Molecular cloning and characterization of a rat sensory nerve Ca\(^{2+}\)-sensing receptor

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Molecular cloning and characterization of a rat sensory nerve Ca\(^{2+}\)-sensing receptor. Am J Physiol Cell Physiol 285: C64–C75, 2003. First published March 12, 2003; 10.1152/ajpcell.00543.2002.—A full-length cDNA encoding a Ca\(^{2+}\)-sensing receptor (CaSR) expressed in rat dorsal root ganglia (DRG) was identified using rapid amplification of cDNA ends and primer extension and then cloned into the plasmid vector pCR3.1. The DNA sequence of the DRG CaSR was 99.9% homologous with published rat kidney CaSR in the coding region and 247 bp upstream of the start site but showed little homology 5’ to this site, which maps to exonic junction I/II, supporting the hypothesis that CaSR message arises as a splice variant and showing tissue-to-tissue heterogeneity. Western blot revealed a doublet of 140 and 160 kDa in a thyroparathyroid preparation and a single 140-kDa band in DRG. Deglycosylation using N-glycanase increased the mobility of CaSR protein from both DRG and thyroparathyroid, whereas endo-H was without effect, indicating that the DGR CaSR is a mature form of the receptor. A DRG CaSR-pE GFP fusion product was constructed, and when transfected into HEK-293 cells, it was distributed at the cell membrane and resulted in extracellular Ca\(^{2+}\) (0.5–3 mM)-evoked increases in intracellular Ca\(^{2+}\), which in some instances exhibited oscillatory behavior. We conclude that DRG CaSR cDNA arises from tissue-specific alternative splicing of a single gene, that the amino acid sequence of DRG CaSR is homologous to other known CaSRs, and that the DRG CaSR undergoes differential posttranslational processing relative to the thyroparathyroid CaSR and is functionally active when transfected into a human-derived cell line.

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CHANGES IN THE CONCENTRATION of interstitial Ca\(^{2+}\) as have been shown to occur in tissues involved in transcellular Ca\(^{2+}\) movement (26, 27) can modulate the function of a variety of cell types by activating a membrane-spanning, G protein-coupled receptor for extracellular Ca\(^{2+}\) (7). The first molecular evidence for a Ca\(^{2+}\)-sensing receptor (CaSR) was reported by Brown et al. (6), who demonstrated that bovine parathyroid cells express mRNA encoding a novel protein with homology to the metabotropic glutamate receptor. The CaSR has a large extracellular domain containing multiple N-linked glycosylation sites, a seven-transmembrane spanning domain, and an intracellular tail with consensus protein kinase phosphorylation sites. This receptor represents a novel member of a family of G protein-coupled receptors, which notably include metabotropic glutamate receptors (21, 24), γ-aminoisobutyric acid receptors (23), and a multigene family of putative pheromone receptors identified in the rat vomeronasal organ (20, 25).

A highly homologous receptor has also been reported in other cell types, including human parathyroid and thyroid C cells (15, 16), antral gastrin cells (29), peripheral blood monocytes (37), keratinocytes (3, 28), renal tubule cells (30), brain (33), and intestinal epithelia (11, 13). Although the CaSR is believed to regulate the release of parathyroid hormone from parathyroid cells and calcitonin from thyroid C cells and differentiation in keratinocytes, its function in other cell types has not been as well defined.

In 1995 we reasoned that given their role in sensing environmental stimuli, sensory afferent nerves might express a CaSR as a means of monitoring Ca\(^{2+}\) in the extracellular milieu. We subsequently demonstrated (9) that dorsal root ganglia (DRG), which house cell bodies of sensory nerves that send efferent processes to multiple tissues including the perivascular adventitia, express mRNA encoding a CaSR. We also showed (8) that protein that is immunoreactive with an antibody raised against the human parathyroid CaSR is present in DRG and in a subpopulation of perivascular nerves. The results of continuing physiological studies have been consistent with the hypothesis that activa-
tion of the perivascular Ca\textsuperscript{2+} receptor results in the release of a hyperpolarizing vasodilator that subsequently relaxes adjacent smooth muscle cells (8, 10, 22).

Because of the potential importance of this novel perivascular sensory nerve CaSR-associated dilator system in the control of sensory nerve and vascular function, we have undertaken experiments to more completely characterize the sensory nerve CaSR.

