. PMA (100 nM) induces ERK1/2 phosphorylation at 5 min, but the PKC inhibitor GF109203x (BIS) has no effect on calcium-induced ERK1/2 phosphorylation. B) A similar time course is seen with stimulation of p38 MAPK phosphorylation by high Ca^{2+}_o, whereas ATF-2 (downstream of p38 MAPK) is maximally phosphorylated at 2 h. The PKC inhibitor GF109203x has no effect on high Ca^{2+}_o-induced p38 MAPK phosphorylation. C) Phosphorylation of SEK1 is already maximal at 5 min and persists for 60 min. GF109203x has no effect on high Ca^{2+}_o-induced SEK1 phosphorylation. The western blots are representative of three or more experiments (259). ATF: activating transcription factor, p38: p38 mitogen-activated protein kinase, SEK: stress-activated protein kinase activator.

and IL-1β (277) and is constitutively active as a homodimer. Of note in the context of calcium, iNOS activity is independent of intracellular calcium and calmodulin, distinguishing it from nNOS and eNOS. IL-1β was found to induce NO production in cultured rat Leydig cells expressing iNOS mRNA (252). In Leydig cells, NO functions as a negative regulator of testosterone production in vitro (63, 275). Furthermore, the role of NO as a negative modulator of steroidogenesis in Leydig cells was shown by the decrease in serum testosterone levels produced by agents promoting NO production and the increase in serum testosterone levels induced by the general NOS inhibitor, L-NAME (3). Immunohistochemistry of rat testis also showed the presence of iNOS in both normal and inflamed Leydig cells (179). These studies suggest that NO has an important role in the physiology and pathophysiology of the Leydig cell.

Since the CaR is functionally expressed on H-500 Leydig cancer cells, and because NO seems to be an important second messenger in Leydig cells, we hypothesized that the CaR would regulate NO production in H-500 cells. Using high-quality oligonucleotide microarray analysis, northern blotting, and real-time PCR, we showed that iNOS mRNA was upregulated by Ca^{2+}_o, with a maximum at 18 hours (256). In contrast, the mRNAs of eNOS and nNOS were not regulated by Ca^{2+}_o. Upregulation of iNOS mRNA was followed by upregulation of iNOS protein, as assessed by western blot analysis. To prove that the Ca^{2+}_o-induced iNOS upregulation was mediated through the CaR, we infected H-500 cells with the dominant-negative CaR (Arg185Gln) delivered in adeno-associated virus as described above; this substantially reduced the stimulation of iNOS mRNA by Ca^{2+}_o as compared with the stimulation of cells infected with virus containing a β-galactosidase construct. Validation of GAPDH as a "house keeping gene" was accomplished by investigating the expression of beta-actin in mRNA from H-500 cells stimulated with increasing calcium; no difference in expression was found between the two. The use of three "house keeping genes" or more is now often used as GAPDH and beta-actin are now known to be regulated by a plethora of stimuli. Using this methodology would have strengthened our work. To investigate whether CaR-induced iNOS upregulation also augments NO production, we used the NO-specific dye DAF, which is a diaminofluorescein (138). With this dye, NO production can be determined in real-time using fluorescence measurements. Figure 6a shows confocal micrographs of H-500 cells on coverslips treated overnight with low or high concentrations of calcium and subsequently loaded with 10 μM DAF-FM in a medium containing low levels of calcium. The cells treated overnight with high calcium had higher fluorescence intensity, with excitation at 488 nm and emission at 500-550 nm. To quantify this increase, we performed spectrofluorimetry under similar conditions. The cells treated with high calcium overnight had a 2.7 ± 0.7-fold higher fluorescence intensity as compared with cells treated with low calcium ($P<0.05$) (Figure 6b). To assess whether the upregulated NO had modulated the growth of the H-500 cells, we challenged the cells with competitive inhibitor of NOS, L-NAME, and a selective iNOS inhibitor, 1400W; neither of the compounds had any effect on Thymidine uptake or PTHrP release (data not shown in the paper). This suggests that the NO produced by iNOS in the H-500 cells by CaR stimulation is not involved in the CaR-mediated DNA synthesis. As the H-500 cells do not produce steroid hormones (212), the function of the NO produced by the CaR does not appear to be the regulation of steroid hormone production. Therefore, overall the function of NO upregulation by the CaR in the H-500 remains to be clarified. It could be speculated that the effect of NO may be in angiogenesis, as it has been demonstrated that NO promotes neovascularization in xenograft tumors, which increases invasiveness, metastatic ability and tumor growth (73, 146). A reasonable question about the design of this study is how we can be sure that elevated Ca^{2+}_o does not act on NO by raising intracellular calcium levels. Two factors argue against this. First, intracellular calcium is not a cofactor or known regulator of iNOS (unlike eNOS and nNOS). Second, intracellular calcium does not increase when H-500 cells are exposed to high extracellular calcium concentrations (259). In support of our observation that the CaR regulates iNOS in cancer cells, a later study showed that a calcilytic could downregulate the iNOS induced by calcium in astrocytomas cells (62). As re-

viewed earlier, the intracellular, downstream mediators of the CaR identified thus far include G-proteins, PLC, PKC, MAPKs, and transactivation of the EGF receptor (254, 301). The data presented here suggest that the CaR may also use NO as a second messenger. While the CaR is ubiquitously expressed in the body, its most important functions are the regulation of PTH release and the renal handling of calcium (254). Nitroprusside, an NO donor, has been reported to abolish PTH release and cAMP accumulation in bovine parathyroid cells in vitro (78, 79). PTH release was inhibited at all calcium concentrations, and a calcium chelator failed to prevent the inhibition. Therefore, we hypothesize that regulation of NO production by the CaR may not be limited to cancer cells such as H-500 cells. In support of this hypothesis, in a paper from the spring of 2006 Ziegelstein et al. found that in human endothelial cells expressing the CaR stimulation of the receptor induced increased NO production, and this could be blocked by siRNA of the CaR. They also measured NO by the DAF-FM method (316). Although the NO producing enzyme in these cells most likely is eNOS, although this was not investigated.

THE ROLE OF THE CALCIUM-SENSING RECEPTOR IN CELL PROLIFERATION AND APOPTOSIS

Cancer accounts for most of the deaths in the western world, and understanding the regulation of tumor growth is crucial to the success of treatment of malignant diseases. An emerging body of literature suggests a role for the CaR in the regulation of the development of cancer. In HMM where a distant tumor is producing a humoral factor, in our model PTHrP, is inducing the hypercalcemia, one line of treatment is to reduce the size of the primary tumor; thus the understanding the regulation of the growth of the tumor is key. Stimulation of the CaR leads to growth arrest in colonic crypt cells, pancreatic carcinoma cells, and keratinocytes, whereas it induces proliferation in astrocytoma cells, osteoblasts, fibroblasts, myeloma, and ovarian surface cancer (17, 43, 130, 166, 167, 207, 293, 297). In 1998, Lin et al. reported that stimulation with $Ca^{2+}{}_o$ prevented the apoptosis of AT-3 prostate cancer cells induced by Sindbis virus (152). Shortly afterwards, Chattopadhyay et al. showed that the CaR induced cellular proliferation in U373 human astrocytoma cells (43). In support of this it was later reported that transfecting an activating mutation of the CaR, Thr151Met, into NIH/3T3 cells supported colony formation in soft agar, in contrast to the non-transfected NIH/3T3 cells (112). We therefore investigated whether stimulating the CaR in H-500 cells would affect cell-cycle regulation (255). We found that $Ca^{2+}{}_o$ stimulation for 24 hours induced the uptake of the [^{3}H]thymidine (a measure of DNA uptake/proliferation) in a dose-dependent manner. We also showed, by the use of the specific type II agonist, NPS R-467, and its less active stereoisomer, NPS S-467 (178), that the effects of $Ca^{2+}{}_o$ were mediated through the CaR. An approach using the virus containing the dominant negative CaR was not considered because the amount of virus need for these studies were very large as the DNA synthesis was done in 24 well plates. In order to state that this is proliferation, as written in paper III (255), the DNA synthesis assay should have been followed by actual counting of cell number, which we have done in later studies (240). One could also argue that the [^{3}H]thymidine may change the endogenous production of thymidine. This was not investigated. Keeping these problems in mind, the DNA synthesis assay is still considered valid to use and the [^{3}H]thymidine uptake is a strong indicator of cell cycle changes. To investigate the intracellular signaling pathway(s) involved, we challenged the H-500 cells with calcium with or without the presence of inhibitors of MEK1/2, p38 MAPK, and AKT. As described earlier, we found that, in contrast to CaR-mediated PTHrP release, p38 MAPK and AKT, but not ERK1/2, were involved in the CaR-mediated proliferation. Several reports have described similar findings: In myoblasts, insulin-induced proliferation occurred through the AKT and ERK1/2 pathways but not through the p38

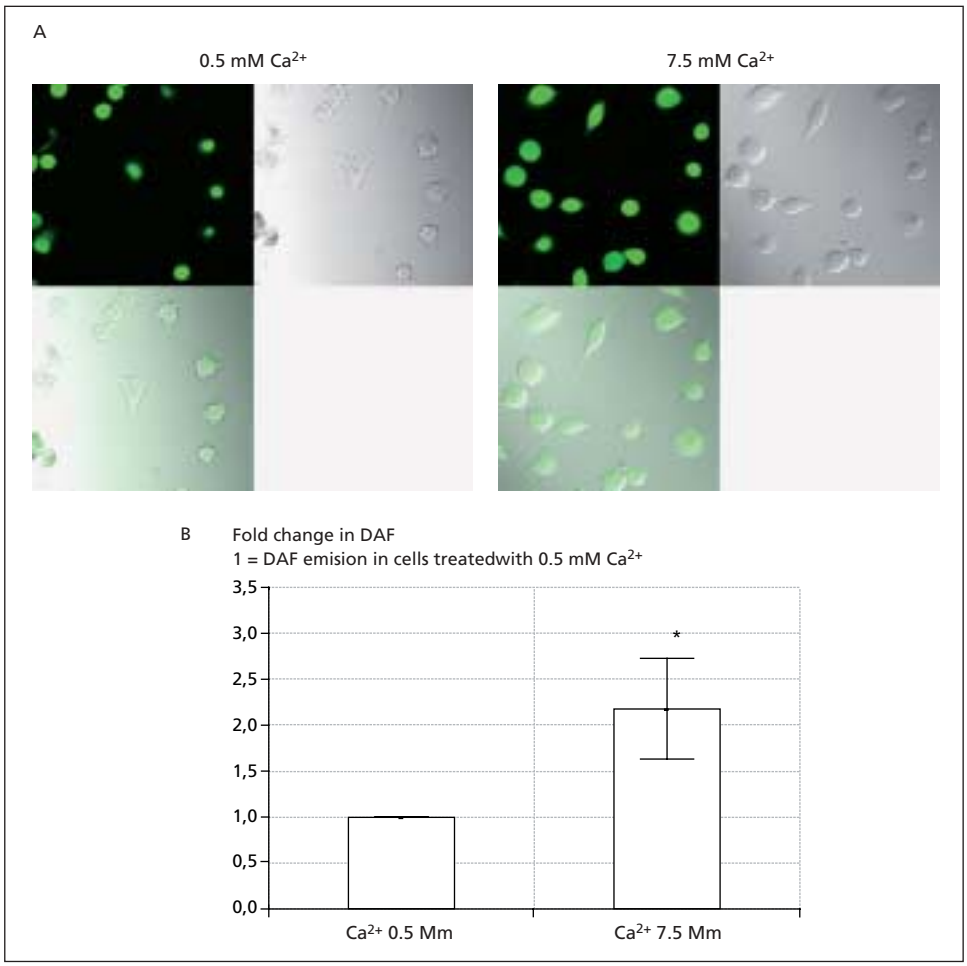

Figure 6. Calcium stimulates NO production in H-500 cells. Cells were plated on coverslips; after 72 h, at 80-90% confluency, the coverslips were treated with 0.5 mM or 7.5 mM calcium for 18 h and washed and loaded with the DAF-FM dye. A) NO production is upregulated in H-500 cells on coverslips treated with 7.5 mM calcium. Green color signifies that the DAF-FM dye is bound to NO. Upper left panel, fluorescence photomicrograph of the cells; right upper panel, light photomicrograph; left lower panel, overlay of the two fluorescent and light photomicrographs. Representative confocal micrographs from four independent experiments. B) Spectrofluorimetric measurements of DAF-FM-loaded H-500 cells on coverslips treated overnight with 0.5 mM or 7.5 mM calcium. The cells were excited at 490 nm, and emission was measured at 520 nm. Pooled data from five independent experiments ($P<0.05$) (256). DAF-FM: 4,5-diaminofluorescein diacetate-FM.

MAPK pathway, whereas anti-apoptotic effects of insulin occurred only through the PI3K/AKT pathway (57). A study investigating the anti-apoptotic effects of EGF and TGFα in hepatocytes found that the prosurvival effect of EGF was mediated through the AKT and ERK1/2 pathways but not through the p38 MAPK pathway. In contrast, the prosurvival effect of EGF was through the ERK1/2 and p38 MAPK pathways, but not through the AKT pathway (216). Perhaps activation of these pathways leads to different outcomes depending on the cell type studied and the stimuli to which that cell is exposed, because of different spatial and temporal patterns of activation. The temporal importance of ERK1/2 activation has been shown in a paper by Murphy et al. that nicely showed that stimulation of EGFR induced a prolonged activation of ERK1/2 and induced proliferation, whereas stimulation of PDGF activated ERK1/2 for a much shorter time and did not produce any proliferatory response (172). The factors that may account for the observed differences are the level of expression of the receptor, its scaffolding proteins and signaling partners, as well as compartmentalization of the involved proteins, but the exact mechanism is currently poorly understood.

Because our previous results showed that the CaR-mediated high Ca^{2+}_o-induced PTHrP release, we speculated that the CaR-mediated stimulation of proliferation was through an autocrine mechanism involving PTHrP, similar to the mechanism we recently described for EGF in CaR-mediated PTHrP release in PC-3 prostate cancer cells (301). Using the PTH peptide (7-34), known as an antagonist of the PTHR1 (173), we found that blocking the PTHR1 had no effect on Ca^{2+}_o-induced proliferation. Thus, CaR-mediated proliferation was not through an autocrine effect of PTHrP. Autocrine effects of PTHrP have been shown to act through a nuclear localization signal, and Sepulveda et al. showed that PTHrP stimulated proliferation of PC-3 prostate cancer cells in an intracrine manner (269). The same group reported that PTHrP, acting in an intracrine fashion, also protected MCF-7 breast cancer cells from apoptosis induced by serum starvation (270). The intracrine action of PTHrP therefore might serve as a mediator of the actions of high calcium on the proliferation (and potentially apoptosis) of H-500 cells. We have data in favor of this notion, but they were not considered sound enough to be included in our paper. The hypothesis was investigated by using two guanosine nucleotides analogs reported as inhibitors of the PTHrP promoter (75). Both 6-thioguanine and 6-thioguanosine abolished the effects of calcium on DNA synthesis (data not shown), but they were omitted from the paper as a reviewer raised valid questions about the specificity of the drugs. Another approach to investigate the possibility of an intracrine effect of PTHrP on calcium-induced DNA synthesis would have been to apply the stable transfection of a PTHrP plasmid with mutated nuclear localization signal (270), but the H-500 cells have in our hands been impossible to stably transfect, whereas others have reported success in performing this method (205). This latter study found that PTHrP takes part in the growth of the tumor using an elegant approach, where antisense PTHrP-transfected H-500 cells displayed reduced cell growth and tumor volume and almost maintained eucalcemia, favouring our hypothesis that PTHrP acts pro-proliferatory through an intracrine signalling mechanism.

