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COMMENTS

Identification and Localization of the Extracellular Calcium-Sensing Receptor in Human Breast*

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ABSTRACT

The extracellular calcium ($Ca^{2+}{}_o$)-sensing receptor (CaR) plays a critical role in maintaining $Ca^{2+}{}_o$ homeostasis in mammals by virtue of its presence in parathyroid gland and kidney. The breast is well recognized as a Ca^{2+}-handling organ, and the effects of altering $Ca^{2+}{}_o$ on the proliferation of breast epithelial cells are well documented. To date there are no data regarding the expression and localization of CaR in breast tissue. In the present study, we assessed the distribution of CaR messenger ribonucleic acid (mRNA) and protein in normal and fibrocystic human breast tissue as well as in ductal carcinoma of the breast using RT-PCR, Northern analysis, and immunohistochemistry with CaR-specific antisera. In all tissues, RT-PCR performed using sense and antisense primers based on the sequence of the human parathyroid CaR complementary DNA amplified a product of the size expected (425 bp) for genuine CaR transcripts. Nucleotide sequencing of RT-PCR products confirmed more than 99% homology with human parathyroid CaR complementary DNA. Although insufficient quantities of mRNA were isolated from normal and fibrocystic tissue for Northern analysis, a single 5.2-kb CaR transcript was expressed in malignant breast tissue similar to the major CaR transcript in human parathyroid. Localization of CaR protein by immunohistochemistry showed specific CaR staining of the ductal epithelial cells of the breast in all three tissue types. These findings indicate the presence of CaR mRNA and protein in the breast, providing indirect evidence that the CaR may have some role(s) in the control of Ca^{2+} transport, epithelial cell proliferation, and/or other processes in normal and abnormal breast tissue. (*J Clin Endocrinol Metab* **83:** 703–707, 1998)

A CELL SURFACE, extracellular calcium ($Ca^{2+}{}_o$)-sensing receptor (CaR) has been cloned from bovine parathyroid (1) and rat kidney (2), which is a key mediator of the direct effects of $Ca^{2+}{}_o$ on several of the tissues that are intimately involved in calcium homeostasis. Subsequently, the same receptor has been cloned from human parathyroid gland (3) and kidney (4), a rat thyroidal C cell line (5), and rat brain (6).

$Ca^{2+}{}_o$ plays an important role in the breast, not only in its normal physiological function, but in pathologic states as well. $Ca^{2+}{}_o$ is obviously an important constituent of milk, and lactating mothers produce breast milk containing about 200 mg $Ca^{2+}{}_o$ daily (7). A diagnostically important characteristic of breast cancer is the tendency to form microcalcifications (8). In addition, *in vitro* studies have shown that increases in $Ca^{2+}{}_o$ within the physiological range induce senescence and terminal differentiation of normal human breast epithelial cells in culture (9, 10). It is not known how $Ca^{2+}{}_o$ is involved in these diverse physiological processes of the breast.

The present study was undertaken to evaluate the expression of CaR in human breast. Specifically, we assayed for CaR transcripts by RT-PCR and Northern analysis in normal breast, fibrocystic breast tissue, and/or ductal carcinoma of the breast. To assess the degree of homology of CaR transcripts expressed in breast tissues with the human parathyroid CaR (HuPCaR) complementary DNA (cDNA), nucleotide sequencing was performed on RT-PCR products amplified from each tissue. In addition, immunohistochemistry was performed using antibodies directed against the extracellular domain of the CaR and provided further localization and confirmation of expression of the CaR in sections of normal, fibrocystic, and malignant human breast tissues.

Materials and Methods

Patients

Normal and fibrocystic tissues were obtained from two patients for each tissue type during surgery for various breast diseases. Ductal carcinoma tissue was procured during lumpectomy in two patients. This study was approved by the investigational review board at Brigham and Women's Hospital (Boston, MA).

Tissues and reagents

Tissue fragments were frozen after surgical removal and stored at −70 C. Histopathological examination by light microscopy was routinely performed on each tissue sample using standard techniques. For immunohistochemistry, tissues were snap-frozen, embedded in OCT

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compound (Miles, Elkhart, IN), and maintained in 2-methylbutane at −70 C until further use.

