Chimeric Human Calcitonin and Glucagon Receptors Reveal Two Dissociable Calcitonin Interaction Sites†

Steven D. Stroop,* Rolf E. Kuestner, Thomas F. Serwold, Lennie Chen, and Emma E. Moore

ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, Washington 98102

Received August 15, 1994; Revised Manuscript Received October 27, 1994®

ABSTRACT: Two chimeric receptors were constructed by transposing the coding regions for the putative N-terminal domains of the human calcitonin (hCTR) and glucagon (hGGR) receptors. These receptors were stably expressed as glycosylated proteins with molecular masses of 80 kDa for the calcitonin receptor N-terminus chimera (NtCTr) and 65 kDa for the glucagon receptor N-terminus chimera (NtGGr). The NtCTr chimera binds salmon calcitonin (sCT) with an apparent K_d of 12 nM relative to 0.3 nM for the native hCTR. However, this chimera does not mediate a cAMP response even with a transfectant expressing 1.8×10^6 cell surface receptors. Stable transfectants expressing the NtGGr chimera show no detectable binding of ^{125}I-sCT or ^{125}I-human glucagon. Surprisingly, adenylate cyclase is activated through the NtGGr chimera by sCT, pCT, and hCT with half-maximal activation at 2.2 ± 0.6, 5.8 ± 2.1, and 810 ± 151 nM, respectively, and the maximum response is similar to that induced by 25 μM forskolin. The rank-order of competition for sCT binding to the NtCTr chimera is similar to the hCTR (sCT > pCT > hCT), but the concentrations required for half-maximal competition are 100- to >2000-fold higher. In addition, salmon calcitonin binds with a much more rapid on-rate and off-rate to the NtCTr chimera relative to the hCTR which binds hormone irreversibly. Cross-linking of ^{125}I-sCT to the NtCTr chimera with bis(sulfosuccinimidyl) suberate is much greater than to the hCTR, suggesting unique conformations for the two receptor−hormone complexes. These studies identify two physically dissociable hormone sites on the calcitonin receptor that likely cooperate in complexing the hormone on the native receptor. Their principal functions are demonstrated in the high-affinity binding of sCT at site-one in the receptor N-terminus and activation of adenylate cyclase at site-two in the remaining receptor C-terminus. Moreover, both sites confer a specificity for ligand interaction similar to the native receptor.

Calcitonin is a 32 amino acid peptide hormone secreted primarily by the thyroid gland in response to increases in serum calcium. Subsequent activation of receptors in the kidney and bone results in the hypocalcemic action of the hormone. Calcitonin also has significant activity in the central nervous system where receptors have been shown to be concentrated in the hypothalamus (Martin & Moseley, 1990; Epand & Caulfield, 1990). Glucagon, a peptide of 29 amino acids, is a pancreatic hormone active in the regulation of blood glucose levels. Binding of glucagon to receptor sites in the liver helps to regulate the production of glucose by glycogenolysis and gluconeogenisis (Premont & Iyengar, 1987). Similarities in the structure−function properties and receptor interaction characteristics of these two nonhomologous peptide hormones have been recognized (Epand, 1983). The hormone and receptor determinants responsible for the specific binding and subsequent signal transduction of calcitonin and glucagon are still largely unknown; however, models have been presented to account for their observed binding and activity characteristics (Unson & Merrifield, 1994; Bharucha & Tager, 1990; Horwitz et al., 1986; Epand et al., 1981, 1986; Epand & Epand, 1986; Findlay et al., 1985).

The recent elucidation of the structures of both the calcitonin and glucagon receptors by expression cloning yielded the surprising observation that both are highly homologous members of a new family of seven-transmembrane G protein-linked receptors. The glucagon, calcitonin, and parathyroid hormone (PTH) receptors have also been shown to activate multiple signal transduction pathways (Segre & Goldring, 1993). Two isoforms of the human calcitonin receptor have been identified which differ by the presence or absence of a 16 amino acid insert within the first putative intracellular loop region (Gorn et al., 1992; Kuestner et al., 1994). The insert-negative form appears predominant (Kuestner et al., 1994). An additional brain-specific isoform has been described in rodents which has a 37 amino acid insert in the first extracellular loop region. This isoform exhibits altered binding properties for the various calcitonins (Sexton et al., 1993; Albrandt et al., 1993; Houssami et al., 1994).

The adrenergic receptor family has served as a prototypical seven-transmembrane receptor model, and chimeric receptors between members of this family led to the identification of binding sites for agonists and antagonists, as well as the structural determinants for G-protein coupling (Dohlmam et al., 1991). Chimeric receptor systems have also been reported for, among others, the muscarinic M1 and M2 receptors (Kubo et al., 1988), neurokinin B and substance P receptors (Huang et al., 1994), and the thyrotropin and chorionic gonadotropin receptors (Akamizu et al., 1993) [for a review, see Schwartz (1994)]. Chimeric PTH receptors made between the rat, human, and opossum receptors

† The nucleic acid sequence encoding the human glucagon receptor has been submitted to GenBank under Accession No. L20316, and that encoding the human calcitonin receptor has been submitted to the EMBL data bank under Accession No. X69920.

* To whom correspondence should be addressed. Telephone: (206) 442-6727. Fax: (206) 442-6608. Email: stroops@zgi.com.

® Abstract published in *Advance ACS Abstracts,* January 1, 1995.

0006-2960/95/0434-1050$09.00/0 © 1995 American Chemical Society

localized a major determinant of high-affinity binding for PTH within the amino-terminal domain of the PTH receptor (Juppner et al., 1994).

The human glucagon receptor (hGGR)[1] (Lok et al., 1994) and the insert-negative form of the human calcitonin receptor (hCTR) (Kuestner et al., 1994) show approximately 30% sequence identity in the transmembrane domain regions, 23% identity in the N-terminal domains, and 16% identity in the C-terminal intracellular domain. There is no significant interaction of calcitonin with the hGGR nor of glucagon with the hCTR. These observations suggested that chimeric proteins of the two receptors might be properly expressed on the cell surface, and therefore allow us to identify and characterize regions of the receptor involved in ligand binding and receptor activation.