As described above, inhibition of the PI3K pathway negatively affected CaR-induced DNA synthesis; thus we investigated by western blotting whether the classical survival pathway the AKT pathway was activated by stimulation with high Ca^{2+}_o in H-500 cells. Indeed, stimulation of the H-500 cells with Ca^{2+}_o induced phosphorylation of AKT from 15 to 60 minutes, with a maximum at 30 minutes. Therefore, we investigated the possibility that elevated Ca^{2+}_o had, in addition to a pro-proliferative effect, an anti-apoptotic/"pro-survival" action. We used TUNEL staining to measure apoptotic cells in the paradigm of apoptosis induced by serum withdrawal. Figure 7a shows two representative photographs of TUNEL-stained H-500 cells at low or high concentrations of Ca^{2+}_o. Quantification by two investigators blinded to the treatments showed that 52.2 ± 8.6% of

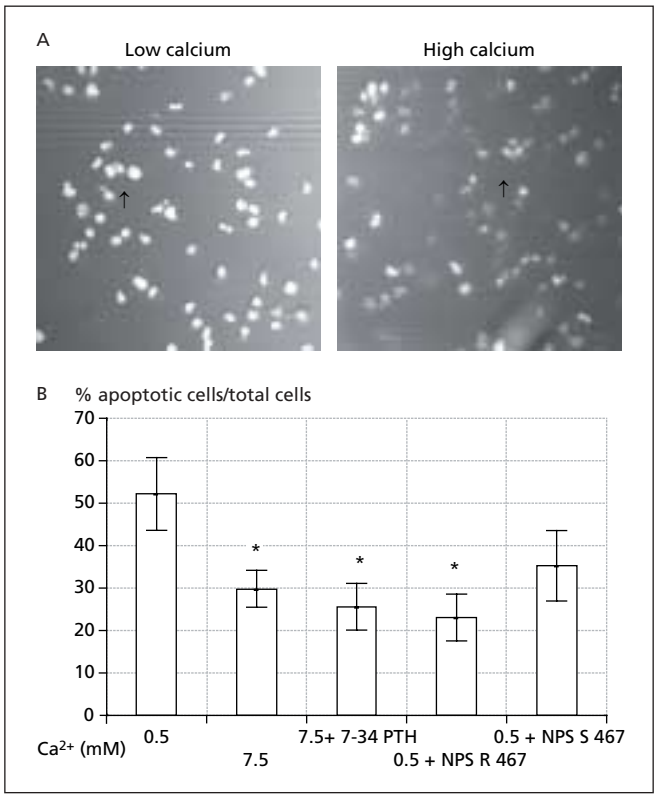

Figure 7. Calcium, acting via the CaR, protects H-500 cells from apoptosis induced by serum deprivation. (A) Representative fluorescent photomicrographs of TUNEL-stained H-500 cells on coverslips that had been incubated with 0.5 or 7.5 mM calcium. Arrow indicates TUNEL-positive cells. (B) TUNEL-positive cells quantified as the percent of the total number of cells. 3 mM NPS R-467 in the presence of 0.5 mM Ca^{2+}_o had the same effect on protection against apoptosis induced by serum starvation as 7.5 mM Ca^{2+}_o, while 3 mM NPS S-467 in the presence of 0.5 mM calcium was not significantly different from 0.5 mM calcium alone. 0.1 mM PTH peptide 7-34 in the presence of 7.5 mM Ca^{2+}_o had no effect on high calcium-induced protection against apoptosis. The results are pooled data from three independent experiments with two data points from each experiment (* P<0.05 compared with low calcium) (255). PTH: parathyroid hormone.

the H-500 cells were TUNEL-positive (apoptotic) at 0.5 mM calcium, whereas 29.8 ± 4.4% of the cells were TUNEL-positive at 7.5 mM calcium (*P<0.05*) (Figure 7b). This confirmed our hypothesis that high Ca^{2+}_o protects cells against apoptosis. Therefore, the increase in DNA synthesis might, in part, be the result of the presence of a greater number of apoptotic cells at low calcium. The addition of the PTH receptor antagonist PTH (7-34) (0.1 μM) to cells grown with high calcium did not reverse the protective effect of high Ca^{2+}_o against apoptosis induced by serum withdrawal (25.6 ± 5.5% TUNEL-positive cells). Therefore, we next studied whether the effect of high Ca^{2+}_o on cell survival was mediated by CaR. H-500 cells under serum-free conditions had 23.3 ± 5.5% apoptotic cells in the presence of 3 μM NPS R-467 plus 0.5 mM Ca^{2+}_o as compared with 52.2 8.6% apoptotic cells at 0.5 mM Ca^{2+}_o alone (*P <0.05*). On the other hand, cells treated with the less potent stereoisomer, NPS S-467 (3 μM plus 0.5 mM Ca^{2+}_o), showed a lower protective action (35.3 ± 8.3% TUNEL positive cells), and the number of apoptotic cells observed was not statistically significantly different from the number observed at 0.5 mM Ca^{2+}_o alone. The TUNEL assay measures DNA fragmentation, a hallmark of apoptosis, but in rare cases of apoptosis this is not seen although this was not the case in our studies. The TUNEL assay could have been followed by the DNA fragmentation method using e.g. 2% agarose gel. A third method to support the DNA fragmentation assays that is widely used is western blotting or ELISA as a means of assessing the activation of caspase proteases. The caspase family of cysteine proteases plays a key role in apoptosis (265). Caspase-3 is a key protease that is activated during

the early stages of apoptosis and is synthesized as an inactive proenzyme that is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. The addition of measuring caspase activation in our study would have strengthened the study. Several studies have examined the effects of calcium with or without calcimimetics on the proliferation/DNA synthesis of nonmalignant and malignant cells. In a study of parathyroid chief cells, NPS R-467 inhibited cellular proliferation, whereas calcium stimulated proliferation (220). The authors suggested that the discrepancies may arise from differences in the level of CaR expression. Thus, stimulation of the CaR in a parathyroid gland cell with high CaR expression inhibits proliferation whereas the same stimulation in a cell expressing a low level of the CaR induces proliferation. Alternatively, it may be explained by biphasic growth responses seen with the activation of many 7TMs. Available reports have shown that the calcimimetics NPS R-467 and NPS R-568 induced proliferation of fibroblasts and myeloma cells, although the authors did not use the inactive isomers NPS S-568 or NPS S-467 as a negative control (158, 297). These findings are compatible with our results in rat H-500 cells. As reviewed earlier for pancreatic adenoma cells and colonic adenocarcinoma cells, high calcium decreased proliferation. Thus the cellular response to high calcium mediated by the CaR depends on the cell type as well as whether it is benign or malignant. Our data on DNA synthesis and apoptosis favor CaR as a promalignant stimulator of growth in H-500 cells that induces growth by prevention from programmed cell death and increases the rate of proliferation after 24 hours of stimulation.

REGULATION OF PITUTARY TUMOR TRANSFORMING GENE IN TWO MALIGNANT MODELS IN VITRO

A hallmark of cancer cells is the transformation of normal cells that divide in a controlled manner with respect to their surrounding cells to malignant cells whose growth and invasion of surrounding tissue are unregulated. Oncogenes are intracellular molecules that are thought to be necessary for the transformation of a normal cell to a malignant cell. Pituitary tumor transforming gene (PTTG) is considered such an oncogene (311).

PTTG IN TESTIS LEYDIG CANCER

The oncogenic roles of PTTG, along with its abundance in the testis, as well as the upregulation of PTHrP release and DNA synthesis by the CaR in our HHM model, led us to hypothesize that the CaR could play a role in the regulation of PTTG in H-500 cells. Our investigation using northern blot analysis revealed that PTTG was expressed in H-500 cells and that stimulation by Ca^{2+}_o induced an upregulation in the level of the PTTG (261). Real-time PCR with specific primers for PTTG showed that Ca^{2+}_o upregulated PTTG messenger RNA in a dose-dependent manner and reached a peak at 18 hours. H-500 cells infected with the dominant-negative CaR, Arg185Gln, exhibited a blunted response to Ca^{2+}_o as compared with H-500 cells infected with β-galactosidase. This suggests that the calcium-induced upregulation of PTTG was mediated through the CaR. To assess the specificity of the CaR for inducing PTTG mRNA, we used ADP, the ligand for another group of 7TMs, the purinergic receptors, some of which, like the CaR, are coupled to $G\alpha_{q11}$. In a previous study, we showed that treatment of H-500 cells with ADP produced a rapid and transient increase in Ca^{2+}_i (259), thus confirming that H-500 cells express a functional ADP-sensitive receptor linked to the PI-PLC system that elevates Ca^{2+}_i in an agonist-dependent manner. Because ADP is degraded by ectonucleotidases within 15 to 30 minutes, we used instead ADPbS, a non-degradable agonist of the ADP receptor. We next examined whether the effect of activation of the purinergic receptor, like activation of the CaR, would increase PTTG expression. Whereas, high Ca^{2+}_o significantly increased the expression of PTTG mRNA (6.06 ± 2.18-fold increase), the cells treated with ADPbS (10^{-6} M) did not show any change in their expression of PTTG mRNA (0.84 ± 0.06). Upregulation of PTTG has been shown to induce proliferation and angiogenesis (121, 191). We therefore investigated whether the high-Ca^{2+}_o-induced CaR-mediated action on PTTG expression also regulated the expression of the potent angiogenic factor, vascular endothelial growth factor (VEGF). We found, using real-time PCR, that high Ca^{2+}_o indeed upregulated the mRNA of the VEGF gene in H-500 cells stimulated for 18 hours as compared with low calcium. Prior to this study, the regulation of PTTG expression was known to be 1) inhibited by cyclosporine A and hydrocortisone in T lymphocytes and 2) upregulated by estrogen and bFGF and downregulated by a peroxisome proliferator-activated receptor γ agonist rosiglitazone in pituitary adenomas (98, 99, 247). Our study showed for the first time that a $G\alpha_{q11}$-coupled 7TM, the CaR, upregulates PTTG expression in testis-derived rat Leydig cancer cells.

PTTG AND ASTROCYTOMA CELLS

The finding the PTTG was upregulated by the CaR in the H-500 cells led us to hypothesize that CaR may be upregulating PTTG in most malignant, CaR-expressing cells. In order to test this hypothesis, we used the U-87 cells. The U-87 astrocytoma cell line was chosen to investigate the effects of CaR stimulation on the PTTG oncogene because of its robust response to calcium in PTHrP release and ion channel regulation (40, 44).

PTTG expression has been positively correlated with several malignancies, including breast, prostate, and thyroid cancer (18, 92). In 2003, PTTG expression was reported to be present in developing neurons and was potentially implicated as an important cell-cycle regulator in human neurogenesis (19). These reports led us to primarily investigate whether PTTG was expressed in astrocytomas, the most common primary tumor of the brain. Astrocytomas, particularly high-grade astrocytoma or glioma, carry a very poor prognosis. Using PCR, we showed that PTTG and its binding protein were expressed in human primary astrocytes, astrocytoma, and glioblastomas, suggesting that astrocyte-derived cells possess the complete functional apparatus for PTTG (263). A comparison of the expression levels showed a trend towards greater expression of PTTG in the malignant samples than in the astrocytes. Similar results was obtained by a German group a year later in a larger sample size. They used mRNA from normal brain as controls and found very low expression of the PTTG mRNA (36). PTTG has been implicated in angiogenesis and proliferation; thus secondarily we studied the proliferative role of PTTG in astrocytomas by designing a small interfering RNA against PTTG mRNA and investigating its effect on bromodeoxyuridine incorporation, a measure of DNA synthesis and proliferation. Silencing PTTG mRNA via the small interfering RNA methodology inhibited serum-induced incorporation of bromodeoxyuridine by approximately 50% in the U-87 astrocytoma cell line as compared with the incorporation by U-87 cells treated with a scrambled oligonucleotide (Figure 8). The incorporation of bromodeoxyuridine was used to measure DNA synthesis and not [^{3}H]thymidine incorporation as used in paper III, because the bromodeoxyuridine incorporation assay requires fewer cells and hence less small interfering mRNA. As in paper III it would have added value to the paper if I had counted the cells as mentioned earlier. Furthermore, making a stable U-87 cell line with a PTTG loss-of-function mutation and implanting them back in to an animal would also have strengthened our hypothesis that PTTG is important in the growth of the tumor. In addition, the use of astrocyte cells from PTTG knock out animals (285) would also be a possible future path to investigating the role of the PTTG in astrocyte cells. Keeping this in mind, we still concluded that PTTG plays a role in regulating the growth of astrocytoma cells. To test our primary hypothesis that the CaR could regulate PTTG expression and to characterize the regulation of PTTG in astrocytomas, we stimulated the U-87 cells with agents known to be important in either the regulation of the astrocytoma cell cycle or of PTTG in other tissues using doses known to be effective based on the literature as well as data from our labora-

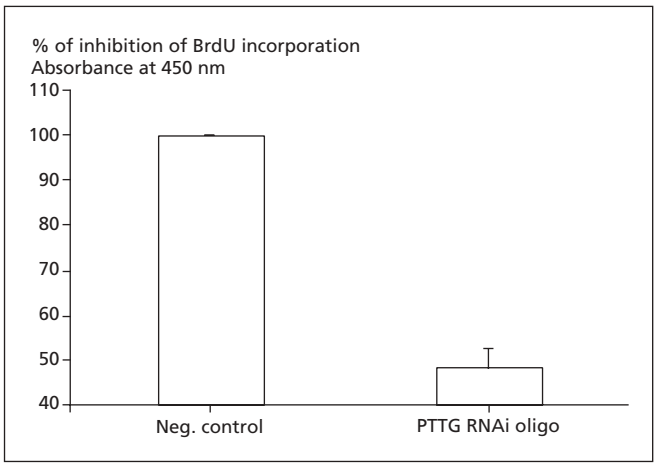

Figure 8. Silencing PTTG mRNA by siRNA inhibits U-87 proliferation. Cells were plated at 60–70% confluence in 96-well plates (six wells each for negative control oligonucleotide and PTTG siRNA). Twenty four hours after plating, cells were transfected with the two oligonucleotides (CaR-specific and scrambled). Forty-eight hours after transfection, cells were pulsed with bromodeoxyuridine, and ELISA was performed to estimate cell proliferation. Data are pooled from three independent experiments ($P < 0.05$) (263). PTTG: pituitary tumor transforming gene.

tory and pilot studies (when no known dose was considered effective). In these cells, in contrast to the H-500 cells, Ca^{2+}_o did not upregulate PTTG expression. Neither estradiol nor ciglitazone, a peroxisome proliferator-activated receptor γ agonist, both of which regulate PTTG expression in pituitary adenoma cells, regulated PTTG mRNA expression. The primary astrocytes as well as in the human U-87 cells and other astrocytoma cell lines have previously been shown to express this receptor and to be responsive to peroxisome proliferator-activated receptor γ agonist (41, 171). Three different peroxisome proliferator-activated receptor γ agonists in suprapharmacologic doses were used in the pilot studies, and none affected PTTG mRNA expression. Estrogen has been found to increase the life of nude rats implanted with U-87 cells, suggesting the existence of an estrogen receptor in the tumor (196). Stimulating the U-87 cells with the two EGFR ligands, EGF and TGFα, upregulated PTTG at both the mRNA and protein levels. EGF and TGFα are known promalignant growth factors in astrocytomas. The effects of the two ligands were through the EGFR, as the EGFR inhibitor, AG1478, abolished the effect of EGF and TGFα on the PTTG transcript. Another promalignant growth factor, hepatocyte growth factor, upregulated PTTG in the U-87 cells. The fact that EGF, TGFα, and hepatocyte growth factor all induced PTTG upregulation but that recognized regulators of astrocytoma growth and PTTG expression, including calcium, had no such effect, points to the cell-selective regulation of the PTTG oncogene. Following the same line of argument, the lack of effect of calcium on PTTG regulation could also be explained by the fact that stimulation of the CaR in U-87 cells did not change their proliferative rate when they were challenged with calcium (44). This observation led us to speculate that PTTG is only regulated by compounds that also affect the cell cycle. This hypothesis is supported by data showing the IGF, a proliferatory cytokine in U-87 cells and U-87 tumor cells in vivo (234), also upregulated PTTG in U-87 cells (36).

