Identification and localization of extracellular Ca\(^{2+}\)-sensing receptor in rat intestine

NAIBEDYA CHATTOPADHYAY,1 IVAN CHENG,1 KIMBERLY ROGERS,2 DANIELA RICCARDI,2 AMY HALL,3 RUBEN DIAZ,1 STEVEN C. HEBERT,3 DAVID I. SOYBEL,4,5 AND EDWARD M. BROWN3

1Endocrine-Hypertension Division, Departments of Medicine and Surgery, Brigham and Women's Hospital and Harvard Medical School, Boston 02115; and 2Department of Surgery, Veterans Affairs Medical Center, West Roxbury, Massachusetts 02132; 3NPS Pharmaceuticals, Inc., Salt Lake City, Utah 84108; and 4Nephrology Division, Vanderbilt University Medical Center, Nashville, Tennessee 37232

Chattopadhyay, Naibedya, Ivan Cheng, Kimberly Rogers, Daniela Riccardi, Amy Hall, Ruben Diaz, Steven C. Hebert, David I. Soybel, and Edward M. Brown. Identification and localization of extracellular Ca\(^{2+}\)-sensing receptor in rat intestine. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G122–G130, 1998.—The extracellular Ca\(^{2+}\)-sensing receptor (CaR) plays vital roles in Ca\(^{2+}\) homeostasis, but no data are available on its expression in small and large intestine. Polymerase chain reaction products amplified from reverse-transcribed duodenal RNA using CaR-specific primers showed >99% homology with the rat kidney CaR. Northern analysis with a CaR-specific cDNA probe demonstrated 4.1- and 7.5-kb transcripts in all intestinal segments. Immunohistochemistry with CaR-specific antisera showed clear basal staining of epithelial cells of small intestinal villi and crypts and modest apical staining of the former, whereas there was both basal and apical staining of colonic crypt epithelial cells. In situ hybridization and immunohistochemistry also demonstrated CaR expression in Auerbach’s myenteric plexus of small and large intestines and in the submucosa in the region of Meissner’s plexus. Our results reveal CaR expression in several cell types of small and large intestine, in which it may modulate absorptive and/or secretomotor functions.

villi; crypts; nerve plexus; Northern analysis; immunohistochemistry; in situ hybridization histochemistry

MAINTENANCE OF A CONSTANT level of the extracellular ionized calcium (Ca\(^{2+}\)) concentration is a vital biological function in a wide variety of organisms (4). The system regulating Ca\(^{2+}\) homeostasis involves several organs and hormones. The former include the parathyroid glands, the kidneys, the small and large intestines, and the skeleton, and the latter comprise parathyroid hormone (PTH), calcitonin, and vitamin D (for review, see Ref. 4). The parathyroid cell is extremely sensitive to alterations in Ca\(^{2+}\) and responds promptly with changes in PTH secretion designed to normalize Ca\(^{2+}\) by acting on its target organs (4). Acute PTH-mediated responses to hypocalcemia include increased reabsorption of Ca\(^{2+}\) from the kidney and resorption of Ca\(^{2+}\) from bone. Protracted hypocalcemia leads to PTH-stimulated 1-hydroxylation and resultant activation of 25-hydroxyvitamin D\(_3\) in the renal proximal tubule. The 1,25-dihydroxyvitamin D\(_3\) produced by this reaction then acts through its receptor in the duodenum to increase the absorption of Ca\(^{2+}\) (4, 10).

