Inhibition of Inositol Phosphate Second Messenger Formation by Intracellular Loop One of a Human Calcitonin Receptor

EXPRESSION AND MUTATIONAL ANALYSIS OF SYNTHETIC RECEPTOR GENES*

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Receptors for calcitonin (CTRs) have been cloned from several species, and two isoforms have been found to be expressed in human tissue. One human CTR isoform (hCTR-1) contains a 16-amino acid insertion in its first intracellular (I1) loop that is not present in porcine CTR (pCTR), rat CTR, or the other human CTR (hCTR-2). To facilitate the study of CTRs by mutational analysis, we have constructed synthetic hCTR-1 and hCTR-2 genes. Activation of hCTR-1 expressed transiently in COS-1 cells stimulates the formation of cAMP but not of inositol phosphates (IPs) whereas pCTR, a chimeric CTR in which the I1 loop of pCTR was substituted for the I1 loop of hCTR-1, and hCTR-2 stimulate cAMP and IP formation. A series of chimeric CTRs in which intracellular loops 1, 2, and 3 and the carboxyl tail of pCTR were substituted individually or in combination for those of hCTR-1 were constructed. All chimeras stimulated cAMP formation whereas chimeras containing the I1 loop of hCTR-1 with its 16-amino acid insertion were incapable of stimulating IP formation. There was no correlation between maximal stimulation of cAMP and IP formation by these CTRs. Thus, an inserted sequence in the I1 loop of hCTR-1 abolishes stimulation of the IP signal transduction pathway while allowing stimulation of the cAMP pathway.

CT* is a polypeptide hormone that reduces plasma calcium by inhibiting bone resorption and stimulating renal calcium clearance and is present in the central nervous system. CT initiates its actions by binding to cell-surface receptors, which putatively contain seven membrane-spanning helices and couple to and activate heterotrimeric GTP-binding regulatory proteins (1-4). Activated GTP-binding proteins may then activate adenyl cyclase and phosphoinositide-specific phospholipase C to generate intracellular second messenger molecules, cAMP and IPs, respectively (5). It has been shown that activation of all CTRs leads to the generation of cAMP, but activation of pCTR has been found to stimulate IP formation also (6, 7). Activation of one form of human CTR (hCTR-2) was reported to stimulate an elevation of intracellular calcium (4), but no evidence was presented as to whether there was concomitant stimulation of IPs. The deduced sequence of another isoform of the human CTR, hCTR-1 (2), contains an insertion of 16 amino acids in its I1 loop, which is between the putative first and second transmembrane helices that is not present in pCTR (1), rCTR (3), or hCTR-2 (8). The functional consequence of the 16-amino acid insertion in the I1 loop was not known.

To facilitate study of CTRs by mutational analysis, we have constructed synthetic hCTR-1 and hCTR-2 genes. Synthetic genes (9) have been constructed for a number of proteins (10, 11) including rhodopsin (12), ß-adrenergic receptor (13), and angiotensin II receptor (14), which are members of the GTP-binding protein-coupled receptor superfamily. Using the synthetic hCTR-1 gene, we constructed a series of chimeric CTRs in which we substituted the intracellular loops and carboxyl tail of hCTR-1 with these domains of pCTR. We show that the presence of the 16-amino acid insertion in I1 loop as in hCTR-1 abolishes stimulation of IPs by all CTRs tested while allowing for stimulation of cAMP.