THE ROLE OF THE CALCIUM-SENSING RECEPTOR AND SIGNALING PATHWAYS IN TWO IN VITRO MODELS OF MALIGNANT HYPERCALCEMIA. EXPRESSION AND REGULATION OF PITUITARY TUMOR TRANSFORMING GENE IN LEYDIG CELL TESTICULAR CANCER AND ASTROCYTOMA CELLS

Hypercalcemia is frequent in patients with malignant disease. More than half of patients with hypercalcemia in a hospital setting have an underlying malignant disease. In Denmark the annual incidence of malignant hypercalcemia is approximately 150 per million inhabitants (253). Survival for more than 6 month after malignant hypercalcemia is diagnosed is unusual (208). Malignant hypercalcemia is most often caused by osteolytic metastases, but less frequently by osteosclerotic metastases and rarely humoral hypercalcemia of malignancy. Humoral hypercalcemia of malignancy (HHM) is often defined by a tumor producing hypercalcemia through a systemic hormone or cytokine (245). In approximately 80% of HHM patients, parathyroid hormone-related peptide (PTHrP) is the causative factor. The complication of hypercalcemia carries a very poor prognosis, and there is only a 50% survival three months after diagnosis (208). The disease has a major impact on bone, and after 3 month of duration 50% of the trabecular bone is lost (246), indicating that there is an uncoupling of the osteoclastic bone degradation and the osteoblastic bone formation. Of note, this uncoupling is not seen in primary hyperparathyroidism, so it remains a conundrum as to whether the hypercalcemia-inducing factors PTH and PTHrP act through the same PTHR1. The pathophysiology of malignant hypercalcemia is often complex and to some extent unknown; it may be caused by humoral factors secreted by the tumor locally or systemically that activate the osteoblast, which, in turn, activates the osteoclast through the RANK/RANK-ligand system to degrade the extracellular bone matrix. TGFβ and other growth factors, such as insulin-like growth factors 1 and 2, are embedded in the bone matrix (96); TGFβ has been shown to activate the tumor to produce more hypercalcemia-inducing cytokines, e.g., PTHrP (49). In case of bony metastasizes, the seed and soil theory by Paget (184) predicts that the tumor cells have to have features that allow them to be seeded and that the tumor cells will then produce factors that induce bone degradation. Secondarily, the bone should have a milieu or soil that is fertile for the tumor to metastasize to bone. The embedded growth factors in the bone matrix represent factors that will favor the growth of the tumor or provide "a fertile soil". In HHM there is by definition no seeding to the bone, but the growth factors produced by the tumor induce the osteoblast's activation of the osteoclastic bone-degradation, and the outcome is to some extent the same (Figure 3). The primary event is the production of factors by the malignant transformed tumor cells that will activate the osteoblast. Secondarily, degradation of the bone will release embedded growth factors and cytokines that in turn will stimulate growth and possibly the release of the bone-degradation-inducing factor by the tumor cells, which in our studies was PTHrP. Most divide malignant hypercalcemia into the two groups suggested by Stewart et al. (245), but it is probably more correct to acknowledge that the pathophysiology of most cases of malignant hypercalcemia is a continuum between the classically defined HHM, where the humoral factor is solely inducing the hypercalcemia, and malignant hypercalcemia that is induced by bony metastases with no systemic humoral impact (54).

Although the CaR was discovered primarily as a regulator of the primary calcium-regulating hormone, PTH, as well as of calcitonin and renal calcium handling, soon after it was cloned, the receptor was found to be expressed in non-calcium homeostatic tissues, such as fibroblasts, brain, and heart. Furthermore the CaR has now been recognized to be widely expressed in malignant tissues. The overall role of the CaR in cancer is diverse and depends on the particular cell type being studied. In colon cancer, for example, the loss of CaR expression carries a poor prognosis; for this reason stimulation of the CaR through a high dietary intake of calcium has been proposed as a chemopreventive measure. In cancer types such as Leydig, prostate, breast, and ovarian cancers, stimulation of the CaR leads overall to expression of more malignant features of the tumor cells. An interesting observation is that stimulating the CaR in normal breast cells induced an inhibition of PTHrP, whereas the opposite was true in breast cancer cells.

DOWNSTREAM SIGNALING APPARATUS, DECIDING THE FATE OF THE CaR STIMULI?

The CaR, like all other cell surface receptors, communicates messages from the surface of the cell to inside the cell. Signals modulating the CaR's activity are generated mainly through changes in systemic or local calcium homeostasis, and changes in $Ca^{2+}{}_o$ will, through the CaR, generate activity in intracellular signaling pathways downstream of the CaR. In our model of HHM, I found that stimulating H-500 cells with high Ca^{2+} activated several major intracellular signaling pathways: the PKC, AKT/PI3K, and MAPK pathways (Figure 9). These three signaling pathways may be seen as information highways inside the cell, although the full impact of the pathways remains to be understood. Kifor et al. first recognized that the CaR activated the MAPK pathway in parathyroid gland cells and HEK-CaR (136). Subsequently, MacLeod et al. described the requirement for MAPK in CaR-mediated PTHrP release in HEK-CaR cells (156). In accordance with these results, I showed that the ERK1/2, p38 MAPK, and SEK1/JNK pathways were required for CaR-mediated PTHrP release in H-500 cells, which naturally express the CaR although at a lower level than the parathyroid cells. Our results on the involvement of the ERK1/2 pathway in CaR signaling is particularly interesting because of the involvement of the ERK1/2 pathway in CaR-mediated PTHrP release but not cell proliferation, whereas p38 MAPK and AKT/PI3K were involved in both CaR-mediated proliferation and PTHrP release. Another intriguing observation regarding the CaR signaling pathways is that PKC seems to work in parallel to MAPKs, but the latter were not distal to PKC, since inhibition of PKC did not change calcium-stimulated ERK1/2, p38 MAPK, or SEK1 activation by high calcium. A valid concern is that the western blotting showing the activation of the MAPK and AKT pathways was carried out after challenging the cells with calcium. This, of course, alone does not prove that the CaR mediated these calcium-induced effects. It would have strengthened the argument if the western blots were performed by challenging the cells with a type II CaR agonist, or if the cells had been infected with DN CaR as in the studies on PTHrP release. But together with the data showing that the CaR-induced PTHrP release can be inhibited by selective inhibitors of the signaling pathways, these data in the conjunction with the literature mentioned above are highly suggestive of that the CaR is utilizing these pathways. Therefore, it is valid to state that the CaR signals through various kinase pathways that lead to different outcomes in our model of HHM. Such differences may be useful in a clinical setting, for example, if one cellular function regulated through a specific pathway is desired whereas another function mediated through a distinct CaR pathway is unwanted. Modulation of the latter could be a pharmaceutical target. One must also keep in mind that the outcome of a signal through a specific intracellular pathway activated by the CaR may depend on the type of cell as well as on whether it is normal or neoplastic. In future studies, the in vitro characterization of patient tumor cells may provide information that will allow the oncologist to design the most successful treatment regime.

The H-500 cells seems to be less dependent of embedded growth factors in the bone to sustain a high level of PTHrP release and unlimited growth as the cells kept their cancer phenotype for at least 10 cell passages in vitro. Therefore, the primary event in this model of HHM could be speculated to be the malignant transformation of the Leydig cells enabling the cells to grow unrestrained and produce PTHrP, and the factors released simultaneously with bone degradation and calcium release may play a lesser secondary role that seems to augment the pace of the development of the disease. PTHrP production seems to be a key factor for the H-500 cells, as H-500 tumor cells treated with antisense to PTHrP showed less growth and did not induce hypercalcemia in vivo (205). The use of a PTHrP neutralizing antibody or a PTHrP promoter approach in theory seems to be a very promising approach to treat rats with implanted H-500 cells. To my knowledge none of these studies have been performed.

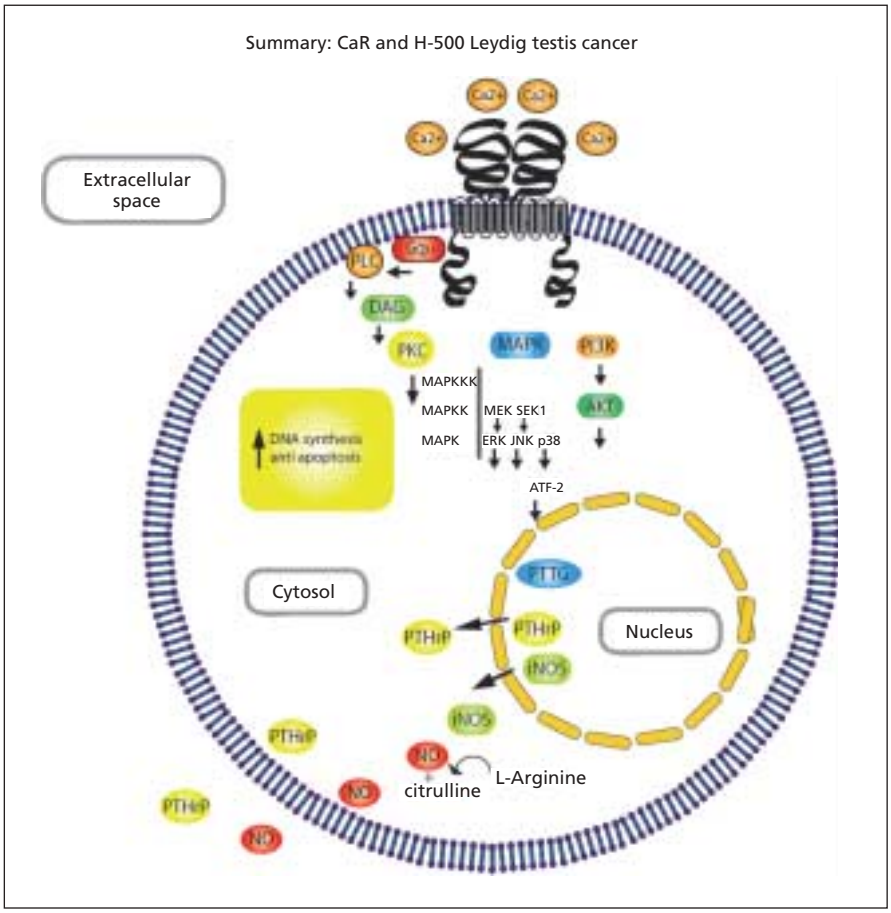

Figure 9. Schematic drawing of our results in the H-500 cells. CaR stimulation induces promalignant features Stimulating the calcium-sensing receptor with extracellular calcium activates the intracellular signaling pathways PKC, MAPK and AKT/PI3K pathways in the H-500 cells. CaR stimulation also leads to upregulation of the oncogene PTTG and the nitric oxide-producing enzyme, inducible nitric oxide synthase (iNOS). CaR activation likewise leads to upregulation of the malignancy-associated hypercalcemic factor, PTHrP. Tumor growth is enhanced, as CaR stimulation will induce proliferation and protect against apoptosis. All features are promalignant and would be expected to worsen the disease. Abbreviations: protein kinase B (AKT), activating transcription factor-2 (ATF-2), calcium-sensing receptor (CaR), extracellular regulated kinase (ERK), alpha subunit of Gq subtype of the heterotrimeric G proteins (Gq/11), inducible nitric oxide synthase (iNOS), Jun amino terminal kinase (JNK), mitogen-activated protein kinase (MAPK), MAPK kinase (MEK), nitric oxide (NO), p38 MAPK (p38), phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), parathyroid hormone-related peptide (PTHrP), pituitary tumor transforming gene (PTTG), and stress-activated protein kinase ERK kinase 1 (SEK1).

The observation that the CaR upregulates the iNOS enzyme opens the door to an entirely new understanding of the CaR in normal physiology both in tissues with high levels of expression of the receptor, such as parathyroid gland and kidney, and in cell types with low expression of the CaR, such as cardiomyocytes and fibroblasts. Two reports have suggested that the regulation of the myogenic tone maybe dependent of a CaR expressed on the endothelial cells (181, 289). And this effect on myogenic tone seems to be through the NO system, as indicated by one study that found that downregulation of the CaR by siRNA decreased NO production in response to calcium in human endothelial cells (316), strongly suggesting that the CaR mediated these effects on NO. These studies support our initial hypothesis that CaR utilizes the NOS/NO system as a downstream signaling molecule. It would be interesting to study whether the effects of the CaR on iNOS and VEGF in H-500 cells will also be present in those tissues with particularly high or low levels of expression of the CaR. Furthermore, studying the role of NOS/NO downstream of the CaR in the parathyroid gland is an important to clarify whether this signaling pathway is important as indicated by the studies by Gardner et al. (78, 79) .

In the literature one report indicates the existence of the CaR by immunohistochemistry in testicular cells (169). It could be of interest to study whether the CaR is functionally expressed in the normal testicular cells and in particular the Leydig cells. The CaR may be sensing calcium in local fashion as described by Hofer et al. (110), and the functions that I have described in this thesis for the CaR in cell cycle and NO regulation could be investigated as they may be of importance in normal Leydig cells.

REGULATION OF PTTG BY THE CaR IN CANCER

Since the cloning of PTTG in 1997 from pituitary tumor cells, our understanding of its structure, function, and clinical importance has made impressive progress (258). A great body of data has enabled us to understand the molecular and cell biological regulation of PTTG and its biological functions. Increasing evidence regarding the roles of PTTG in various cancers, including pituitary adenomas, is making PTTG a candidate marker of malignancy and a chemotherapeutic target (100, 222, 313). I found that the CaR upregulated the PTTG mRNA in the testicular H-500 cells, whereas this was not the case in astrocytoma U-87 cells; Moreover, the CaR stimulates growth in H-500 cells but not in U-87 cells. In U-87 cells I found TGF and HGF pathways to upregulate the PTTG, and others have found that IGF-1 is also contributing to the upregulation (36). These data indicate that the CaR uses PTTG in a proproliferative response. Hence further studies are needed to analyze whether the CaR utilizes the PTTG pathway to promote growth in the H-500 cells and other cancer cells with a functional CaR. If this hypothesis is valid, one could speculate that the PTTG mRNA or protein may be used as a marker of therapeutic effect of chemotherapy or even a drug target, especially when the CaR is stimulating growth.

CaR IN CANCER: THEORETICAL AND CLINICAL IMPLICATIONS

In this thesis I propose that the calcium released during malignant bone degradation is a possible second candidate, in addition to TGFβ, that may stimulate the tumor to increase its production of hypercalcemia-inducing agents-in our studies, PTHrP. Our findings in H-500 cells are summarized schematically in Figure 9. Stimulation of the CaR by Ca^{2+}_o leads to enhanced PTHrP release, through the PKC, MAPKs, and PI3K pathways. The oncogene PTTG, known to be necessary for normal testis development, is also upregulated when the CaR is activated. This may be merely an association that indicates that the rate of growth of the tumor cells is upregulated but may also be an important signaling pathway in the cell downstream of the CaR but proximal to proliferation. Thus PTTG may be a drug target in the vicious cycle of our HHM model. To validate this hypothesis, I could have used the siRNA approach as used in paper IV. Furthermore in paper II, I found that Ca^{2+}_o upregulates VEGF, perhaps the most potent growth factor in the stimulation of new vessel formation. I speculate that the VEGF upregulation may be mediated through the CaR. This has to my knowledge not been tested. In H-500 cells, stimulating the CaR likewise upregulated the constitutively active NOS enzyme, inducible NOS, but not the mRNA of endothelial or neuronal NOS. The upregulation of iNOS by the CaR seems to increase NO production, as high calcium concentration increased NO levels in the H-500 cells. A key characteristic of the cancer cell is its capacity to grow, and the growth of a tumor is decided primarily by the increase in its rate of proliferation and the reduction in its apoptosis. In the case of proliferation, my data could not confirm that the upregulated NO participated in the CaR-induced DNA synthesis. I found that stimulating the CaR in the H-500 cells leads to increased DNA synthesis through the p38 MAPK and PI3K signaling pathways, but not through the ERK1/2 signaling pathway or NO production. Last, stimulation of the CaR protects H-500 cells from undergoing programmed cell death. Thus, stimulation of the CaR induces several promalignant features in the Leydig tumor, and blocking the CaR seems to be a promising drug target in the treatment of this disease. On the other hand, since the CaR is so ubiquitously expressed, and especially because of its key role in calcium homeostasis, the positive effects of blocking the receptor pharmaceutically may be overshadowed by its adverse effects, even in cells in which the CaR has a documented promalignant role, such as H-500 cells. In PC-3 prostate cancer cells, we found that EGFR mediates some of the effects of the CaR on PTHrP release; thus, the EGFR is another possible membrane-bound target in the treatment of hypercalcemia-inducing cancers in which a functional CaR is expressed. Targeting the EGFR in colon cancer in which its growth was induced by prostaglandin, acting through its respective 7TM, has also had promising experimental results (185). The triple membrane spanning signaling or transactivation by CaR through the EGFR was latter shown also to be present in HEK-CaR cells, fibroblast and H-500 cells (157, 264, 266). The importance of this newly characterized pathway has not been established in parathyroid cells, which would be an important future study as the full story of CaR's signalling apparatus in the parathyroid cells has yet to be elucidated.