Isolation of total ribonucleic acid (RNA)

Total RNA was isolated as described previously (1, 2, 11). Briefly, 1 g tissue was homogenized in 8 mL 4 mol/L guanidine isothiocyanate, 25 mmol/L sodium citrate, and 1.12 mg/mL β-mercaptoethanol. The homogenate was layered onto 4 mL of a 5.7 mol/L CsCl-25 mmol/L sodium acetate cushion and sedimented at 32,000 rpm at 18 C for 18 h. The RNA pellet was resuspended in water and concentrated by precipitation with sodium acetate and ethanol.

RT-PCR of CaR transcripts

A 425-bp product was amplified from RNA isolated from breast tissues by RT-PCR using primers specific for the human parathyroid cDNA based on previously described methodology (5). Total RNA (5 μg) for each of the tissues was reverse transcribed at 42 C (1 h) using random primers and the avian myeloblastoma virus reverse transcriptase from the cDNA Cycle Kit (Invitrogen, San Diego, CA). Sense and antisense primers from the 5′-end of the human parathyroid CaR cDNA were used to amplify a 425-bp DNA fragment. The PCR buffer contained 29.75 μL water, 5 μL 10 Thermo DNA poly 10× reaction buffer (Promega), 3.75 μL $MgCl_2$ (25 mmol/L), 2 μL deoxynucleotide triphosphates (2.5 mmol/L each), 3 μL single-stranded cDNA, 3 μL each of the sense and antisense primers based on the sequence of the HuPCaR cDNA [primer 2009, 5′-CGGGGTACCTTAAGCACCTACGGCATCTAA-3′ (bp 1745–1774); primer 2017, 5′-GCTCTAGAGTTAACGCGATCCCAAAGGG-CTC-3′ (bp 2200–2230), respectively] and 0.5 μL Taq polymerase (2.5 U; Pharmacia LKB Biotechnology, Piscataway, NJ). Optimal cycling conditions were as follows: 92 C for 1 min, 55 C for 1 min, and 72 C for 1.5 min; cycling under these conditions was repeated 29 times, followed by a final extension step at 72 C for 8 min.

Nucleotide sequencing of CaR RT-PCR products

Primers 2009 and 2017 contained restriction sites for *Afl*II and *Hpa*I, respectively. Before sequencing, PCR products were digested with *Afl*II and *Hpa*I and ligated into the plasmid, pBluescript II SK^- (Stratagene, La Jolla, CA). Bidirectional sequencing was performed by the dideoxy chain termination method (2) using an Applied Biosystems model 373A automated sequencer (Department of Genetics, Children's Hospital, Boston, MA). Further nucleotide and amino acid analyses were carried out using GeneWorks software (version 2.3.1, IntelliGenetics, Mountain View, CA).

Northern analysis of messenger RNA (mRNA) encoding for the CaR

Polyadenylated [poly$(A)^+$] RNA selection was carried out using oligo(deoxythymidine)-cellulose chromatography (1, 2). Aliquots of 5 μg denatured poly$(A)^+$ RNA were electrophoresed on 2.2 mol/L formaldehyde-1% agarose gels and transferred overnight to nylon membranes (Duralon, Stratagene) (1, 2). A 486-bp *Kpn*I-*Xba*I fragment corresponding to nucleotides 1745–2230 of the HuPCaR cDNA was subcloned into the pBluescript SK^+ vector (Stratagene). The plasmid was then linearized with *Kpn*I, and a [^{32}P]UTP-labeled riboprobe was synthesized using the T3 polymerase site in the vector with the MAXIscript T3 kit (Ambion, Austin, TX). Nylon membranes were prehybridized for 2 h at 65 C in a solution consisting of 50% formamide, 4 × Denhardt's solution (50 × Denhardt's solution = 5 g Ficoll, 5 g polyvinylpyrrolidone, and 5 g BSA), 5 × SSPE (20 × SSPE = 2.98 mol/L NaCl and 0.02 mol/L ethylenediamine tetraacetate in 0.2 mol/L phosphate buffer, pH 7.0), 0.5% SDS, 25 mmol/L Tris (pH 7.5), 10.7 μg/mL yeast transfer RNA, and 250 μg/mL salmon sperm DNA. Labeled probe (2×10^6 cpm/mL) was then added, and the membranes were hybridized overnight at the same temperature. Washing was carried out twice at low stringency (2 × SSC [20 × SSC = 3 mol/L NaCl and 0.3 mol/L Na_3-citrate($2H_2O$)] and 0.1% SDS at room temperature) for 20–25 min and once at high stringency (0.1 × SSC-0.1% SDS at 65 C) for 15 min. ^{32}P-Labeled bands were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) (12).