EXPERIMENTAL PROCEDURES

Design, Chemical Synthesis, and Assembly of the Synthetic hCTR-1 Gene—The gene for hCTR-1 was synthesized by the method of Khorana (9). The amino acid sequence of hCTR-1 deduced from the sequence of the native cDNA (2) was reverse translated into a fully degenerate DNA sequence, and a complete restriction endonuclease map was obtained using the software package MacVector (Eastman Kodak). A maximum number of evenly spaced unique restriction endonuclease recognition sequences was selected as follows. The changes introduced in the nucleotide sequence would not result in a change in amino acid sequence (silent mutations); the few unique restriction endonuclease recognition sites present in the native gene were retained except when they were too close to each other, in which case they were relocated in the nucleotide sequence; when there were multiple sites in the native sequence all but one site were removed; new unique sites were introduced. In addition, the consensus sequence CCACC for initiation of translation (15) was placed immediately upstream of the initiation codon, and two contiguous stop codons, TGA at codon 491 and TAG at codon 492, were placed at the end of the open reading frame.

The gene was subdivided into three fragments: fragment A was 532 base pairs, fragment B was 583 base pairs, and fragment C was 471 base pairs (see Fig. 1). Fragment A was constructed with 10 pairs of complementary oligonucleotides ranging from 40 to 54 nucleotides, fragment B with 10 pairs of complementary oligonucleotides of 48-66 bases, and fragment C with 7 pairs of complementary oligonucleotides of 47-63 bases. Each fragment was cloned and amplified as an inter-
FIG. 1. Nucleotide sequence of the synthetic hCTR-1 gene. Fifty-four oligonucleotides (A-1...A-20, B-1...B-20 and C-1...C-14), which are numbered above and below the sequence, were synthesized containing 5' cohesive overhangs of 4 nucleotides, which are shown by the brackets. Ligation of the three separate groups of oligonucleotides yielded fragments A, B, and C, which were cloned and amplified in plasmid Bluescript. These three fragments were subcloned and ligated together into a mammalian expression vector. Asterisks denote nucleotides that were changed from the native cDNA sequence.
mediate in plasmid Bluescript. Fragment A had cohesive ends that corresponded to the sites for restriction enzymes EcoRI and HindIII; fragment B had ends for HindIII and BamHI; and fragment C had ends for BamHI and NotI. Except for the ends of the fragments, the complementary pairs of oligonucleotides produced 5' cohesive overhangs of 4 residues that were not self-complementary and were distinct from each other, thereby precluding incorporation of more than one copy of a single pair of oligonucleotides and formation of concatemers. Except for the oligonucleotides whose 5' cohesive overhangs were part of restriction endonuclease sequences (oligonucleotides A-1, A-20, B-1, B-20, C-1, and C-14), all other oligonucleotides were chemically phosphorylated at their 5' ends. Oligonucleotides were purified by high performance liquid chromatography.

Hybridization and Enzymatic Ligation of Oligonucleotides—The reaction consisted of 6-8 oligonucleotides (2 μm, final concentration) representing three to four contiguous DNA duplexes mixed in a final volume of 50 μl containing 20 mM Tris-Cl, pH 7.6, and 10 μM MgCl₂. The mixtures were brought to 95 °C in a programmable thermal controller (MJ Research, Inc.) for 10 min, and then the temperature was reduced at a rate of 1 °C every 10 min until 4 °C. The annealed mixtures were supplemented to a final concentration of 20 mM Tris-Cl, pH 7.6, 10 μM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 50 μg/ml bovine serum albumin, and 10 μM 32P-DNA ligase in a final volume of 100 μl. The reaction was incubated at room temperature for 4 h, and the expected double-stranded DNA fragments, ranging from 149 to 232 base pairs, were purified by agarose gel electrophoresis (2% Bio-Gel) followed by purification using the Mermaid system (Bio 101).

The purified DNA fragments were combined (three for fragment A, three for fragment B, and two for fragment C) and were ligated with plasmid Bluescript digested with the appropriate restriction enzymes using the same conditions described above in a final volume of 10–15 μl.

The clones obtained for each fragment were verified by dideoxy sequencing modified to use a thermostable DNA polymerase (Circumvent kit, New England Biolabs) with primers based in the vector (M13 universal primer (–20) and T3) and the sequence of the synthetic hCTR-1.