Regulation of PTH secretion by Ca\(^{2+}\) is dependent on a process of Ca\(^{2+}\) sensing that is afforded by a recently identified Ca\(^{2+}\)-sensing receptor (CaR), which is a G protein-coupled heptahelical receptor (5). Initially cloned from bovine parathyroid (5), CaR has subsequently been isolated from various other organs that participate in Ca\(^{2+}\) homeostasis, such as rat (28) and rabbit kidney (7), as well as human (20) and chicken parathyroid gland (15). It has also been cloned from the brain (30) and is likewise expressed in several other tissues that are not directly involved in the mineral ion homeostatic mechanism (e.g., keratinocytes (1) and lens epithelial cells (11)). The intestine is an important site for the maintenance of Ca\(^{2+}\) homeostasis because of its capacity for regulated absorption of dietary Ca\(^{2+}\). The intestinal tract exhibits functional heterogeneity along its length, and each intestinal segment has specialized absorptive and/or secretory functions (23), analogous to the nephron, which is also involved in the secretion and absorption of solutes. The proximal portion of the small intestine (i.e., the duodenum) absorbs large volumes of fluid against a relatively small electrochemical gradient (31), similar to the proximal tubule of the nephron. In addition, the duodenum is the major site for absorption of Ca\(^{2+}\), the jejunum and ileum, in addition to absorbing lesser amounts of Ca\(^{2+}\), are known to secrete Ca\(^{2+}\) by a nonsaturable paracellular mechanism (3, 23). Serosal-to-mucosal flux of Ca\(^{2+}\) in these intestinal segments may enable chelation of fatty acids and bile salts, thereby forming insoluble “calcium soaps” that preclude potential damage to colonic epithelial cells resulting from the actions of soluble, unchelated fatty acids and bile salts. Although the duodenum, jejunum, and ileum exhibit regional differences in their transport of various nutrients, vitamins, minerals, and bile acids, there are less marked regional differences in electrolyte transport (3). Even though the major function of colon is to absorb water and sodium (Na\(^{+}\)), which is again reminiscent of the function of certain portions of the nephron, it also absorbs significant amounts of
Ca\(^{2+}\) by both vitamin D-dependent and -independent mechanisms (17).

In addition to being absorbed and secreted by the intestine, Ca\(^{2+}\), along with vitamin D, exerts several direct actions on intestinal function. Ca\(^{2+}\) and vitamin D both increase the level of expression of calbindin in duodenal explants (2). In addition, there are several reports (6, 13, 14) that Ca\(^{2+}\) and/or 1,25(OH)\(_2\) vitamin D\(_3\) inhibit the proliferation of colonic epithelial cells in vivo and in vitro. Similar effects have been observed in cultured Caco-2 cells, a cell line derived from a human colon carcinoma (13, 14). Moreover, we have recently shown that these cells express CaR, which may mediate the effects of Ca\(^{2+}\) on the apical surface of the cells to inhibit cellular proliferation and expression of c-myc through activation of protein kinase C (PKC) (22). These data led us to study the localization of CaR in the small intestine and colon of the rat. We observed that it is widely distributed and exhibits region-specific variations in localization that are of potential physiological significance.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley CD rats (weighing 225–250 g) were obtained from Charles River Laboratories (Wilmington, MA). They were housed in a controlled environment with a 12:12-h light-dark cycle and had free access to standard rat chow and tap water. They were handled according to the humane practices of animal care established by the Standing Committee on Animals at Harvard University. Rats were killed after we induced anesthesia with pentobarbital sodium.

For reverse transcription-polymerase chain reaction (RT-PCR) and Northern analysis, tissues were harvested from the duodenal mucosa and muscularis as well as from the jejunum, ileum, and colon by gross dissection and were snap frozen in liquid nitrogen and stored at −70°C until further use. For immunohistochemistry, rats were perfused transcardially with 4% buffered paraformaldehyde via the descending aorta, followed by a solution containing phosphate-buffered saline (PBS) and sucrose having an osmolality of 700 mosM. After perfusion, tissues were quickly removed, postfixed in 4% paraformaldehyde for 3 h on ice, and cryoprotected by incubation in PBS-buffered 20% sucrose at 4°C overnight. The tissues were then embedded in optimum-cutting compound (Miles, Elkhart, IN), snap frozen in 2-methylbutane and liquid nitrogen, and stored at −70°C until use.