Immunohistochemistry

Immunohistochemistry was performed using techniques modified from those described previously (12–14). Sections (5 μm thick) were prepared on a cryostat and were postfixed in acetone for 10 min at −20 C. Endogenous peroxidases were inhibited by incubating the sections in Dako peroxidase blocking reagent (Dako Corp., Carpenteria, CA) for 10 min, followed by treatment with Dako protein block serum-free solution (Dako Corp.) for 1 h. The sections were then incubated overnight at 4 C with 10 μg/mL primary anti-CaR antisera in blocking solution (Dako Corp.; either affinity-purified rabbit polyclonal antiserum 4637 raised against a peptide corresponding to amino acids 345–359 of the bovine parathyroid CaR or protein A-purified rabbit polyclonal antiserum 4641 raised against a peptide corresponding to residues 215–237 of BoPCaR). Characterization of these antisera has been detailed previously (12–14). In both cases, the corresponding sequences of the immunogenic peptides were identical in the human and bovine CaRs. Control sections were prepared by incubation with protein A-purified preimmune serum or with anti-CaR antiserum preabsorbed with synthetic CaR peptide (10 μg Ab/20 μg peptide for antiserum 4637; 10 μg Ab/40 μg peptide for antiserum 4641) against which the antibody was raised. After washing the sections three times with 0.5% BSA in PBS for 10 min each time, peroxidase-coupled, goat antirabbit IgG (1:100; Sigma) was added and incubated for 1 h at room temperature. The slides were then washed in PBS three times for 10 min each time, and the color reaction was developed using the Dako AEC Substrate System (Dako Corp.) for about 5 min. The color reaction was stopped by washing three times in water.

Results

Amplification and sequencing of RT-PCR products derived from human breast CaR

In RNA prepared from normal and fibrocystic breast tissue as well as from ductal carcinoma of the breast, RT-PCR amplified 425-bp DNA fragments of the expected size for CaR transcripts, as shown in Fig. 1A. These PCR products were of the same size as those obtained from the HuPCaR cDNA as a positive control. The primers that were employed spanned at least one intron, precluding amplification of products of the same size from contaminating genomic DNA; furthermore, no PCR products were amplified when the reverse transcriptase was omitted from the reaction (not shown). Nucleotide sequencing of RT-PCR products obtained from each tissue revealed more than 99% nucleotide identity with the corresponding HuPCaR clone, indicating that this PCR product was derived from *bona fide* CaR transcripts (Fig. 1B).

Northern analysis

Northern analysis (Fig. 2) performed at high stringency on the poly$(A)^+$ RNA isolated from the ductal carcinoma tissue that was used for RT-PCR revealed a single 5.2-kilobase (kb) transcript, similar in size to one of the principal transcripts observed in human parathyroid gland and kidney. The latter organs, however, expressed an additional 4.0-kb transcript in prior studies (3, 4).

