

# Regulation of secretion of PTHrP by $\text{Ca}^{2+}$ -sensing receptor in human astrocytes, astrocytomas, and meningiomas

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**Chattopadhyay, Naibedya, Cetin Evliyaoglu, Oliver Heese, Rona Carroll, Jennifer Sanders, Peter Black, and Edward M. Brown.** Regulation of secretion of PTHrP by  $\text{Ca}^{2+}$ -sensing receptor in human astrocytes, astrocytomas, and meningiomas. *Am J Physiol Cell Physiol* 279: C691–C699, 2000.—Parathyroid hormone-related protein (PTHrP) is the major mediator of the humoral hypercalcemia of malignancy and of malignant osteolysis associated with skeletal metastases of common epithelial cancers. PTHrP secretion is regulated by the extracellular calcium concentration ( $[\text{Ca}^{2+}]_o$ ) in several types of normal and malignant cells. Because the  $[\text{Ca}^{2+}]_o$ -sensing receptor (CaR) is a key mediator of  $[\text{Ca}^{2+}]_o$ -regulated hormone secretion [e.g., of parathyroid hormone (PTH) by parathyroid chief cells], we investigated the expression of the CaR and PTHrP in normal and neoplastic glial cells and studied the effects of  $[\text{Ca}^{2+}]_o$  on PTHrP secretion. Our results show that primary embryonic human astrocytes (HPA) express CaR mRNA and protein as detected by RT-PCR and Western analysis, respectively. Furthermore, astrocytomas and meningiomas also express the CaR at similar levels as assessed by RT-PCR and Northern and Western blot analyses. HPA and astrocytomas express transcripts encoding all three known isoforms of PTHrP [PTHrP(139), PTHrP(141), and PTHrP(173), comprising 139, 141, and 173 predicted amino acid residues, respectively] as assessed by RT-PCR, whereas meningiomas express only the first two of these. Finally, elevated levels of  $[\text{Ca}^{2+}]_o$  and other polycationic CaR agonists dose dependently stimulate PTHrP secretion from HPA, astrocytomas, and meningiomas, although both basal and high  $[\text{Ca}^{2+}]_o$ -stimulated rates of PTHrP secretion are ~2.5-fold higher in HPA than in the glial tumors studied here. Therefore, our results show that HPA, astrocytomas, and meningiomas express both the CaR and PTHrP and that CaR agonists stimulate PTHrP secretion.

brain tumor; glioma; proliferation; humoral hypercalcemia of malignancy

PARATHYROID HORMONE-RELATED PROTEIN (PTHrP) was originally discovered as a tumor-derived, adenylate cyclase-stimulating factor associated with the endocrine neoplastic syndrome, humoral hypercalcemia of

malignancy (HHM) (39, 17). Features of HHM can be observed with tumors of the respiratory, gastrointestinal, and urogenital tracts as well as with hematological malignancies and breast cancer (12, 32, 34). Excessive PTHrP secretion from these various tumor cells produces hypercalcemia by stimulating bone resorption as well as by increasing renal tubular reabsorption of calcium (18). Further investigations have revealed that numerous normal cells produce PTHrP, which serves as an important paracrine/autocrine regulator of growth, differentiation, transepithelial calcium transport (1, 14), smooth muscle relaxation, and diverse other cellular functions, many of which are mediated by the PTH/PTHrP receptor, a G protein-coupled receptor (32, 27). PTHrP expression in normal and neoplastic cells is regulated by many factors, including glucocorticoids (24, 28), epidermal growth factor, (21), transforming growth factor- $\beta$  (30), interleukin-6 (34), tumor necrosis factor- $\alpha$  (34), vitamin D (2), and 9-*cis*-retinoic acid (2).

Elevations in the extracellular calcium concentration ( $[\text{Ca}^{2+}]_o$ ) are known to stimulate PTHrP secretion from squamous (30) and cervical cancer cells (26) and normal human keratinocytes (22). The cloning of an extracellular calcium-sensing receptor (CaR) has defined an important mediator of the direct actions of  $[\text{Ca}^{2+}]_o$  on various aspects of cellular function (5, 6). The CaR is well recognized to play key roles in systemic calcium homeostasis by enabling  $[\text{Ca}^{2+}]_o$  to act as an “extracellular first messenger” that regulates target organs such as parathyroid gland (6), kidney (33), and bone (8). More recently, the CaR has also been demonstrated to modulate numerous cellular processes in several cell types apparently uninvolved in systemic calcium homeostasis, including proliferation and differentiation, apoptosis, and maintenance of membrane potential (5). Notable among these latter cell types are several that are derived from the central nervous system (CNS). The CaR has been cloned from rat striatum (36) and is also expressed in neurons of the CNS (7, 35)

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as well as in perivascular sensory nerves (42). In the CNS, the CaR has also been shown to be functionally expressed in oligodendrocytes (9) and microglia (10) as well as in the human astrocytic tumor cell line U87 (11). Aspects of cellular function that are modulated by the CaR in these cells include [Ca<sup>2+</sup>]<sub>o</sub>-activated K<sup>+</sup> channels, nonselective cation channels, and cellular proliferation (9–11, 41, 47).

Rat meningeoepithelium expresses PTHrP transcripts and peptides, whereas astrocytes express the PTH/PTHrP receptor, thereby strongly suggesting that PTHrP could provide a mechanism through which meningeoepithelial cells can communicate in a paracrine manner with astrocytes via the PTHrP receptor (38). In rat astrocytes, PTHrP, possibly acting via activation of adenylate cyclase, elicits astrocytic process extension, an *in vitro* correlate of the formation of the glial-limiting membrane *in situ* (19). Thus PTHrP could be an important mediator of astrocytic differentiation. To date, however, there are no data regarding PTHrP secretion and its potential regulation by the CaR in normal human glial cells and in gliomas.

