The Deletion of 14 Amino Acids in the Seventh Transmembrane Domain of a Naturally Occurring Calcitonin Receptor Isoform Alters Ligand Binding and Selectively Abolishes Coupling to Phospholipase C*

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The cDNA that encodes the rabbit calcitonin receptor was cloned by screening a rabbit osteoclast library. Reverse transcription-polymerase chain reaction amplification of calcitonin receptor sequences from rabbit osteoclast RNA yielded cDNAs that encode two isoforms of the calcitonin receptor. One isoform is homologous to the C1a isoform previously identified in multiple cell types and species, while the second, designated CTRα13, is a previously unidentified isoform that is apparently generated by alternative splicing during mRNA processing that deletes exon 13, resulting in the absence of 14 amino acids in the predicted seventh transmembrane domain. Expression of mRNA transcripts encoding the two isoforms varies in a tissue-specific manner, with CTRα13 accounting for less than 15% of the total calcitonin receptor mRNA in osteoclasts, kidney, and brain, but comprising at least 50% of the transcripts in skeletal muscle and lung. The two isoforms were expressed, and the ligand binding and signal transduction properties were characterized. Deletion of the residues in the seventh transmembrane domain in CTRα13 reduced the binding affinity for salmon and human calcitonin by more than 10-fold and approximately 2-fold, respectively, resulting in a receptor that failed to discriminate between the two forms of calcitonin. Both isoforms activated adenylyl cyclase, with EC50 values consistent with the difference in ligand affinities. In contrast, only the C1a isoform, but not the CTRα13 isoform, activated phospholipase C. Thus, while the CTRα13 remains active despite the deletion of a significant portion of its seventh transmembrane domain, it has significantly altered ligand recognition and signal transduction properties.

Calcitonin (CT)1 is a 32-amino acid peptide hormone that acts to reduce serum calcium levels by inhibiting bone resorption and promoting renal calcium excretion. Besides this hypocalcemic effect, CT modulates the renal transport of water and several ions other than calcium, and acts on the central nervous system to induce analgesia, anorexia, and gastric secretion (1). The CT receptor (CTR) belongs to a family of G protein-coupled peptide receptors that includes receptors for parathyroid hormone/parathyroid hormone-related peptide, secretin, vasoactive intestinal peptide, growth hormone-releasing hormone, glucagon, glucagon-like peptide, pituitary adenyl cyclase-activating peptide, corticotropin-releasing factor, and calcitonin gene-related peptide (2–4). The CTR, like other members of this family, is known to couple to multiple trimeric G proteins that activate several signaling molecules, thereby producing diverse biologic responses in different cell types. For example, we and others have shown that some aspects of the osteoclast (OC) response to CT are mediated by the cAMP pathway, while others are mediated by protein kinase C (5, 6), and studies in our laboratory, using synchronized LLC-PK1 kidney proximal tubule cells, have shown that the mode of receptor coupling and the biological effects of CT vary with the stage of the cell cycle (7, 8). The coupling of a recombinant CTR to both the adenylyl cyclase and phospholipase C signaling pathways has been demonstrated (9, 10). However, the mechanisms by which the coupling to individual G proteins might be differentially regulated are not defined. Cloning of the CTR gene and cDNAs has revealed the presence of several alternative spliced cassettes, resulting in the expression of different isoforms of the receptor. Four isoforms have so far been identified. The most common isoform, C1a, was originally cloned from porcine kidney epithelial cells (11) and is also expressed in human, mouse, and rat cells (12–16). A second isoform, which contains a 16-amino acid cassette in the putative first intracellular loop, is expressed in both human and pig cells (12, 17, 18). Compared to the C1a, it has been reported to have a similar (19) or higher (20) binding affinity for salmon CT (sCT), but less potent ligand-dependent cAMP responses and no coupling to phospholipase C (19, 21). A third isoform, C1b, contains a 37-amino acid cassette in the putative first extracellular loop and is found in mouse brain and OC-like cells and rat brain (13, 14, 22). It shows lower ligand binding affinity than C1a, but no difference in the ability of the receptor to couple to signaling pathways. The fourth isoform, cloned from human breast carcinoma cells, lacks the first 47 amino acids of the N-terminal extracellular domain, but shows no change in ligand affinity to sCT or in ligand-dependent cAMP response relative to C1a (23). Taken together, these data suggest that the function of functionally distinct isoforms of the CTR by alternative splicing of mRNA contributes to the modulation...
of cellular responses to CT.