We have constructed two chimeric receptors by transposing the amino-terminal domains of these receptors and generated stable transfectants that express high levels of each chimeric receptor on their cell surface. Our studies reveal two dissociable hormone sites on the calcitonin receptor. One site is in the receptor amino terminus and has high-affinity calcitonin binding while a second site located within the remaining receptor carboxy terminus has low affinity for calcitonin and is coupled to activation of adenylate cyclase. Both of these hormone sites exhibit a specificity for hormone interaction similar to the hCTR. Collectively, these data suggest a two-site hormone−receptor interaction model. Both sites apparently cooperate to irreversibly bind calcitonin and activate the receptor which may include a ligand-induced conformational change in the native calcitonin receptor.

MATERIALS AND METHODS

Peptide hormones and ^{125}I-labeled peptide hormones were obtained from Peninsula Laboratories. Cell culture media and reagents were from Life Technologies. Bis(sulfosuccinimidyl) suberate (BS3) was obtained from Pierce Chemical Co. Except where otherwise indicated, all other reagents were obtained through Sigma Chemical Co.

Hybrid Construction and Transfections. Hybrid receptor DNA constructs were prepared using the cDNAs encoding the human glucagon receptor (GenBank Accession No. L20316) and human calcitonin receptor (EMBL Accession No. X69920). These cDNAs were inserted into the pHZ-1 mammalian expression vector behind a modified metallothionein-1 promoter (Lok et al., 1994). The selective marker on this vector is neomycin. One chimera (NtCTr) was generated by transposing residues 1−156 of the hCTR onto residues 150−477 of the hGGR. This was accomplished using synthetic oligonucleotides to join an *Nsi*I site in the hCTR cDNA to an *Apa*I site in the hGGR cDNA. A second chimera (NtGGr) was generated by transposing residues 1−139 of the hGGR onto residues 147−474 of the hCTR. This was done using synthetic oligonucleotides to join an *Xma*I site in the hGGR cDNA to an *Eco*57I site in the hCTR cDNA. The junctions of these constructs were verified by sequencing. The constructs were transfected by calcium phosphate precipitation into a baby hamster kidney (BHK) cell line (ATCC CRL 1632) which had been previously engineered to stably express a cAMP-responsive reporter construct. Selection was done in 500 μg/mL G418 (Life Technologies).

Fusion Proteins. A fusion protein expression and purification system (New England Biolabs) was used to generate fragments of the calcitonin receptor fused to the *Escherichia coli* maltose binding protein (MBP). The DNA fragments encoding the N-terminal 146 amino acids (1−146) and the C-terminal amino acids (392−474) of the human hCTR were ligated to the 3′ end of the MBP DNA in the pMAL-p2 and pMAL-c2 expression vectors, respectively, and constructs were verified by sequencing. The N-terminal-containing vector (pIP2) and the C-terminal-containing vector (pCYT) were transformed into *E. coli* strain JM1019. Fusion proteins were purified by maltose affinity chromatography.

Antibody Production. Mice were injected intraperitoneally with 100 μg of fusion protein mixed in Freund's complete adjuvant. All subsequent injections were at 14 day intervals with 50 μg of protein mixed in Freund's incomplete adjuvant. First bleeds were taken 5−7 days after the third injection. Rabbits were injected with 500 μg of protein mixed in Freund's complete adjuvant. All subsequent injections were with 200 μg of protein mixed in incomplete Freund's adjuvant. First bleeds were taken 10 days after the third injection. Antisera specifically recognizing the recombinant human calcitonin receptor were confirmed by comparison of immunoblots of receptor-positive and receptor-negative BHK cell membranes.

Gel Electrophoresis and Immunoblots. Gel electrophoresis was performed using precast (Novex) 4−20%, 1.5 mm Tris/glycine gels. Some gels were transferred to nitrocellulose for immunoblotting. Protein prestained standards (Life Technologies) contained myosin H chain, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, lysozyme, and bovine trypsin inhibitor. Nitrocellulose blots were blocked with 50 mM Tris, pH 7.4, 5 mM EDTA, 0.05% NP-40, 100 mM NaCl, and 0.25% gelatin (WBA). They were incubated with antisera (1:250) for 1 h, washed 3 times, and incubated with a 1:1000 dilution of secondary antibody (either goat anti-rabbit HRP or goat anti-mouse HRP, Boehringer Mannheim) in WBA for 1 h. The blots were washed, and antigen bands were visualized using 120 mL of developer (0.05% 4-chloronaphthol, 17% methanol, 40 mM Tris, pH 7.4, 13 mM NaCl, and 0.025% hydrogen peroxide).

Binding and Cross-Linking of Hormone. Binding of radiolabeled hormones to membranes, whole cells, or detergent-solubilized membranes was done as previously described (Kuestner et al., 1994; Stroop et al., 1993a). For cross-linking, membranes were washed with 100 mM HEPES, pH 7.4, and resuspended in the same buffer at about 4 mg of protein/mL. Membranes (0.2 mL) were incubated for 30 min with ^{125}I-sCT (300 000 cpm, 1000 Ci/mmol) or ^{125}I-glucagon (1×10^6 cpm, 1460 Ci/mmol). Labeled peptides were reconstituted in 0.1 M sodium phosphate, pH

[1] Abbreviations: BHK, baby hamster kidney; BSA, bovine serum albumin; BS3, bis(sulfosuccinimidyl) suberate; cAMP, adenosine cyclic 3′,5′-monophosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CNBr, cyanogen bromide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; hGGR, human glucagon receptor; hCTR, human calcitonin receptor; hCT, human calcitonin; hCGRP, human calcitonin gene-related peptide; MBP, bacterial maltose binding protein; NtCTr, N-terminal calcitonin receptor chimera; NtGGr, N-terminal glucagon receptor chimera; PAGE, polyacrylamide gel electrophoresis; pCT, porcine calcitonin; PNGase F, peptide *N*-glycosidase F; sCT, salmon calcitonin; SDS, sodium dodecyl sulfate; SPA, scintillation proximity assay; TFA, trifluoroacetic acid.