Although direct blocking of the CaR in malignant disease may be hazardous, I believe that understanding the mechanism of tumor growth is crucial for success in treatment of malignant diseases. Therefore, knowledge on the role of the CaR in malignant cells from this series of studies may be useful in selecting appropriate and optimal treatments of malignant hypercalcemia. The clinical treatment of malignant hypercalcemia follows two approaches: the first priority is treatment of the underlying malignant disease by a surgeon or an oncologist, and the second is correction of the hypercalcemia by rehydration and bisphosphonates, and sometimes glucocorticoids, in order to decrease the degradation of bone by osteoclasts. The use of bisphosphonates is in accord with my results, as well as with the work of Guise et al., which may be treating not only the hypercalcemia but also the underlying disease because of the decreased stimulation of the CaR or TGFβ receptor on the tumor cell surface. On another note, a third effect of the bisphosphonates seems to be a potential direct inhibitory effect on cancer cell growth (49). This, of course, raises the question of whether the bisphosphonates should be introduced in a treatment regimen for patients with malignant tumors proven to be sensitive to bisphosphonates even before hypercalcemia is present. Although this potential effect has not been fully established, as improved survival of patients with bone metastasis treated bisphosphonates is still debatable (28). Most of the models described in this thesis are based on cell lines or primary cultures that are very homogeneous and in which it is possible to modify experimental variables one at a time. In the complex clinical situation with heterogeneous tumors, perhaps only some cells express the CaR, and there is great variety in patients' responses, e.g.,

their immune responses. Accordingly, the next step is to test my hypothesis in animal studies and, ultimately, in clinical trials.

SUMMARY

The calcium-sensing receptor (CaR) is a seven transmembrane receptor incorporated into the cell membrane that is sensitive to extracellular calcium and other cations. The finding that the CaR is expressed on cancer cells has opened the door to a new understanding of the role of extracellular calcium as a promalignant stimulus through the CaR and its signaling apparatus as demonstrated in this thesis. I found, in a model of humoral hypercalcemia of malignancy (HHM), that stimulation of the CaR worsens the promalignant features of the testicular H-500 Leydig cancer cells that were used in my studies. The CaR upregulated the release of parathyroid hormone-related peptide (PTHrP), the main mediator of hypercalcemia in HHM. The growth rate of the tumor was also increased by stimulation of the CaR, as DNA synthesis and protection against apoptosis were enhanced. The oncogene, pituitary tumor-transforming gene (PTTG), was found to be upregulated by the CaR in the H-500 cells, whereas calcium had no effect on PTTG expression in the U-87 astrocytoma cell line, but other proproliferative agents did upregulate PTTG in the U-87 cells. This makes PTTG a potential marker of malignancy and a therapeutic target in cancer, where the CaR is promalignant. Nitric oxide synthase (NOS) exists in three isoforms, and I found that the CaR upregulated the inducible NOS but not the two other isoforms. This upregulation was accompanied by an increased production of NO. NO has been shown to be potentially promalignant, although such a role was not established in the H-500 cells. Therefore, the CaR stimulates several promalignant features in the H-500 cells. In turn, blocking these effects by targeting a proximal downstream signaling molecule of the CaR may be a future clinical approach, since blocking the CaR might have too many adverse effects on calcium homeostasis. In conclusion, the CaR plays diverse roles in cancer-acting as an inhibitor of cell proliferation in the colon crypt cells giving rise to colon cancer but as a promalignant receptor in most other cancer types, including Leydig cell cancers.

ABBREVATIONS

7TM: seven transmembrane receptor/G protein-coupled receptor
AA: Arachidonic acid
AC: adenylate cyclase
AKT: protein kinase B
ATF-2: activating transcription factor-2
ATP: adenosine trisphosphate
bFGF: basic fibroblast growth factor
cAMP: cyclic adenosine monophosphate
Ca^{2+}_i: intracellular calcium
Ca^{2+}_o: extracellular calcium
CaR: calcium-sensing receptor
CNS: central nervous system
DAF: nitric oxide binding dye in the group of diaminofluoresceins
DAG: diacylglycerol
DNA: deoxyribonucleic acid
ECD: extracellular domain
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
ERK1/2: extracellular signal-regulated kinases 1 and 2
GDP: guanosine diphosphate
$G\alpha i$: alpha subunit of Gi subtype of the heterotrimeric G proteins
$G\alpha q_{11}$: alpha subunit of Gq/11 subtype of the heterotrimeric G proteins
H-500 cells: primary cultured rat Rice H-500 Leydig testis cancer cells
HB-EGF: heparin-binding EGF
HEK: human embryonic kidney
HGF: hepatocyte growth factor
HHM: humoral hypercalcemia of malignancy
HPT: hyperparathyroidism
ICD: intracellular domain
IGF-1: Insulin-like growth factor 1
IL: interleukin
IP_3: inositol-1,4,5-trisphosphate
JNK: c-jun NH_2-terminal kinase
MAPK: mitogen-activated protein kinase
MEK: MAPK kinase
NO: nitric oxide
NOS: nitric oxide synthase
p38: p38 MAPK
PBF: PTTG binding factor
PC-3 cells: human prostate cancer PC-3 cells
PCR: polymerase chain reaction
PDGFR: platelet-derived growth factor receptor
PI3K: phosphatidylinositol 3-kinase
PI4K: phosphatidylinositol 4-kinase
PIP_2: phosphatidylinositol 4,5-bisphosphate
PKC: protein kinase C
PL: phospholipase
PPARγ: peroxisome proliferator-activated receptor gamma
PTH: parathyroid hormone
PTHR: PTH receptor 1
PTHrP: parathyroid hormone-related peptide
PTTG: pituitary tumor transforming gene
RNA: ribonucleic acid
SEK1: stress-activated protein kinase ERK kinase 1
TGFα: transforming growth factor α
TMD: transmembrane domain
VEGF: vascular endothelial growth factor.
U-87 cells: human U-87 astrocytoma cell line

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I. Tfelt-Hansen J., R. J. MacLeod, S. Chattopadhyay, S. Yano, S. Quinn, X. Ren, E. F. Terwilliger, P.Schwarz and E. M. Brown. Calcium-sensing receptor stimulates PTHrP secretion by PKC-, SEK1, p38 MAPK- and ERK1/2-dependent pathways in H-500 cells. Am J Physiol Endocrinol Metab 2003; 285: E329-37
II. Tfelt-Hansen J., E. M. Brown, E. F. Terwilliger, P. Schwarz, N. Chattopadhyay. Calcium-sensing receptor induces mRNA of human securin, pituitary tumor transforming gene, in rat testicular cancer. Endocrinology 2003; 144: 5188- 93
III. Tfelt-Hansen J, Chattopadhyay N, Yano S, Kanuparthi D, Rooney P, Schwarz P, and Brown EM. Calcium-sensing receptor induces proliferation through p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase but not extracellularly regulated kinase in a model of humoral hypercalcemia of malignancy. Endocrinology. 2004; 145: 1211-1217
IV. Tfelt-Hansen J, Yano S, Bandyopadhyay S, Carroll R, Brown EM, Chattopadhyay N. Expression of pituitary tumor transforming gene and its binding protein in human astrocytes and astrocytoma cells: Function and regulation of PTTG in U-87 astrocytoma cells. Endocrinology. 2004; 145: 4222-31
V. Yano S., J. Macleod, N. Chattopadhyay, J. Tfelt-Hansen, O. Kifor, R. Butters, E. Brown. Calcium sensing receptor activation stimulates parathyroid hormone related protein secretion in prostate cancer cells: role of epidermal growth factor receptor transactivation. Bone 2004; 35: 664-72
VI. Tfelt-Hansen J., A. Ferreira, S. Yano, D. Kanuparthi, J. R. Romero, E. M. Brown, N. Chattopadhyay. Calcium-sensing receptor activation induces nitric oxide production in H-500 Leydig cancer cells. Am J Physiol Endocrinol Metab. 2005; 288: E1206-1213

REFERENCES

1. Abbud RA, Takumi I, Barker EM, Ren SG, Chen DY, Wawrowsky K, and Melmed S. Early multipotential pituitary focal hyperplasia in the alpha-subunit of glycoprotein hormone-driven pituitary tumor-transforming gene transgenic mice. Mol Endocrinol 19: 1383-1391, 2005.
2. Adams GB, Chabner KT, Alley IR, Olson DP, Szczepi orkowski ZM, Poznansky MC, Kos CH, Pollak MR, Brown EM, and Scadden DT. Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature 439: 599-603, 2006.
3. Adams ML, Meyer ER, Sewing BN, and Cicero TJ. Effects of nitric oxide-related agents on rat testicular function. J Pharmacol Exp Ther 269: 230-237, 1994.
4. Adebanjo OA, Igietseme J, Huang CL, and Zaidi M. The effect of extracellularly applied divalent cations on cytosolic Ca2+ in murine leydig cells: evidence for a Ca2+-sensing receptor. J Physiol 513 (Pt 2): 399-410, 1998.
5. Ahn S, Shenoy SK, Wei H, and Lefkowitz RJ. Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. J Biol Chem 279: 35518-35525, 2004.
6. Aida K, Koishi S, Tawata M, and Onaya T. Molecular cloning of a putative Ca(2+)-sensing receptor cDNA from human kidney. Biochem Biophys Res Commun 214: 524-529, 1995.
7. Alderton WK, Cooper CE, and Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J 357: 593-615, 2001.
8. Amling M, Priemel M, Holzmann T, Chapin K, Rueger JM, Baron R, and Demay MB. Rescue of the skeletal phenotype of vitamin D receptor-ablated mice in the setting of normal mineral ion homeostasis: formal histomorphometric and biomechanical analyses. Endocrinology 140: 4982-4987, 1999.
9. Asa SL, Henderson J, Goltzman D, and Drucker DJ. Parathyroid hormone-like peptide in normal and neoplastic human endocrine tissues. J Clin Endocrinol Metab 71: 1112-1118, 1990.
10. Awata H, Huang C, Handlogten ME, and Miller RT. Interaction of the calcium-sensing receptor and filamin, a potential scaffolding protein. J Biol Chem 276: 34871-34879, 2001.
11. Bai M, Pearce SH, Kifor O, Trivedi S, Stauffer UG, Thakker RV, Brown EM, and Steinmann B. In vivo and in vitro characterization of neonatal hyperparathyroidism resulting from a de novo, heterozygous mutation in the Ca2+-sensing receptor gene: normal maternal calcium homeostasis as a cause of secondary hyperparathyroidism in familial benign hypocalciuric hypercalcemia. J Clin Invest 99: 88-96, 1997.
12. Bai M, Quinn S, Trivedi S, Kifor O, Pearce SH, Pollak MR, Krapcho K, Hebert SC, and Brown EM. Expression and characterization of inactivating and activating mutations in the human Ca2+o-sensing receptor. J Biol Chem 271: 19537-19545, 1996.
13. Bai M, Trivedi S, and Brown EM. Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. J Biol Chem 273: 23605-23610, 1998.
14. Bai M, Trivedi S, Lane CR, Yang Y, Quinn SJ, and Brown EM. Protein kinase C phosphorylation of threonine at position 888 in Ca2+o-sensing receptor (CaR) inhibits coupling to Ca2+ store release. J Biol Chem 273: 21267-21275, 1998.
15. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, and Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A 98: 13681-13686, 2001.
16. Bevilacqua M, Dominguez LJ, Righini V, Valdes V, Toscano R, Sangaletti O, Vago T, Baldi G, Barrella M, and Bianchi-Porro G. Increased Gastrin And Calcitonin Secretion After Oral Calcium Or Peptones Administration In Patients With Hypercalciuria: A Clue To An Alteration In Calcium Sensing Receptor Activity. J Clin Endocrinol Metab, 90: 1489-94. 2005 .
17. Bikle DD, Ng D, Tu CL, Oda Y, and Xie Z. Calcium- and vitamin D-regulated keratinocyte differentiation. Mol Cell Endocrinol 177: 161-171, 2001.
18. Boelaert K, McCabe CJ, Tannahill LA, Gittoes NJ, Holder RL, Watkinson JC, Bradwell AR, Sheppard MC, and Franklyn JA. Pituitary tumor transforming gene and fibroblast growth factor-2 expression: potential prognostic indicators in differentiated thyroid cancer. J Clin Endocrinol Metab 88: 2341-2347, 2003.
19. Boelaert K, Tannahill LA, Bulmer JN, Kachilele S, Chan SY, Kim D, Gittoes NJ, Franklyn JA, Kilby MD, and McCabe CJ. A potential role for PTTG/securin in the developing human fetal brain. Faseb J 17: 1631-1639, 2003.
20. Brauner-Osborne H, Jensen AA, Sheppard PO, O'Hara P, and Krogsgaard-Larsen P. The agonist-binding domain of the calcium-sensing receptor is located at the amino-terminal domain. J Biol Chem 274: 18382-18386, 1999.
21. Brent GA, LeBoff MS, Seely EW, Conlin PR, and Brown EM. Relationship between the concentration and rate of change of calcium and serum intact parathyroid hormone levels in normal humans. J Clin Endocrinol Metab 67: 944-950, 1988.
22. Brown AJ, Ritter CS, Finch JL, and Slatopolsky EA. Decreased calcium-sensing receptor expression in hyperplastic parathyroid glands of uremic rats: role of dietary phosphate. Kidney Int 55: 1284-1292, 1999.
23. Brown EM. Physiology and pathophysiology of the extracellular calcium-sensing receptor. Am J Med 106: 238-253, 1999.
24. Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J, and Hebert SC. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. Nature 366: 575-580, 1993.
25. Brown EM, Leombruno R, Thatcher J, and Burrowes M. The acute secretory response to alterations in extracellular calcium concentration and dopamine in perifused bovine parathyroid cells. Endocrinology 116: 1123-1132, 1985.
26. Brown EM and MacLeod RJ. Extracellular calcium sensing and extracellular calcium signaling. Physiol Rev 81: 239-297, 2001.
27. Brown EM, Watson EJ, Thatcher JG, Koletsky R, Dawson HB, Posillico JT, and Shoback DM. Ouabain and low extracellular potassium inhibit PTH secretion from bovine parathyroid cells by a mechanism that does not involve increases in the cytosolic calcium concentration. Metabolism 36: 36-42, 1987.
28. Brown JE, Neville-Webbe H, and Coleman RE. The role of bisphosphonates in breast and prostate cancers. Endocr Relat Cancer 11: 207-224, 2004.
29. Buchan AM, Squires PE, Ring M, and Meloche RM. Mechanism of action of the calcium-sensing receptor in human antral gastrin cells. Gastroenterology 120: 1128-1139, 2001.
30. Buchs N, Manen D, Bonjour JP, and Rizzoli R. Calcium stimulates parathyroid hormone-related protein production in Leydig tumor cells through a putative cation-sensing mechanism. Eur J Endocrinol 142: 500-505, 2000.
31. Butters RR, Jr., Chattopadhyay N, Nielsen P, Smith CP, Mithal A, Kifor O, Bai M, Quinn S, Goldsmith P, Hurwitz S, Krapcho K, Busby J, and Brown EM. Cloning and characterization of a calcium-sensing receptor from the hypercalcemic New Zealand white rabbit reveals unaltered responsiveness to extracellular calcium. J Bone Miner Res 12: 568-579, 1997.
32. Carling T, Szabo E, Bai M, Ridefelt P, Westin G, Gustavsson P, Trivedi S, Hellman P, Brown EM, Dahl N, and Rastad J. Familial hypercalcemia and hypercalciuria caused by a novel mutation in the cytoplasmic tail of the calcium receptor. J Clin Endocrinol Metab 85: 2042-2047, 2000.
33. Caroppo R, Gerbino A, Debellis L, Kifor O, Soybel DI, Brown EM, Hofer AM, and Curci S. Asymmetrical, agonist-induced fluctuations in local extracellular [Ca(2+)] in intact polarized epithelia. Embo J 20: 6316-6326, 2001.
34. Chakrabarty S, Radjendirane V, Appelman H, and Varani J. Extracellular calcium and calcium sensing receptor function in human colon carcinomas: promotion of E-cadherin expression and suppression of beta-catenin/TCF activation. Cancer Res 63: 67-71, 2003.
35. Chakrabarty S, Wang H, Canaff L, Hendy GN, Appelman H, and Varani J. Calcium sensing receptor in human colon carcinoma: interaction with Ca(2+) and 1,25-dihydroxyvitamin D(3). Cancer Res 65: 493-498, 2005.
36. Chamaon K, Kirches E, Kanakis D, Braeuninger S, Dietzmann K, and Mawrin C. Regulation of the pituitary tumor transforming gene by insulin-like-growth factor-I and insulin differs between malignant and non-neoplastic astrocytes. Biochem Biophys Res Commun 331: 86-92, 2005.
37. Chang W, Tu C, Pratt S, Chen TH, and Shoback D. Extracellular Ca(2+)-sensing receptors modulate matrix production and mineralization in chondrogenic RCJ3.1C5.18 cells. Endocrinology 143: 1467-1474, 2002.
38. Chattopadhyay N, Butters RR, and Brown EM. Agonists of the retinoic acid- and retinoid X-receptors inhibit hepatocyte growth factor secretion and expression in U87 human astrocytoma cells. Brain Res Mol Brain Res 87: 100-108, 2001.
39. Chattopadhyay N, Cheng I, Rogers K, Riccardi D, Hall A, Diaz R, Hebert SC, Soybel DI, and Brown EM. Identification and localization of extracellular Ca(2+)-sensing receptor in rat intestine. Am J Physiol 274: G122-130, 1998.
40. Chattopadhyay N, Evliyaoglu C, Heese O, Carroll R, Sanders J, Black P, and Brown EM. Regulation of secretion of PTHrP by Ca(2+)-sensing receptor in human astrocytes, astrocytomas, and meningiomas. Am J Physiol Cell Physiol 279: C691-699, 2000.
41. Chattopadhyay N, Singh DP, Heese O, Godbole MM, Sinohara T, Black PM, and Brown EM. Expression of peroxisome proliferator-activated receptors (PPARS) in human astrocytic cells: PPARgamma agonists as inducers of apoptosis. J Neurosci Res 61: 67-74, 2000.
42. Chattopadhyay N, Ye C, Singh DP, Kifor O, Vassilev PM, Shinohara T, Chylack LT, Jr., and Brown EM. Expression of extracellular calcium-sensing receptor by human lens epithelial cells. Biochem Biophys Res Commun 233: 801-805, 1997.
43. Chattopadhyay N, Ye CP, Yamaguchi T, Kerner R, Vassilev PM, and