METHODS

Animals. All procedures using animals were approved by the Institutional Animal Care and Use Committee. Male Wistar rats 10–12 wk of age were obtained from Harlan Sprague Dawley (Indianapolis, IN) and, upon arrival, were the Institutional Animal Care and Use Committee. Male completely characterize the sensory nerve CaSR.

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Rapid amplification of 5'-cDNA ends. Full-length double-stranded cDNA for use in the rapid amplification of cDNA ends (RACE) reaction was generated using the Marathon cDNA amplification kit following the manufacturer's instructions (Clontech, Palo Alto, CA). Briefly, total RNA was extracted as described (8), and poly(A) mRNA was isolated using an oligo(dT) cellulose spin column (United States Biochemical, Cleveland, OH). Reverse transcription of the poly(A) RNA was performed using Moloney murine leukaemia virus reverse transcriptase and dNTPs; second-strand cDNA was synthesized using dNTPs, RNaseH, Escherichia coli DNA polymerase I, and T4 DNA polymerase. The double-stranded cDNA was then ligated, using T4 DNA ligase, to the cDNA adapter, and a reverse primer (5'-CGCGGAGGGTGG-3') (Clontech, Palo Alto, CA).

Double-stranded cDNA was then amplified using KlenTaq polymerase (Clontech), the AP1 forward primer contained in the cDNA adapter, and a reverse primer (5'-TGGCCTTTCGAGGAGGGTGG-3') equivalent to base pairs +1647 through +1665 of the published rat kidney CaSR sequence (30). The resulting amplifier, which was ~1,500 bp, was then subcloned into the pT7BlueR vector (Novagen) for sequence analysis using the dideoxy method. Once the sequence for the 5'-UTR of the DRG CaSR is unique to the DRG or is also expressed in thyroparathyroid tissue and the kidney. Total RNA isolated from DRG, thyroparathyroid, and kidney, was used as template in reactions using primers designed to amplify the 722-bp region of the published rat kidney 5'-UTR and the 712-bp region of the DRG 5'-UTR that we identified. A common reverse primer with the sequence 5'-TCTGCAGGGTGG-3' and an internal forward primer with the sequence 5'-TGTCAGACGGATCTT-3' (bp 551 to bp 127 149) of the published kidney sequence. The PCR reaction was carried out for 30 cycles of denaturation (94°C, 1 min), annealing, and extension (68°C, 3 min). The products were separated on 1.5% agarose gels. Restriction mapping using MseI was used to analyze the reaction products.

Western blot. Protein was extracted from minced parathyroid gland and DRG by homogenization using a glass-glass homogenizer in buffer containing 10 mM Tris, pH 7.5, 0.25 M sucrose, 3 mM MgCl\textsubscript{2} containing dithiothreitol (1 mM), Pefabloc (1 mM), leupeptin (10 \mu M), bestatin (130 \mu M), pepstatin (1 \mu M), and calpain inhibitor II (10 \mu g/ml). The homogenate was then centrifuged at 10,000 g for 10 min, and a microsomal fraction was prepared from the supernatant by centrifugation at 100,000 g for 90 min. The pellet was dissolved in buffer containing 10 mM Tris, pH 7.5, and 1% Triton X-100, size separated using 8% SDS-PAGE, and electroblotted onto nitrocellulose membrane as described (8). The membrane was incubated with either a commercially available polyclonal antiserum raised against a synthetic peptide of the rat CaSR with the sequence ALAWHSSAYGPDPQRAQ (Affinity Bioreagents, Golden, CO) or a polyclonal antibody that recognizes enhanced green fluorescent protein (EGFP; Clonetics). The primary antibody-protein complex was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h and visualized using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Piscataway, NJ). In some experiments, the antibody was preadsorbed with excess antigen to establish specificity.

Deglycosylation. The effect of deglycosylating the protein was determined as previously described (2). For cleavage with peptide N-glycosidase F (PNGase F; Boehringer Mannheim), the protein extract was denatured using SDS and ß-mercaptoethanol (BME) and then incubated in a buffer containing 50 mM Tris, pH 8.0, and a cocktail of protease inhibitors with or without 1 unit of PNGase F for 4 h at 37°C. For cleavage with endoglycosidase H (EndoH; Boehringer Mannheim), the protein extract was denatured using SDS and BME and incubated in a buffer containing 50 mM sodium citrate, pH 5.5, and the protease inhibitor cocktail with or without 0.1 mU of EndoH for 4 h at 37°C. The treated proteins were subsequently separated using 8% SDS-PAGE,
electroblotted onto nitrocellulose membranes, and probed with polyclonal anti-CaSR.