7.4, 50 mM NaCl, 0.1% BSA, and 0.01% NaN_3. Control incubations included excess unlabeled sCT or glucagon (4 μM). Bis(sulfosuccinimidyl) suberate (BS3, Pierce) (15 μL of 60 mg/mL in DMSO) was added to each for 30 min. The reaction was stopped by addition of 1 mL of 1 M glycine/50 mM Tris, pH 9.0, and membranes were centrifuged and then washed with 2 mL of PBS/acetic acid, pH 2.5, and then washed again with 1 mL of water. For some experiments, membranes of the NtCTr chimera (clone G) cross-linked with ^{125}I-sCT were treated with cyanogen bromide (CNBr) in order to digest labeled receptor at methionine residues. Salmon calcitonin contains no methionine. The labeled and washed pellet (about 2 mg of protein) was dissolved in 0.25 mL of 70% trifluoroacetic acid (TFA), and CNBr (4 mg) was added, mixed, and incubated overnight in the dark at room temperature. This reaction was evaporated to dryness and then dried twice more after addition of water. Half the digest was redissolved in 75 μL of buffer (60 mM ammonium bicarbonate, pH 7.8, 10 μM EDTA, 0.5% octyl β-glucoside, and 1% β-mercaptoethanol) and further treated with 1 unit of peptide *N*-glycosidase F (PNGase F, Boehringer Mannheim) overnight at 37 °C.

Adenylate Cyclase Activation. Two methods were used to measure cAMP levels. In both cases, forskolin was added (25 μM) to determine maximum synthesis levels of cAMP. The first method utilized a scintillation proximity−cAMP system (cAMP−SPA, Amersham) as previously described (Kuestner et al., 1994). All points were done in duplicate or triplicate. The second method measured cAMP induction of the firefly luciferase gene under control of multiple cAMP responsive elements, essentially as described by Himmler et al. (1993). The chimeric receptors were transfected into a BHK cell line containing this reporter gene which shows a 10−20-fold induction of luciferase in response to forskolin. Transfectants were plated at 2×10^4 cells per 96-well plate (Microlite, Dynatech Laboratories). The next day, cells were stimulated with increasing amounts of hormone or forskolin for 4 h at 37 °C. The reaction was terminated by lysing cells in 25 μL of lysis reagent (Promega, E1501). After 15 min at room temperature, 50 μL of luciferase assay reagent (Promega) was added per well. Luminescence was monitored in a Luminoskan luminometer (Labsystems), and values are reported as arbitrary units directly from the instrument. The direct correlation of cAMP-induced luminescence with elevated cAMP levels was confirmed by comparison with cAMP levels measured using the cAMP-SPA assay.

RESULTS

Generation of Receptor Transfectants. The N-terminal domains, with predicted glycosylation sites, of the hCTR and hGGR were transposed onto the corresponding C-terminal region of the homologous receptor (Figure 1) generating two human chimeric receptors. The calcitonin receptor N-terminus chimera (NtCTr) and the glucagon receptor N-terminus chimera (NtGGr) constructs were stably transfected into BHK cells which contain a cAMP-responsive luciferase reporter construct (Himmler et al., 1993; Spengler et al., 1993). The NtCTr transfectant pool bound ^{125}I-sCT but showed no elevated cAMP response. Conversely, the NtGGr transfectant pool showed no detectable ^{125}I-sCT binding but, surprisingly, did show an elevated cAMP response on addition of sCT which was equivalent to 30% of the maximal forskolin response. No specific binding or elevated cAMP signal was detected with human glucagon with either transfectant pool (data not shown).

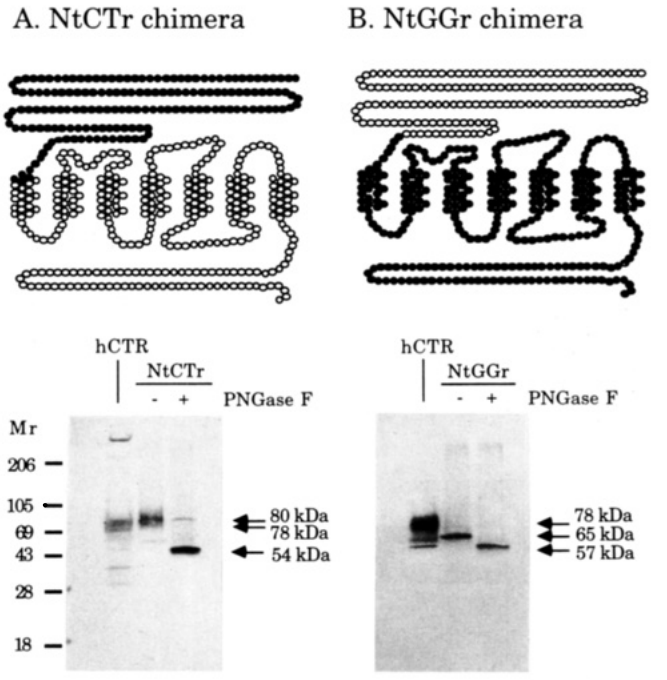

FIGURE 1: Structure and immunoblots of chimeric receptors. Closed circles represent the calcitonin receptor sequence, and open circles represent the glucagon receptor sequence. Transmembrane regions were predicted from a multiple alignment of all known homologous receptors. (**Panel A**) The N-terminus of the calcitonin receptor was transposed onto the glucagon receptor (NtCTr) as described above. Membranes from cells expressing a recombinant human calcitonin receptor (clone Hx1) were loaded on SDS−PAGE (25 μg of total protein) next to membranes expressing the NtCTr chimera (clone G, 1 μg of protein). An aliquot of membranes from the NtCTr chimera was treated overnight with PNGase F as described above to remove carbohydrates. After electrophoresis and transfer to nitrocellulose, this blot was developed with antisera from mice injected with the N-terminal calcitonin−receptor fusion protein "pIP2" as described above. (**Panel B**) The N-terminus of the glucagon receptor was transposed onto the calcitonin receptor (NtGGr) as described above. Human calcitonin receptor (clone Hx1, 5 μg of protein) and membranes expressing NtGGr chimera (clone F, 25 μg of protein) were treated, electrophoresed, and transferred as for panel A. This blot was developed using antisera from a rabbit injected with a C-terminal calcitonin−receptor fusion protein "pCYT" as described above.