Detection of CaR transcripts in isolated gastrointestinal segments by RT-PCR. Total RNA was isolated as previously described by Chirgwin et al. (12). We used 5 µg of total RNA for the synthesis of sense-stranded cDNA. Briefly, RNA samples were reverse transcribed at 42°C for 1 h by incubation with 20 µl of an RT mixture containing the following constituents: 25 pmol random hexamer primers, 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl\(_2\), 20 U RNasin, 0.5 mM deoxyribonucleotide triphosphates (dNTP), and 200 U reverse transcriptase (Stratagene, La Jolla, CA) as well as 0.25 µM of sense and antisense primers based on the sequence of the rat kidney Ca\(^{2+}\)-sensing receptor (RaKCaR) (forward primer, 5'-ACCTTTAACCCTGGCCCTGA-3' (RaKCaR bp 597–616) and reverse primer, 5'-GGGCAACAAAATTCTAGT-3' (RaKCaR bp 964–980), respectively).

The primer pairs amplified a 383-bp fragment encoding a region located within the predicted NH\(_2\)-terminus of RaKCaR. The optimum temperature cycling protocol was determined to be 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 30 cycles using a programmable thermal cycler (Omnigen, Hybaid Instruments, Holbrook, NY). Positive and negative control experiments were performed, respectively, by using 5 µg of RNA extracted from rat kidney and RNA samples from various gastrointestinal segments processed similarly but without RT.

PCR products obtained from duodenal mucosa were subcloned into the pCR-Script SK(+) vector using the pCR-Script Amp SK(+) cloning kit (Stratagene). Bidirectional sequencing was performed using the dye-deoxy chain termination method with an Applied Biosystems model 373A automated sequencer (Department of Genetics, Children's Hospital, Boston, MA). Further nucleotide analyses were carried out using GeneWorks software (version 2.3.1, Intelligenetics, Mountain View, CA).

Detection of CaR transcripts by Northern blot analysis. For the purpose of determining the size of the CaR transcripts in various intestinal segments, we employed Northern blot analysis on aliquots of 5 µg poly(A)\(^+\) RNA obtained using oligo(dT) cellulose chromatography of total RNA. Poly(A)\(^+\) -enriched RNA samples were denatured and electrophoresed in 2.2 M formaldehyde-1% agarose gels along with a 0.24- to 9.5-kb RNA ladder (Life Technologies) and transferred overnight to nylon membranes (Duralon, Stratagene). A 577-bp Bgl II-Sac I fragment corresponding to nucleotides 721–1,298 of the RaKCaR cDNA was subcloned into the pBluescript SK(+) vector. The plasmid was then linearized with Bgl II, and a \(^{32}P\)-labeled riboprobe was synthesized with the MAXIscript T\(_7\) kit (Ambion, Austin, TX) using T\(_7\) polymerase and \(^{32}P\)UTP. Nylon membranes were prehybridized for 2 h at 42°C in a solution consisting of 50% formamide, 4× Denhardt’s solution [50× Denhardt’s solution is 5 g Ficoll, 5 g polyvinylpyrrolidone, and 5 g bovine serum albumin (BSA)], 5× SSPE (20× SSPE is 2.98 M NaCl and 0.02 M EDTA in 0.2 M phosphate buffer, pH 7.0), 0.5% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 250 µg/ml yeast tRNA, and 200 µg/ml calf thymus DNA. Labeled probe (2× 10⁶ cpm/ml) was then added, and the membranes were hybridized overnight at the same temperature. Washing was carried out at high stringency [in 0.25× SSC (20× SSC is 3 M NaCl, 0.3 M Na\(_2\)citrate-2H\(_2\)O), 0.5% SDS at 60°C] for 20 min. Membranes were then exposed to X-ray film (Kodak XAR-5, Eastman Kodak, Rochester, NY) for 4 days at –70°C. Integritvity of RNA samples was confirmed by reprobing the blot with a β-actin cDNA probe.