**Cultured DRG neurons.** DRG were isolated under aseptic conditions, trimmed of axons, and dispersed using a collagenase-based method as described by Supowit et al. (35). The dispersed cells were initially seeded on polyornithine-coated coverslips in F-12 medium supplemented with 10% heat-inactivated horse serum. After 48 h, the medium was replaced with serum-free F-12 enriched with N2 supplement (GIBCO). After an additional 48 h, the cells were fixed with paraformaldehyde and immunostained by using polyclonal anti-CaSR at 1:250 as primary antibody and Texas red-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) at 1:750 as secondary antibody. Negative controls were stained with secondary antibody alone. After they were mounted, the cells were imaged using a Zeiss LSM 510 laser scanning confocal microscope (Thorwood, NY) with a ×40 oil-immersion objective.

**CaSR-pEGFP fusion construct.** In-frame fusion of the cDNA encoding full-length DRG CaSR with pEGFP was performed as described by Gama and Breitwieser (14) using a commercially available kit (pEGFP-N3 vector; Clontech). Total RNA was extracted and used in an RT-PCR reaction in which primers were designed to introduce a HindIII site −211 bp upstream from the CaSR start site and to replace the stop codon with a BamHI site. The forward primer was 5′-CTCTACATTCCTCACACGGAGAAGCAGC-3′, and the reverse primer was 5′-GCTATGGATCTTAAATACGTTTCTCCGTACAGACG-3′ (restriction sites in bold type). The resulting PCR product was gel purified and initially cloned into a TA cloning vector, after which the BamHI and HindIII fragments were excised and ligated into the pEGFP-N3 vector and transformed into Top 10 competent cells. A total of 20 bacterial colonies were isolated and expanded, and plasmids were purified with the use of a Qiagen miniprep kit and tested for the presence of the CaSR-pEGFP insert by using restriction analysis with HindIII and BamHI.

**Functional analysis.** Functional expression of the CaSR-pEGFP fusion protein was assessed using HEK-293 cells. A mixture of 4 μg of DNA plus 2 μl of Lipofectamine (LF-2000; GIBCO-BRL) was added to HEK-293 cells plated in 60-mm dishes. Protein isolated from cell extracts taken from 60-mm dishes was used in SDS-PAGE and Western blot analysis of the CaSR and EGFP protein as previously described in Western blot. Cells were also grown on glass coverslips for laser confocal microscopic analysis of EGFP protein expression and intracellular Ca2+ responses (Zeiss LSM 510) or conventional confocal fluorescence measurements were performed at room temperature.

**RESULTS**

**Sequence analysis of DRG cDNA.** 5′-RACE with a primer extension strategy was used to isolate a 4,107-bp cDNA from rat DRG (GenBank accession numbers: bankit511314, AY214122). Sequence analysis showed that the cDNA consists of a 3,237-bp open reading frame that predicts a 1,079-amino acid protein that is >99% homologous with the rat kidney CaSR (30). Only three base pair differences were detected that could not be attributed to sequencing error, and only one, 2705C→T, would result in a change in amino acid sequence (proline to leucine). The 3′-UTR is also completely homologous with the sequence reported for the rat kidney CaSR. In contrast, the 563-bp 5′-UTR showed only weak (45%) homology with rat CaSR and little or no homology to other known sequences (Fig. 1).

**Tissue distribution of the splice variants.** Because of the marked heterogeneity between the 5′-UTRs of the DRG and kidney CaSR messages, RT-PCR was performed to determine the tissue distribution of CaSR 5′-UTR. An aliquot of poly(A) mRNA from rat DRG, kidney, and the thyroparathyroid glands was reverse transcribed and amplified by PCR using the primers outlined in Fig. 1. The 5′-UTR of the DRG CaSR message was present only in the DRG, whereas the 5′-UTR of the kidney CaSR message was present in both kidney and thyroparathyroid glands (Fig. 2A). The identity of RT-PCR product was confirmed by restriction enzyme digestion using MseI (Fig. 2B). These data indicate that relative to at least two other major sites of expression (kidney and thyroparathyroid), the DRG CaSR mRNA is a tissue-specific variant.