The full-length synthetic gene was finally assembled in a four-part ligation reaction and cloned into the expression vector pMT4 (16) using EcoR1 and NotI sites. Except for the ends of the fragments, the complementary pairs of oligonucleotides produced 3' cohesive overhangs that were part of restriction endonuclease sequences representing three to four contiguous DNA duplexes mixed in a final volume of 50 μl containing 20 mM Tris-Cl, pH 7.6, and 10 μM MgCl₂.

The oligonucleotides whose 3' cohesive overhangs were part of restriction endonuclease sequences (oligonucleotides A-1, A-20, B-1, B-20, C-1, and C-14) and the sequence of the full-length gene was verified.

Operon Technologies, Inc. (Alameda, CA).

We constructed a synthetic gene of hCTR-1 (Fig. 1). The gene is 1493 nucleotides in length and does not contain any of the original 5'- or 3'-untranslated regions. We made 83 nucleotide changes from the native hCTR-1 cDNA nucleotide sequence, affecting 70 codons (silent mutations) in the open reading frame. Although no care was taken to avoid changes in codon usage, the synthetic gene does not differ substantially from the native gene or other mammalian proteins in codon usage frequency. The changes in codons have permitted an increase in the number of unique restriction endonuclease recognition sequences from 23 in the native cDNA to 74 in the synthetic gene. These new restriction sites are present at approximately equal distances along the entire sequence of DNA and are especially useful for cassette mutagenesis, that is, the substitution of restriction fragments by synthetic DNA duplexes containing site-specific codon change(s).

We compared the characteristics of hCTR-1s expressed in CHO-1 and HEK-293 cells transfected either with the synthetic hCTR-1 gene or with the native cDNA. ScT exhibited equal potency and efficacy in stimulating elevations of cAMP and equal affinity and maximal extents of binding of 125I-sCT in cells expressing hCTR-1s encoded by the synthetic gene or the native cDNA (see below). Cells transfected with vector alone did not respond to ScT nor exhibit specific binding of 125I-sCT.

We determined whether activation of hCTR-1 would stimulate IP formation. In a series of eight experiments in which hCTR-1 was expressed in COS-1 cells using the native cDNA, activation by a maximally effective dose of ScT of hCTR-1s stimulated a 3.2 ± 0.71-fold increase in cellular cAMP but did not increase IPs (1.0 ± 0.05-fold). In these same experiments, ScT stimulated elevations of both cAMP and IPs in cells expressing pCTRs (see below) as reported previously (6, 7). We, therefore, decided to replace the entire intracellular domain of hCTR-1 with pCTR sequences (hCTR-1/p1(I1+I2+I3+COOH)) and determine whether this chimeric CTR would stimulate IP formation. Fig. 2 confirms that hCTR-1 does not stimulate IP formation and illustrates that hCTR-1/p1(I1+I2+I3+COOH) is nearly as effective as pCTR in stimulating elevations of CAMP and IP formation.