Six high-expressing clones were isolated from the NtCTr pool by sCT binding and from the NtGGr pool by their luciferase signal in response to sCT. Membrane expression of chimeric receptors was confirmed in all clones by immunoblots using antisera directed to either the N-terminus or the C-terminus of the human calcitonin receptor. One clone of each chimera was selected for further study. Immunoblot analysis of membrane preparations of the NtCTr (clone G) and NtGGr (clone F) (Figure 1) shows that the NtCTr migrates as a 80 kDa protein similar to the recombinant hCTR at 78 kDa (Figure 1A) and the NtGGr migrates at 65 kDa (Figure 1B). Both are significantly glycosylated and migrate at lower molecular mass after deglycosylation with PNGase F (Figure 1). Comparison of the NtGGr to native hGGR was not possible due to a lack of antibody recognizing hGGR.

Ligand Binding Studies. Membranes prepared from the NtCTr chimera transfectant bind sCT with a high affinity (apparent $K_d = 12 \pm 0.6$ nM, $n = 3$) which is about 40-fold

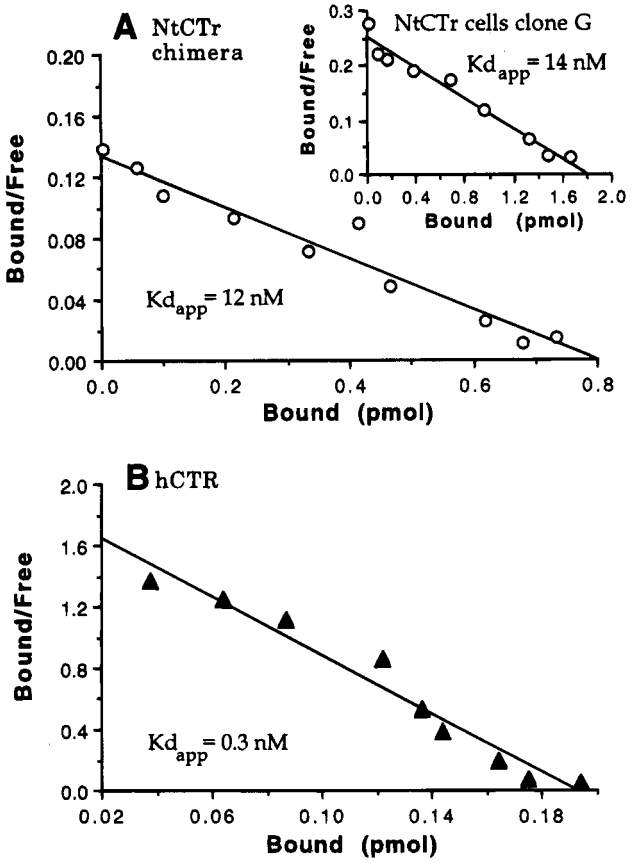

FIGURE 2: Binding of ^{125}I-sCT to human calcitonin receptor and NtCTr chimera. Membranes of BHK cells expressing the NtCTr chimera (clone G) (**panel A**) and membranes of cells expressing a recombinant human calcitonin receptor (clone Hx1) (**panel B**) were used for parallel incubations with ^{125}I-sCT and increasing concentrations of unlabeled sCT at room temperature. Specific binding was plotted by the method of Scatchard. Representative curves are presented and apparent dissociation constants were determined as indicated from three experiments. (**Panel A, inset**) Binding of ^{125}I-sCT to whole cells expressing the NtCTr chimera (clone G).

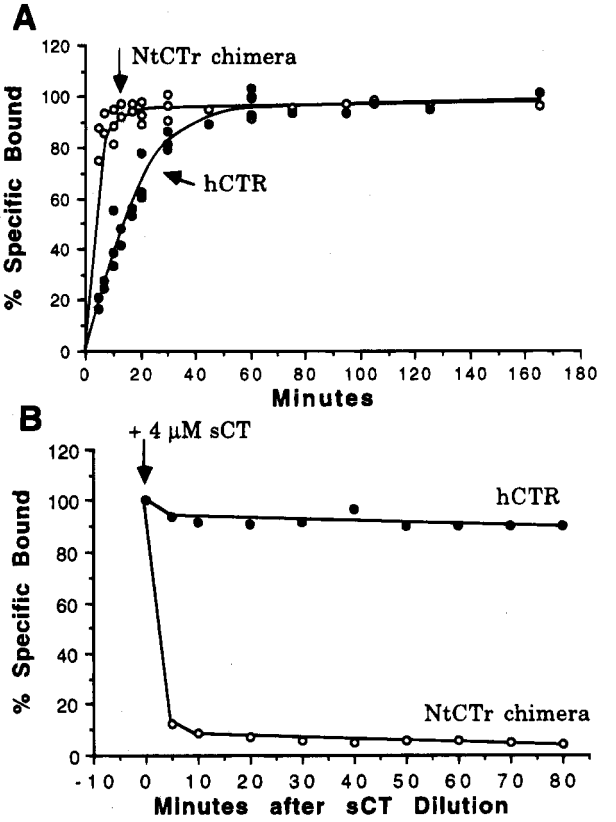

FIGURE 3: Kinetics of ^{125}I-sCT binding to human calcitonin receptor and NtCTr chimera. (**Panel A**) Membranes of cells expressing hCTR and NtCTr chimera were incubated with ^{125}I-sCT as described in Figure 2. Incubation was started at time zero by addition of ^{125}I-sCT and terminated at the indicated times by centrifugation for 5 min. (**Panel B**) Membranes were incubated for 1 h at room temperature with ^{125}I-sCT, at which time, designated time zero, an excess of unlabeled sCT (4 μM) was added and incubations were terminated as indicated after sCT addition by centrifugation. Results are combined from two representative experiments. Duplicate determinations at each time point varied less than 5%. Rates of association and dissociation were graphically estimated.

lower than the apparent K_d of the hCTR in a parallel experiment (0.3 ± 0.1 nM, n = 3) (Figure 2A,B). Specific high-affinity binding (apparent K_d = 8 nM) is also observed after solubilizing NtCTr membranes with CHAPS detergent (data not shown). Salmon calcitonin binds to whole cells expressing the NtCTr (clone G) with similar affinity (apparent K_d = 14 ± 1 nM, n = 3) (Figure 2A, inset). A high cell-surface receptor density of (1.8 ± 0.1) × 10^6 (n = 3) receptors per cell was determined for this clone. Specific binding of ^{125}I-labeled human calcitonin or ^{125}I-labeled human glucagon was not observed with these cells or with transfectants of the NtGGr (clone F) which also did not bind ^{125}I-sCT (data not shown).