In situ hybridization histochemistry. In situ hybridization of transverse sections from rat duodenum and proximal colon was performed as previously described (29). Briefly, sense and antisense riboprobes corresponding to nucleotides 199 to +994 of the RaKCaR cDNA were transcribed using \(^{35}S\)UTP. Ten-micrometer frozen sections were fixed in 4% paraformaldehyde-0.1 M PBS, rinsed in PBS, and acetylated with acetic anhydride in 0.1 M triethanolamine. Sense or antisense probes were mixed in hybridization buffer (10⁵ cpm/ml) [50% deionized formamide, 250 µg/ml yeast tRNA, 100 µg/ml denatured salmon sperm DNA, 10 mM Tris, pH 7.5, 1 mM EDTA, 0.6 M NaCl and 1× Denhardt’s solution] and applied to the sections, which were then covered with Parafilm and incubated in
a humidified chamber at 55°C overnight. After hybridization, the sections were washed in 1× SSC and treated with 20 µg/ml ribonuclease A. The sections were subsequently washed in 0.1× SSC at 55°C, dehydrated in a graded ethanol series, and air dried overnight before being dipped in NTB-2 photographic emulsion (Eastman Kodak) and stored in light-tight boxes at 4°C for 7 days. The slides were then developed (D-19 developer, Eastman Kodak) and fixed (Eastman Kodak fixative). The sections, covered by glass coverslips, were photographed under dark-field microscopy.

Immunohistochemistry for CaR. Before immunohistochemistry, 2-µm-thick sections were subjected to "antigen rescue," according to the supplier's recommended procedure (9). Briefly, slides containing the sections were placed in a Tissue-TekR slide holder filled with 1× antigen-retrieval citra solution (concentrated format, 10×; BioGenex, San Ramon, CA) and microwaved at high power (800 W) until the solution came to a rapid boil, at which point the oven was reset at 500 W for 8 min. The slides were then allowed to cool for 30 min and were rinsed several times with distilled water, dried, and processed for immunohistochemistry as described below.

Endogenous peroxidases were inhibited by incubating the sections in peroxidase blocking reagent (DAKO, Carpenteria, CA) for 5 min followed by treatment with protein block serum-free solution (DAKO) for 15 min (7–9). The sections were then incubated overnight at 4°C with 10 µg/ml of affinity-purified anti-CaR antiserum (4637) raised against a peptide corresponding to amino acids 345–359, which resides within the predicted amino-terminal extracellular domain of the bovine parathyroid CaR. This antiserum has been characterized previously (9). Similar results (not shown) were obtained using another polyclonal antiserum raised against a peptide corresponding to residues 215–237 of the bovine CaR, which has likewise been characterized fully in our previous studies (8). Control sections were prepared by incubation with anti-CaR antiserum preabsorbed with the specific CaR peptide (10 µg/ml) against which it was raised. After washing the sections three times with 0.5% BSA in PBS for 10 min, we added peroxidase-coupled, goat anti-rabbit immunoglobulin G (1:100; Sigma Chemical, St. Louis, MO) for 1 h at room temperature. The slides were then washed in PBS three times for 10 min each, and the color reaction was developed using the DAKO AEC substrate system for ~5 min. The reaction was stopped by washing three times in water.

RESULTS

Distribution of CaR mRNA in small and large intestine by RT-PCR. RT-PCR was performed separately on RNA isolated from the muscularis and mucosa of the duodenum as well as on RNA isolated from whole jejenum, ileum, and several regions of the colon to screen for the presence of CaR transcripts. Figure 1A shows that PCR products of the expected size, i.e., 383 bp, were amplified by CaR-specific primers from reverse-transcribed RNA isolated from all of the segments of small and large intestine studied. Moreover, these PCR products were of the same size as that obtained from the RaKCaR cDNA as a positive control (not shown). 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 a PCR product subcloned from duodenal mucosa revealed >99% nucleotide identity with the corresponding sequence of the RaKCaR cDNA, indicating that this PCR product was derived from bona fide CaR transcript(s) (Fig. 1B).