**Immunocytochemistry and Western blot analysis.** Immunocytochemistry was performed on primary cultures of DRG neurons to assess for expression of CaSR protein by these cells. As shown in Fig. 3A, staining with anti-CaSR revealed CaSR protein in DRG neurons that is consistent with earlier demonstration of the receptor in perivascular sensory nerves (8, 10). In contrast, omission of the primary antibody resulted only in background staining of the cells (Fig. 3B).

SDS-PAGE and Western blot analysis of microsomal fractions of rat DRG, thyroparathyroid, and kidney homogenates were performed to assess the molecular mass of the DRG protein relative to the other tissues. The results showed that the thyroparathyroid fraction migrated as a doublet of ~140 and 160 kDa and a dense band of unknown identity at a higher molecular mass. In contrast, the DRG extract migrated as a single band of 140 kDa (Fig. 4A, control). Preadsorption of the antibody with excess antigen resulted in a complete loss of signal for the DRG CaSR (not shown). It has been postulated that the low-molecular-mass 140-kDa band seen in HEK-293 cells stably transfected with cDNA encoding the human parathyroid CaSR is a high-mannose-containing immature form of the receptor that is restricted to the endoplasmic reticulum (2). We therefore performed experiments to learn whether the single 140-kDa band seen in the DRG and mesenteric artery preparations is sensitive to EndoH, which
Fig. 1. Comparison of the NH$_2$-terminal coding sequence and nonhomologous untranslated regions (UTR) of the dorsal root ganglia (DRG) and published rat kidney (Kid) (30) Ca$^{2+}$-sensing receptor (CaSR) cDNA sequences. M indicates the start codon for the open reading frame; * indicates the putative splice junction of exons 1 and 2 (15). The underlined sequence labeled “R-common” shows the position of the common reverse primer, and the underlined sequences labeled “F-DRG” and “F-Kid” show the two different forward primers used for RT-PCR analysis of the DRG and kidney 5'-UTRs. MseI indicates restriction site used for mapping the 5'-UTRs.
cleaves mannose sugars. Incubation with PNGaseF, which cleaves N-linked oligosaccharides, caused a molecular mass shift of both bands in the thymoparathyroid fraction and in the 140-kDa band of the DRG to an estimated mass of 120 kDa (Fig. 4A). In contrast, neither the upper or lower bands of the thymoparathyroid doublet nor the 140-kDa band of the DRG fraction shifted after treatment with EndoH, which cleaves N-linked high-mannose oligosaccharides (Fig. 4B). EndoH did, however, cause a significant increase in the mobility of carboxypeptidase Y, which was used as a positive control for the EndoH assay (Fig. 4C).

**Functional analysis of the DRG CaSR.** As shown in Fig. 5A, the full-length cDNA encoding the DRG CaSR was fused in-line with the pEGFP vector to facilitate visual tracking of subsequently expressed CaSR protein. Western blot analysis of cell extracts from control and CaSR-EGFP-transfected HEK-293 cells using anti-CaSR demonstrated expression of a protein doublet with molecular masses of 185 and 165–170 kDa, which corresponds to the predicted CaSR-EGFP fusion protein. These proteins were not present in the control transfected cells (Fig. 5B). Similarly, when the blot was probed with anti-GFP, extracts of HEK cells transfected with the CaSR-EGFP fusion protein showed the same doublet as seen with the anti-CaSR, and these bands were absent in the control transfected cells.

When examined using laser scanning confocal microscopy, cells transfected with the CaSR-EGFP fusion product showed EGFP fluorescence at the cell membrane (Fig. 6A), whereas HEK-293 cells transfected with the EGFP vector showed a pattern of cytosolic expression of the EGFP protein (Fig. 6B). We noted that transient transfection of HEK-293 cells with the CaSR-EGFP vector resulted in heterogeneous expression of the EGFP-labeled protein (Fig. 7). For example, cells 1 and 2 showed high levels of fluorescence intensity, cells 3 and 4 showed a lower level of green fluorescence, and other cells (e.g., cells 5 and 6) showed no basal fluorescence at all. After cells were loaded with fluo 4, we found that increasing extracellular Ca\(^{2+}\) from 1 to 3 mM and higher resulted in an increase in intracellular Ca\(^{2+}\) in cells that showed green fluorescence before fluo 4 loading but not in nonfluorescent cells. Moreover, we found that a subpopulation of HEK-293 cells transfected with the CaSR-EGFP construct responded to the addition of extracellular Ca\(^{2+}\) with an initial rise in intracellular Ca\(^{2+}\) that became oscillatory in nature. The oscillatory activity was observed up to 20 min after the increase in extracellular Ca\(^{2+}\) and had a frequency of 2–2.5 cycles per minute (Fig. 7).