Despite the lower binding affinity of sCT, the on-rate of sCT binding to the NtCTr chimera membranes is more rapid (apparent $t_{1/2}$ of <5 min) compared to the hCTR (apparent $t_{1/2}$ ~20 min) (Figure 3A). Even more striking is the rapid off-rate of sCT bound to NtCTr (apparent $t_{1/2}$ <5 min) (Figure 3B) compared to the well-documented irreversible calcitonin binding to the native calcitonin receptor [Figure 3B and Martin and Moseley (1990)].

The rank-order for competition of binding of sCT to the native hCTR (Figure 4) is sCT > pCT > hCT > hAmylin > hCGRP with half-maximal competition (IC_{50}) at approximately 0.2, 0.3, 5, 40, and 500 nM, respectively. A similar, but less inclusive, rank-order for competition of sCT binding to the NtCTr chimera is observed with IC_{50}s of approximately 20 nM, 100 nM, and >10 μM, respectively, for sCT, pCT, and hCT. The IC_{50}s for competition with the NtCTr relative to hCTR have been increased by approximately 100-, 333-, and >2000-fold for sCT, pCT, and hCT, respectively. Glucagon and secretin do not compete for binding of sCT to either receptor.

Cross-Linking Studies. Using a water-soluble cross-linker (BS3), we observed a significant specific cross-linking of ^{125}I-sCT to the NtCTr chimera (Figure 5A, lanes 1 and 2) that migrates at 70 kDa on SDS−PAGE. In contrast, cross-linking to an equivalent amount of hCTR is almost undetectable (Figure 5A, lane 6), although a specifically labeled band at 70 kDa can reproducibly be seen with longer exposure times (data not shown). Both the NtCTr and hCTR have an equal number of lysines available for cross-linking in their predicted extracellular regions. Cross-linking of hCTR membranes prior to ^{125}I-sCT addition results in no labeled bands (Figure 5A, lanes 3 and 4). Cross-linked NtCTr membranes were digested with cyanogen bromide in order to localize the receptor sites of cross-linking (Figure 5C). The majority of cross-linked peptides migrate at <6 kDa, but two fragments were observed at 45 and 17 kDa which subsequently migrate at 11 kDa after deglycosylation with

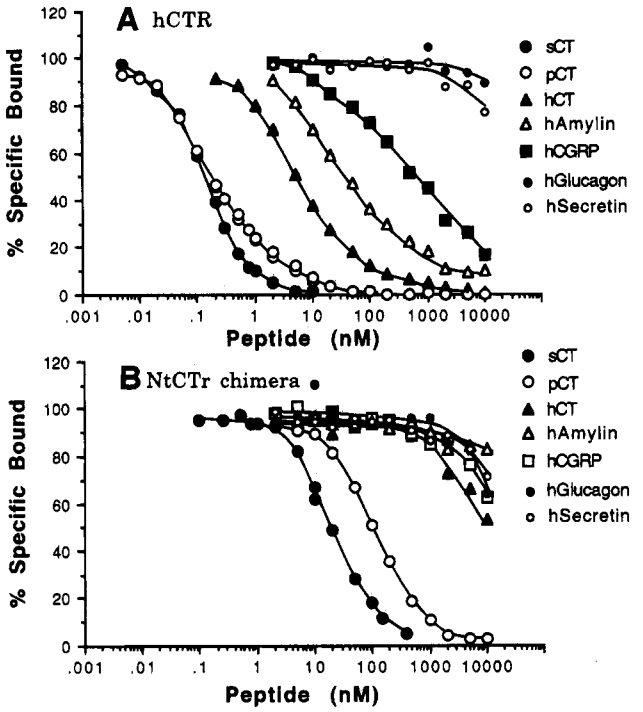

FIGURE 4: Competition for binding of ^{125}I-sCT to human calcitonin receptor and NtCTr chimera. Membranes of cells expressing hCTR (clone Hx1) (**panel A**) and NtCTr chimera (clone G) (**panel B**) were incubated with ^{125}I-sCT for 1 h as described in Figure 2 in the presence of increasing concentrations of unlabeled peptides as indicated. Concentrations of peptides resulting in 50% inhibition of specific binding (IC_{50}s) were estimated graphically. Results are shown for representative experiments with duplicate determinations that varied less than 5%.

PNGase F. These data suggest that the NtCTr chimera may be valuable for studies of the interaction of hormone with specific residues in the calcitonin receptor N-terminus.

Despite the fact that no specific binding of ^{125}I-sCT or ^{125}I-human glucagon was observed with the NtGGr (clone F), we found that cross-linking of ^{125}I-glucagon to membranes of these cells with BS3 results in a specifically labeled band at 50 kDa after SDS−PAGE (Figure 5B). Labeling of the 50 kDa band is not particularly efficient but is inhibited by addition of excess unlabeled glucagon while other nonspecifically labeled bands are unaffected. No cross-linking of ^{125}I-sCT was observed to NtGGr membranes under similar conditions (data not shown). These data suggest that glucagon can bind to the NtGGr chimera but with such a low affinity that the bound ligand can only be detected after covalent "trapping" on the receptor and separation from nonspecific background by electrophoresis.

Signal Transduction Studies. Six stable transfectants of the NtGGr were isolated on the basis of a strong cAMP-dependent luciferase signal, and one (clone F) was selected for further study. Using the cAMP-responsive luciferase assay, we found that sCT, pCT, and hCT induce elevated cAMP signals with effective half-maximal concentrations (EC_{50}s) of 2.2 ± 0.6 nM (n = 5), 5.8 ± 2.1 nM (n = 4), and 810 ± 151 nM (n = 4), respectively (Figure 6A). The maximum response of these cells to 100 nM sCT was typically 75−115% of that induced by 25 μM forskolin. An EC_{50} of 7 ± 1 nM for sCT was also determined using a direct assay for cAMP (Figure 6B). These EC_{50}s are higher than the EC_{50}s of 0.06 nM (sCT) and 0.08 nM (hCT) with the recombinant hCTR (clone Hx1) (Kuestner et al., 1994). The observed EC_{50} values are expected to be much lower than their associated binding affinities due to the spare receptor effect which results in a full activation of adenylate cyclase via a small fraction of occupied receptors. Human amylin, human CGRP, and human glucagon at concentrations up to 1000 nM did not elevate cAMP signals in the NtGGr (clone F) cells (Figure 6A, and data not shown).