To better understand the molecular basis of the function of the CTR in OCs, we have investigated the expression of CTR isoforms in isolated rabbit OCs. In addition to the widely expressed C1a isoform, we found a novel isoform, CTRε13, that is generated by deletion of the cassette that corresponds to exon 13 of the porcine CTR gene (17) and encodes 14 amino acids in the putative seventh transmembrane domain. The CTRε13 mRNA is present in all rabbit tissues that express the C1a isoform. Despite the deletion of more than half of the putative seventh transmembrane domain, which could have been expected to have a profound negative effect on the CTR expression or activity, characterization of the CTRε13 isoform demonstrated that the protein is a functional CTR, albeit with unique differences in ligand binding and signal transduction properties relative to those of C1a.

**EXPERIMENTAL PROCEDURES**

**Osteoclast Isolation—**Osteoclasts were isolated as described previously (24), with minor modifications. Long bones were isolated from 1-week-old rabbits (body weight, 90–120 g). After removal of muscle and cartilage, the bones were minced in minimum essential medium-α modification (MEM) (Sigma) (pH 6.9) containing 5% fetal bovine serum, 1% penicillin-streptomycin, 26 mM sodium bicarbonate, and 10 mM HEPES. Cells were dissociated from bone fragments by gentle vortexing, then bone fragments were allowed to settle under normal gravity. The supernatant was removed and saved, and the mining and sedimentation were repeated three more times. The supernatants were pooled and centrifuged for 5 min at 60 × g. The pellet cells obtained from the bones of one rabbit were resuspended in 40 ml of α-MEM and plated into four 10-cm culture dishes. After 18 h of culture, the adherent cells were washed three times with phosphate-buffered saline and then treated with phosphate-buffered saline containing 0.02% EDTA and 0.001% Pronase E (Sigma) for 10 min at 37°C to remove contaminating cells. The highly enriched osteoclasts (~90%) were washed three times in phosphate-buffered saline and cultured in α-MEM for 18 h prior to isolation of RNA.

**Cloning of the Receptor cDNA—**Standard molecular biology techniques were performed as described elsewhere (25) unless otherwise noted. RNA was isolated from purified osteoclasts using the guanidine isothiocyanate method (Stratagene, Menasha, WI). A cDNA library was generated from rabbit OC mRNA in the αEXloX vector. Oligonucleotide primers were designed based on sequences that are highly conserved among C1 members from other species (11, 12, 14, 15). For RT-PCR, first strand cDNA was synthesized by reverse transcription using a gene-specific primer at the C-terminal end of the coding region, and the CTR cDNA was amplified by PCR with various pairs of primers chosen to correspond to sense and antisense sequences in the N-terminal extracellular domain (nucleotides 458–484 of human C1a cDNA; 5′-TATTGCGACCCCGATGGATGGATGG-3′ (primer 1), the fifth transmembrane domain (primer 2, antisense complementary to nucleotides 1187–1213 of human C1a cDNA; 5′-CATGACAGGTCCATGGATGGATGG-3′ (primer 3), the sense corresponding to nucleotides 1139–1165 of human C1a cDNA; 5′-GCCAATTCACCTCAATGACCACTGTCCGG-3′ (primer 4)). The PCR product was cloned and sequenced to confirm that the amplified DNA fragment corresponded to the expected sequence.

**Ligand Binding Assay—**Both saturation and competition binding assays were performed as described previously (19). Cells were rinsed with binding medium (DMEM containing 1 mg/ml bovine serum albumin) and incubated in this medium for 15 min at room temperature. In saturation binding assays, the medium was then removed and replaced with binding medium containing increasing concentrations of [3H]-labeled human calcitonin (Peninsula, Belmont, CA) with or without a 100-fold excess of unlabeled calcitonin. Cells were incubated at 37°C for 60 min. After washing, the cells were incubated in assay medium containing various concentrations of salmon CT at 37°C for 30 min. The reaction was stopped by the addition of 0.75 ml of ice-cold 20 mM formic acid. Inositol phosphates were separated from myo-inositol by ion exchange chromatography using Dowex columns (Bio-Rad AG 1-X8, 100–200 mesh, formate form). Data are expressed as the percentage of the total recovered [3H]inositol that is found in the inositol phosphate (IP) fraction.