A comparison of the sequences of the intracellular domains of all CTRs showed a striking difference in the I1 loop in which hCTR-1 contains an insertion of 16 amino acids that is not present in pCTR, rCTR, or hCTR-2 (Fig. 3). We, therefore, constructed a chimeric CTR in which the I1 loop of pCTR was substituted for the I1 loop of hCTR-1 (hCTR-1/p1(I1)). hCTR-1/p1(I1) was capable of stimulating IP formation when expressed in COS-1 cells (Fig. 4). We compared stimulation by ScT in cells expressing pCTRs, hCTR-1s, and hCTR-1/p1(I1). In cells expressing pCTRs, ScT stimulated a maximal elevation of cellular CAMP of 17 ± 2.9-fold and of IPs of 8.3 ± 0.92-fold (n = 13).
The EC50 values for stimulation of cAMP and IPs were 0.47 ± 17 nM (n = 4) and 4.7 ± 2.1 nM (n = 3), respectively. These values are not different from those reported for sCT stimulation of HEK-293 cells expressing pCTRs (7). In contrast, in cells expressing hCTR-1s in these experiments, sCT maximally stimulated CAMP 3.5 ± 0.42-fold (n = 17) but had no effect on the levels of IPs (1.0 ± 0.05, n = 18). The EC50 for sCT stimulation of CAMP in cells expressing hCTR-2 was 1.5 ± 0.29 nM (n = 4), which is not different from the potency of sCT in a human ovarian small cell carcinoma line (BIN-67) from which hCTR-1 was cloned (2). In cells expressing hCTR-1/pIls, sCT stimulated a maximal increase in CAMP of 7.5 ± 1.0-fold and in IPs of 4.6 ± 0.47-fold (n = 6). The fold-stimulation by sCT of CAMP and IPs in cells expressing hCTR-1/pIls varied between 50 and 85% of those for sCT stimulation in cells expressing pCTRs. The EC50 values for sCT stimulation of CAMP and IPs were 0.28 ± 0.07 and 7.2 ± 1.7 nM, respectively (n = 4), which are not different from the EC50 values for sCT stimulation in cells expressing pCTRs. Thus, hCTR-1/pIls are as sensitive to sCT for stimulation of the CAMP and IP signaling pathways but are not as effective as pCTRs, whereas hCTR-1s are as sensitive to sCT for stimulation of CAMP but are not as effective as either hCTR-1/pIls or pCTRs and do not stimulate IP formation. hCTR-2 showed characteristics of stimulation of CAMP and IP formation that were indistinguishable from hCTR-1/pIls (data not shown).

One explanation for the differences in apparent efficacies of pCTRs, hCTR-1s, and hCTR-1/pIls (or hCTR-2s) could have been that the different receptors were expressed to different levels on the surfaces of COS-1 cells. The binding of 125I-sCT to cells expressing pCTRs, hCTR-1s, and hCTR-1/pIls was, therefore, measured (Fig. 5). Cells expressing pCTRs, hCTR-1s, and hCTR-1/pIls bound sCT with apparent Kd values of 9 ± 7, 9 ± 4, and 12 ± 4 nM, respectively. The value for pCTR is similar to that reported (1) whereas that for hCTR-1 is higher than that observed previously (2). The levels of cell-surface receptors were similar in the three cell populations. The maximal binding of 125I-sCT was 8,160 ± 80, 6,610 ± 1,900, and 7,280 ± 94 cpm/well in cells expressing pCTRs, hCTR-1s, and hCTR-1/pIls, respectively. In some experiments, the maximal binding in cells expressing hCTR-1s was 50% of that in cells expressing pCTRs. With cells expressing hCTR-2s, the Kd for 125I-sCT binding was 14 ± 0.58 nM, and the maximal binding was similar also. Hence, the differences in the magnitude of second messenger stimulation in cells expressing the three CTRs were not caused by differences in levels of receptor expression and, therefore, represent differences in the intrinsic efficacies (21) of these CTRs.

To further support the idea that the observed lack of stimulation by sCT of IP formation in cells expressing hCTR-1s was not an artifact of a lower level of stimulation overall, we re-
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produced the level of stimulation in cells expressing hCTR-1/pI1s and pCTRs by transfecting COS-1 cells with lower amounts of CTR expression plasmids. Our goal was to compare the effectiveness of the three CTRs in stimulating IP formation at equal levels of stimulation of cAMP. Fig. 6 illustrates, as expected, that the magnitude of stimulation by sCT of cAMP and IP formation in cells expressing hCTR-1/pI1s or pCTRs was decreased as the plasmid levels used for transfection were decreased. In cells transfected with the lowest amount of plasmid containing the cDNAs for hCTR-1/pI1s and pCTRs, sCT stimulated cAMP to the same level, approximately 4-fold, as in cells transfected with 100 times as much plasmid containing the hCTR-1 complementary DNA. At this level of cAMP stimulation, sCT stimulated IP formation 2.2- and 2.8-fold in cells expressing hCTR-1/pI1s and pCTRs, respectively, but did not stimulate IP formation in cells expressing hCTR-1s (1.0-fold). We do not know whether the decreased stimulation of second messenger formation in cells transfected with reduced amounts of plasmids was because fewer CTRs were expressed in all CTR-expressing cells or, more likely, because fewer cells in the population were expressing similar numbers of CTRs, that is because of reduced efficiencies of transfection.