In addition to confocal analysis, the response of HEK-CaSR-EGFP cells to increasing concentrations of extracellular Ca\(^{2+}\) was also assessed using a fura 2-based system. After cells were loaded with fura 2, they were incubated in physiological salt solution containing 0.5 mM Ca\(^{2+}\). When HEK cells transfected with the DRG CaSR-EGFP fusion protein were studied, increasing extracellular Ca\(^{2+}\) from 0.5 to 1 mM resulted in an increase in intracellular Ca\(^{2+}\) (Fig. 8A) that persisted in the presence of 1 μM nifedipine (Fig. 8B), indicating that the rise in intracellular Ca\(^{2+}\) was not the result of an influx of Ca\(^{2+}\) through voltage-operated Ca\(^{2+}\) channels. In contrast, HEK cells transfected with the EGFP vector alone did not respond to increasing concentrations of extracellular Ca\(^{2+}\) (Fig. 8C).

**DISCUSSION**

The present experiments were performed to characterize the sensory nerve CaSR protein. The results reveal several new findings. One finding is that the
mRNA that gives rise to the DRG CaSR is significantly different in the 5’-UTR from those of the thyroparathyroid and kidney, suggesting that the DRG CaSR mRNA is the product of expression of alternate 5’-UTR exons. A second finding is that there is different posttranslational processing of the DRG CaSR protein from that of the rat thyroparathyroid protein, which results in altered migration of the protein on SDS-PAGE. Our data also confirm the recent report of Breitwieser and Gama (4) that expression of a CaSR-EGFP fusion product in HEK-293 cells results in the spontaneous development of oscillatory changes in intracellular Ca\(^{2+}\) concentration.

One observation that merits discussion is our finding that although the coding sequence as well as a span of 242 nucleotides 5’ to the translation start site of the

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**Fig. 3.** A: confocal image of a field of primary cultures of DRG neurons that have been stained with polyclonal anti-CaSR (1:250) and secondary antibody conjugated with Texas red (1:750). B: DRG neurons stained with secondary antibody conjugated with Texas red (1:750) alone (control).

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**Fig. 4.** Glycosylation pattern of the DRG and TPTH CaSRs. A: effect of digestion with peptide N-glycosidase F (PNGase) on the migration on 8% SDS PAGE of DRG and TPTH CaSR protein. PNGase shifted the DRG singlet and the TPTH doublet to a single lower molecular mass species. B: effect of digestion with endoglycosidase H (EndoH) on the migration of CaSR protein of DRG and TPTH. EndoH was without effect on either protein extract, but caused a significant shift in the migration of carboxypeptidase Y (C), which was used as a positive control. Similar results were observed in at least 3 different experiments.
DRG mRNA are homologous with that of the kidney CaSR, there is significant variation in the DNA sequence further upstream. Restriction enzyme analysis of RT-PCR products derived from the 5′-UTR of the thyroparathyroid, kidney, and DRG indicates that the thyroparathyroid and kidney share a common 5′-UTR sequence that is different from that of the DRG. Of interest, the site where the 5′ sequence diverges maps to the junction of exons 1 and 2 observed by Garrett et al. (15) for the human parathyroid CaSR. Thus it appears that alternative 5′-UTR exons have been spliced into a common coding region of the CaSR. This conclusion is supported by the recent report of Chikatsu et al. (12), who showed two different 5′ exons in the human system. When viewed in the context of the report of Oda et al. (28) that dermal cells express yet another splice variant of the CaSR lacking exon 5, it seems clear that the CaSR gene and its products have a high degree of complexity of both transcriptional and translational control.