Immunohistochemical localization of CaR in human breast

Immunohistochemistry using two specific anti-CaR antisera directed at highly conserved epitopes within the extracellular domain identified strong CaR immunoreactivity along the epithelial cells lining the ducts of the breast as well as in neoplastic breast epithelial cells in two separate ductal carcinomas (Fig. 3). Staining was abolished by preincubation

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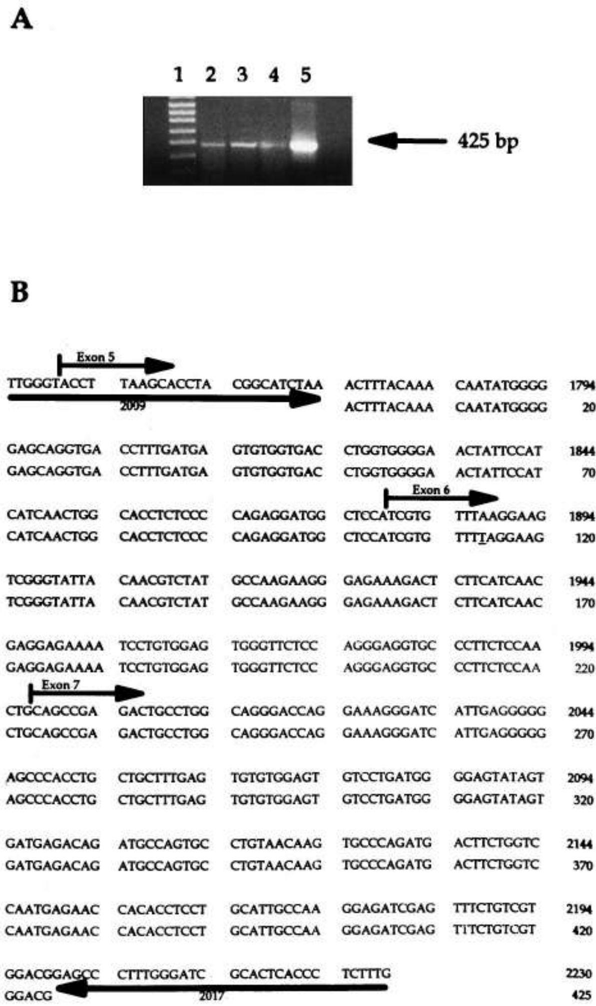

FIG. 1. A, RT-PCR amplification of CaR-related sequences from human breast, performed as described in *Materials and Methods*. The size of the CaR product was estimated to be 425 bp, as indicated. Lane 1, One hundred-base pair DNA ladder; lane 2, PCR product from normal breast; lane 3, PCR product from fibrocystic breast; lane 4, PCR product from ductal carcinoma of the breast; lane 5, PCR product from HuPCaR cDNA. B, The nucleotide sequence of a PCR product amplified from breast tissue (designated HuBrCaR) is aligned with the sequence of the 4.0-kb HuPCaR cDNA clone (3). Mismatches are indicated by *underlined letters*. Primer sequences are represented as *solid bars with arrowheads* under the corresponding sequence of the CaR cDNA, and nucleotide numbering corresponds to the coding sequence of HuPCaR.

of the antibody with an excess of the peptide against which the respective antisera were raised, thereby confirming the specificity of the reaction.

Discussion

In this study we have demonstrated the presence of a CaR in human breast similar or identical to that cloned from human parathyroid gland. We used RT-PCR to amplify a fragment of the human CaR from normal, fibrocystic, and malignant mammary tissue, which revealed more than 99% identity in its nucleotide sequence to that of the HuPCaR cDNA. The low level of nonidentity most likely resulted from errors in proof reading by *Taq* polymerase, but might con-