In a number of other GPCRs, regions of loops I2 and I3 and of the carboxyl tail have been shown to be involved in coupling to GTP-binding proteins (22, 23). We, therefore, constructed a series of chimeric CTRs in which we substituted these domains of hCTR-1 with those from pCTR alone or in combination and expressed them in COS-1 cells. Most of these CTRs were expressed to levels between 40 and 110% of pCTRs (data not shown), and we studied these with regard to their efficacies in stimulating cAMP and IP formation. Fig. 7 illustrates the stimulation by a maximally effective dose of sCT of cAMP and IP formation in COS-1 cells. Although these substitutions had effects on the magnitude of stimulation of cAMP formation by these chimeric receptors, there was no consistent pattern as to which intracellular domain was most important. The striking finding, however, was that no chimeric CTR that contained the I1 loop of hCTR-1 was capable of stimulating IP formation whereas every chimeric CTR that contained the I1 loop of pCTR was capable of stimulating IP formation. Fig. 8 compares the maximal stimulation of cAMP and IP formation by these chimeric CTRs and shows that there was no correlation between them.

DISCUSSION

We report the total synthesis of genes that encode the two isoforms of hCTR. We carried out this synthesis in order to facilitate our studies of the structure-function relationships of these receptors. Using these genes, we constructed a series of chimeric receptors in which the intracellular domains of hCTR were replaced by homologous sequences from pCTR. We characterized these chimeric receptors in transiently transfected cells. Our data show that a naturally occurring, 16-amino acid insertion in the I1 loop of hCTR-1 inhibits this receptor from stimulating IP formation even though hCTR-1 is effective in stimulating cAMP formation. These findings are consistent with the idea that CTRs couple more effectively to Gs (5) and then to adenyl cyclase to form cAMP than to Gi and then to

Fig. 6. Maximal sCT stimulation of cAMP and IP accumulation in mock-transfected COS-1 cells, in cells expressing different levels of pCTRs and hCTR-1/pI1s, and in cells expressing a single level of hCTR-1s. Cells were transfected with the indicated amounts of plasmids. Bars represent mean ± S.D. of triplicate determinations in two experiments.

Fig. 7. Maximal sCT stimulation of cAMP and IP accumulation in COS-1 cells expressing hCTR-1, hCTR-1/pCOOH, hCTR-1/p(COOH+I2), or hCTR-1/p(COOH+I3) without (striped bars) or with pI1 (hatched bars). Bars represent mean ± S.D. of triplicate determinations in two experiments.
phosphoinositide-specific phospholipase C to form IPs. This idea is supported by the finding that sCT is 10-fold more potent in stimulating cAMP formation than in stimulating IP formation in cells expressing hCTR-2s (Fig. 4) or pCTRs (6) (Fig. 4). Similarly increased potencies in stimulating cAMP versus IP formation are exhibited by GPCRs for thyroid-stimulating hormone (24) and parathyroid hormone/parathyroid hormone-related peptide (25). Whether these differences in potency are because of an intrinsic property of these receptors that allows them to bind to Gs with higher affinity than to Go, or whether it is because there is relatively more Gs than Go, in these cells has not been determined.