Brown EM. Extracellular calcium-sensing receptor induces cellular proliferation and activation of a nonselective cation channel in U373 human astrocytoma cells. Brain Res 851: 116-124, 1999.
44. Chattopadhyay N, Ye CP, Yamaguchi T, Vassilev PM, and Brown EM. Evidence for extracellular calcium-sensing receptor mediated opening of an outward K+ channel in a human astrocytoma cell line (U87). Glia 26: 64-72, 1999.
45. Chen CJ, Barnett JV, Congo DA, and Brown EM. Divalent cations suppress 3',5'-adenosine monophosphate accumulation by stimulating a pertussis toxin-sensitive guanine nucleotide-binding protein in cultured bovine parathyroid cells. Endocrinology 124: 233-239, 1989.
46. Cheng I, Klingensmith ME, Chattopadhyay N, Kifor O, Butters RR, Soybel DI, and Brown EM. Identification and localization of the extracellular calcium-sensing receptor in human breast. J Clin Endocrinol Metab 83: 703-707, 1998.
47. Chien W and Pei L. A novel binding factor facilitates nuclear translocation and transcriptional activation function of the pituitary tumor-transforming gene product. J Biol Chem 275: 19422-19427, 2000.
48. Chikatsu N, Fukumoto S, Takeuchi Y, Suzawa M, Obara T, Matsumoto T, and Fujita T. Cloning and characterization of two promoters for the human calcium-sensing receptor (CaSR) and changes of CaSR expression in parathyroid adenomas. J Biol Chem 275: 7553-7557, 2000.
49. Chirgwin JM, Mohammad KS, and Guise TA. Tumor-bone cellular interactions in skeletal metastases. J Musculoskelet Neuronal Interact 4: 308-318, 2004.
50. Chomczynski P and Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159, 1987.
51. Christakos S, Dhawan P, Liu Y, Peng X, and Porta A. New insights into the mechanisms of vitamin D action. J Cell Biochem 88: 695-705, 2003.
52. Cifuentes M, Albala C, and Rojas C. Calcium-sensing receptor expression in human adipocytes. Endocrinology 146: 2176-2179, 2005.
53. Clapham DE. Calcium signaling. Cell 80: 259-268, 1995.
54. Clines GA and Guise TA. Hypercalcaemia of malignancy and basic research on mechanisms responsible for osteolytic and osteoblastic metastasis to bone. Endocr Relat Cancer 12: 549-583, 2005.
55. Cole DE, Janicic N, Salisbury SR, and Hendy GN. Neonatal severe hyperparathyroidism, secondary hyperparathyroidism, and familial hypocalciuric hypercalcemia: multiple different phenotypes associated with an inactivating Alu insertion mutation of the calcium-sensing receptor gene. Am J Med Genet 71: 202-210, 1997.
56. Coleman RE. Skeletal complications of malignancy. Cancer 80: 1588-1594, 1997.
57. Conejo R and Lorenzo M. Insulin signaling leading to proliferation, survival, and membrane ruffling in C2C12 myoblasts. J Cell Physiol 187: 96-108, 2001.
58. Conigrave AD, Franks AH, Brown EM, and Quinn SJ. L-amino acid sensing by the calcium-sensing receptor: a general mechanism for coupling protein and calcium metabolism? Eur J Clin Nutr 56: 1072-1080, 2002.
59. Conigrave AD, Mun HC, Delbridge L, Quinn SJ, Wilkinson M, and Brown EM. L-amino acids regulate parathyroid hormone secretion. J Biol Chem 279: 38151-38159, 2004.
60. Conigrave AD, Quinn SJ, and Brown EM. L-amino acid sensing by the extracellular Ca2+-sensing receptor. Proc Natl Acad Sci U S A 97: 4814-4819, 2000.
61. Corbett TH, Griswold DP, Jr., Roberts BJ, Peckham JC, and Schabel FM, Jr. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Res 35: 2434-2439, 1975.
62. Dal Pra I, Chiarini A, Nemeth EF, Armato U, and Whitfield JF. Roles of Ca(2+) and the Ca(2+)-sensing receptor (CASR) in the expression of inducible NOS (nitric oxide synthase)-2 and its BH(4) (tetrahydrobiopterin)-dependent activation in cytokine-stimulated adult human astrocytes. J Cell Biochem, 2005.
63. Del Punta K, Charreau EH, and Pignataro OP. Nitric oxide inhibits Leydig cell steroidogenesis. Endocrinology 137: 5337-5343, 1996.
64. Dudley DT, Pang L, Decker SJ, Bridges AJ, and Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A 92: 7686-7689, 1995.
65. Eftekhari F and Yousefzadeh DK. Primary infantile hyperparathyroidism: clinical, laboratory, and radiographic features in 21 cases. Skeletal Radiol 8: 201-208, 1982.
66. Emanuel RL, Adler GK, Kifor O, Quinn SJ, Fuller F, Krapcho K, and Brown EM. Calcium-sensing receptor expression and regulation by extracellular calcium in the AtT-20 pituitary cell line. Mol Endocrinol 10: 555-565, 1996.
67. Endo K, Katsumata K, Iguchi H, Kubodera N, Teramoto T, Ikeda K, Fujita T, and Ogata E. Effect of combination treatment with a vitamin D analog (OCT) and a bisphosphonate (AHPrBP) in a nude mouse model of cancer-associated hypercalcemia. J Bone Miner Res 13: 1378-1383, 1998.
68. Enslen H, Raingeaud J, and Davis RJ. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. J Biol Chem 273: 1741-1748, 1998.
69. Fan GF, Ray K, Zhao XM, Goldsmith PK, and Spiegel AM. Mutational analysis of the cysteines in the extracellular domain of the human Ca2+ receptor: effects on cell surface expression, dimerization and signal transduction. FEBS Lett 436: 353-356, 1998.
70. Farnebo F, Enberg U, Grimelius L, Backdahl M, Schalling M, Larsson C, and Farnebo LO. Tumor-specific decreased expression of calcium sensing receptor messenger ribonucleic acid in sporadic primary hyperparathyroidism. J Clin Endocrinol Metab 82: 3481-3486, 1997.
71. Freichel M, Zink-Lorenz A, Holloschi A, Hafner M, Flockerzi V, and Raue F. Expression of a calcium-sensing receptor in a human medullary thyroid carcinoma cell line and its contribution to calcitonin secretion. Endocrinology 137: 3842-3848, 1996.
72. Fudge NJ and Kovacs CS. Physiological studies in heterozygous calcium sensing receptor (CaSR) gene-ablated mice confirm that the CaSR regulates calcitonin release in vivo. BMC Physiol 4: 5, 2004.
73. Fukumura D, Kashiwagi S, and Jain RK. The role of nitric oxide in tumour progression. Nat Rev Cancer 6: 521-534, 2006.
74. Gaich G and Burtis WJ. Measurement of circulating parathyroid hormone-related protein in rats with humoral hypercalcemia of malignancy using a two-site immunoradiometric assay. Endocrinology 127: 1444-1449, 1990.
75. Gallwitz WE, Guise TA, and Mundy GR. Guanosine nucleotides inhibit different syndromes of PTHrP excess caused by human cancers in vivo. J Clin Invest 110: 1559-1572, 2002.
76. Gama L, Baxendale-Cox LM, and Breitwieser GE. Ca2+-sensing receptors in intestinal epithelium. Am J Physiol 273: C1168-1175, 1997.
77. Gardella TJ and Juppner H. Interaction of PTH and PTHrP with their receptors. Rev Endocr Metab Disord 1: 317-329, 2000.
78. Gardner DG, Brown EM, and Aurbach GD. Inhibition of adenosine 3',5'-monophosphate accumulation and parathyroid hormone release by sodium nitroprusside. Endocrinology 105: 360-366, 1979.
79. Gardner DG, Brown EM, and Aurbach GD. Sodium nitroprusside inhibition of parathyroid hormone release is not mediated through cyclic GMP. Metabolism 30: 1179-1184, 1981.
80. Garner SC, Hinson TK, McCarty KS, Leight M, Leight GS, Jr., and Quarles LD. Quantitative analysis of the calcium-sensing receptor messenger RNA in parathyroid adenomas. Surgery 122: 1166-1175, 1997.
81. Garrett JE, Capuano IV, Hammerland LG, Hung BC, Brown EM, Hebert SC, Nemeth EF, and Fuller F. Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. J Biol Chem 270: 12919-12925, 1995.
82. Garrett JE, Tamir H, Kifor O, Simin RT, Rogers KV, Mithal A, Gagel RF, and Brown EM. Calcitonin-secreting cells of the thyroid express an extracellular calcium receptor gene. Endocrinology 136: 5202-5211, 1995.
83. Goebel SU, Peghini PL, Goldsmith PK, Spiegel AM, Gibril F, Raffeld M, Jensen RT, and Serrano J. Expression of the calcium-sensing receptor in gastrinomas. J Clin Endocrinol Metab 85: 4131-4137, 2000.
84. Gogusev J, Duchambon P, Hory B, Giovannini M, Goureau Y, Sarfati E, and Drueke TB. Depressed expression of calcium receptor in parathyroid gland tissue of patients with hyperparathyroidism. Kidney Int 51: 328-336, 1997.
85. Goodman WG, Hladik GA, Turner SA, Blaisdell PW, Goodkin DA, Liu W, Barri YM, Cohen RM, and Coburn JW. The Calcimimetic agent AMG 073 lowers plasma parathyroid hormone levels in hemodialysis patients with secondary hyperparathyroidism. J Am Soc Nephrol 13: 1017-1024, 2002.
86. Grant FD, Conlin PR, and Brown EM. Rate and concentration dependence of parathyroid hormone dynamics during stepwise changes in serum ionized calcium in normal humans. Journal of clinical endocrinology and metabolism 71: 370-378, 1990.
87. Grantmyre EB. Roentgenographic features of “primary” hyperparathyroidism in infancy. J Can Assoc Radiol 24: 257-260, 1973.
88. Gray E, Muller D, Squires PE, Asare-Anane H, Huang GC, Amiel S, Persaud SJ, and Jones PM. Activation of the extracellular calcium-sensing receptor initiates insulin secretion from human islets of Langerhans: involvement of protein kinases. J Endocrinol 190: 703-710, 2006.
89. Grill V, Ho P, Body JJ, Johanson N, Lee SC, Kukreja SC, Moseley JM, and Martin TJ. Parathyroid hormone-related protein: elevated levels in both humoral hypercalcemia of malignancy and hypercalcemia complicating metastatic breast cancer. J Clin Endocrinol Metab 73: 1309-1315, 1991.
90. Guise TA and Mundy GR. Cancer and bone. Endocr Rev 19: 18-54, 1998.
91. Guise TA, Yoneda T, Yates AJ, and Mundy GR. The combined effect of tumor-produced parathyroid hormone-related protein and transforming growth factor-alpha enhance hypercalcemia in vivo and bone resorption in vitro. J Clin Endocrinol Metab 77: 40-45, 1993.
92. Hamid T and Kakar SS. PTTG and cancer. Histol Histopathol 18: 245-251, 2003.