In the present study, we report that human astrocytomas and meningiomas as well as human embryonic primary astrocytes (HPA) express and secrete PTHrP, unlike their normal rodent counterparts (38). We also show that HPA secrete substantially more PTHrP than do the tumor cells in culture, these normal and neoplastic cells both express the CaR, and polycationic CaR agonists dose dependently stimulate PTHrP secretion in all cell types that we studied.

## MATERIALS AND METHODS

**Tissues.** HPA were purchased from Clonetics-BioWhittaker (Walkersville, MD). Brain tumor tissues, e.g., astrocytomas and meningiomas, were obtained from operations performed at the Brigham and Women's Hospital and Children's Hospital in Boston during the years 1995–1999. These discarded human tissues were obtained and studied under a protocol approved by the Committee for the Protection of Human Subjects in Research at the Brigham and Women's Hospital. A portion of the tissue was immediately fixed in 4% Formalin for 48 h, embedded in paraffin, and used for immunohistochemistry and routine histopathological studies. Immunohistochemical studies were performed on 16 astrocytomas (5 low-grade astrocytomas, 5 anaplastic astrocytomas, and 6 glioblastoma multiforme) and 2 meningiomas. Other portions of the tumor tissues were immediately frozen and stored in liquid nitrogen or embedded in OCT compound (Miles, Elkhart, IN) and stored at –70°C for RT-PCR, Northern analysis, and/or Western blot analysis.

**Cell culture.** All routine culture media were obtained from GIBCO-BRL (Grand Island, NY). HPA were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1× G-5 astrocyte supplement diluted from a 100× stock as per the supplier's recommendations (Life Technologies, Gaithersburg, MD). Tumor cells were enzymatically dispersed from tumor specimens immediately after surgical removal using trypsin and DNase. Cells were cultured from astrocytomas (2 anaplastic astrocytoma and 3 glioblastoma multiforme) under conditions similar to those used for fetal HPA. Cells were cultured from meningiomas (*n* = 5) in α-MEM supplemented with 10% FBS.

**RT-PCR of CaR and PTHrP transcripts in HPA and tumor cells.** Total RNA was prepared from monolayers of HPA as well as from primary astrocytoma and meningioma cells grown in 25-cm<sup>2</sup> culture flasks using the TRIzol Reagent (GIBCO-BRL). Total RNA (2 μg) was used for the synthesis of single-stranded cDNA (cDNA synthesis kit, GIBCO-BRL). The resultant first-stranded cDNA was used for the PCR procedure. PCR was performed in a buffer with the following constituents (in mM): 20 Tris·HCl (pH 8.4), 50 KCl, 1.8 MgCl<sub>2</sub>, 0.2 dNTP, and 0.4 μM of forward primer, 0.4 μM of reverse primer, and 1 μl of *Taq* DNA polymerase (GIBCO BRL).

Primers based on the sequence of the human parathyroid CaR were used to detect CaR transcripts in HPA and tumor cells, and their sequences are given below. This set of primers was designed to span two introns of the human CaR gene to avoid confusion arising from amplification of a similar-sized product arising from contaminating genomic DNA: sense, 5'-CGGGGTACCTTAAGCACCTACGGCATCTAA-3'; and antisense, 5'-GCTCTAGAGTTAACGCGATCCCAAAGGGCTC-3'.

For detection of transcripts of PTHrP, the following primer pairs were used. For all isoforms: sense, 5'-ATGCAGCGAGACTGGTTCAG-3'; and antisense, 5'-TCAATGCCTCCGTGAATCGAGCTCCAGCGACGT-3'.

For PTHrP(139): sense, 5'-ATGCAGCGGAGACTGGTTCAG-3'; and antisense, 5'-AAGGGAGGCAGCTGAGAGCAC-3'.

For PTHrP(141): sense, 5'-ATGCAGCGGAGACTGGTTCAG-3'; and antisense, 5'-GTCCTTGGAAGGTCTCTGCTG-3'.

For PTHrP(173): sense, 5'-ATGCAGCGGAGACTGGTTCAG-3'; and antisense, 5'-TTCTAGTGCCACTGCCATTG-3'.

To perform "hot start" PCR, the enzyme was added during the initial 3-min denaturation, which was followed by 35 cycles of amplification (30-s denaturation at 94°C, 30-s annealing at 55°C, and 1-min extension at 72°C). The reaction was completed with an additional 10-min incubation at 72°C to allow completion of extension. PCR products were fractionated on 1.2% agarose gels. The presence of a 485-bp amplified product was indicative of a positive PCR reaction arising from CaR-related sequence within cDNA. PCR products in the reaction mixture were purified using the QIAquick PCR purification kit (Qiagen, Santa Clarita, CA) and subjected to direct, bidirectional sequencing employing the same primer pairs used for PCR by means of an automated sequencer (AB377, Applied Biosystems, Foster City, CA) in the DNA Sequence Facility of the University of Maine (Orono, ME) using dideoxy terminator *Taq* technology.