Distribution of CaR mRNA in various intestinal segments by Northern blot analysis. Figure 2 shows Northern blot analysis on poly(A)+ RNA isolated from

![Fig. 1. A: reverse transcription-polymerase chain reaction (RT-PCR) amplification of the extracellular Ca2+-sensing receptor (CaR) sequences from various intestinal segments. The size of the CaR RT-PCR product was estimated to be 383 bp, as indicated. Lane 1: DNA ladder; lane 2: duodenal mucosa; lane 3: duodenal muscularis; lane 4: jejenum; lane 5: ileum; lane 6: proximal colon. B: the nucleotide sequence of a subcloned RT-PCR product from duodenal mucosa (designated as DuoCaR) is aligned with the sequence of the rat kidney CaR (RaKCaR) cDNA done. Primer sequences are described in MATERIALS AND METHODS, and numbering of the nucleotide sequence corresponds to that of the coding sequence of RaKCaR. Differences in the nucleotide sequences of DuoCaR and RaKCaR are underlined.]
the same regions of small and large intestine utilized for RT-PCR, which revealed two transcripts with sizes of 7.5 and 4.1 kb in all of the intestinal segments studied (Fig. 2, lanes c-g). The duodenum showed an additional 3.0-kb transcript (Fig. 2, lanes c and d). The 4.1-kb transcript was the predominant CaR mRNA species in all of the intestinal segments studied (Fig. 2, lanes c-g), whereas in rat kidney and brain (Fig. 2, lanes a and b), the 7.5-kb transcript was expressed at the highest levels.

Immunohistochemistry of CaR in small and large intestine. Immunohistochemistry using a specific anti-CaR antiserum (antiserum 4637, see MATERIALS AND METHODS) directed at a highly conserved epitope within the extracellular domain of the CaR (residues 345–359) identified CaR immunoreactivity in the duodenum that was localized predominantly on the basal aspects of the villus absorptive cells, the epithelial cells of the crypts and Brunner’s glands, as well as in the region of the submucosa containing Meissner’s plexus; there was also strong CaR immunostaining in the serosa (Fig. 3, A, B, and D–F). The specificity of the immunostaining was confirmed by its abolition after preabsorption of the anti-CaR antiserum with the peptide against which it was raised (Fig. 3C). No CaR immunoreactivity was observed in the smooth muscle layers. Staining of nerve fibers extending from the submucosa into Auerbach’s plexus between the circular and longitudinal layers of the duodenal muscularis could be visualized in some sections (see also Fig. 5A). There was no change in the pattern of staining when the tissue sections were pretreated with collagenase before immunostaining; moreover, similar results were observed using another anti-CaR antiserum raised against a different epitope within the extracellular domain [antiserum 4641, raised against a peptide corresponding to amino acids 215–237 in the bovine CaR (8), which are identical to the corresponding residues in RαKCaR; not shown].

The jejunum and ileum showed a pattern of immunostaining for CaR that was similar to that observed in the duodenum (Fig. 4). In these regions of the small intestine as well as in the duodenum, the intensity of CaR immunoreactivity was greater in the bases than at the tips of individual villi. In all cases, the specificity of CaR immunoreactivity in the jejunum and ileum was confirmed by demonstrating ablation of staining after preabsorption of the primary, anti-CaR antiserum with its specific peptide (data not shown). In addition to the CaR immunoreactivity in surface epithelial cells of the small intestine, there were also isolated, intensely CaR-immunoreactive cells as well as tubular structures that stained for CaR within the interior of the villi. The identity of the former is uncertain, whereas the latter may represent lacteals.

In transverse sections of the entire colon, strong immunostaining for the CaR was observed in the serosa, on the basal surfaces of the crypt cells, in the submucosa, and in what appeared to be nerve fibers extending between the submucosa and Auerbach’s myenteric plexus (Fig. 5A). In cross sections of some crypts obtained by sectioning parallel to the surface of the colon, there was clear staining of the apical plasma membrane of the crypt cells and the underlying cytoplasm (Fig. 5, B and D). As in the small intestine, a similar pattern of CaR immunoreactivity was observed with both anti-CaR antisera, 4637 and 4641, and the specificity of the immunostaining was confirmed by the marked reduction in the intensity of the staining when the anti-CaR antisera were preabsorbed with their respective immunogenic peptides (e.g., Fig. 5C).