93. Handlogten ME, Huang C, Shiraishi N, Awata H, and Miller RT. The Ca2+-sensing receptor activates cytosolic phospholipase A2 via a Gq-alpha-dependent ERK-independent pathway. J Biol Chem 276: 13941-13948, 2001.
94. Hansen JL, Theilade J, Haunso S, and Sheikh SP. Oligomerization of wild type and nonfunctional mutant angiotensin II type I receptors inhibits galphaq protein signaling but not ERK activation. J Biol Chem 279: 24108-24115, 2004.
95. Harper JW, Burton JL, and Solomon MJ. The anaphase-promoting complex: it's not just for mitosis any more. Genes Dev 16: 2179-2206, 2002.
96. Hauschka PV, Mavrakos AE, Iafrati MD, Doleman SE, and Klagsbrun M. Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose. J Biol Chem 261: 12665-12674, 1986.
97. Hawkins D, Enyedi P, and Brown E. The effects of high extracellular Ca2+ and Mg2+ concentrations on the levels of inositol 1,3,4,5-tetrakisphosphate in bovine parathyroid cells. Endocrinology 124: 838-844, 1989.
98. Heaney AP, Fernando M, and Melmed S. PPAR-gamma receptor ligands: novel therapy for pituitary adenomas. J Clin Invest 111: 1381-1388, 2003.
99. Heaney AP, Horwitz GA, Wang Z, Singson R, and Melmed S. Early involvement of estrogen-induced pituitary tumor transforming gene and fibroblast growth factor expression in prolactinoma pathogenesis. Nat Med 5: 1317-1321, 1999.
100. Heaney AP, Singson R, McCabe CJ, Nelson V, Nakashima M, and Melmed S. Expression of pituitary-tumour transforming gene in colorectal tumours. Lancet 355: 716-9, 2000.
101. Heath H, 3rd. Familial benign (hypocalciuric) hypercalcemia. A troublesome mimic of mild primary hyperparathyroidism. Endocrinol Metab Clin North Am 18: 723-740, 1989.
102. Hellman P, Hellman B, Juhlin C, Juppner H, Rastad J, Ridefelt P, and Akerstrom G. Regulation of proliferation in JEG-3 cells by a 500-kDa Ca2+ sensor and parathyroid hormone-related protein. Arch Biochem Biophys 307: 379-385, 1993.
103. Henderson J, Bernier S, D'Amour P, and Goltzman D. Effects of passive immunization against parathyroid hormone (PTH)-like peptide and PTH in hypercalcemic tumor-bearing rats and normocalcemic controls. Endocrinology 127: 1310-1318, 1990.
104. Henderson J, Sebag M, Rhim J, Goltzman D, and Kremer R. Dysregulation of parathyroid hormone-like peptide expression and secretion in a keratinocyte model of tumor progression. Cancer Res 51: 6521-6528, 1991.
105. Hendy GN, D'Souza-Li L, Yang B, Canaff L, and Cole DE. Mutations of the calcium-sensing receptor (CASR) in familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia. Hum Mutat 16: 281-296, 2000.
106. Hilgard P, Schmitt W, Minne H, and Ziegler R. Acute hypercalcemia due to Walker carcinosarcoma 256 in the rat. Horm Metab Res 2: 255-256, 1970.
107. Hjalm G, MacLeod RJ, Kifor O, Chattopadhyay N, and Brown EM. Filamin-A binds to the carboxyl-terminal tail of the calcium-sensing receptor, an interaction that participates in CaR-mediated activation of mitogen-activated protein kinase. J Biol Chem 276: 34880-34887, 2001.
108. Ho C, Conner DA, Pollak MR, Ladd DJ, Kifor O, Warren HB, Brown EM, Seidman JG, and Seidman CE. A mouse model of human familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. Nat Genet 11: 389-394, 1995.
109. Hobson SA, McNeil SE, Lee F, and Rodland KD. Signal transduction mechanisms linking increased extracellular calcium to proliferation in ovarian surface epithelial cells. Exp Cell Res 258: 1-11, 2000.
110. Hofer AM, Curci S, Doble MA, Brown EM, and Soybel DI. Intercellular communication mediated by the extracellular calcium-sensing receptor. Nat Cell Biol 2: 392-398, 2000.
111. Hoff AO, Catala-Lehnen P, Thomas PM, Priemel M, Rueger JM, Nasonkin I, Bradley A, Hughes MR, Ordonez N, Cote GJ, Amling M, and Gagel RF. Increased bone mass is an unexpected phenotype associated with deletion of the calcitonin gene. J Clin Invest 110: 1849-1857, 2002.
112. Hoff AO, Cote GJ, Fritsche HA, Jr., Qiu H, Schultz PN, and Gagel RF. Calcium-induced activation of a mutant G-protein-coupled receptor causes in vitro transformation of NIH/3T3 cells. Neoplasia 1: 485-491, 1999.
113. Holick MF and Clark MB. The photobiogenesis and metabolism of vitamin D. Fed Proc 37: 2567-2574, 1978.
114. Hosokawa Y, Pollak MR, Brown EM, and Arnold A. Mutational analysis of the extracellular Ca(2+)-sensing receptor gene in human parathyroid tumors. J Clin Endocrinol Metab 80: 3107-3110, 1995.
115. House MG, Kohlmeier L, Chattopadhyay N, Kifor O, Yamaguchi T, Leboff MS, Glowacki J, and Brown EM. Expression of an extracellular calcium-sensing receptor in human and mouse bone marrow cells. J Bone Miner Res 12: 1959-1970, 1997.
116. Hu J, McLarnon SJ, Mora S, Jiang J, Thomas C, Jacobson KA, and Spiegel AM. A region in the seven-transmembrane domain of the human Ca2+ receptor critical for response to Ca2+. J Biol Chem 280: 5113-5120, 2005.
117. Huang C, Handlogten ME, and Miller RT. Parallel activation of phosphatidylinositol 4-kinase and phospholipase C by the extracellular calcium-sensing receptor. J Biol Chem 277: 20293-20300, 2002.
118. Igarashi T, Ogata E, Maruyama K, and Fukuda T. Effect of calcimimetic agent, KRN568, on gastrin secretion in healthy subjects. Endocr J 47: 517-523, 2000.
119. Ikeda I, Miura T, and Kondo I. Pyridinium cross-links as urinary markers of bone metastases in patients with prostate cancer. BrJUrol 77: 102-106, 1996.
120. Insogna KL, Stewart AF, Vignery AM, Weir EC, Namnum PA, Baron RE, Kirkwood JM, Deftos LM, and Broadus AE. Biochemical and histomorphometric characterization of a rat model for humoral hypercalcemia of malignancy. Endocrinology 114: 888-896, 1984.
121. Ishikawa H, Heaney AP, Yu R, Horwitz GA, and Melmed S. Human pituitary tumor-transforming gene induces angiogenesis. J Clin Endocrinol Metab 86: 867-874, 2001.
122. Itami A, Kato M, Komoto I, Doi R, Hosotani R, Shimada Y, and Imamura M. Human gastrinoma cells express calcium-sensing receptor. Life Sci 70: 119-129, 2001.
123. Jacobs B and Huseby R. Neoplasms occuring in aged Fisher rats with special reference to testicular, uterine and thyroid tumors. J Natl Cancer Inst 39: 303-309, 1967.
124. Jensen AA, Hansen JL, Sheikh SP, and Brauner-Osborne H. Probing intermolecular protein-protein interactions in the calcium-sensing receptor homodimer using bioluminescence resonance energy transfer (BRET). Eur J Biochem 269: 5076-5087, 2002.
125. Jiang ZY, Zhou QL, Chatterjee A, Feener EP, Myers MG, Jr., White MF, and King GL. Endothelin-1 modulates insulin signaling through phosphatidylinositol 3-kinase pathway in vascular smooth muscle cells. Diabetes 48: 1120-1130, 1999.
126. Journe F, Dumon JC, Kheddoumi N, Fox J, Laios I, Leclercq G, and Body JJ. Extracellular calcium downregulates estrogen receptor alpha and increases its transcriptional activity through calcium-sensing receptor in breast cancer cells. Bone 35: 479-488, 2004.
127. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, and Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol 17: 16-23, 1979.
128. Kallay E, Bajna E, Wrba F, Kriwanek S, Peterlik M, and Cross HS. Dietary calcium and growth modulation of human colon cancer cells: role of the extracellular calcium-sensing receptor. Cancer Detect Prev 24: 127-136, 2000.
129. Kallay E, Bonner E, Wrba F, Thakker RV, Peterlik M, and Cross HS. Molecular and functional characterization of the extracellular calcium-sensing receptor in human colon cancer cells. Oncol Res 13: 551-559, 2003.
130. Kallay E, Kifor O, Chattopadhyay N, Brown EM, Bischof MG, Peterlik M, and Cross HS. Calcium-dependent c-myc proto-oncogene expression and proliferation of Caco-2 cells: a role for a luminal extracellular calcium-sensing receptor. Biochem Biophys Res Commun 232: 80-83, 1997.
131. Kaneko C, Mizunashi K, Tanaka M, Uzuki M, Kikuchi M, Sawai T, and Goto MM. Relationship between Ca-dependent change of serum PTH and extracellular Ca2+-sensing receptor expression in parathyroid adenoma. Calcif Tissue Int 64: 271-272, 1999.
132. Kato M, Doi R, Imamura M, Furutani M, Hosotani R, and Shimada Y. Calcium-evoked insulin release from insulinoma cells is mediated via calcium-sensing receptor. Surgery 122: 1203-1211, 1997.
133. Kato M, Doi R, Imamura M, Okada N, Shimada Y, Hosotani R, and Miyazaki JI. Response of human insulinoma cells to extracellular calcium is different from normal B cells. Dig Dis Sci 43: 2429-2438, 1998.
134. Kifor O, Diaz R, Butters R, and Brown EM. The Ca2+-sensing receptor (CaR) activates phospholipases C, A2, and D in bovine parathyroid and CaR-transfected, human embryonic kidney (HEK293) cells. J Bone Miner Res 12: 715-725, 1997.
135. Kifor O, Diaz R, Butters R, Kifor I, and Brown EM. The calcium-sensing receptor is localized in caveolin-rich plasma membrane domains of bovine parathyroid cells. J Biol Chem 273: 21708-21713, 1998.
136. Kifor O, MacLeod RJ, Diaz R, Bai M, Yamaguchi T, Yao T, Kifor I, and Brown EM. Regulation of MAP kinase by calcium-sensing receptor in bovine parathyroid and CaR-transfected HEK293 cells. Am J Physiol Renal Physiol 280: F291-302, 2001.
137. Kifor O, Moore-FD J, Wang P, Goldstein M, Vassilev P, Kifor I, Hebert SC, and Brown EM. Reduced immunostaining for the extracellular Ca2+-sensing receptor in primary and uremic secondary hyperparathyroidism. JClin EndocrinolMetab 81: 1598-1606, 1996.
138. Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y, and Nagano T. Detection and imaging of nitric oxide with

novel fluorescent indicators: diaminofluoresceins. Anal Chem 70: 2446-2453, 1998.
139. Kolakowski LF, Jr. GCRDb: a G-protein-coupled receptor database. Receptors Channels 2: 1-7, 1994.
140. Komoto I, Kato M, Itami A, Shimada Y, Doi R, Hosotani R, and Imamura M. Expression and function of the calcium-sensing receptor in pancreatic islets and insulinoma cells. Pancreas 26: 178-184, 2003.
141. Kovacs CS, Lanske B, Hunzelman JL, Guo J, Karaplis AC, and Kronenberg HM. Parathyroid hormone-related peptide (PTHrP) regulates fetal-placental calcium transport through a receptor distinct from the PTH/PTHrP receptor. Proc Natl Acad Sci U S A 93: 15233-15238, 1996.
142. Kovalenko M, Gazit A, Bohmer A, Rorsman C, Ronnstrand L, Heldin CH, Waltenberger J, Bohmer FD, and Levitzki A. Selective platelet-derived growth factor receptor kinase blockers reverse sis-transformation. Cancer Res 54: 6106-6114, 1994.
143. Kremer R, Shustik C, Tabak T, Papavasiliou V, and Goltzman D. Parathyroid-hormone-related peptide in hematologic malignancies. Am J Med 100: 406-411, 1996.
144. Kronenberg HM. Developmental regulation of the growth plate. Nature 423: 332-336, 2003.
145. Kuestner RE, Elrod RD, Grant FJ, Hagen FS, Kuijper JL, Matthewes SL, O'Hara PJ, Sheppard PO, Stroop SD, Thompson DL, and et al. Cloning and characterization of an abundant subtype of the human calcitonin receptor. Mol Pharmacol 46: 246-255, 1994.
146. Lala PK and Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol 2: 149-156, 2001.
147. Lamprecht SA and Lipkin M. Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. Nat Rev Cancer 3: 601-614, 2003.
148. Lawson DE, Wilson PW, and Kodicek E. New vitamin D metabolite localized in intestinal cell nuclei. Nature 222: 171-172, 1969.
149. Lewin E, Garfia B, Recio FL, Rodriguez M, and Olgaard K. Persistent downregulation of calcium-sensing receptor mRNA in rat parathyroids when severe secondary hyperparathyroidism is reversed by an isogenic kidney transplantation. J Am Soc Nephrol 13: 2110-2116, 2002.
150. Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R, and Demay MB. Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. Proc Natl Acad Sci U S A 94: 9831-9835, 1997.
151. Lin HY, Harris TL, Flannery MS, Aruffo A, Kaji EH, Gorn A, Kolakowski LF, Jr., Lodish HF, and Goldring SR. Expression cloning of an adenylate cyclase-coupled calcitonin receptor. Science 254: 1022-1024, 1991.
152. Lin KI, Chattopadhyay N, Bai M, Alvarez R, Dang CV, Baraban JM, Brown EM, and Ratan RR. Elevated extracellular calcium can prevent apoptosis via the calcium-sensing receptor. Biochem Biophys Res Commun 249: 325-331, 1998.
153. Lindberg JS, Culleton B, Wong G, Borah MF, Clark RV, Shapiro WB, Roger SD, Husserl FE, Klassen PS, Guo MD, Albizem MB, and Coburn JW. Cinacalcet HCl, an oral calcimimetic agent for the treatment of secondary hyperparathyroidism in hemodialysis and peritoneal dialysis: a randomized, double-blind, multicenter study. J Am Soc Nephrol 16: 800-807, 2005.
154. Liu B, Goltzman D, and Rabbani SA. Regulation of parathyroid hormone-related peptide production in vitro by the rat hypercalcemic Leydig cell tumor H-500. Endocrinology 132: 1658-1664, 1993.
155. Luttrell LM, Della Rocca GJ, van Biesen T, Luttrell DK, and Lefkowitz RJ. Gbetagamma subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. J Biol Chem 272: 4637-4644, 1997.
156. MacLeod RJ, Chattopadhyay N, and Brown EM. PTHrP stimulated by the calcium-sensing receptor requires MAP kinase activation. Am J Physiol Endocrinol Metab 284: E435-442, 2003.
157. MacLeod RJ, Yano S, Chattopadhyay N, and Brown EM. Extracellular calcium-sensing receptor transactivates the epidermal growth factor receptor by a triple-membrane-spanning signaling mechanism. Biochem Biophys Res Commun 320: 455-460, 2004.
158. Mailland M, Waelchli R, Ruat M, Boddeke HG, and Seuwen K. Stimulation of cell proliferation by calcium and a calcimimetic compound. Endocrinology 138: 3601-3605, 1997.
159. Mallya SM, Gallagher JJ, Wild YK, Kifor O, Costa-Guda J, Saucier K, Brown EM, and Arnold A. Abnormal parathyroid cell proliferation precedes biochemical abnormalities in a mouse model of primary hyperparathyroidism. Mol Endocrinol, 2005.
160. Mannstadt M, Juppner H, and Gardella TJ. Receptors for PTH and PTHrP: their biological importance and functional properties. Am J Physiol 277: F665-675, 1999.
161. Marti A, Luo Z, Cunningham C, Ohta Y, Hartwig J, Stossel TP, Kyriakis JM, and Avruch J. Actin-binding protein-280 binds the stress-activated protein kinase (SAPK) activator SEK-1 and is required for tumor necrosis factor-alpha activation of SAPK in melanoma cells. J Biol Chem 272: 2620-2628, 1997.
162. Martin-Salvago M, Villar-Rodriguez JL, Palma-Alvarez A, Beato-Moreno A, and Galera-Davidson H. Decreased expression of calcium receptor in parathyroid tissue in patients with hyperparathyroidism secondary to chronic renal failure. Endocr Pathol 14: 61-70, 2003.
163. Marx SJ, Attie MF, Spiegel AM, Levine MA, Lasker RD, and Fox M. An association between neonatal severe primary hyperparathyroidism and familial hypocalciuric hypercalcemia in three kindreds. N Engl J Med 306: 257-264, 1982.
164. McCabe CJ, Khaira JS, Boelaert K, Heaney AP, Tannahill LA, Hussain S, Mitchell R, Olliff J, Sheppard MC, Franklyn JA, and Gittoes NJ. Expression of pituitary tumour transforming gene (PTTG) and fibroblast growth factor-2 (FGF-2) in human pituitary adenomas: relationships to clinical tumour behaviour. Clin Endocrinol (Oxf) 58: 141-150, 2003.
165. McGehee DS, Aldersberg M, Liu KP, Hsuing S, Heath MJ, and Tamir H. Mechanism of extracellular Ca2+ receptor-stimulated hormone release from sheep thyroid parafollicular cells. J Physiol 502 (Pt 1): 31-44, 1997.
166. McNeil L, Hobson S, Nipper V, and Rodland KD. Functional calcium-sensing receptor expression in ovarian surface epithelial cells. Am J Obstet Gynecol 178: 305-313, 1998.
167. McNeil SE, Hobson SA, Nipper V, and Rodland KD. Functional calcium-sensing receptors in rat fibroblasts are required for activation of SRC kinase and mitogen-activated protein kinase in response to extracellular calcium. J Biol Chem 273: 1114-1120, 1998.
168. Merryman JI, Capen CC, McCauley LK, Werkmeister JR, Suter MM, and Rosol TJ. Regulation of parathyroid hormone-related protein production by a squamous carcinoma cell line in vitro. Lab Invest 69: 347-354, 1993.
169. Mitsuma T, Rhue N, Kayama M, Mori Y, Adachi K, Yokoi Y, Ping J, Nogimori T, and Hirooka Y. Distribution of calcium sensing receptor in rats: an immunohistochemical study. Endocr Regul 33: 55-59, 1999.
170. Mizobuchi M, Hatamura I, Ogata H, Saji F, Uda S, Shiizaki K, Sakaguchi T, Negi S, Kinugasa E, Koshikawa S, and Akizawa T. Calcimimetic compound upregulates decreased calcium-sensing receptor expression level in parathyroid glands of rats with chronic renal insufficiency. J Am Soc Nephrol 15: 2579-2587, 2004.
171. Morosetti R, Servidei T, Mirabella M, Rutella S, Mangiola A, Maira G, Mastrangelo R, and Koeffler HP. The PPARgamma ligands PGJ2 and rosiglitazone show a differential ability to inhibit proliferation and to induce apoptosis and differentiation of human glioblastoma cell lines. Int J Oncol 25: 493-502, 2004.
172. Murphy LO, Smith S, Chen RH, Fingar DC, and Blenis J. Molecular interpretation of ERK signal duration by immediate early gene products. Nat Cell Biol 4: 556-564, 2002.
173. Nagasaki K, Yamaguchi K, Miyake Y, Hayashi C, Honda S, Urakami K, Miki K, Kimura S, Watanabe T, Abe K, and et al. In vitro and in vivo antagonists against parathyroid hormone-related protein. Biochem Biophys Res Commun 158: 1036-1042, 1989.
174. Nasmyth K. Segregating sister genomes: the molecular biology of chromosome separation. Science 297: 559-565, 2002.
175. Nasmyth K, Peters JM, and Uhlmann F. Splitting the chromosome: cutting the ties that bind sister chromatids. Science 288: 1379-1385, 2000.
176. Naylor SL, Sakaguchi AY, Szoka P, Hendy GN, Kronenberg HM, Rich A, and Shows TB. Human parathyroid hormone gene (PTH) is on short arm of chromosome 11. Somatic Cell Genet 9: 609-616, 1983.
177. Nemeth EF. Regulation of cytosolic calcium by extracellular divalent cations in C-cells and parathyroid cells. Cell Calcium 11: 323-327, 1990.
178. Nemeth EF, Steffey ME, Hammerland LG, Hung BC, Van Wagenen BC, DelMar EG, and Balandrin MF. Calcimimetics with potent and selective activity on the parathyroid calcium receptor. Proc Natl Acad Sci U S A 95: 4040-4045, 1998.
179. O'Bryan MK, Schlatt S, Gerdprasert O, Phillips DJ, de Kretser DM, and Hedger MP. Inducible nitric oxide synthase in the rat testis: evidence for potential roles in both normal function and inflammation-mediated infertility. Biol Reprod 63: 1285-1293, 2000.
180. Oda Y, Tu CL, Pillai S, and Bikle DD. The calcium sensing receptor and its alternatively spliced form in keratinocyte differentiation. J Biol Chem 273: 23344-23352, 1998.
181. Ohanian J, Gatfield KM, Ward DT, and Ohanian V. Evidence for a Functional Calcium-Sensing Receptor that Modulates Myogenic Tone in Rat Subcutaneous Arteries. Am J Physiol Heart Circ Physiol, 2004.
182. Okazaki R, Chikatsu N, Nakatsu M, Takeuchi Y, Ajima M, Miki J, Fujita T, Arai M, Totsuka Y, Tanaka K, and Fukumoto S. A novel activating mutation in calcium-sensing receptor gene associated with a family of autosomal dominant hypocalcemia. J Clin Endocrinol Metab 84: 363-366, 1999.
183. Osherov N and Levitzki A. Epidermal-growth-factor-dependent activation of the src-family kinases. Eur J Biochem 225: 1047-1053, 1994.
184. Paget S. The distribution of secondary growths in cancer of the breast. Lancet 1: 571-572, 1889.
185. Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, and Tarnawski AS. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for