**Northern blot analysis of the CaR in HPA and tumor cells.** To determine the size(s) of CaR transcripts in HPA and tumor cells, Northern blot analysis was employed on aliquots of 5.0 μg poly(A<sup>+</sup>) RNA obtained using oligo(dT) cellulose chromatography of total RNA. RNA samples were denatured and electrophoresed in 2.2 M formaldehyde-1% agarose gels along with an 0.24–9.5 kb RNA ladder (GIBCO-BRL) and transferred overnight to nylon membranes (Duralon, Stratagene, La Jolla, CA). A 486-bp *Kpn* I-*Xba* I fragment corresponding to nucleotides 1745 to 2230 of the human parathyroid CaR cDNA was subcloned into the pBluescript(SK<sup>+</sup>) vector. The plasmid was then linearized with *Kpn* I, and a <sup>32</sup>P-labeled riboprobe was synthesized with the MAXIScript T3 kit (Pharmacia Biotech, Piscataway, NJ) using T3 polymerase and <sup>32</sup>P-UTP. Nylon membranes were prehybridized for 2 h at 60°C in a solution consisting of 50% formamide, 4× Denhardt's solution (50× Denhardt's = 5 g Ficoll, 5 g polyvinylpyrrolidone, and 5 g BSA per 50 ml), 5× SSPE (20× SSPE = 2.98 M NaCl and 0.02 M EDTA in 0.2 M phosphate buffer, pH 7.0), 0.5% SDS, 10% dextran sulfate, 250 μg/ml

yeast tRNA, and 200 µg/ml calf thymus DNA. The labeled cRNA probe ( $2 \times 10^6$  cpm/ml) was then added, and the membranes were hybridized overnight at 65°C. Washing was carried out at high stringency [ $0.1 \times$  SSC ( $20 \times$  SSC = 3 M NaCl and 0.3 M Na<sub>3</sub>-citrate·2H<sub>2</sub>O), 0.1% SDS at 68°C] for 30 min. The membranes were sealed in plastic and exposed to a PhosphorImager screen. The screens were analyzed on a Molecular Dynamics, PhosphorImager (Sunnyvale, CA) with the ImageQuant program.

**Western analysis of the CaR in HPA and tumor cells.** Monolayers of HPA or of primary astrocytic or meningioma tumor cells cultured in six-well plates were rinsed twice with 1 mM EDTA in PBS and lysed with 1.0 ml of a lysis solution (1% SDS, 10 mM Tris·HCl, pH 7.4) heated to 65°C. The cells were scraped from the flasks, transferred to microcentrifuge tubes, and heated for an additional 5 min at 65°C. The viscosity of the samples was reduced by brief sonication, and insoluble material was removed by centrifugation for 5 min. The resultant whole cell lysate in the supernatant was stored at -20°C until Western blot analysis was carried out.

Aliquots of 50 µg of cell proteins were dissolved in SDS-Laemmli gel loading buffer containing 100 mM dithiothreitol, incubated at 37°C for 15 min, and resolved electrophoretically on 6–12% gradient SDS-polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose blots at 240 mA for 40 min in transfer buffer containing 19 mM Tris·HCl, 150 mM glycine, 0.015% SDS, and 20% methanol. The blots were blocked overnight with 5% carnation milk, 1.5% goat serum, and 0.25% Triton X-100 in PBS (blocking solution). The blots were incubated at room temperature for 3 h with 1:250 dilution of 1 mg/ml of an affinity-purified polyclonal anti-CaR antiserum in blocking solution (4637; a generous gift of Drs. Forrest Fuller and Karen Krapcho, NPS Pharmaceuticals, Salt Lake City, UT), which was raised to a peptide corresponding to residues 345–359 within the bovine CaR's extracellular domain (3, 7, 9–11, 25). The specificity of immunoreactive bands was assessed by performing the same procedure with peptide-blocked antiserum [the same concentration of the 4637 antiserum preincubated at room temperature for 30 min with twice the amount (wt/vol) of the peptide to which the antibody was raised]. The blots were then washed three times with PBS containing 0.25% Triton X-100 (washing solution) at room temperature for 30 min each. The blots were further incubated with a 1:1,000 dilution of horseradish peroxidase-coupled goat anti-rabbit IgG (Sigma Chemical) in blocking solution for 1 h at room temperature. The blots were then washed three times with washing solution at room temperature for 20 min each, and specific protein bands were detected using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

**Immunohistochemistry of CaR and PTHrP in tumor tissues.** Immunohistochemistry for PTHrP was performed on 6-µm paraffin-embedded sections that were deparaffinized in xylene for 20 min, washed with ethanol at progressively lower concentrations (100, 95, 80, and 60%) for 1 min each, and then washed with deionized water for 5 min. Slides were then subjected to "antigen retrieval" by heating in a microwave for 10 min in 10 mM sodium citrate buffer, washed three times with PBS, incubated for 20 min in 0.5% H<sub>2</sub>O<sub>2</sub>, and rinsed again in PBS. After incubation with 1% horse serum as a blocking solution for 30 min, the primary monoclonal antibody against PTHrP at a 1:100 dilution [antibody 9H7; raised against human PTHrP-(109–141); a generous gift from Dr. L. J. Deftos (San Diego, CA)] was added, and the slides were incubated overnight at 4°C. Anti-mouse secondary antiserum was then added for 30 min, and color development of the avidin-biotin-horseradish peroxidase complex

was performed using the ABC kit (Vector Laboratories, Burlingame, CA). Slides were washed twice in PBS for 5 min each between each step. The 3,3'-diaminobenzidine tetrahydrochloride chromagen produced brown staining of positive cells. Tissue sections were counterstained with hematoxylin, dehydrated in ethanol, and mounted. Negative controls were carried out by incubating primary antibody against PTHrP with the immunogenic peptide against which it was raised. Frozen sections of human placenta and umbilical cord served as positive controls for expression of PTHrP (1) (data not shown).

Determination of the expression of the CaR by immunohistochemistry in tumor specimens was performed on frozen sections (5 µm) fixed in cold acetone, washed three times with PBS, incubated for 20 min in 0.3% H<sub>2</sub>O<sub>2</sub>, and rinsed again in PBS. After incubation with 5% BSA for 20 min as a blocking reagent, the primary antibody was added (5 µg/ml anti-CaR antiserum, 4637), and the slides were incubated overnight at 4°C. This was followed by incubation with goat anti-rabbit IgG secondary antibody for 30 min and then the same procedures as described above for immunohistochemistry for PTHrP. Negative controls were carried out by performing the same procedures, except that the primary anti-CaR antiserum was first preabsorbed with the peptide against which it was raised, as described above for Western analysis.