**RESULTS**

**Molecular Cloning of the CTR Isoforms in Rabbit Osteoclasts—**Other laboratories have identified several isoforms of CTR. The full-length of CTR exon 13 was amplified using a pair of sense (nucleotides 1383–1403 of rabbit C1a; 5′-GGAGATCCTAGTGGTACCTCAGTCCG-3′) and antisense primers (nucleotides 1601–1622 of rabbit C1a; 5′-CTACCCACGAGGGCCACCGAAAC-3′). Thirty-five cycles of amplification were performed using AmpliTaq DNA polymerase (Perkin-Elmer) with 1 min of cycling at 94°C, 1 min at 60°C, 30 s at 72°C and a final extension for one cycle at 72°C for 1 min. The amplified PCR products were run on an 8% agarose gel, transferred to a nylon membrane and probed with a 32P-end-labeled oligonucleotide that complexed to sequence common to both isoforms (nucleotides 1486–1508 of rabbit C1a).

**RNA protection assay** was performed using the RPA II kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Total RNA (20 μg from OC and 100 μg from the other tissues) was mixed with the [32P]-labeled (100,000 cpm) sense RNA probe in 20 μl of hybridization buffer and incubated at 42°C overnight. The following morning, 200 μl of 1:100 diluted RNAse solution was added, and the mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of 300 μl of RNAse inactivation/precipitation mixture, and the RNA precipitated at −20°C for 30 min. Protected RNA fragments were separated on a 8% denaturing polyacrylamide gel and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) overnight.

**Cell Culture and Transfections—**The COS-7 cells were maintained in Dulbecco’s minimum essential medium (DMEM) (Life Technologies, Inc.) containing 4500 mg/liter glucose, 5% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. The HEK-293 cells were maintained in the same medium except that 10% heat-inactivated fetal bovine serum was used. Cells grown to 50% confluence in 10-cm tissue culture dishes were transfected with 8 μg of plasmid DNA and 60 μg of Lipofectin reagent (Life Technologies, Inc.) in 5 ml of DMEM (27) and incubated at 37°C in a 5% CO2 atmosphere for 6 h, after which 5 ml of DMEM containing 10% fetal bovine serum were added to transfection medium. Cells were cultured for an additional 18 h, at which time the transfection medium was replaced with regular culture medium. 24 h later, cells were replated into 24-well plates at a density of 5 × 104 cells/well and cultured for an additional 48 h before assaying the receptor function. Stable transfectants were generated by culturing transfected HEK-293 cells with 500 μg/ml G418 (Life Technologies, Inc.). Clones were isolated using cloning rings and were maintained in the presence of G418 (500 μg/ml).

**Ligand Binding Assay—**Both saturation and competition binding assays were performed as described previously (19). Cells were rinsed with binding medium (DMEM containing 1 mg/ml bovine serum albumin) and incubated in this medium for 15 min at room temperature. In saturation binding assays, the medium was then removed and replaced with binding medium containing increasing concentrations of [3H]-labeled human calcitonin (Peninsula, Belmont, CA). In competition binding assays, 20,000–25,000 cpm/well of [3H]-labeled human calcitonin (1 nm for C1a and 3 nm for CTRε13-transfected cells) were added to the wells in the presence of increasing concentrations of unlabeled ligands. The cells were incubated for 1 h at room temperature, washed three times with phosphate-buffered saline, and solubilized with 0.5 N NaOH. The samples were collected from each well and counted in a gamma counter.