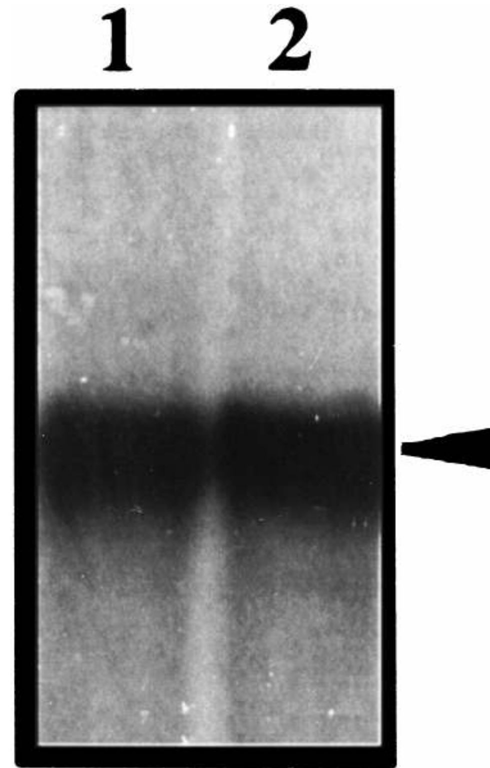

FIG. 2. Northern blot analysis of CaR transcripts in human breast ductal carcinoma. Total RNA was extracted, poly(A)$^{+}$ enriched, and analyzed by Northern analysis with a ^{32}P-labeled riboprobe derived from the 5′-region of HuPCaR as described in *Materials and Methods*. Lane 1, Patient 1; lane 2, patient 2. The *arrowhead* indicates the major transcript at ~5.2 kb.

ceivably represent polymorphisms. Specificity of the amplification of CaR transcripts by RT-PCR was confirmed by negative control experiments in which the reverse transcriptase was omitted from the RT-PCR reaction, as PCR products were not amplified under these conditions. In addition, the use of intron-spanning primers precluded amplification of the same sized products as a result of priming from contaminating genomic DNA. Because the RT-PCR primers were designed from the 5′-end of the CaR cDNA, which codes for the extracellular domain of the receptor (2), the observed sequence homology provides evidence that a portion of the putative Ca^{2+}-binding domain (1, 2) is conserved in the CaR expressed in breast.

Our Northern analysis clearly demonstrates that CaR mRNA is present in the breast. The 5.2-kb transcript is similar in size to one of the major transcripts seen in human parathyroid. The parathyroid gland, however, expresses an additional 4.0-kb band. It is interesting to note that the sequence of the PCR product that was amplified from reverse transcribed CaR transcripts in all three types of breast tissue, including the 5.2-kb transcript observed in ductal carcinoma tissue, was the same as that of the 4.0-kb HuPCaR cDNA. The latter exhibits an in-frame 30-nucleotide deletion compared to the corresponding region of the sequence of the 5.2-kb clone isolated from human parathyroid (3). As the functions of the CaRs encoded by the 4.0- and 5.2-kb human parathyroid cDNA clones exhibited no obvious differences when