Astrocytic tumor specimens were classified according to the World Health Organization histological classification criteria. Immunoreactive staining was graded on a scale of weak to strong depending on the percentage of the tumor cells in the section that stained positively in the manner of Iezzoni et al. (23). A score of 0 indicated the total absence of positively stained cells compared with the control; +, indicated patchy immunostaining (1–30% cells positive); ++, moderate immunostaining (31–60% cells positive); and +++, strong immunostaining (61–100% cells positive).

**In vitro PTHrP release.** PTHrP secretion studies were performed within the first four passages in culture, because in preliminary studies we observed that cells maintained in culture for five or more passages exhibited greatly reduced secretion of PTHrP (data not shown). For studying PTHrP secretion, HPA or tumor cells cultured by the methods described above were first incubated for 16–18 h in DMEM (containing 1.8 mM Ca<sup>2+</sup>) supplemented with 10% FBS and then DMEM (containing 0.5 mM Ca<sup>2+</sup> and 4.0 mM L-glutamine) with 0.2% BSA for 6 h. The effects of polycationic CaR agonists on PTHrP secretion were subsequently studied as follows: DMEM (containing 0.5 mM Ca<sup>2+</sup>, 4.0 mM L-glutamine, and 0.2% BSA) was supplemented with 2.0, 3.0, or 4.0 mM CaCl<sub>2</sub>, 100 µM neomycin or 50 µM [Gd<sup>3+</sup>]<sub>o</sub>, and maintained in a cell culture incubator for 24 h in 24-well plates. PTHrP was measured in the conditioned media using a two-site immunoradiometric assay (Nichols Institute Diagnostics, San Juan, CA) that employs two polyclonal antisera specific for the NH<sub>2</sub>-terminal (1–40) and midregions (60–72) of the PTHrP molecule. Assay sensitivity was 1.9 mIU/ml. The PTHrP content of each well was normalized by determining protein concentration.

**Statistics.** Results are expressed as the mean ± SE. Statistical evaluation for differences between groups was done using one-way ANOVA followed by Fisher's protected least significant difference. For all statistical tests, values of *P* < 0.05 were considered to indicate a statistically significant difference between groups.

## RESULTS

*Identification and characterization of PTHrP and CaR proteins in HPA, glioblastomas, and meningiomas by immunohistochemistry and Western blotting.* We initially performed immunohistochemistry to screen for expression of the CaR and of PTHrP in gliomas and meningiomas. Abundant expression of PTHrP immunoreactivity was observed in glioblastomas and meningiomas, and examples are shown in Fig. 1, A and B (illustrating representative data from the total of 16 astrocytomas and 2 meningiomas that were studied in this manner). Adjacent sections incubated with primary antibody preabsorbed with its immunogenic peptide served as negative controls (Fig. 1, C and D). The PTHrP immunoreactivity was localized in the cytoplasm and associated with the cell membrane. All astrocytic tumors and meningiomas also expressed specific CaR immunoreactivity within the cytoplasm and cell membrane of tumor cells, and representative examples are illustrated in Fig. 2, A and B. The specificity of the staining was documented by showing that the immunoreactivity was abolished after preabsorption of the antiserum with the peptide against which it was raised (Fig. 2, C and D). Table 1 shows the relative levels of expression of the CaR and of PTHrP in various

astrocytomas, including low-grade astrocytomas, anaplastic astrocytomas, and glioblastoma multiforme, which were graded in the manner of Iezzoni et al. (23).

To further document expression of the CaR protein, we performed Western blot analysis on proteins isolated from HPA, astrocytic tumors, and meningiomas using the same affinity-purified, polyclonal anti-CaR antiserum (4637) used for immunohistochemistry. Several closely apposed CaR-immunoreactive bands were observed with apparent molecular weights between ~150 and 160 kDa (Fig. 3A), which in other tissues have been shown to represent species of the CaR protein glycosylated to varying extents (3). The specificity of the bands identified with the anti-CaR antiserum was confirmed by the abolition of their immunoreactivity after preabsorption of the antiserum with the peptide against which it was raised (Fig. 3B). There were no obvious differences in the levels of expression of the CaR in the various tissues and cells examined.

*Identification and characterization of transcripts for PTHrP and CaR.* We next identified and characterized the transcripts for PTHrP and the CaR in HPA as well as in selected glioblastomas and meningiomas using RT-PCR and Northern analysis. Total RNA extracted from HPA and from various glioblastomas and menin-