**cAMP Measurements—**cAMP was measured as described elsewhere (10). Cells were incubated with varying concentrations of ligand in DMEM containing 10 mM isobutylmethylxanthine (Sigma) for 10 min. The reaction was stopped by the addition of 95% ethanol containing 3 mM HCl. The cAMP was quantitated using a scintillation proximity assay (Amersham Corp.) following the manufacturer’s instructions. Total RNA (300 μg/ml). Data are expressed as the percentage of the total recovered [3H]inositol that is found in the inositol phosphate (IP) fraction.
the CTR that differ in their ligand binding and signaling properties in a variety of tissues and cells from human, pig, rat, and mouse (11–14, 16, 22, 23, 28, 29). We therefore sought to identify the CTR isoforms expressed in rabbit OC. An 0.8-kb fragment was amplified from total rabbit OC RNA by RT-PCR using primers 1 and 2, and used to screen a rabbit OC cDNA library. Four cDNA clones, approximately 3.5 kb in length, were obtained. All four clones contained the complete coding region for the C1a isoform, as well as 5′ and 3′ untranslated sequences (Fig. 1). The rabbit C1a CTR is 71% identical at the nucleic acid level and 79% identical at the amino acid level to the porcine C1a. The putative translation initiation site was assigned at position 276 based on its similarity to predicted porcine, human, and rodent CTR initiation sites. In addition, it encompasses nucleotides 1425–1466 (GGA...GAG).

**Fig. 1.** Nucleotide and deduced amino acid sequences of the rabbit OC CTR cDNA. Two additional upstream in-frame initiation codons are underlined. The predicted seven transmembrane domains have been underlined and numbered (I–VII). The arrow indicates a potential signal sequence cleavage site. Open circles indicate potential N-linked glycosylation sites. The closed square indicates a potential protein kinase C phosphorylation site. The deletion in CTR-D13 encompasses nucleotides 1425–1466 (GGA...GAG).
has adenosine residues at the +4 and the −3 positions, consistent with the requirements for translation initiation (30). The N-terminal domain encoded by the sequence on the 3′ side of the assigned initiation site included a hydrophobic domain flanked by polar regions, consistent with the general outline of a signal peptide. The rabbit OC CTR displays the cardinal features of the CTR family, including a large N-terminal extracellular domain with six conserved cysteine residues and three N-linked glycosylation sites (Asn-X-Ser/Thr) and the seven transmembrane domain topography shared by all G protein-coupled receptors. It also contains a motif in the third intracellular loop (R-X$_{11}$-K-A-V-K$^{648}$) postulated to be involved in coupling to $G_{	ext{s}}$ (31) and a potential protein kinase C phosphorylation site in the C-terminal tail (Thr$^{601}$).

In parallel with screening the cDNA library, RT-PCR was used to determine if alternatively spliced CTR mRNAs were present in rabbit OCs. The expression of CTR isoforms that contain the inserts in the first intracellular loop and the first extracellular loop was examined using primers 1 and 2 which amplified the region from near the N terminus to the fifth transmembrane domain (756 bp in the human C1a sequence). A single product was observed in this reaction and sequence analysis indicated that it corresponded to the C1a isoform. None of the other isoforms previously reported in pig, human, and rodent were observed. However, amplification of the regions from the fifth transmembrane domain to near C terminus (primers 3 and 4) and from near the N terminus to near C terminus (primers 1 and 4) each produced two fragments, one with the size predicted from the C1a cDNA sequence (501 and 1182 bp, respectively) and a second slightly shorter fragment. For both amplification reactions, restriction digestion of the two PCR products with NcoI and PstI restriction enzymes generated a fragment of 382 bp and a shorter fragment of 340 bp, indicating a deletion of a DNA sequence between the NcoI and PstI sites (data not shown). Sequence analysis of these two PCR products indicated that the deletion corresponded to the 42 bp that encode 14 amino acids (Gly$^{604}$-Glu$^{607}$) in the putative seventh transmembrane domain. This 42-bp cassette corresponds exactly to exon 13 of the porcine CTR gene (17), suggesting that this novel isoform of CTR, “CTR$\Delta$e13,” is generated by alternative splicing of exon 13.

**Tissue Distribution of the C1a and CTR$\Delta$e13 Isoforms**—The tissue distribution of the C1a and CTR$\Delta$e13 transcripts was investigated by amplification of RNA from several tissues, using a pair of primers flanking the region of exon 13 that produce a 242-bp product for C1a and a 200-bp product for CTR$\Delta$e13. Fig. 2A shows that both the C1a and CTR$\Delta$e13 were expressed in OC, kidney, brain, cerebellum, heart, and muscle. Only the CTR$\Delta$e13 was weakly detectable in lung, and neither isoform was detectable in liver.