promoting colon cancer growth and gastrointestinal hypertrophy. Nat Med 8: 289-293, 2002.
186. Parfit A and Kleerekoper M. Clinical disorders of calcium, phosphorus and magnesium metabolism. In: Clinical Disorders of Fluid and Electrolyte Metabolism. New York: McGraw-Hill, 1980.
187. Parkash J, Chaudhry MA, and Rhoten WB. Calbindin-D28k and calcium sensing receptor cooperate in MCF-7 human breast cancer cells. Int J Oncol 24: 1111-1119, 2004.
188. Peacock M, Bilezikian JP, Klassen PS, Guo MD, Turner SA, and Shoback D. Cinacalcet hydrochloride maintains long-term normocalcemia in patients with primary hyperparathyroidism. J Clin Endocrinol Metab 90: 135-141, 2005.
189. Pearce SH, Bai M, Quinn SJ, Kifor O, Brown EM, and Thakker RV. Functional characterization of calcium-sensing receptor mutations expressed in human embryonic kidney cells. J Clin Invest 98: 1860-1866, 1996.
190. Pei L. Genomic organization and identification of an enhancer element containing binding sites for multiple proteins in rat pituitary tumor-transforming gene. J Biol Chem 273: 5219-5225, 1998.
191. Pei L and Melmed S. Isolation and characterization of a pituitary tumor-transforming gene (PTTG). Mol Endocrinol 11: 433-441, 1997.
192. Peters JM. The anaphase-promoting complex: proteolysis in mitosis and beyond. Mol Cell 9: 931-943, 2002.
193. Peters U, Chatterjee N, Yeager M, Chanock SJ, Schoen RE, McGlynn KA, Church TR, Weissfeld JL, Schatzkin A, and Hayes RB. Association of genetic variants in the calcium-sensing receptor with risk of colorectal adenoma. Cancer Epidemiol Biomarkers Prev 13: 2181-2186, 2004.
194. Pi M, Faber P, Ekema G, Jackson PD, Ting A, Wang N, Fontilla-Poole M, Mays RW, Brunden KR, Harrington JJ, and Quarles LD. Identification of a novel extracellular cation sensing G-protein coupled receptor. J Biol Chem, 2005.
195. Pi M, Oakley RH, Gesty-Palmer D, Cruickshank RD, Spurney RF, Luttrell LM, and Quarles LD. Beta-arrestin- and G protein receptor kinase-mediated calcium-sensing receptor desensitization. Mol Endocrinol 19: 1078-1087, 2005.
196. Plunkett RJ, Lis A, Barone TA, Fronckowiak MD, and Greenberg SJ. Hormonal effects on glioblastoma multiforme in the nude rat model. J Neurosurg 90: 1072-1077, 1999.
197. Pollak MR, Brown EM, Estep HL, McLaine PN, Kifor O, Park J, Hebert SC, Seidman CE, and Seidman JG. Autosomal dominant hypocalcaemia caused by a Ca(2+)-sensing receptor gene mutation. Nat Genet 8: 303-307, 1994.
198. Ponten J and Macintyre EH. Long term culture of normal and neoplastic human glia. Acta Pathol Microbiol Scand 74: 465-486, 1968.
199. Ponthier R and Rice B. Hypercalcemia of neoplasia, parathyroid hormone and vitamin D: Studies in parabiosis. Acta Endocrinol (Copenh) 77: 527-539, 1974.
200. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, and Ullrich A. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402: 884-888, 1999.
201. Qian J, Colbert MC, Witte D, Kuan CY, Gruenstein E, Osinska H, Lanske B, Kronenberg HM, and Clemens TL. Midgestational lethality in mice lacking the parathyroid hormone (PTH)/PTH-related peptide receptor is associated with abrupt cardiomyocyte death. Endocrinology 144: 1053-1061, 2003.
202. Quinn SJ, Bai M, and Brown EM. pH sensing by the calcium receptor. J Biol Chem 279: 37241-37249, 2004.
203. Quinn SJ, Kifor O, Trivedi S, Diaz R, Vassilev P, and Brown E. Sodium and ionic strength sensing by the calcium receptor. J Biol Chem 273: 19579-19586, 1998.
204. Quinn SJ, Ye CP, Diaz R, Kifor O, Bai M, Vassilev P, and Brown E. The Ca2+-sensing receptor: a target for polyamines. Am J Physiol 273: C1315-1323, 1997.
205. Rabbani SA, Gladu J, Liu B, and Goltzman D. Regulation in vivo of the growth of Leydig cell tumors by antisense ribonucleic acid for parathyroid hormone-related peptide. Endocrinology 136: 5416-5422, 1995.
206. Rabbani SA, Mitchell J, Roy DR, Kremer R, Bennett HP, and Goltzman D. Purification of peptides with parathyroid hormone-like bioactivity from human and rat malignancies associated with hypercalcemia. Endocrinology 118: 1200-1210, 1986.
207. Racz GZ, Kittel A, Riccardi D, Case RM, Elliott AC, and Varga G. Extracellular calcium sensing receptor in human pancreatic cells. Gut 51: 705-711, 2002.
208. Ralston SH, Gallacher SJ, Patel U, Campbell J, and Boyle IT. Cancer-associated hypercalcemia: morbidity and mortality. Clinical experience in 126 treated patients. Ann Intern Med 112: 499-504, 1990.
209. Ray K and Northup J. Evidence for distinct cation and calcimimetic compound (NPS 568) recognition domains in the transmembrane regions of the human Ca2+ receptor. J Biol Chem 277: 18908-18913, 2002.
210. Riccardi D, Park J, Lee WS, Gamba G, Brown EM, and Hebert SC. Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation-sensing receptor. Proc Natl Acad Sci U S A 92: 131-135, 1995.
211. Rice B, Ponthier R, and Miller M. Hypercalcemia and neoplasmia: a model system. Endocrinology 88: 1210-1216, 1971.
212. Rice B, Roth L, Cole F, MacPhee A, Davis K, Ponthier R, and Sternberg W. Hypercalcemia and neoplasia: biochemical and ultrastructural studies of a hypercalcemia producing Leydig cell tumor of the rat. Lab Invest 33: 428-439, 1975.
213. Ritter CS, Finch JL, Slatopolsky EA, and Brown AJ. Parathyroid hyperplasia in uremic rats precedes down-regulation of the calcium receptor. Kidney Int 60: 1737-1744, 2001.
214. Ritter CS, Martin DR, Lu Y, Slatopolsky E, and Brown AJ. Reversal of secondary hyperparathyroidism by phosphate restriction restores parathyroid calcium-sensing receptor expression and function. J Bone Miner Res 17: 2206-2213, 2002.
215. Rizzoli R and Bonjour JP. High extracellular calcium increases the production of a parathyroid hormone-like activity by cultured Leydig tumor cells associated with humoral hypercalcemia. J Bone Miner Res 4: 839-844, 1989.
216. Roberts RA, James NH, and Cosulich SC. The role of protein kinase B and mitogen-activated protein kinase in epidermal growth factor and tumor necrosis factor alpha-mediated rat hepatocyte survival and apoptosis. Hepatology 31: 420-427, 2000.
217. Rodan SB, Insogna KL, Vignery AM, Stewart AF, Broadus AE, D'Souza SM, Bertolini DR, Mundy GR, and Rodan GA. Factors associated with humoral hypercalcemia of malignancy stimulate adenylate cyclase in osteoblastic cells. J Clin Invest 72: 1511-1515, 1983.
218. Rodland KD. The role of the calcium-sensing receptor in cancer. Cell Calcium 35: 291-295, 2004.
219. Romoli R, Lania A, Mantovani G, Corbetta S, Persani L, and Spada A. Expression of calcium-sensing receptor and characterization of intracellular signaling in human pituitary adenomas. J Clin Endocrinol Metab 84: 2848-2853, 1999.
220. Roussanne MC, Lieberherr M, Souberbielle JC, Sarfati E, Drueke T, and Bourdeau A. Human parathyroid cell proliferation in response to calcium, NPS R-467, calcitriol and phosphate. Eur J Clin Invest 31: 610-616, 2001.
221. Ruat M, Molliver ME, Snowman AM, and Snyder SH. Calcium sensing receptor: molecular cloning in rat and localization to nerve terminals. Proc Natl Acad Sci U S A 92: 3161-3165, 1995.
222. Saez C, Japon MA, Ramos-Morales F, Romero F, Segura DI, Tortolero M, and Pintor-Toro JA. hpttg is over-expressed in pituitary adenomas and other primary epithelial neoplasias. Oncogene 18: 5473-5476, 1999.
223. Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, and Brown EM. Ca(2+)-sensing receptor expression and PTHrP secretion in PC-3 human prostate cancer cells. Am J Physiol Endocrinol Metab 281: E1267-1274, 2001.
224. Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, and Brown EM. Extracellular calcium-sensing receptor (CaR) expression and its potential role in parathyroid hormone-related peptide (PTHrP) secretion in the H-500 rat Leydig cell model of humoral hypercalcemia of malignancy. Biochem Biophys Res Commun 269: 427-432, 2000.
225. Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, Butters RR, and Brown EM. Extracellular calcium-sensing receptor expression and its potential role in regulating parathyroid hormone-related peptide secretion in human breast cancer cell lines. Endocrinology 141: 4357-4364, 2000.
226. Sands JM, Flores FX, Kato A, Baum MA, Brown EM, Ward DT, Hebert SC, and Harris HW. Vasopressin-elicited water and urea permeabilities are altered in IMCD in hypercalcemic rats. Am J Physiol 274: F978-985, 1998.
227. Sands JM, Naruse M, Baum M, Jo I, Hebert SC, Brown EM, and Harris HW. Apical extracellular calcium/polyvalent cation-sensing receptor regulates vasopressin-elicited water permeability in rat kidney inner medullary collecting duct. J Clin Invest 99: 1399-1405, 1997.
228. Schwarz P. Dose response dependency in regulation of acute PTH (1-84) release and suppression in normal humans: a citrate and calcium infusion study. Scand J Clin Lab Invest 53: 601-605, 1993.
229. Schwarz P, Hyldstrup L, and Transbol I. Cica clamp evaluation of parathyroid responsiveness in chronic hypoparathyroidism: a sequential citrate and calcium clamp study. Miner Electrolyte Metab 20: 135-140, 1994.
230. Schwarz P, Sorensen HA, McNair P, and Transbol I. Cica-clamp technique: a method for quantifying parathyroid hormone secretion: a sequential citrate and calcium clamp study. Eur J Clin Invest 23: 546-553, 1993.
231. Schwarz P, Sorensen HA, Momsen G, McNair P, and Transbol I. Normal pattern of parathyroid response to blood calcium lowering in primary hyperparathyroidism: a citrate clamp study. Clin Endocrinol (Oxf) 37: 344-348, 1992.
232. Schwarz P, Sorensen HA, and Transbol I. Inter-relations between the