Although we have not performed studies to assess the functional significance of the alternative splice variant that is present in the DRG CaSR transcript, the finding raises questions regarding its possible consequences. It is well established that there is regulatory information in the 5′-UTR of some mRNAs. For example, the 5′-UTR of cDNA encoding the iron storing protein-ferritin has an iron-responsive element that mediates iron-dependent control of its translation (1). Moreover, Wu and Bag (36) have shown that poly(A)-rich regions in the 5′-UTR of the poly(A)-binding protein contribute to message stability. Whether the heterogeneous 5′-UTRs for the CaSR play a role in regulating expression or message stability is unknown at the present time. Rogers and colleagues (32) have reported that the parathyroid CaSR message is not transcriptionally regulated by 1,25-dihydroxyvitamin D\(_3\) [1,25(OH)\(_2\)D\(_3\)] and suggest that the protein is constitutively expressed. In contrast, Brown et al. (5) have provided evidence that is consistent with regulation of CaSR message and protein by 1,25(OH)\(_2\)D\(_3\) in parathyroid gland (5). Moreover, Bikle et al. (3) have shown that CaSR message expression is enhanced by
1,25(OH)2D3 in noncommitted keratinocytes, and Riccardi and colleagues (31) have shown that dietary phosphate alters CaSR expression in the kidney tubule. Moreover, there appear to be disease states in which expression of the receptor is altered (18, 19). Whether the 5'-UTR of the CaSR mRNA is functionally significant is unknown at the present time.

A second finding that warrants discussion is the difference in the apparent molecular mass that was observed using SDS-PAGE for the CaSR protein extracted from the different tissues. On the basis of the cDNA sequence, the predicted molecular mass of the translation product is 116 kDa. Reports from multiple laboratories, however, have demonstrated that protein...

Fig. 7. Top: image of a field of HEK-293 cells transfected with the CaSR-pEGFP vector described in text. After we established whether cells exhibited EGFP fluorescence (cells 1-4) or not (cells 5 and 6), the cells were loaded with fluo 4 and the intracellular Ca2+ response to extracellular Ca2+ was determined. Bottom: time course of the intracellular Ca2+ response to increasing extracellular Ca2+ from 1 to 3 mM for cells exhibiting a high level of the CaSR-EGFP fluorescence (cells 1 and 2) and a low level of CaSR-EGFP fluorescence (cells 3 and 4), as well as the absence of an intracellular Ca2+ response in cells that exhibited no detectable fluorescence (cells 5 and 6).
isolated from the bovine parathyroid gland, as well as the human parathyroid CaSR cDNA expressed as protein in HEK-293 cells, migrates on SDS-PAGE as a doublet with molecular masses of 140 and 160 kDa. On the basis of enzyme-catalyzed deglycosylation experiments, it has been shown that the heavier-than-predicted variants reflect glycosylated species of the receptor (2, 17). Moreover, the pattern has emerged that the oligosaccharides in the lower molecular mass band of the parathyroid message expressed in HEK-293 cells have a high mannose content and are cleaved by both PNGase F and EndoH. This finding has been interpreted to indicate that the 140-kDa variant of the receptor is immature and restricted to the endoplasmic reticulum. In contrast, the oligosaccharides of the higher molecular mass variant of the receptor are cleaved only by PNGase F, and it has therefore been proposed that the heavier band represents the mature form of the receptor that is inserted into the cell membrane.

On the basis of these findings obtained using the HEK-293 expression model, our observation (8) that CaSR protein in membrane preparations of both the DRG and the mesenteric artery comigrate with the 140-kDa band can be interpreted to indicate that only the immature, non-plasma membrane-inserted form of the receptor is expressed in sensory nerves. In contrast, our finding that the DRG CaSR protein is shifted by PNGase F but not by EndoH, indicating that the receptor is not a high-mannose form of the receptor. These data support the hypothesis that a functional CaSR is present in the perivascular sensory nerve network and suggest that there is tissue-specific posttranslational processing of the DRG CaSR compared with that of the thyroparathyroid extract and that the DRG species has N-linked, EndoH-insensitive sugars. Of additional interest is the finding that the common DRG CaSR-EGFP fusion product results in a doublet when transfected into HEK-293 cells, indicating that DRG message is processed by these cells in a manner similar to the human CaSR message. Although we do not know whether this differential pattern of glycosylation results in functionally different receptors, there are examples in the literature where glycosylation of a protein significantly alters affinity or efficacy for particular ligands (34).