The relative expression of C1a and CTR$\Delta$e13 was then quantified by RNase protection assay using a radio-labeled antisense RNA probe that spans the region of exon 13. The predicted 253-base protected fragment for C1a and the predicted 170-base protected fragment for CTR$\Delta$e13 transcripts was detectable in all tissues except liver, lung, and heart (Fig. 2B). The relative levels of the mRNAs for the two isoforms, i.e. CTR$\Delta$e13/C1a, were $0.04 \pm 0.02$, $0.12 \pm 0.03$, $0.09 \pm 0.02$, $0.10 \pm 0.02$, and $1.93 \pm 0.14$ in OC, kidney, brain, cerebellum, and muscle, respectively ($n = 2$). Only the CTR$\Delta$e13 transcript was detectable in lung, and neither transcript was detectable in liver and heart. These data are consistent with the results of targeted PCR, again demonstrating that the CTR$\Delta$e13 was expressed in various tissues of rabbit, and indicating that CTR$\Delta$e13 represented a much higher proportion of total CTR transcripts in lung and muscle than in the other tissues.

**Ligand Binding Characteristics of the C1a and CTR$\Delta$e13 Isoforms**—The ligand binding properties of the two isoforms were determined using a transient expression system. COS-7 cells transfected with cDNA constructs that encoded C1a or CTR$\Delta$e13 receptors with and without N-terminal HA epitope tags were used for saturation binding assays with either 125I-labeled salmon or human CT as described under “Experimental Procedures.” Preliminary studies demonstrated that the presence of the N-terminal HA epitope tag had no effect on the binding characteristics of either isoform, and the epitope-tagged receptors were used for subsequent studies. Saturable binding of 125I-sCT (Fig. 3A) and 125I-hCT (Fig. 3B) was observed with both the C1a- and CTR$\Delta$e13-transfected cells. No specific binding was detected in cells transfected with the empty pBK-CMV vector. Scatchard analysis of the binding data (Table I) showed that the affinity of the C1a isoform for sCT was more than 10-fold greater than the affinity of the same isoform for hCT. Deletion of the exon 13 cassette reduced the binding affinity of the CTR for both sCT and hCT, but with a substantially greater effect on sCT binding, resulting in a receptor that no longer distinguished between sCT and hCT.

Similar results were obtained in competitive binding assays analyzing the displacement of 125I-hCT by unlabeled sCT, hCT, or calcitonin gene-related peptide (CGRP) (Fig. 4 and Table I). sCT displaced 125I-hCT from the C1a isoform nearly 10 times more efficiently that hCT ($K_i = 0.21$ nM and 1.82 nM, respect
tively), but was only slightly more potent than hCT in displacing the labeled ligand from the CTRΔe13 isoform (K_i = 1.23 nM and 1.65 nM for sCT and hCT, respectively). CGRP did not displace 125I-hCT from either of the isoforms at concentrations up to 10 μM.

**Coupling of the C1a and CTRΔe13 Isoforms to Adenylyl Cyclase**—CT induces increased levels of intracellular cAMP, presumably by coupling to G_s, which activates adenylyl cyclase (2). The CT-dependent accumulation of cAMP was measured in both transiently transfected COS-7 cells and HEK-293 cells lines expressing the HA-tagged C1a and CTRΔe13 isoforms, where the ligand binding characteristics of the two isoforms were similar (data not shown). The relative potencies of CT for inducing adenylyl cyclase activity were consistent with the

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**FIG. 3.** Specific binding of 125I-sCT (A) and 125I-hCT (B) to COS-7 cells transiently expressing the C1a and CTRΔe13 isoforms. The average receptor number per transfected cell was 3.15 × 10^5 for C1a (○) and 2.34 × 10^5 for CTRΔe13 (●) when 125I-sCT was used as a ligand. Note the greater difference between the binding curves in A compared to that in B. No specific binding to cells transfected with the empty pBK-CMV vector (△) was detected. Data represent the means of triplicate measurements and are representative of three separate experiments.