In situ hybridization histochemistry of CaR mRNA in small and large intestine. In situ hybridization was performed using an antisense probe derived from a portion of the CaR cDNA corresponding to a region of the extracellular domain of the receptor, as described in MATERIALS AND METHODS. Cells expressing CaR mRNA were present in Auerbach’s myenteric plexus between the circular and longitudinal muscle layers (Fig. 6, A and B). These cells appeared as intense clusters of silver grains (see arrows in Fig. 6A). There was additional, diffuse labeling of cells within the submucosa and in the crypt and villus cells of the duodenum. Strong labeling could also be seen in Auerbach’s plexus of the colon (Fig. 6B). Specificity of the labeling was confirmed by incubating sections with a sense probe (data not shown).

DISCUSSION

The CaR has been found in a variety of organs and cell types (10). It is highly abundant in the parathyroid gland and kidney, presumably reflecting the key roles of these tissues in the maintenance of Ca\textsuperscript{2+} homeostasis (10). Its presence in several additional organs or cell types, including numerous regions of the brain (30), AtF-20 cells (16), keratinocytes (1) and lens epithelial cells (11), which, however, are not directly involved in mineral ion homeostasis, suggests that CaR might have a role in regulating other types of processes as well. CaR has also been demonstrated to be present in several human intestinal epithelial cell lines in recent studies (19). Although the intestine plays an important role in Ca\textsuperscript{2+} homeostasis because of its capacity for regulated absorption of dietary Ca\textsuperscript{2+} and phosphate, the potential
role(s) of the CaR in these processes has not been explored. In addition, various physiological processes in both the small and large intestine, such as exocrine and endocrine secretion, proliferation, and maturation of the mucosal epithelial cells as well as contraction of smooth muscle, are Ca^{2+}-dependent. Therefore, we studied CaR localization in the small and large intestine as a first step toward investigating the potential physiological functions of CaR in the gastrointestinal tract.

Northern blot analysis demonstrated the presence of CaR transcripts with molecular sizes of 7.5 and 4.1 kb in all of the intestinal segments examined, whereas the duodenum alone expressed an additional, smaller transcript of 3.0 kb. CaR transcripts of 7.5 and 4.1 kb are also present in rat kidney (8) and brain (9); however, the relative ratios of the abundance of these two transcripts in kidney and brain varies from that of the various segments of intestine studied here. The ratio of the relative abundances of the 7.5- and 4.1-kb transcripts in kidney and brain is ~2.0, whereas it is ~0.3 throughout the intestinal tract. Because the 4.1-kb transcript encodes the entire functional CaR protein (28), the significance of the larger 7.5-kb transcript remains uncertain. However, the possibility of organ-specific, posttranscriptional regulation of CaR expression as a result of variations in the stabilities of the

Fig. 3. Immunohistochemistry on sections obtained from rat duodenum using a specific anti-CaR antiserum and the immunoperoxidase technique as described in MATERIALS AND METHODS. Photographs were taken by light microscopy. A: full-thickness section showing CaR immunoreactivity in the villus (arrow head), crypt (arrow), and submucosa (open arrow). Double-headed arrow shows muscle layers, which do not exhibit any CaR staining. B: villus at high power. C: peptide-preabsorbed control of villus. D: crypts and submucosal layer at high power. E: crypts and bases of villi at high power. F: area of duodenal submucosa showing Brunner’s glands (clear circular areas) with staining of the basal surface of epithelial cells (arrows). Scale bar represents 150 µM in A, 20 µM in B and C, and 40 µM in D-F.
Fig. 4. Immunohistochemistry of rat small intestinal segments using a specific anti-CaR antiserum and the immunoperoxidase technique as described in MATERIALS AND METHODS. Photographs were taken by light microscopy. A: full-thickness section from jejunum. B: full-thickness section from ileum. Scale bar represents 150 µm in A and 100 µM in B.

Fig. 5. Immunohistochemistry of rat proximal colon using specific anti-CaR antiserum and the immunoperoxidase technique as described in MATERIALS AND METHODS. Photographs were taken by light microscopy. A: full-thickness section from proximal colon (antiserum 4637). B: cross section of colonic crypts (antiserum 4641). C: peptide-preabsorbed control of colonic crypts with slight residual staining indicated by arrow (antiserum 4641). D: higher power magnification of colonic crypts in B showing apical staining for CaR as well as diffuse cytosolic staining under the apical membrane (antiserum 4641). Scale bar represents 150 µM in A, 40 µM in B and C, and 20 µM in D.
various CaR transcripts cannot be ruled out. Moreover, the presence of a distinct, lower molecular weight transcript of 3.0 kb might indicate expression of a different form of this or a related CaR in the duodenum. Even smaller (e.g., ~2 kb), CaR-related transcripts have also been described in the parathyroid (5), but their functional relevance is likewise unknown at present.