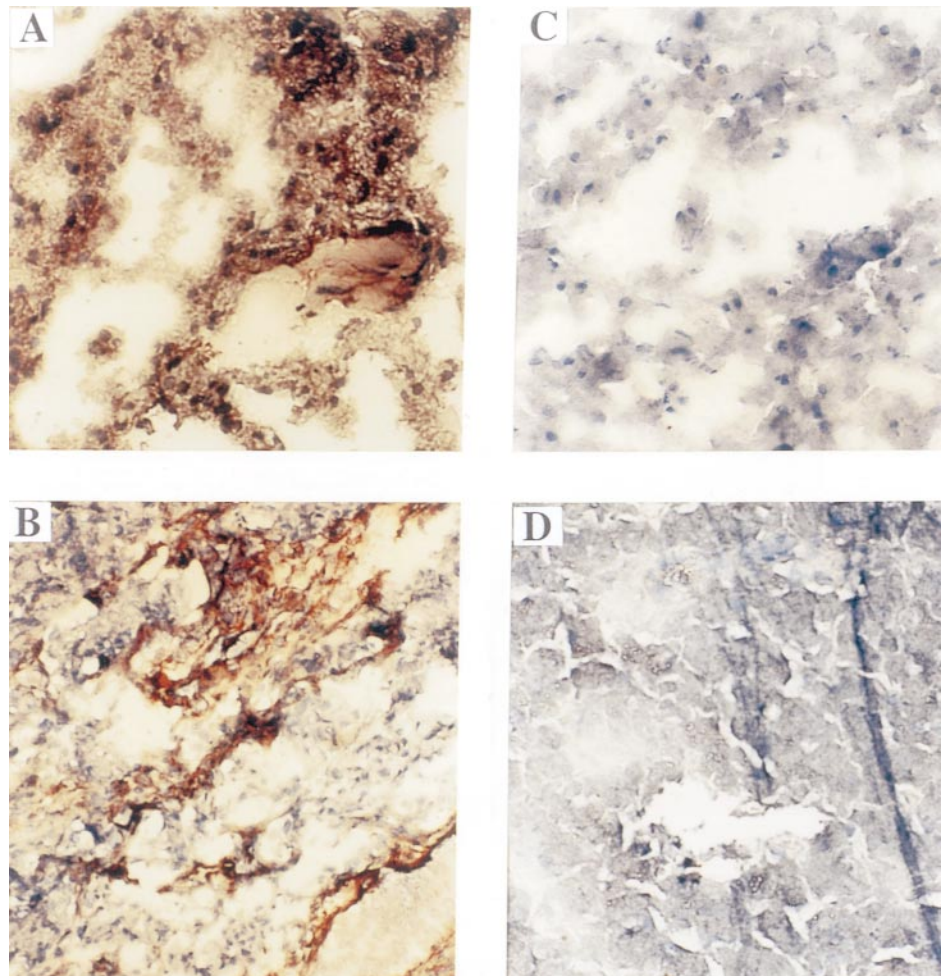


Fig. 1. Representative examples of the immunohistochemical localization of parathyroid hormone-related peptide (PTHrP) in human astrocytomas ( $n = 19$ ) and meningiomas ( $n = 32$ ) using a monoclonal anti-PTHrP antibody as described in MATERIALS AND METHODS. A: astrocytoma; abundant immunoreactivity was observed in neoplastic astrocytes. B: meningioma; abundant immunoreactivity was observed especially in concentric cells. Negative controls were performed by preabsorbing the PTHrP antibody with its immunogenic peptide before carrying out the immunohistochemical staining of astrocytoma (C) and meningioma (D). All photographs were taken at  $\times 200$  magnification.

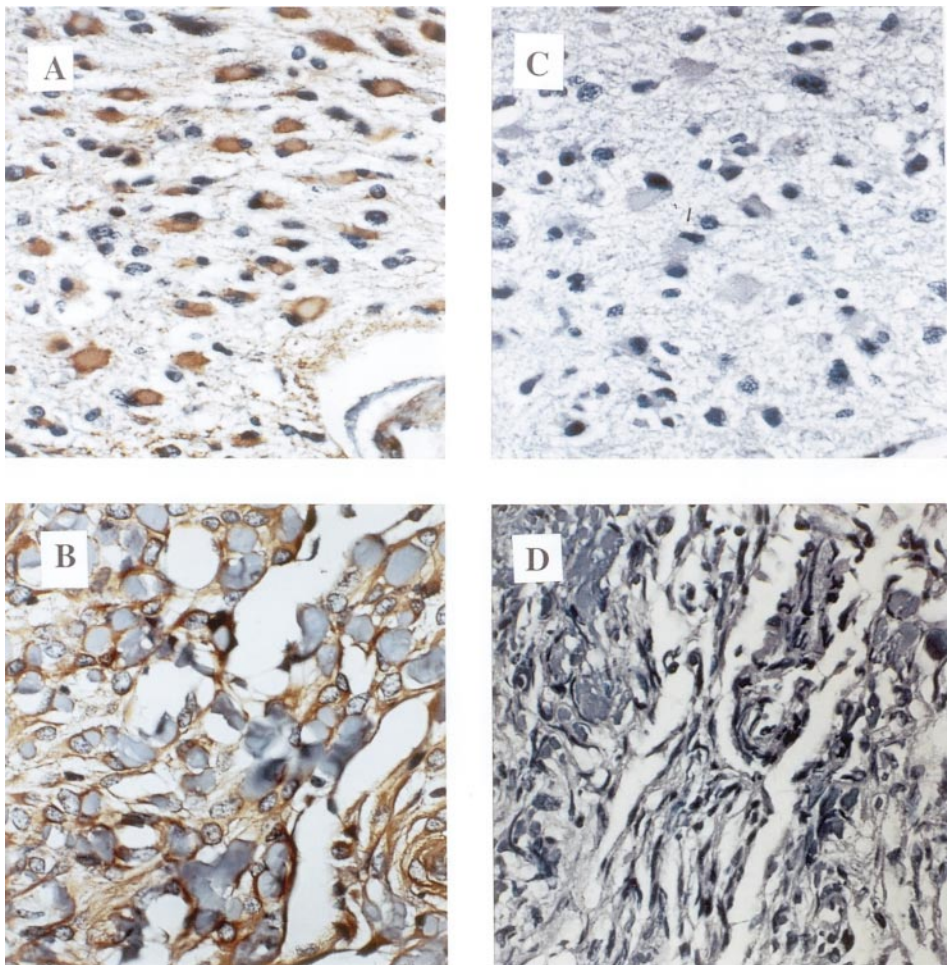


Fig. 2. Representative examples of the immunohistochemical localization of calcium-sensing receptor (CaR) in human astrocytomas ( $n = 19$ ) and meningiomas ( $n = 2$ ) using an affinity-purified anti-CaR antiserum as described in MATERIALS AND METHODS. *A*: astrocytoma; staining of scattered astrocytoma cells was observed, with both diffuse immunoreactivity within the cytoplasm as well as stronger immunostaining of the cell membrane. *B*: meningioma; there is strong and widespread CaR immunoreactivity. Negative controls were performed by preabsorbing the anti-CaR antiserum with its immunogenic peptide before carrying out the immunohistochemical staining of astrocytoma (*C*) and meningioma (*D*). All photographs were taken at  $\times 200$  (original) magnification.