Activation of adenylate cyclase by the NtCTr chimera (clone G) was not observed with sCT, hCT, or glucagon (10 μM) even though these cells expressed nearly two million cell-surface receptors per cell and bind sCT with a high affinity (Figure 6C). In addition, we examined transfectants of both chimeras for hormone-induced increase in intracellular calcium as previously described for the hCTR (Stroop et al., 1993b) but did not observe any activity with sCT or human glucagon at concentrations up to 1 μM.

DISCUSSION

These studies identify two physically separable extracellular sites on the calcitonin receptor that interact with calcitonin. The first site resides on the receptor N-terminus (site-one) and binds hormone with high affinity while the second site is located within the remaining receptor carboxy terminus (site-two). Although site-two has a low, undetectable affinity for calcitonin, it can fully activate adenylate cyclase. Moreover, both of these sites possess an apparent specificity of hormone interaction similar to the native calcitonin receptor. Neither chimera, however, completely mimics the binding or activation observed with the native receptor, suggesting that the two sites normally function in concert to complex calcitonin. For example, although site-one is not required for full activation of adenylate cyclase on the NtGGr chimera, it apparently contributes to the lower EC_{50}s observed with the native hCTR (~100-fold for sCT) presumably by facilitating a high-affinity binding interaction. Likewise, site-two is not required for high-affinity binding to the NtCTr chimera but apparently contributes to a greatly increased calcitonin affinity and irreversible binding on the native hCTR primarily by decreasing the off-rate of bound hormone.

It is interesting to compare the behavior of our chimeric receptors with two isoforms of the rat calcitonin receptor that have been recently characterized (Houssami et al., 1994). A brain-specific form of the receptor (C1b) contains a 37 amino acid insert within the first extracellular loop which results in an approximately 40-fold lower affinity for sCT and increased EC_{50} values for adenylate cyclase activation compared to the C1a receptor lacking the insert. Although the kinetics of hormone binding are more rapid and reversible for the C1b isoform, the rank-order for specificity of hormone interaction is essentially unaltered. Thus, the C1b isoform behaves in similar fashion to the NtCTr chimera for hormone binding and the NtGGr chimera for adenylate cyclase activation. These observations suggest that the large first extracellular loop of the C1b receptor may cause a partial, functional dissociation of the two receptor hormone sites and an "uncoupling" of their interaction with calcitonin.

Calcitonin peptides with lower affinity and potency for the native receptor show greater fold reduction for interaction with both chimeric receptors, and this may be related to their reduced probability to form an amphipathic helix structure in solution (Martin & Moseley, 1990; Epand et al., 1985).

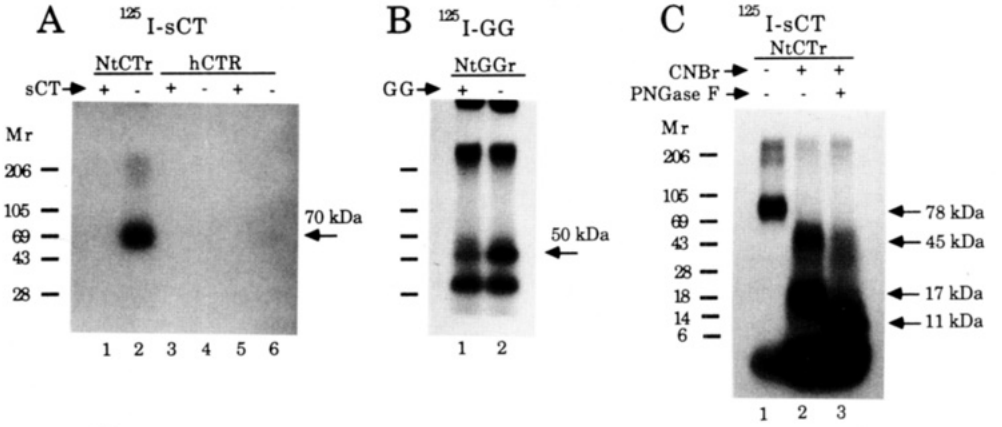

FIGURE 5: Cross-linking of ^{125}I-hormone to human calcitonin receptor and chimeric receptors. (**Panel A**) Membranes of cells (about 100 μg of protein) expressing NtCTr chimera (lanes 1 and 2) and hCTR (lanes 5 and 6) were incubated with 40 000 cpm of ^{125}I-sCT with or without excess unlabeled sCT (1.2 μM). Membranes expressing hCTR (lanes 3 and 4) were pretreated with BS3 and washed prior to addition of ^{125}I-sCT. Cross-linking and SDS−PAGE were followed by autoradiography for 3 days at −80 °C for all three panels. (**Panel B**) Membranes expressing the NtGGr chimera were cross-linked in the presence of ^{125}I-glucagon (1×10^6 cpm) with and without excess glucagon (4 μM). (**Panel C**) Membranes of cells (about 2 mg of protein) expressing NtCTr chimera were cross-linked with BS3 in the presence of ^{125}I-sCT (300 000 cpm) and washed as described above. Labeled membranes (lane 1, 10 μg) were dissolved and digested with CNBr as described above. This digest was divided into two aliquots and resolved on 4−20% SDS−PAGE before (lane 2) and after (lane 3) treatment with PNGase F.