Sequencing of RT-PCR products from the duodenal muscularis revealed >99% sequence identity with RaKCaR cDNA. 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, since PCR products were not amplified under these conditions. In addition, the use of intron-spanning primers precluded amplification of a product of the size expected from bona fide CaR transcripts as a result of priming from contaminating genomic DNA. The low level of nonidentity between the sequences of the rat intestinal CaR transcripts and the RaKCaR cDNA most likely reflects PCR artifacts, since different nonidentities in nucleotide sequences were observed in RT-PCR products amplified from RNA isolated from the rat stomach (I. Cheng, N. Chattopadhyay, D. Soybel, and E. M. Brown, unpublished observations). Therefore, our data show that the CaR transcripts in the rat intestinal tract are essentially identical to those in the kidney and brain, at least in the 5' region that harbors the putative binding site for Ca\textsuperscript{2+} and other polycationic ligands. Because the probe used for Northern analysis was also derived from the 5' portion of the RaKCaR cDNA, the possibility that the intestinal tract expresses transcripts containing different transmembrane and/or cytosolic domains of the CaR cannot be ruled out without cloning the full-length transcript(s). The latter possibility appears unlikely in view of the similarity between the predominant CaR transcripts observed on Northern analysis and those observed previously from the rat kidney and brain.

In the duodenum, CaR immunoreactivity was principally localized on the basal aspects of the epithelial cells of the villi and crypts as well as in the submucosa, which consists of densely packed Brunner's glands and Meissner's plexus. The intensity of the CaR immunostaining was greater near the bases of the villi and within the crypts than at the tips of the villi. In the duodenum, epithelial proliferation occurs in crypt cells, but these latter cells also serve important secretory functions. Undifferentiated cells originating from proliferating stem cells within the crypt migrate up the villi and are eventually extruded into the gut lumen at the villus tips. Therefore, the abundance of the CaR in the crypts and at the bases of the villi and the lower levels of receptor expression at the tips of the villi suggest a potential role(s) for the receptor in the process of crypt-to-villus differentiation, wherein a signal for differentiation might also act as a signal for inhibiting CaR expression at the tip of the villus.

The duodenum manifests the greatest absorptive flux of Ca\textsuperscript{2+} per unit length. Therefore, the presence of CaR immunoreactivity at higher levels on the basal side of the epithelial cells of the villi relative to their apical surface might indicate some role for the CaR in absorptive processes involving sensing of Ca\textsuperscript{2+} on the systemic or blood side of the absorptive cells. In addition, further studies using costaining with specific markers will be needed to identify the additional CaR-expressing cells within the villi, which might potentially include enterochromaffin cells and/or lacte-
The presence of the receptor on the basal side of the crypts is also of interest in terms of a possible direct regulation of intestinal secretion by Ca$^{2+}$. Recent studies have shown that raising the level of Ca$^{2+}$ perfusing the blood supply to the small intestine inhibits intestinal absorption and reduces secretion of Ca$^{2+}$ (24), raising the possibility of a physiological role for CaR in mineral ion homeostasis via regulation of intestinal secretion. The localization of the CaR protein on the basal surface of the epithelial cells, without any apparent CaR immunoreactivity on the lateral cell surface, may indicate specific interactions of the receptor with molecular components present in the basal plasma membrane per se and/or the basement membrane. The presence of the CaR protein in Brunner’s glands may suggest some role for the receptor in control of the secretion of the alkaline, mucus-rich solution that is produced by these glands. Additional studies using immunolectron microscopy will be necessary to determine whether the CaR on the basal surface of Brunner’s glands and/or the epithelial cells of the crypts and villi is localized solely on epithelial cells per se or is also present on CaR-expressing nerve endings that innervate these cells.