gliomas was subjected to RT-PCR using primers designed to amplify 576- and 426-bp fragments of the mRNAs for PTHrP and the CaR, respectively (Fig. 4). RT-PCR revealed that primer pairs specific for the CaR and for a nucleotide sequence common to all PTHrP transcripts each amplified a single fragment in all cases that was of the expected size for the product derived from the respective mRNA(s). Nucleotide sequencing of products amplified by both primer pairs revealed  $>99\%$  sequence homology with the corresponding regions of the known sequences of the transcripts encoding the human isoforms of the CaR and PTHrP (not shown).

To determine which of the various isoforms of PTHrP is expressed in HPA, astrocytomas, and meningiomas, we used an RT-PCR-based approach that amplified a distinct product from each of the three alternatively spliced PTHrP transcripts. The use of this approach revealed that HPA and the three astrocytomas examined in this manner express all three isoforms, namely those encoding PTHrP(139), PTHrP(141), and PTHrP(173) (Fig. 5). In meningiomas, in contrast, only the 139 and 141 isoforms of PTHrP were expressed. Northern blot analysis revealed expression of a major,  $\sim 5.0$ -kb CaR transcript in all of the tissues that were studied with no apparent differences in its expression level (Fig. 6).

***In vitro PTHrP release.*** The use of a two-site immunoradiometric assay specific for human PTHrP revealed that HPAs secrete readily detectable amounts of PTHrP ( $\sim 9.0 \text{ mIU} \cdot \text{ml}^{-1} \cdot 24 \text{ h}^{-1}$ ) and secrete PTHrP at a basal rate that was between 55 and 80% greater than that exhibited by the tumor cells that were studied in the same manner (Table 2 and Fig. 7). Figure 7 also shows that elevation of  $[\text{Ca}^{2+}]_o$  from 0.5 to 4.0 mM dose dependently increased PTHrP secretion, with a maximal secretory rate at 3.0 mM that was  $\sim 2.5$ -fold greater than the basal rate of secretion for both HPA and the glial tumors. The polycationic CaR agonists,  $[\text{Gd}^{3+}]_o$  (50  $\mu\text{M}$ ) and neomycin (100  $\mu\text{M}$ ), each stimulated PTHrP secretion to a similar extent, further supporting the CaR's involvement in the high  $[\text{Ca}^{2+}]_o$ -evoked secretion of PTHrP from HPA and glial-derived tumors.

## DISCUSSION

Previous studies have shown that changes in  $[\text{Ca}^{2+}]_o$  modulate PTHrP secretion from several types of tumors as well as from keratinocytes (26, 22), although the mechanism(s) underlying this action of  $[\text{Ca}^{2+}]_o$  has not been defined. More recently, it has been demonstrated that rat oligodendrocytes (9) and microglia (10) as well as human astrocytoma cell lines (11) express a

Table 1. Results of staining of tumors for the CaR and PTHrP using the immunoperoxidase technique

	CaR	PTHrP
Low-grade astrocytoma (Sample number)		
1	++	++
2	++	++
3	+	++
4	+	+++
5	+	+
Anaplastic astrocytoma (Sample number)		
7	++	+
8	+	+++
9	+	+++
10	+++	+
11	++	++
Glioblastoma multiforme (Sample number)		
11	+	++
12	+	+++
13	+	+
14	+	++
15	+	+++
16	+	+

CaR, calcium-sensing receptor; PTHrP, parathyroid hormone-related protein. Technique described in MATERIALS AND METHODS. +, Patchy immunostaining (1–30% cells positive); ++, moderate immunostaining (31–60% cells positive); +++, strong immunostaining (61–100% cells positive).

functional CaR similar to that originally cloned from bovine parathyroid. Furthermore, there are reports showing expression of transcripts or immunoreactivity for PTHrP in rodent neurons (43) and meningeal epithelial cells (38) as well as expression of a functional PTH/PTHrP receptor in human cerebellar neurons (13) and rat astrocytes (19). Thus PTHrP, acting through

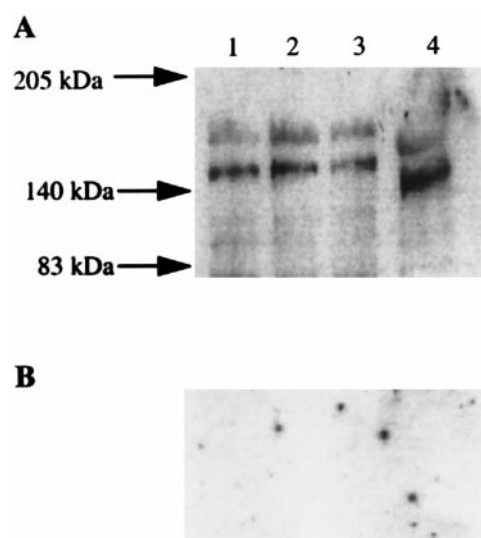


Fig. 3. A: Western blot analysis of CaR protein in human primary astrocytes (HPA), astrocytomas, and meningiomas using an affinity purified anti-CaR antiserum as described in MATERIALS AND METHODS. Lane 1, HPA; lanes 2 and 3, astrocytomas; and lane 4, meningioma. B: the use of peptide-preabsorbed anti-CaR antiserum demonstrates the specificity of the immunoreactive bands.