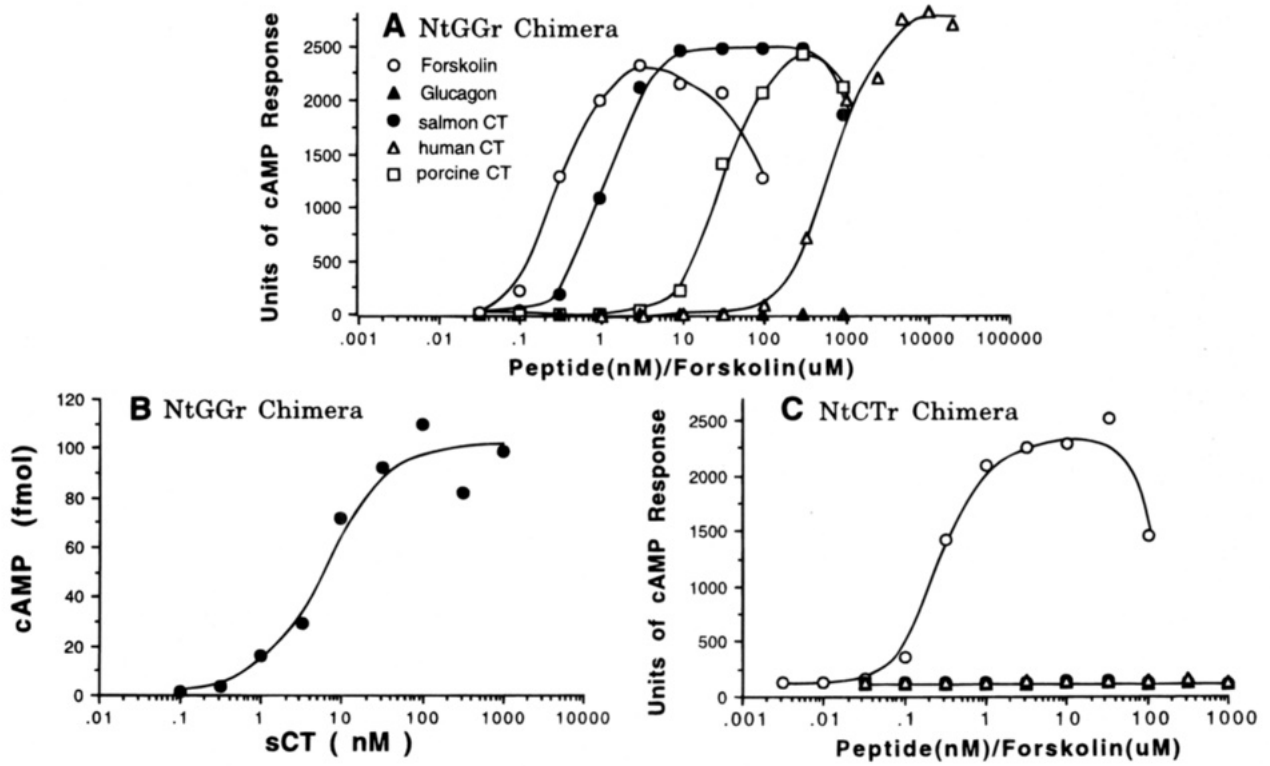

FIGURE 6: Activation of adenylate cyclase by chimeric receptors. (**Panel A**) Transfectants expressing the NtGGr chimera (clone F) were grown in 96-well plates and assayed for induction of a cAMP-responsive luciferase signal as described above. Each curve is representative of four to five experiments with each peptide used to determine EC_{50} concentrations (see text). Duplicate determinations at each peptide concentration varied less than 15% within a single experiment. Arbitrary units of luminescence are shown on the vertical axis. (**Panel B**) Cells (NtGGr chimera, clone F) were grown in 24-well plates and treated with salmon calcitonin. Elevation of total cAMP per well was determined using a cAMP−SPA assay as described above. Results are duplicate determinations from a single representative experiment. An EC_{50} of 7 ± 1 nM was determined from three experiments. (**Panel C**) Transfectants expressing the NtCTr chimera (clone G) were assayed as described in panel A with forskolin, sCT, hCT, and human glucagon.

Hormone potency and biological activity are also dependent on other peptide features such as conformational flexibility [see Martin and Moseley (1990), Epand and Caulfield (1990), and Epand (1983)]. Nevertheless, some hormone determinants may preferentially interact with the N-terminal (site-one) or C-terminal (site-two) hormone sites of the receptor. For example, the N-terminus of calcitonin, which exhibits the highest degree of homology between species, has been implicated in receptor activation and may, therefore, preferentially interact with the receptor site coupled to adenylate cyclase (site-two). If so, it is surprising then that sCT, pCT, and hCT have the same rank-order for binding to site-one as they have for activation of adenylate cyclase at site-two even though the first nine amino acids of these peptides are essentially identical. It seems likely that other peptide structural features contribute to calcitonin interaction at site-

two and these features are also responsible for the similar binding specificity to site-one. Modification of some of these peptide features may, however, produce agonist and antagonist peptides where binding at site-one and adenylate cyclase activation at site-two are dissociated. Some calcitonin analogs with these characteristics have been reported (Orlowski et al., 1987; Epand et al., 1985, 1988; Nakamuta et al., 1990; Feyen et al., 1992).

One possible interpretation for the differential cross-linking of ^{125}I-sCT to the NtCTr chimera compared to the native hCTR is that the calcitonin receptor undergoes a conformational change so that the bound hormone is partially protected from cross-linking. The greatly increased cross-linking of the NtCTr is consistent with the conformation of these two receptor−hormone complexes being significantly different since both receptors have equal numbers of cross-linkable lysines in their predicted extracellular regions.

There are two other recent examples of seven-transmembrane receptors where high-affinity binding sites are separated from an activation site. These receptors have no structural homology to the calcitonin receptor. Ligand binding and activation of the glycoprotein C5a receptor consist of two physically separable domains (Siciliano et al., 1994). The first includes the N-terminus and possibly the first extracellular loop whereas the second site lies in the remaining C-terminal portion of the receptor. Only interaction with the second site is required for receptor activation. A similar situation is reported for the thyrotropin receptor where chimeric studies of the extracellular domain suggest that this domain is involved in high-affinity hormone binding but that high-affinity binding is not required for signal transduction (Akamizu et al., 1993). An aspartate residue in the first extracellular loop of this receptor has been identified as being necessary for activation but not for binding (Ji & Ji, 1993).

These studies describe two hormone sites on the calcitonin receptor that can be physically dissociated. The dissociated sites retain their principal functions of binding at site-one and adenylate cyclase activation at site-two. They also retain the ability to discriminate between salmon, porcine, and human calcitonins. These two sites likely cooperate on the native receptor to complex calcitonin. One possible model would be that calcitonin first binds to site-one on the N-terminus. These reversible interactions are followed by a slower interaction of hormone with both of the receptor hormone sites to form a high-affinity complex in which hormone is bound with apparent irreversibility and multiple signaling pathways are activated. Slow conversion to the irreversible hormone−receptor complex may be accompanied by a conformational change as discussed above.