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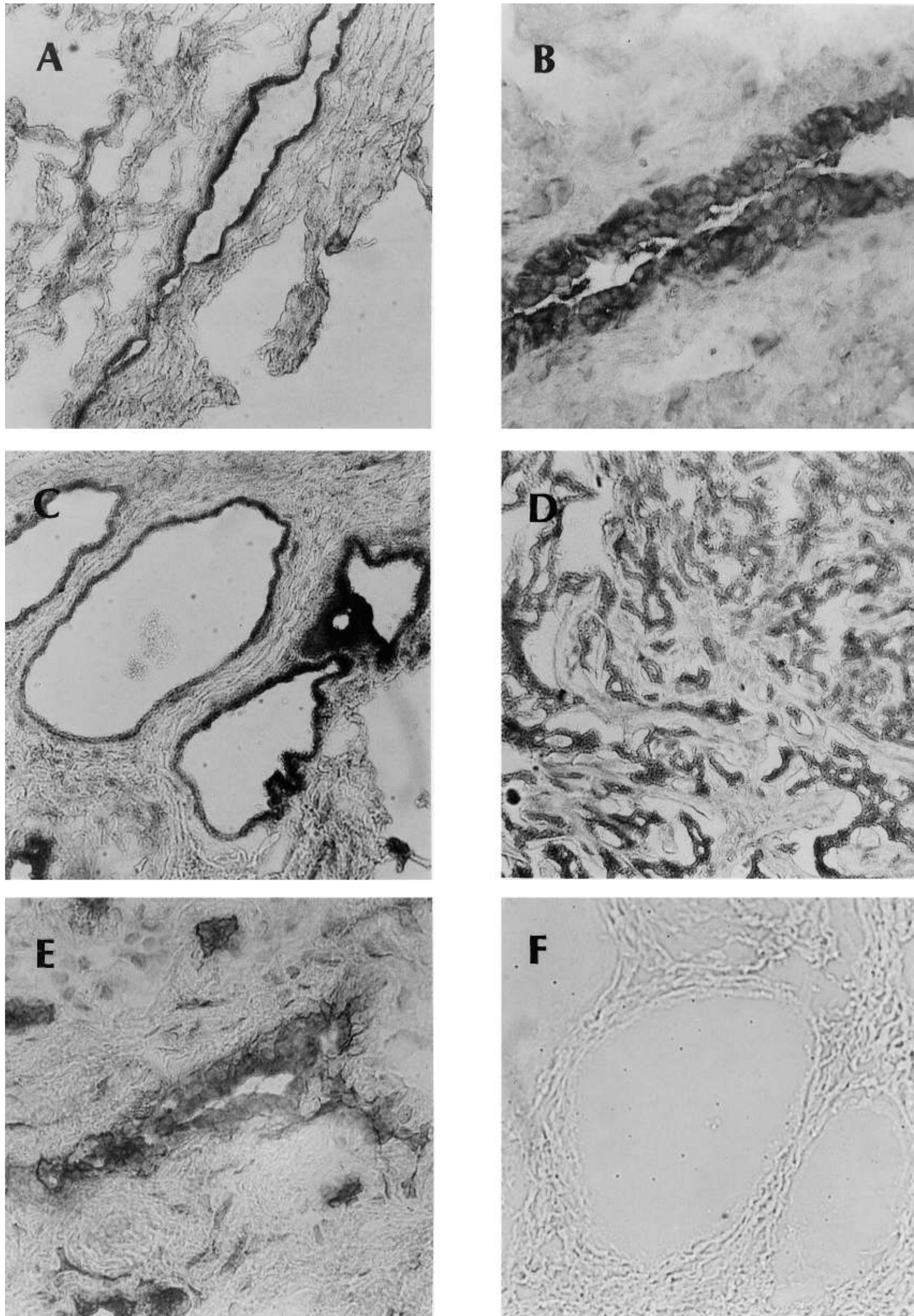

FIG. 3. Immunohistochemical staining of human breast tissue with a specific polyclonal antiserum directed against the extracellular domain of the CaR, performed as described in *Materials and Methods*. A, Normal breast tissue at ×40 magnification. B, Normal breast tissue at ×100 magnification. C, Fibrocystic breast tissue at ×40 magnification. D, Ductal carcinoma at ×40 magnification. E, Ductal carcinoma at ×100 magnification. F, A representative negative control from fibrocystic breast tissue using antiserum preabsorbed with the peptide against which it was raised. Similar results were observed using a second anti-CaR antiserum directed against a different epitope within the receptor's extracellular domain as described in *Materials and Methods*.

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expressed in *Xenopus laevis* oocytes (3), the 30-nucleotide deletion present in the transcripts amplified from human breast tissue by RT-PCR is of uncertain significance.

By immunohistochemistry we demonstrated the presence of CaR protein in all three breast tissue types. Strong immunostaining was observed along the epithelial cells of the ducts in normal and fibrocystic breast tissue. Although there is a dearth of data about the regulation of the transport of Ca^{2+} into milk, the presence in the receptor in ductal cells raises the possibility of a role for the CaR in such transport processes. Furthermore, metastatic cancer of the breast is known to spread readily to bone (15). We have recently shown that numerous normal cells within the bone marrow express the CaR (16). Perhaps the presence of the CaR on normal and malignant mammary cells contributes to the tendency of these and other malignant cells to metastasize to bone, in which there are high local levels of $Ca^{2+}{}_o$ related to bone resorption (17).

In summary, our data demonstrate the presence of CaR mRNA and protein in normal and malignant human breast tissue. Given the importance of $Ca^{2+}{}_o$ in the breast, it is not difficult to imagine that the CaR may play diverse roles in the physiology and pathophysiology of breast tissue. Further studies are required to evaluate the role(s) of the CaR in the ductal epithelial cells shown to express it in this study.

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