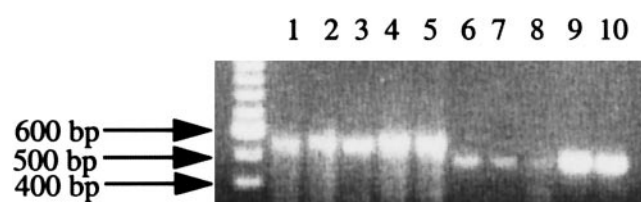


Fig. 4. mRNA expression of PTHrP (lanes 1–5) and CaR (lanes 6–10) in HPA, astrocytomas, and meningiomas. RT-PCR analysis for PTHrP (all isoforms) and CaR transcripts using the primer pairs described in MATERIALS AND METHODS. Lanes 1 and 6, HPA; lanes 2 and 3 and 7 and 8, astrocytomas; and lanes 4 and 5 and 9 and 10, meningiomas.

its receptor, may function in an autocrine/paracrine manner in the CNS. In the present study we demonstrated that the CaR and PTHrP are both expressed in HPA and in the most frequently occurring brain tumors, e.g., astrocytomas and meningiomas. Moreover, the polycationic agonists of the CaR, neomycin and Gd<sup>3+</sup> (3, 6), each stimulated PTHrP secretion from these various normal and neoplastic cells.

RT-PCR performed on total RNA isolated from HPA as well as from glioblastomas and meningiomas, performed using primer pairs specific for the CaR and for the region of the PTHrP transcripts common to all of its isoforms, amplified products of the expected sizes. The latter, in turn, on sequencing revealed >99% identity with the previously published sequences of the CaR (15, 16) and PTHrP (39) in humans. Moreover, Northern blot analysis revealed expression of a single ~5.0-kb CaR mRNA with a similar abundance in HPA, glioblastomas, and meningiomas, providing additional evidence for the expression of readily detectable and similar levels of mRNA for the receptor by these various tissues.

CaR protein was also expressed in HPA as well as in histological sections and primary cultures derived from the various brain tumors studied here as assessed by immunohistochemistry and Western analysis using an affinity-purified antiserum directed against the extra-

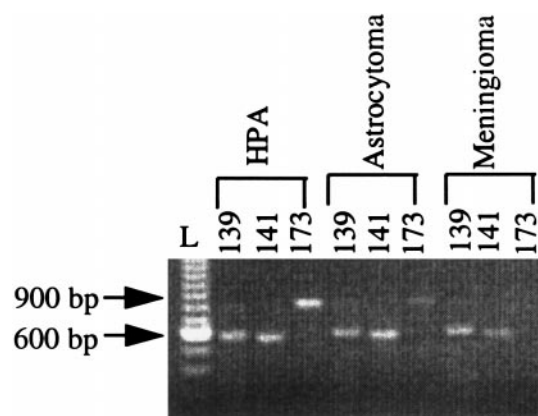


Fig. 5. Representative figure showing analysis of PTHrP isoform expression (139, 141, 173) in HPA ( $n = 2$ ), astrocytomas ( $n = 2$ ), and meningiomas ( $n = 3$ ). The primer pairs employed and the PCR conditions are as described in MATERIALS AND METHODS. L, 100-bp DNA ladder.

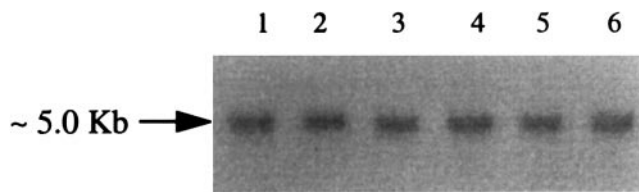


Fig. 6. Northern blot analysis of CaR transcripts using a cRNA probe as described in MATERIALS AND METHODS. Lane 1, HPA; lanes 2 and 3, astrocytomas; lanes 4–6, meningiomas.

cellular domain of the receptor. Multiple specific CaR bands were observed in all of the tissues studied here by Western analysis. We observed a similar pattern of CaR protein expression on Western blot analysis in U87 human astrocytoma cell lines (11). Moreover, we observed that cells expressing comparatively low levels of the CaR, i.e., CNS-derived cells (7) and bone cells (45, 46), exhibit more intracellular CaR immunoreactivity than those expressing high levels of the receptor, e.g., parathyroid (5, 25) and kidney cells (33), in which immunoreactivity is localized predominantly on the cell membrane. Such differences in the patterns of CaR protein distribution may be explained by differing rates, extents and/or patterns of posttranslational processing of the receptor protein, and/or other factors. Moreover, as for its transcript, the level of CaR protein expression did not differ appreciably between HPA and the two tumor types as assessed by Western blotting. Taken together, these data show unequivocally by nucleotide- and antibody-based approaches that the CaR is expressed at readily detectable levels apparently sufficient for mediating a biological response, i.e., regulation of PTHrP secretion, in primary human astrocytes, at least in early life, and also in malignant astrocytes and meningeal cells. Unfortunately, it is not currently possible to culture astrocytes from normal human adult brain, precluding a direct comparison of the expression and function(s) of the CaR in these cells with that in the various cell types examined in this study.