ACKNOWLEDGMENT

We gratefully acknowledge Drs. D. Durnam and W. Kindsvogel for comments and discussion and Dr. J. Kelley and P. McKernan for the use of BHK cells expressing a cAMP-reporter construct.

REFERENCES

Akamizu, T., Inoue, D., Kosugi, S., Ban, T., Kohn, L. D., Imura, H., & Mori, T. (1993) *Endocri. J. 40*, 363−372.

Albrandt, K., Mull, E., Brady, E. M. G., Herich, J., Moore, C. X., & Beaumont, K. (1993) *FEBS Lett. 325*, 225−230.

Bharucha, D. B., & Tager, H. S. (1990) *J. Biol. Chem. 265*, 3070−3079.

Dohlmam, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653−688.

Epand, R. M. (1983) *Mol. Cell. Biochem. 57*, 41−47.

Epand, R. M., & Epand, R. F. (1986) *Biochemistry 25*, 1964−1968.

Epand, R. M., & Caulfield, M. P. (1990) in *Comprehensive Medicinal Chemistry* (Emmett, J. C., Ed.,) Vol. 3, pp 1023−1045, Pergamon Books Ltd., Oxford.

Epand, R. M., Rosselin, G., Hoa, D. H. B., Cote, T. E., & Laburthe, M. (1981) *J. Biol. Chem. 256*, 1128−1132.

Epand, R. M., Epand, R. F., & Orlowski, R. C. (1985) *Int. J. Pept. Protein Res. 25*, 105−111.

Epand, R. M., Epand, R. F., & Orlowski, R. C. (1988) *Biochem. Biophys. Res. Commun. 152*, 203−207.

Feyen, J. H. M., Cardinaux, F., Gamse, R., Bruns, C., Azria, M., & Trechsel, U. (1992) *Biochem. Biophys. Res. Commun. 187*, 8−13.

Findlay, D. M., Michelangeli, V. P., Martin, T. J., Orlowski, R. C., & Seyler, J. K. (1985) *Endocrinology 117*, 801−805.

Gorn, A. H., Lin, H. Y., Yamin, M., Auron, P. E., Flannery, M. R., Tapp, D. R., Manning, C. A., Lodish, H. F., Krane, S. M., & Goldring, S. R. (1992) *J. Clin. Invest. 90*, 1726−1735.

Himmler, A., Stratowa, C., & Czernilofsky, A. P. (1993) *J. Recept. Res. 13*, 79−94.

Horwitz, E. M., Wyborski, R. J., & Gurd, R. S. (1986) *J. Biol. Chem. 261*, 13670−13676.

Houssami, S., Findlay, D. M., Brady, C. L., Myers, D. E., Martin, T. J., & Sexton, P. M. (1994) *Endocrinology 135*, 183−190.

Huang, R.-R. C., Yu, H., Strader, C. D., & Fong, T. M. (1994) *Biochemistry 33*, 3007−3013.

Ji, I., & Ji, T. H. (1993) *J. Biol. Chem. 268*, 20851−20854.

Juppner, H., Schipani, E., Bringhurst, F. R., McClure, I., Keutmann, H. T., Potts, J. T., Jr., Kronenberg, H. M., Abou-Samra, A. B., Segre, G. V., & Gardella, T. J. (1994) *Endocrinology* 134, 879−884.

Kubo, T., Bujo, H., Akiba, I., Nakai, J., Mishina, M., & Numa, S. (1988) *FEBS Lett. 241*, 119−125.

Kuestner, R. E., Elrod, R., Grant, F. J., Hagen, F. S., Kuijper, J. L., Matthewes, S. L., O'Hara, P. J., Sheppard, P. O., Stroop, S. D., Thompson, D. L., Whitmore, T. E., Findlay, D. M., Houssami, S., Sexton, P. M., & Moore, E. E. (1994) *Mol. Pharmacol. 46*, 246−255.

Lok, S., Kuijper, J. L., Jelinek, L. J., Kramer, J. M., Whitmore, T. E., Sprecher, C. A., Mathewes, S., Grant, F. J., Biggs, S. H., Rosenberg, G. B., Sheppard, P. O., O'Hara, P. J., Foster, D. C., & Kindsvogel, W. (1994) *Gene 140*, 203−209.

Martin, T. J., & Moseley, J. M. (1990) in *Metabolic Bone Disease and Clinically Related Disorders* (Avioli, L. V., & Krane, S. M., Eds.) 2nd ed., pp 131−154, W. B. Saunders Co., Harcourt Brace Jovanovich, Inc., Philadelphia.

Nakamuta, H., Orlowski, R. C., & Epand, R. M. (1990) *Endocrinology 127*, 163−169.

Orlowski, R. C., Epand, R. M., & Stafford, A. R. (1987) *Eur. J. Biochem. 162*, 399−402.

Premont, R. T., & Iyengar, R. (1987) in *Peptide Hormone Receptors* (Kalimi, M. Y., & Hubbard, J. R., Eds.) pp 129−177, Walter de Gruyter, Berlin.

Schwartz, T. W. (1994) *Curr. Opin. Biotechnol. 5*, 434−444.

Segre, G. V., & Goldring, S. R. (1993) *Trends Endocrinol. Metab. 4*, 309−314.

Sexton, P. M., Houssami, S., Hilton, J. M., O'Keeffe, L. M., Center, R. J., Gillespie, M. T., Darcy, P. K., & Findlay, D. M. (1993) *Mol. Endocrinol. 7*, 815−821.

Siciliano, S. J., Rollins, T. E., DeMartino, J., Konteatis, Z., Malkowitz, L., Riper, G. V., Bondy, S., Rosen, H., & Springer, M. S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1214−1218.

Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H., & Journot, L. (1993) *Nature 365*, 170−175.

Stroop, S. D., Moore, E. E., Kuestner, R. E., & Thompson, D. L. (1993) *J. Recept. Res. 13*(8), 1173−1179.

Stroop, S. D., Thompson, D. L., Kuestner, R. E., & Moore, E. E. (1993) *J. Biol. Chem. 268*, 19927−19930.

Unson, C. G., & Merrifield, R. B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 454−458.

BI9418784