The jejunum and ileum are sites where Ca$^{2+}$ secretion takes place, although a minor fraction of the total Ca$^{2+}$ entering in the small intestine is also absorbed in these segments (23–25). Intense staining in Auerbach’s and perhaps in Meissner’s plexus in jejunum and ileum raises the possibility that the CaR has some function(s) in secretory or motor functions of these segments of the small intestine. Therefore, it is possible that the CaR could play diverse role(s) in both absorption of Ca$^{2+}$ and in secretion of ions and mucus by various segments of the small intestine as well as in the motor functions of the intestine. It is a long-standing clinical observation that hypercalcemia reduces gastrointestinal motility, whereas hypocalcemia is associated with increased gastrointestinal motility. In view of the presence of the CaR within the enteric nervous system throughout the small and large intestine, it is possible that these effects of alterations in systemic levels of Ca$^{2+}$ are mediated by the CaR. Direct investigation of the actions of specific CaR agonists on gastrointestinal motility, however, will be needed to address this latter point further. In addition, studies directed at colocalizing the CaR with specific neuronal markers, including specific neurotransmitters, will be important to address the specific type(s) of neurons expressing the receptor and its functional importance in the enteric nervous system.

Absorption of Ca$^{2+}$ in the rat colon is thought to contribute significantly to the overall intestinal absorption of this mineral ion (17). Our results show the presence of CaR on both the apical and basal aspects of the epithelial cells of the colonic crypts as well as in the submucosa in the region of Meissner’s plexus by immunohistochemistry. CaR localization within the enteric nervous system of the colon was confirmed by in situ hybridization histochemistry, which revealed intense radioactive labeling of neuronal cell bodies within Auerbach’s myenteric plexus. A large body of data has shown that Ca$^{2+}$ inhibits proliferation of colonic epithelial cells (6, 13, 14, 22), and recent studies have suggested that this process is CaR mediated (22). We have demonstrated that reduction in luminal Ca$^{2+}$ stimulates PKC activity and c-myc expression in the Caco-2 cell line, which are associated with increased cellular proliferation (22). Moreover, expression of the CaR was apparent on the apical cell surface of Caco-2 cells. The difference in the apparent polarity of the receptor in normal rat colonic crypt cells vs. Caco-2 cells might be contributed to by 1) species variation, 2) neoplastic transformation, and 3) changes occurring in culture with the latter. It is likewise known that phorbol esters inhibit Ca$^{2+}$-dependent Cl$^{-}$ secretion by colonic epithelial cells (21). It is also possible that the CaR could modulate the activity of the Na$^{+}$–K$^{+}$–2Cl$^{-}$ cotransporter in the colon, as has recently been shown to be the case in the thick ascending limb of the nephron (32).

Our present results, therefore, show that the same CaR that is present in the parathyroid, kidney, and a variety of other cell types (10), both those involved and those uninvolved in systemic mineral ion homeostasis, is also expressed widely throughout the intestinal tract. CaR is located in epithelial cells, in which it could be involved in the control of intestinal absorptive and/or secretory functions, as well as in the enteric nervous system, where it could potentially regulate secretomotor functions. Additional studies are needed to define the functional implications of the CaR along the length of the gastrointestinal tract.

We gratefully acknowledge Dr. Olga Kifor’s suggestions and expertise on immunohistochemistry. We also gratefully acknowledge generous grant support from the National Institute of Diabetes and Digestive and Kidney Diseases (Grants DK-41415 and DK-48330 to E. M. Brown), the St. Giles Foundation (E. M. Brown and S. C. Hebert), NPS Pharmaceuticals, Inc. (E. M. Brown and S. C. Hebert), and the National Dairy Council (E. M. Brown).

Address for reprint requests: N. Chattopadhyay, Endocrine-Hypertension Division, Brigham and Women’s Hospital, Harvard Medical School, 221 Longwood Ave., Boston, MA 02115.

Received 15 July 1997; accepted in final form 6 October 1997.

REFERENCES