The human PTHrP gene can generate via alternative splicing three different species of mRNA that encode three initial translational isoforms, comprising 139, 141, and 173 amino acids (4, 32). The splicing pattern of the PTHrP gene is likely to be highly tissue specific and responsive to external factors, such as hormones and growth factors, as well as to the presence of abnor-

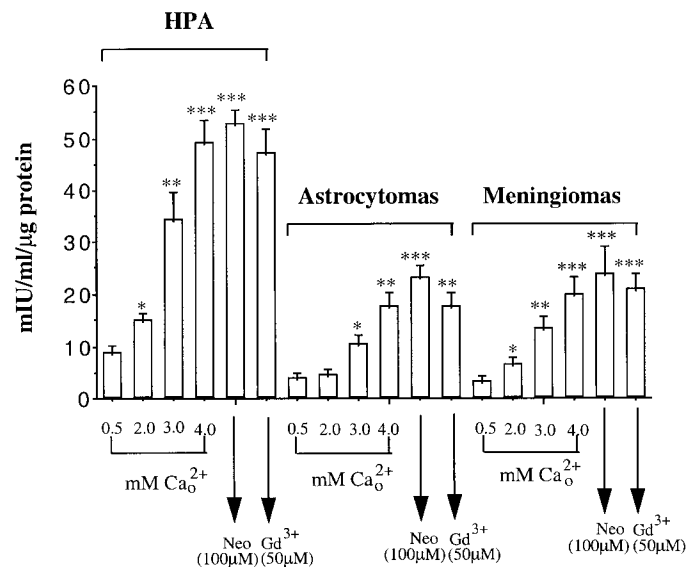


Fig. 7. Secretion of PTHrP and its stimulation by polycationic CaR agonists in HPA ( $n = 3$ ), astrocytic tumors ( $n = 5$ ), and meningiomas ( $n = 5$ ). The various cell types were cultured for 24 h with or without the various CaR agonists shown, and PTHrP in the conditioned medium was measured as described in MATERIALS AND METHODS. \*Significantly greater than values without \*; \*\*, significantly greater than values with or without \*; \*\*\*, significantly greater than values with or without \* or \*\*.

mal states, including malignancy (37, 44). Therefore we were interested to determine if there were any differences in the patterns of PTHrP isoform expression between normal and malignant cells. We also used isoform-specific primers for PTHrP to show that HPA and astrocytic tumors express all three of the known isoforms of PTHrP, e.g., 139, 141, and 173, whereas meningiomas express only the first two of these. It would be of interest to determine which of the isoforms of PTHrP are expressed by the normal counterparts of these malignant cells, although such studies were beyond the scope of the present work.

The present investigations also revealed that HPA secrete nearly twofold more immunoreactive PTHrP under basal conditions than astrocytomas despite the fact that the expression profile of the transcripts for the various PTHrP isoforms is similar in the two cell types. It is possible that fetal HPA are characterized by an elevated rate of PTHrP expression and hence may not provide an ideal model for adult astrocytes or an optimal basis for comparison of the properties of astrocytes with those of astrocytic tumors. Given the lack of data for the secretion of PTHrP from normal adult human astrocytes in the present study, appropriate caution must be exercised in extrapolating to possible differences between adult astrocytes and astrocyte-derived tumors. Thus, although it is possible that normal astrocytes of adult origin secrete more PTHrP than their malignant counterparts and this difference is of pathophysiological significance (e.g., via PTHrP promoting differentiation of astrocytes), further studies are needed in this regard. Alternatively, it is possible that upregulation of PTHrP secretion during fetal life plays

Table 2. Basal PTHrP secretion

HPA	Anaplastic Astrocytoma	Glioblastoma Multiforme	Meningioma
11	6	8	7
8	5	4	5
9		6	4
			4
			6

PTHrP secretion was measured (in mIU·ml<sup>-1</sup>·mg protein<sup>-1</sup>) in duplicate in conditioned medium from each of 2 culture wells for the indicated tumors as described in MATERIALS AND METHODS. HPA, human primary astrocytes.

some role in the normal function of astrocytes during brain development. It is also of interest in this regard that PTHrP exerts a growth inhibitory effect in UMR 106 rat osteoblastic osteosarcoma cells (40), rat type II pneumocytes (20), and hamster ovary cells stably transfected with PTH/PTHrP receptor (31). Finally, future studies could address the relative rates of basal PTHrP secretion in normal human meningo-epithelial cells with that of meningiomas and whether the receptor modulates the growth and/or differentiation of these cell types.

What are the possible roles of the CaR in normal and neoplastic astrocytes and meningeal cells? The CaR has been shown to have roles in promoting proliferation, differentiation, and secretion in various tissues outside of the CNS and could modulate the functions of normal and neoplastic glial cells in similar ways (8). Moreover, in all of the tissues studied here, the CaR, or at least its polycationic agonists, exerted a clear stimulatory action on PTHrP secretion, although further studies are needed using approaches such as the use of transfection with dominant negative constructs of the CaR to prove unequivocally that the effects of these agonists are actually CaR mediated. Thus the CaR may be one of the mechanisms through which PTHrP secretion is regulated in these cells, although there were no apparent differences in the levels of CaR expression between HPA and the tumors studied here that could account for the observed higher rate of PTHrP secretion in HPA. Alterations in neuronal activity are known to modulate the level of extracellular calcium within the brain microenvironment, and such changes in [Ca<sup>2+</sup>]<sub>o</sub> could conceivably participate in the control of PTHrP secretion, acting via the CaR, under normal conditions as well as in the setting of tumors of the CNS. Alternatively, the CaR might modulate the function(s) of astrocytes and other glial cells through additional, direct actions on the cells, as we have demonstrated in rat oligodendrocytes (9), microglia (10), and neurons (36, 41), via changes in the activities of various ion channels.

Therefore, in summary, our data show that the CaR is expressed in human astrocyte-derived cells (both normal and malignant) as well as in meningiomas. We also showed for the first time that HPA and malignant astrocytes secrete PTHrP, unlike their rodent counterpart, which expresses only the PTHrP receptor. However, our finding that human meningiomas secrete PTHrP corroborates previous results showing that rat meningo-epithelial cells have the capacity to secrete this hormone. Finally, we have provided evidence that the CaR is not only expressed in these various cell types but also positively modulates PTHrP secretion.

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