**TABLE I**

|                  | K_d a          | K_b b          |
|------------------|----------------|----------------|
|                  | C1a            | CTRΔe13        | C1a            | CTRΔe13        |
| Salmon calcitonin| 0.23 ± 0.02    | 8.79 ± 2.37    | 0.21 ± 0.01    | 1.23 ± 0.54    |
| Human calcitonin | 3.17 ± 0.12    | 8.43 ± 0.95    | 1.82 ± 0.12    | 1.65 ± 0.14    |
| Human CGRP       | ND             | ND             | > 1 μM        | > 1 μM        |

a Determined by Scatchard analysis of three experiments. Each value is the mean ± S.E.
b Determined by curve fitting competition binding data to a four-parameter general binding program. Each value is the mean ± range of two experiments.
c ND, not determined.

**FIG. 4.** Competition binding curves for 125I-hCT in COS-7 cells transiently transfected with C1a (A) and CTRΔe13 (B). The cells were incubated with 125I-hCT (1 nM for C1a and 3 nM for CTRΔe13-transfected cells) in the presence of increasing amounts of unlabeled sCT (○), hCT (●), and human CGRP (△). Data are the means of triplicate measurements and are representative of two separate experiments.
of the C1a and CTR glycerol (9, 10, 32). We therefore examined the relative abilities inositol-specific phospholipase C, generating IPs and diacylglycerol adenylyl cyclase, the CTR also activates phosphatidyl

dose-dependent accumulation of IP with the EC50 value of 0.12 nM in HEK293 cells expressing the C1a and CTR isoform (Fig. 5B). The cells expressing C1a (○) and CTRα13 (●), as well as cells transfected with the empty pBK-CMV vector (△), were labeled with [3H]inositol 24 h before assay. The cells were washed and incubated with culture medium containing 10 mM LiCl in the presence of increasing concentrations of sCT. cAMP and IP levels were measured as described under "Experimental Procedures." Data are the means of triplicate measurements and are representative of three separate experiments.

Table II

|            | C1a | CTRα13 |
|------------|-----|--------|
| Salmon calcitonin | 0.06 ± 0.02 | 0.56 ± 0.08 |
| Human calcitonin   | 0.54 ± 0.05 | 0.84 ± 0.14 |

Deletion of 14 amino acids from a transmembrane domain of the seven-transmembrane receptor might be expected to markedly reduce or even abolish expression or function of the resulting protein. We found, however, that the CTRα13 protein is a functional receptor, although the properties of the new isoform differ in certain ways from those of the C1a isoform. Thus, while the binding of both sCT and hCT to CTRα13 is somewhat weaker than to C1a (Figs. 3 and 4, and Table I), the reduction in affinity is much greater for sCT than for hCT, resulting in a receptor with the same affinity for the two ligands. Furthermore, unlike C1a, which couples to both adenyl cyclase and phospholipase C, CTRα13 couples to adenyl cyclase with an EC50 slightly higher than that of C1a, but is less sensitive to phospholipase C (Fig. 5).

The stronger binding affinity and hypocalcemic activity that normally characterize sCT relative to mammalian CTs (1) correlate with the strong propensity of the central region of sCT to form an amphipathic helix when placed in a relatively hydrophobic environment (33), a property that is not shared with mammalian CTs such as hCT (34). The relatively greater decrease in affinity for CTRα13 that is seen with sCT and the resulting failure of CTRα13 to discriminate between sCT and hCT suggest that the seventh transmembrane domain of the C1a isoform interacts either with the helix-forming residues of sCT or with other residues of CT that may be positioned adjacent to the seventh transmembrane domain only when the helix is formed. It is well established that residues in the