In other GPCRs, the carboxyl-terminal region of the I2 loop adjacent to transmembrane helix 4, the amino terminus of the I3 loop adjacent to transmembrane helix 5, the carboxyl terminus of the I3 loop near transmembrane helix 7 have been shown to be involved in coupling to G proteins (22, 23). In general, the I1 loop has not been found to be involved in G protein coupling. It has been reported that mutations in the I1 loop of the thyroid-stimulating hormone receptor lead to decreased efficacies in stimulating cAMP formation and abolish stimulation of IP formation (26). These findings, however, appear to result from lowered levels of expression of the receptors mutated in the I1 loop and the lower effectiveness of the thyroid-stimulating hormone receptor in stimulating IP versus cAMP formation (see above). Our observation of the inhibitory effect of the naturally occurring 16-amino acid insertion in the I1 loop of hCTR-1 leading to inhibition of IP signaling is, to our knowledge, a novel finding. We cannot deduce from our data whether the I1 loop of hCTR-1 is involved in binding to G proteins or whether the additional amino acids in the I1 loop may interfere with coupling to Go or Go, even though the I1 loop does not interact directly with G proteins. Based on data from other GPCR systems, we think the second possibility is more likely. It appears, moreover, that the extended I1 loop does not interfere with coupling to Go as there was no correlation between stimulation of IP and cAMP formation (Fig. 8).

CTRs are members of the subfamily of GPCRs that includes receptors for parathyroid hormone/parathyroid hormone-related peptide, secretin, glucagon, glucagon-like peptide, vasoactive intestinal peptide, pituitary adenyly cyclase-activating polypeptide, growth hormone-releasing hormone, and corticotropin-releasing hormone (27). These receptors exhibit significant sequence homology, and all have been shown to elevate intracellular cAMP upon binding their cognate agonist. Several of these receptors have been found to stimulate IP formation also (25, 28). The structures of the genes for some members of this receptor subfamily have begun to be delineated, and several have been found to contain introns within their coding regions (28–32). Alternative splicing of the gene for the receptor for pituitary adenyly cyclase-activating polypeptide has been reported to lead to receptor isoforms with different patterns of signal transduction (28). Of particular interest with regard to our findings is the observation that there are two isoforms of the receptor for corticotropin-releasing hormone, which differ by the inclusion of a 29-amino acid sequence in the I1 loop (29). No functional differences in signaling were reported for the corticotropin-releasing hormone receptor isoforms, but IP generation was not studied. As has been shown for several members of this receptor subfamily, we think it is likely that hCTR-1 and hCTR-2 arise by alternative splicing of adjacent human CTR genes, and we have recently isolated a genomic clone of hCTR and have found that the sequence encoding the 16-amino acid insertion resides on a distinct exon.

We suggest that coordinated regulation of alternative splicing in different tissues may allow for differential expression of the two isoforms of hCTR leading to differential signaling by CT.

In conclusion, we constructed synthetic genes that proved to be useful reagents with which to rapidly and reliably mutate hCTRs for structure-function studies. Our data show that a sequence in the I1 loop of hCTR-1 abolishes stimulation of the IP signal transduction pathway while permitting stimulation of the cAMP pathway. To our knowledge, this is the first example of an inhibitory domain within the I1 loop of any GPCR. We suggest that alternative splicing of the CTR gene is the likely cause of expression of the two hCTR isoforms and that regulation of CTR gene splicing may be a mechanism to differentially modulate CT signaling via the cAMP and IP pathways. We suggest, furthermore, that this mechanism may be used by other members of the GPCR superfamily to regulate stimulation of different signal transduction pathways.

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FIG. 8. Comparison of maximal sCT stimulation of cAMP and IP accumulation in COS-1 cells expressing hCTR-(A), hCTR-1/pCOOH (B), hCTR-1/p1(p2+COOH) (C), hCTR-1/p1(p3+COOH) (D), hCTR-1/p1(p4+COOH) (E), hCTR-1/p1(p6+COOH) (F), hCTR-1/p1(p11+COOH) (G), hCTR-1/p1(p11+12+13+COOH) (H), hCTR-1/p1(p11+12+13+COOH) (I), or hCTR-1/p1(p11+12+13+COOH) (J) or pCTR (K).
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