calcium set-points of Parfitt and Brown in primary hyperparathyroidism: a sequential citrate and calcium clamp study. Eur J Clin Invest 24: 553-558, 1994.
233. Schwarz P, Sorensen HA, Transbol I, and McNair P. Regulation of acute parathyroid hormone release in normal humans: combined calcium and citrate clamp study. Am J Physiol 263: E195-198, 1992.
234. Seely BL, Samimi G, and Webster NJ. Retroviral expression of a kinase-defective IGF-I receptor suppresses growth and causes apoptosis of CHO and U87 cells in-vivo. BMC Cancer 2: 15, 2002.
235. Sheinin Y, Kallay E, Wrba F, Kriwanek S, Peterlik M, and Cross HS. Immunocytochemical localization of the extracellular calcium-sensing receptor in normal and malignant human large intestinal mucosa. J Histochem Cytochem 48: 595-602, 2000.
236. Shimada T, Mizutani S, Muto T, Yoneya T, Hino R, Takeda S, Takeuchi Y, Fujita T, Fukumoto S, and Yamashita T. Cloning and characterization of FGF23 as a causative factor of tumor-induced osteomalacia. Proc Natl Acad Sci U S A 98: 6500-6505, 2001.
237. Shoback DM, Bilezikian JP, Turner SA, McCary LC, Guo MD, and Peacock M. The calcimimetic cinacalcet normalizes serum calcium in subjects with primary hyperparathyroidism. J Clin Endocrinol Metab 88: 5644-5649, 2003.
238. Sica D, Martodam R, Aronow J, and Mundy G. The hypercalcemic rat Leydig cell tumor- a model of the humoral hypercalcemia of malignancy. Calcified Tissue Int 35: 287-293, 1983.
239. Silver IA, Murrills RJ, and Etherington DJ. Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. Exp Cell Res 175: 266-276, 1988.
240. Smajilovic S, Hansen JL, Christoffersen TE, Lewin E, Sheikh SP, Terwilliger EF, Brown EM, Haunso S, and Tfelt-Hansen J. Extracellular calcium sensing in rat aortic vascular smooth muscle cells. Biochem Biophys Res Commun 348: 1215-1223, 2006.
241. Speer G, Cseh K, Mucsi K, Takacs I, Dworak O, Winkler G, Szody R, Tisler A, and Lakatos P. Calcium-sensing receptor A986S polymorphism in human rectal cancer. Int J Colorectal Dis 17: 20-24, 2002.
242. Spiegel AM, Saxe AW, Deftos LJ, and Brennan MF. Humoral hypercalcemia caused by a rat Leydig-cell tumor is associated with suppressed parathyroid hormone secretion and increased urinary cAMP excretion. Horm Metab Res 15: 299-304, 1983.
243. Squires PE, Harris TE, Persaud SJ, Curtis SB, Buchan AM, and Jones PM. The extracellular calcium-sensing receptor on human beta-cells negatively modulates insulin secretion. Diabetes 49: 409-417, 2000.
244. Squires PE, Persaud SJ, Jones PM, and Buchan AM. The extracellular calcium-sensing receptor in PHHI beta cells: does reduced auto-inhibitory input contribute to hypersecretion of insulin? Diabetologia 43: 1078-1080, 2000.
245. Stewart AF, Horst R, Deftos LJ, Cadman EC, Lang R, and Broadus AE. Biochemical evaluation of patients with cancer-associated hypercalcemia: evidence for humoral and nonhumoral groups. N Engl J Med 303: 1377-1383, 1980.
246. Stewart AF, Vignery A, Silverglate A, Ravin ND, LiVolsi V, Broadus AE, and Baron R. Quantitative bone histomorphometry in humoral hypercalcemia of malignancy: uncoupling of bone cell activity. J Clin Endocrinol Metab 55: 219-227, 1982.
247. Stoika R and Melmed S. Expression and function of pituitary tumour transforming gene for T-lymphocyte activation. Br J Haematol 119: 1070-1074, 2002.
248. Strewler GJ. The physiology of parathyroid hormone-related protein. N Engl J Med 342: 177-185, 2000.
249. Suva LJ, Winslow GA, Hammonds RJ, Moseley JM, Diefenbach-Jagger H, Rodda CP, Kemp BE, Rodriguez H, and Chen EY. A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. Science 237: 893-896, 1987.
250. Takeno S, Osada R, Furukido K, Chen JH, and Yajin K. Increased nitric oxide production in nasal epithelial cells from allergic patients – RT-PCR analysis and direct imaging by a fluorescence indicator: DAF-2 DA. Clin Exp Allergy 31: 881-888, 2001.
251. Takeuchi S, Arai K, Saitoh H, Yoshida K, and Miura M. Urinary pyridinoline and deoxypyridinoline as potential markers of bone metastasis in patients with prostate cancer. JUrol 156: 1691-1695, 1996.
252. Tatsumi N, Fujisawa M, Kanzaki M, Okuda Y, Okada H, Arakawa S, and Kamidono S. Nitric oxide production by cultured rat Leydig cells. Endocrinology 138: 994-998, 1997.
253. Tfelt-Hansen J, Brixen K, Mosekilde L, and Schwarz P. Parathyroidea-hormonrelateret peptid-induceret humoral hyperkalkæmi ved malignitet. Ugeskr Læger 167: 924-926, 2005.
254. Tfelt-Hansen J and Brown EM. The calcium-sensing receptor in normal physiology and pathophysiology: a review. Crit Rev Clin Lab Sci 42: 35-70, 2005.
255. Tfelt-Hansen J, Chattopadhyay N, Yano S, Kanuparthi D, Rooney P, Schwarz P, and Brown EM. Calcium-sensing receptor induces proliferation through p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase but not extracellularly regulated kinase in a model of humoral hypercalcemia of malignancy. Endocrinology 145: 1211-1217, 2004.
256. Tfelt-Hansen J, Ferreira A, Yano S, Kanuparthi D, Romero JR, Brown EM, and Chattopadhyay N. Calcium-Sensing Receptor Activation Induces Nitric Oxide Production in H-500 Leydig Cancer Cells. Am J Physiol Endocrinol Metab 288: E1206-1213, 2005.
257. Tfelt-Hansen J, Hansen JL, Smajilovic S, Terwilliger EF, Haunso S, and Sheikh SP. The Calcium Receptor is Functionally Expressed in Rat Neonatal Ventricular Cardiomyocytes. Am J Physiol Heart Circ Physiol 290: H1165-1171, 2006.
258. Tfelt-Hansen J, Kanuparthi D, and Chattopadhyay N. The emerging role of pituitary tumor transforming gene in tumorigenesis. Clin Med Res 4: 130-137, 2006.
259. Tfelt-Hansen J, MacLeod RJ, Chattopadhyay N, Yano S, Quinn S, Ren X, Terwilliger EF, Schwarz P, and Brown EM. Calcium-sensing receptor stimulates PTHrP release by pathways dependent on PKC, p38 MAPK, JNK, and ERK1/2 in H-500 cells. Am J Physiol Endocrinol Metab 285: E329-337, 2003.
260. Tfelt-Hansen J, Schwarz P, Brown EM, and Chattopadhyay N. The calcium-sensing receptor in human disease. Front Biosci 8: s377-390, 2003.
261. Tfelt-Hansen J, Schwarz P, Terwilliger EF, Brown EM, and Chattopadhyay N. Calcium-sensing receptor induces messenger ribonucleic acid of human securin, pituitary tumor transforming gene, in rat testicular cancer. Endocrinology 144: 5188-5193, 2003.
262. Tfelt-Hansen J, Schwarz P, and Torring O. Rapid suppression of S-PTH by oral calcitriol and calcium in healthy premenopausal women. Scand J Clin Lab Invest 61: 395-400, 2001.
263. Tfelt-Hansen J, Yano S, Bandyopadhyay S, Carroll R, Brown EM, and Chattopadhyay N. Expression of pituitary tumor transforming gene (PTTG) and its binding protein in human astrocytes and astrocytoma cells: function and regulation of PTTG in U87 astrocytoma cells. Endocrinology 145: 4222-4231, 2004.
264. Tfelt-Hansen J, Yano S, John Macleod R, Smajilovic S, Chattopadhyay N, and Brown EM. High calcium activates the EGF receptor potentially through the calcium-sensing receptor in Leydig cancer cells. Growth Factors 23: 117-123, 2005.
265. Thornberry NA and Lazebnik Y. Caspases: enemies within. Science 281: 1312-1316, 1998.
266. Tomlins SA, Bolllinger N, Creim J, and Rodland KD. Cross-talk between the calcium-sensing receptor and the epidermal growth factor receptor in Rat-1 fibroblasts. Exp Cell Res 308: 439-445, 2005.
267. Torring O, Bucht E, and Sjoberg HE. Plasma calcitonin response to a calcium clamp. Influence of sex and age. Horm Metab Res 17: 536-539, 1985.
268. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, and et al. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266: 15771-15781, 1991.
269. Tovar Sepulveda VA and Falzon M. Parathyroid hormone-related protein enhances PC-3 prostate cancer cell growth via both autocrine/paracrine and intracrine pathways. Regul Pept 105: 109-120, 2002.
270. Tovar Sepulveda VA, Shen X, and Falzon M. Intracrine PTHrP protects against serum starvation-induced apoptosis and regulates the cell cycle in MCF-7 breast cancer cells. Endocrinology 143: 596-606, 2002.
271. Trechsel U, Bonjour JP, and Fleisch H. Regulation of the metabolism of 25-hydroxyvitamin D3 in primary cultures of chick kidney cells. J Clin Invest 64: 206-217, 1979.
272. Tu CL, Chang W, and Bikle DD. The extracellular calcium-sensing receptor is required for calcium-induced differentiation in human keratinocytes. J Biol Chem 276: 41079-41085, 2001.
273. Tu Q, Pi M, and Quarles LD. Calcyclin mediates serum response element (SRE) activation by an osteoblastic extracellular cation-sensing mechanism. J Bone Miner Res 18: 1825-1833, 2003.
274. Ushio-Fukai M, Griendling KK, Becker PL, Hilenski L, Halleran S, and Alexander RW. Epidermal growth factor receptor transactivation by angiotensin II requires reactive oxygen species in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 21: 489-495, 2001.
275. Valenti S, Cuttica CM, Giusti M, and Giordano G. Nitric oxide modulates Leydig cell function in vitro: is this a way of communication between the immune and endocrine system in the testis? Ann N Y Acad Sci 876: 298-300, 1999.
276. Valimaki S, Farnebo F, Forsberg L, Larsson C, and Farnebo LO. Heterogeneous expression of receptor mRNAs in parathyroid glands of secondary hyperparathyroidism. Kidney Int 60: 1666-1675, 2001.
277. Vallance P and Leiper J. Blocking NO synthesis: how, where and why? Nat Rev Drug Discov 1: 939-950, 2002.
278. VanHouten J, Dann P, McGeoch G, Brown EM, Krapcho K, Neville M, and Wysolmerski JJ. The calcium-sensing receptor regulates mammary gland parathyroid hormone-related protein production and calcium transport. J Clin Invest 113: 598-608, 2004.
279. Vlahos CJ, Matter WF, Hui KY, and Brown RF. A specific inhibitor of

phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem 269: 5241-5248, 1994.
280. Wang R, Xu C, Zhao W, Zhang J, Cao K, Yang B, and Wu L. Calcium and polyamine regulated calcium-sensing receptors in cardiac tissues. Eur J Biochem 270: 2680-2688, 2003.
281. Wang W, Lewin E, and Olgaard K. 1,25(OH)2D3 only affects long-term levels of plasma Ca2+ but not the rapid minute-to-minute plasma Ca2+ homeostasis in the rat. Steroids 64: 726-734, 1999.
282. Wang W, Lewin E, and Olgaard K. Role of calcitonin in the rapid minute-to-minute regulation of plasma Ca2+ homeostasis in the rat. Eur J Clin Invest 32: 674-681, 2002.
283. Wang Z and Melmed S. Characterization of the murine pituitary tumor transforming gene (PTTG) and its promoter. Endocrinology 141: 763-771, 2000.
284. Wang Z, Moro E, Kovacs K, Yu R, and Melmed S. Pituitary tumor transforming gene-null male mice exhibit impaired pancreatic beta cell proliferation and diabetes. Proc Natl Acad Sci U S A 100: 3428-3432, 2003.
285. Wang Z, Yu R, and Melmed S. Mice lacking pituitary tumor transforming gene show testicular and splenic hypoplasia, thymic hyperplasia, thrombocytopenia, aberrant cell cycle progression, and premature centromere division. Mol Endocrinol 15: 1870-1879, 2001.
286. Ward DT and Riccardi D. Renal physiology of the extracellular calcium-sensing receptor. Pflugers Arch 445: 169-176, 2002.
287. Wellendorph P and Brauner-Osborne H. Molecular cloning, expression, and sequence analysis of GPRC6A, a novel family C G-protein-coupled receptor. Gene 335: 37-46, 2004.
288. Wellendorph P, Hansen KB, Balsgaard A, Greenwood JR, Egebjerg J, and Brauner-Osborne H. Deorphanization of GPRC6A: a promiscuous L-alpha-amino acid receptor with preference for basic amino acids. Mol Pharmacol 67: 589-597, 2005.
289. Weston AH, Absi M, Ward DT, Ohanian J, Dodd RH, Dauban P, Petrel C, Ruat M, and Edwards G. Evidence in Favor of a Calcium-Sensing Receptor in Arterial Endothelial Cells. Studies With Calindol and Calhex 231. Circ Res, 2005.
290. Wetzker R and Bohmer FD. Transactivation joins multiple tracks to the ERK/MAPK cascade. Nat Rev Mol Cell Biol 4: 651-657, 2003.
291. Won JG, Tseng HS, Yang AH, Tang KT, Jap TS, Kwok CF, Lee CH, and Lin HD. Intra-arterial calcium stimulation test for detection of insulinomas: detection rate, responses of pancreatic peptides, and its relationship to differentiation of tumor cells. Metabolism 52: 1320-1329, 2003.
292. Wu P, Phillips MI, Bui J, and Terwilliger EF. Adeno-associated virus vector-mediated transgene integration into neurons and other nondividing cell targets. J Virol 72: 5919-5926, 1998.
293. Yamaguchi T, Chattopadhyay N, Kifor O, and Brown EM. Extracellular calcium (Ca2+(o))-sensing receptor in a murine bone marrow-derived stromal cell line (ST2): potential mediator of the actions of Ca2+(o) on the function of ST2 cells. Endocrinology 139: 3561-3568, 1998.
294. Yamaguchi T, Chattopadhyay N, Kifor O, Butters RR, Jr., Sugimoto T, and Brown EM. Mouse osteoblastic cell line (MC3T3-E1) expresses extracellular calcium (Ca2+o)-sensing receptor and its agonists stimulate chemotaxis and proliferation of MC3T3-E1 cells. J Bone Miner Res 13: 1530-1538, 1998.
295. Yamaguchi T, Chattopadhyay N, Kifor O, Ye C, Vassilev PM, Sanders JL, and Brown EM. Expression of extracellular calcium-sensing receptor in human osteoblastic MG-63 cell line. Am J Physiol Cell Physiol 280: C382-393, 2001.
296. Yamaguchi T, Kifor O, Chattopadhyay N, Bai M, and Brown EM. Extracellular calcium (Ca2+o)-sensing receptor in a mouse monocyte-macrophage cell line (J774): potential mediator of the actions of Ca2+o on the function of J774 cells. J Bone Miner Res 13: 1390-1397, 1998.
297. Yamaguchi T, Yamauchi M, Sugimoto T, Chauhan D, Anderson KC, Brown EM, and Chihara K. The extracellular calcium Ca2+o-sensing receptor is expressed in myeloma cells and modulates cell proliferation. Biochem Biophys Res Commun 299: 532-538, 2002.
298. Yamamoto M, Akatsu T, Nagase T, and Ogata E. Comparison of hypocalcemic hypercalciuria between patients with idiopathic hypoparathyroidism and those with gain-of-function mutations in the calcium-sensing receptor: is it possible to differentiate the two disorders? J Clin Endocrinol Metab 85: 4583-4591, 2000.
299. Yan Y, Shirakabe K, and Werb Z. The metalloprotease Kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors. J Cell Biol 158: 221-226, 2002.
300. Yano S, Brown EM, and Chattopadhyay N. Calcium-sensing receptor in the brain. Cell Calcium 35: 257-264, 2004.
301. Yano S, Macleod RJ, Chattopadhyay N, Tfelt-Hansen J, Kifor O, Butters RR, and Brown EM. Calcium-sensing receptor activation stimulates parathyroid hormone-related protein secretion in prostate cancer cells: role of epidermal growth factor receptor transactivation. Bone 35: 664-672, 2004.
302. Yano S, Sugimoto T, Tsukamoto T, Chihara K, Kobayashi A, Kitazawa S, Maeda S, and Kitazawa R. Association of decreased calcium-sensing receptor expression with proliferation of parathyroid cells in secondary hyperparathyroidism. Kidney Int 58: 1980-1986, 2000.
303. Yano S, Sugimoto T, Tsukamoto T, Chihara K, Kobayashi A, Kitazawa S, Maeda S, and Kitazawa R. Decrease in vitamin D receptor and calcium-sensing receptor in highly proliferative parathyroid adenomas. Eur J Endocrinol 148: 403-411, 2003.
304. Ye C, Chattopadhyay N, Brown EM, and Vassilev PM. Defective extracellular calcium (Ca(o))-sensing receptor (CaR)-mediated stimulation of a Ca(2+)-activated potassium channel in glioblastoma cells transfected with a dominant negative CaR. Brain Res Mol Brain Res 80: 177-187, 2000.
305. Ye C, Ho PC, Kanazirska M, Quinn S, Rogers K, Seidman CE, Seidman JG, Brown EM, and Vassilev PM. Amyloid-beta proteins activate Ca(2+)-permeable channels through calcium-sensing receptors. J Neurosci Res 47: 547-554, 1997.
306. Ye C, Kanazirska M, Quinn S, Brown EM, and Vassilev PM. Modulation by polycationic Ca(2+)-sensing receptor agonists of nonselective cation channels in rat hippocampal neurons. Biochem Biophys Res Commun 224: 271-280, 1996.
307. Ye CP, Yamaguchi T, Chattopadhyay N, Sanders JL, Vassilev PM, and Brown EM. Extracellular calcium-sensing-receptor (CaR)-mediated opening of an outward K(+) channel in murine MC3T3-E1 osteoblastic cells: evidence for expression of a functional CaR. Bone 27: 21-27, 2000.
308. Ye CP, Yano S, Tfelt-Hansen J, MacLeod RJ, Ren X, Terwilliger E, Brown EM, and Chattopadhyay N. Regulation of a Ca2+-activated K+ channel by calcium-sensing receptor involves p38 MAP kinase. J Neurosci Res 75: 491-498, 2004.
309. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, and Kato S. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet 16: 391-396, 1997.
310. Yu H, Peters JM, King RW, Page AM, Hieter P, and Kirschner MW. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science 279: 1219-1222, 1998.
311. Yu R and Melmed S. Pituitary tumor transforming gene: an update. Front Horm Res 32: 175-185, 2004.
312. Zhang M and Breitwieser GE. High affinity interaction with filamin A protects against calcium sensing receptor degradation. J Biol Chem, 2005.
313. Zhang X, Horwitz GA, Heaney AP, Nakashima M, Prezant TR, Bronstein MD, and Melmed S. Pituitary tumor transforming gene (PTTG) expression in pituitary adenomas. J Clin Endocrinol Metab 84: 761-767, 1999.
314. Zhang Z, Jiang Y, Quinn SJ, Krapcho K, Nemeth EF, and Bai M. L-phenylalanine and NPS R-467 synergistically potentiate the function of the extracellular calcium-sensing receptor through distinct sites. J Biol Chem 277: 33736-33741, 2002.
315. Zhang Z, Qiu W, Quinn SJ, Conigrave AD, Brown EM, and Bai M. Three adjacent serines in the extracellular domains of the CaR are required for L-amino acid-mediated potentiation of receptor function. J Biol Chem 277: 33727-33735, 2002.
316. Ziegelstein RC, Xiong Y, He C, and Hu Q. Expression of a functional extracellular calcium-sensing receptor in human aortic endothelial cells. Biochem Biophys Res Commun 342: 153-163, 2006.
317. Zou H, McGarry TJ, Bernal T, and Kirschner MW. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science 285: 418-422, 1999.