Our strategy for analyzing the functional properties of the DRG CaSR was to create an EGFP fusion protein as described by Gama and Breitwieser (14) and to study it after transient transfection into HEK-293 cells. Western blot analysis of the resulting fusion protein revealed a doublet with molecular masses of 185 and 165 kDa, consistent with the fusion of the 29-kDa EGFP protein onto the COOH terminus of the CaSR. Confocal analysis of HEK-293 cells transfected with the CaSR-EGFP construct showed localization of green fluorescence at the cell membrane, whereas fluorescence of cells transfected with the empty vector was widely distributed in the cytoplasm, indicating cell membrane localization of the CaSR-EGFP fusion product.

Confocal microscopy was used to study intracellular Ca$^{2+}$ signaling in fields of HEK-293 cells that had been transiently transfected with the CaSR-EGFP vector 48 h previously. Several findings are of note. One such finding is that cells that did not have detectable EGFP fluorescence before being loaded with fluo 4 did not respond to increasing concentrations of extracellular Ca$^{2+}$ with a mobilization of fluo 4 did not respond to increasing concentrations of extracellular Ca$^{2+}$ with a mobilization of Ca$^{2+}$ into the cytoplasm. In contrast, cells that showed basal EGFP fluorescence, and were thus expressing the CaSR protein, responded to increasing levels of extracellular Ca$^{2+}$ with a mobilization...

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**Fig. 8.** Changes in intracellular Ca$^{2+}$ in HEK-293 cells expressing the CaSR-EGFP fusion protein in response to increasing extracellular Ca$^{2+}$ from 0.5 to 1 mM under control conditions (A) and after preincubation with 1 μM nifedipine (B). C: lack of intracellular Ca$^{2+}$ responses in HEK-293 cells expressing EGFP protein in response to increasing levels of extracellular Ca$^{2+}$. $I_{340}/I_{380}$, ratio of fluorescence intensity at 340 and 380 nm; iono, ionomycin.
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|       | VI.  | RPNSVRSKKSSGSGTGGSSSISSKSSNSDRPQPERQOQPLQLSTQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPLQOQOPL
tion of intracellular Ca\(^{2+}\). Moreover, a subpopulation of these cells, which appeared to be expressing a lower level of CaSR protein, responded to the increase in extracellular Ca\(^{2+}\) with spontaneous oscillations in intracellular Ca\(^{2+}\). These findings confirm the recent report of Breitwieser and Gama (4) that HEK-293 cells transfected with a human parathyroid CaSR-EGFP fusion protein undergo oscillatory Ca\(^{2+}\) wave activity.

In addition to the confocal analysis, we also assessed extracellular Ca\(^{2+}\)-evoked changes in intracellular Ca\(^{2+}\) by using a fura 2-based system. HEK-293 cells expressing the DRG CaSR-EGFP fusion protein responded to increasing concentrations of Ca\(^{2+}\) within the physiologic range (0.5–3 mM). This high sensitivity to extracellular Ca\(^{2+}\) differs from the report of Breitwieser and Gama (4), who used a human parathyroid CaSR-EGFP fusion protein in which there is only 82% amino acid homology (Fig. 9). This region would likely be involved in extracellular Ca\(^{2+}\)–intra cellular Ca\(^{2+}\) coupling.

Although we do not know the basis for these differential observations, one possibility is a structural difference between the human parathyroid CaSR-EGFP fusion protein and the rat DRG CaSR-EGFP in the COOH-terminal tail of the protein, in which there is only 82% amino acid homology (Fig. 9). This region would likely be involved in G protein–coupled signaling.

In summary, we have cloned and sequenced the DRG CaSR and found that there is significant heterogeneity in the 5′-UTR compared with the rat kidney cDNA. The difference in the 5′-UTRs appears to be the result of splicing of two different exons to the coding region, and tissue analysis indicates that there is a distinct pattern of tissue-specific expression of these variants. In addition to this variation, there also appears to be tissue-specific posttranslational processing of the thyroparathyroid and DRG CaSRs, which is revealed by enzymatic digestion with two different glycosidases. Expression analysis of a EGFP fusion protein showed that the DRG CaSR transfected into the HEK-293 cell lines incorporates into the cell membrane and functionally links increases in extracellular Ca\(^{2+}\) with both sustained and oscillatory rises in intracellular Ca\(^{2+}\).

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REFERENCES