DISCUSSION

In the present study, we have cloned and characterized the cDNA encoding the CTR from highly purified rabbit OC. Our results demonstrate that rabbit OCs express at least two CTR isoforms. One, which accounts for the majority of CTR mRNA in OC, corresponds to the C1α isoform previously described in porcine, human, mouse, and rat (11–14, 16, 22, 23, 28, 29) that lacks inserts in the first intracellular loop and the first extracellular loop. Analysis of the structural features of the rabbit C1α CTR (Fig. 1) revealed that it is highly homologous (71–87% identical to the amino acid level) to the same isoform in other species and has the conserved structural motifs of the CTR receptor family. The second, CTRα13, is apparently generated by alternative splicing that results in the deletion of exon 13 and hence a 14-amino acid deletion in the C-terminal two thirds of the putative seventh transmembrane domain (Fig. 6).
transmembrane helical domains contribute to forming the ligand-binding sites of G protein-coupled receptors for structurally diverse ligands, including a variety of peptides (35). The seventh transmembrane helix is the site of the covalent attachment of 11-cis-retinal to rhodopsin (36) and contributes to the binding of antagonists to several receptors for biogenic amines and to the binding of both neurokinin peptides and some nonpeptide antagonists to neurokinin receptors (37, 38). Our results suggest that, as in the case of these other G protein-coupled receptors, the seventh transmembrane helix of the CTR also contributes to the receptor’s interaction with ligands.

The deletion of the amino acids encoded by exon 13 changes not only the ligand binding properties but also the signal transduction potential of the receptor. While the C1a activates cAMP-dependent pathways at low ligand concentrations and diacylglycerol- and Ca\(^{2+}\)-dependent pathways at higher ligand concentrations, CTR\(\Delta e13\) activates only the cAMP-dependent pathways. The structural determinants of CTR coupling to the adenyl cyclase and phospholipase C signaling pathways have not been completely defined. Previous studies of CTR isoforms revealed that an inserted sequence in the first intracellular loop interfered with coupling of both neurokinin peptides and some nonpeptide antagonists to neurokinin receptors (37, 38). Our results suggest that, as in the case of these other G protein-coupled receptors, the seventh transmembrane helix of the CTR also contributes to the receptor’s interaction with ligands.

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The genomic structure of many members of the CTR family has been characterized (17, 18, 40–46). The genes of the CTR family are characterized by the presence of numerous introns within the coding region, and there are already several examples of functionally distinct CTR isoforms, some with tissue-specific patterns of expression, that are generated by alternative splicing (12–14, 17, 18, 22, 23). Subtypes of other G protein-coupled receptors generated by alternative splicing that are expressed in a tissue-specific fashion and have different pharmacological properties have also been reported (47, 48). The protein sequence encoded by exon 13 of the porcine CTR gene is one of the most conserved regions in the CTR family, to the point where it is used as a signature sequence in data bases. Furthermore, in all the genes of the CTR family that have been characterized to date, with the exception of the rat glucagon receptor (43), the boundaries of the small exon that corresponds to the porcine CTR exon 13 are also highly conserved, suggesting that this sequence and possibly the ability to selectively exclude it from the final gene product are under a high degree of evolutionary constraint. It might, therefore, be expected that the presence or absence of this portion of the CTR or other receptors in the CTR family would be of considerable physiological importance. To our knowledge, however, this is the only description of an isoform of any member of the CTR family that is generated by alternative splicing of the exon that is homologous to exon 13 of the CTR, other than a preliminary report of a similar alternatively spliced PTH/PTHrP receptor mRNA identified in immortalized human renal tubular cells (49).

The CTR\(\Delta e13\) mRNA is expressed in several tissues. Analysis of the tissue distribution of the C1a and CTR\(\Delta e13\) transcripts by RT-PCR (Fig. 2A) and quantitative RNase protection assay (Fig. 2B) revealed that the C1a isoform accounts for 85–90% of the transcripts in OC, kidney, brain, and cerebellum. In contrast, the CTR\(\Delta e13\) transcripts comprise more than half of the CTR mRNA in lung and skeletal muscle, indicating that the expression of the two CTR isoforms is regulated in a tissue-specific manner. The significantly higher levels of expression of the CTR\(\Delta e13\) mRNA in skeletal muscle, and to a lesser extent in lung, is intriguing. Although these tissues are
not classically thought of as targets for CT, sCT binding and stimulation of adenyl cyclase activity have been reported in membranes derived from muscle (50), and sCT inhibits insulin-stimulated glucose incorporation into glycogen in rat soleus muscle (51). The failure of the CTRαe13 to activate phospholipase C suggests that the pattern of second messengers and signal transduction properties of the two CTR isoforms, together with their importance. The differences in ligand binding and signal transduction of the cDNA library. and Dr. Richard J. Rickles, Ariad Pharmaceuticals, for constructing the cDNA library.

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