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# Divergent Structural Requirements Exist for Calcitonin Receptor Binding Specificity and Adenylate Cyclase Activation

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## SUMMARY

The basis of the high potency of salmon calcitonin (sCT) in radioligand binding competition and cAMP accumulation studies with cloned calcitonin (CT) receptors from rats, pigs, and humans was examined using two sets of CT analogues, i.e., chimeric sCT/human CT (hCT) analogues and analogues of sCT with differing capacities to form an amphipathic $\alpha$-helix. In competition for $^{125}$I-sCT binding the following relative specificities were observed for the chimeric peptides: rat C1a CT receptor, sCT $\geq$ (1–16)hCT/(17–32)sCT (ACT-15) > (1–16)sCT/(17–32)hCT (ACT-27); rat C1b CT receptor, sCT $\gg$ ACT-15 > ACT-27; hCT receptor, sCT = ACT-15 > ACT-27; porcine CT receptor, sCT > ACT-27 > ACT-15. In contrast, in ligand-induced cAMP accumulation studies the relative efficacies were as follows: rat C1a CT receptor, sCT = ACT-15 > ACT-27; rat C1b CT receptor, sCT = ACT-15 > ACT-27; hCT receptor, sCT = ACT-15 $\geq$ ACT-27; porcine CT receptor, sCT = ACT-15 = ACT-27. The data demonstrate that residues present in the carboxyl-terminal half of sCT are more important for binding competition with the rat C1a, rat C1b, and human CT receptors, whereas residues in the amino-terminal half of sCT are more important for binding competition with the porcine CT receptor. Carboxyl-terminal sCT residues are also important for full potency in adenylate cyclase activation with the rat C1a and rat C1b CT receptors but are less important for activation via the hCT receptor. The disparity in the relative potencies of the peptides in studies of binding competition and cAMP accumulation is suggestive of significant differences in the relative affinities of the peptides for active and inactive conformations of the CT receptor. The use of sCT analogues with varying capacities to form $\alpha$-helices also revealed divergence in the responses of different receptors. This was most apparent for the stimulation of cAMP production by the rat receptor isoforms C1a and C1b. In cells expressing the C1a receptor, the helical analogues sCT and des-Ser$^2$-sCT were equipotent with [Gly$^8$]-des-Leu$^{19}$-sCT and des-1-amino-[Ala$^{1,7}$,Gly$^8$]-des-Leu$^{19}$ sCT, analogues that have reduced or absent helical structure, respectively. In contrast, the nonhelical analogues were 100-1000-fold less potent than sCT and des-Ser$^2$-sCT at the C1b receptor. In general, reduction in the ability of sCT analogues to form helix structures had a greater impact on the potency of the analogues in competition for $^{125}$I-sCT binding than in cAMP accumulation. The absence or reduction of helix-forming potential had much less impact on the interaction of the analogues with the cloned hCT receptor in both assay systems but also had minimal impact on the activation of the rat C1a receptor, as assessed by cAMP responses.

The CTs are 32-amino acid peptide hormones whose best recognized action is on osteoclasts, to inhibit bone resorption (1). CT also acts on receptors in the kidney, where it may affect ion secretion (2–4), as well as in the central nervous system, where exogenous CT has potent effects including modification of pain perception, appetite, and gastric acid secretion (5).

CT receptors have been cloned from pigs (6), humans (7, 8), and rats (9, 10). The human and rat receptors exist as multiple isoforms with insert sequences in the first intracellular

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**ABBREVIATIONS:** CD, circular dichroism; CT, calcitonin; sCT, salmon calcitonin; pCT, porcine calcitonin; hCT, human calcitonin; sCTA1, [Gly$^8$,Ala$^{16}$]-des-Leu$^{19}$-salmon calcitonin; sCTA2, des-1-amino-[Ala$^{1,7}$,Gly$^8$]-des-Leu$^{19}$-salmon calcitonin; sCTA3, des-Ser$^2$-salmon calcitonin; sCTA4, [Gly$^{2,3,4,5,6}$]-salmon calcitonin; sCTA5, [Gly$^8$]-des-Leu$^{19}$-salmon calcitonin; sCTA6, [Gly$^8$,D-Arg$^{24}$]des-Leu$^{16}$-salmon calcitonin; DME, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HEK, human embryonic kidney; BHK, baby hamster kidney; ACT-15, (1–16)human calcitonin/(17–32)salmon calcitonin; ACT-27, (1–16)salmon calcitonin/(17–32)human calcitonin; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); BSA, bovine serum albumin.



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loop (7, 8) and the second extracellular domain (9, 10), respectively. Based on nucleotide sequence identity, these are likely to arise from alternative splicing of receptor mRNA. Indeed, the recent characterization of the pCT receptor gene reveals two alternatively spliced isoforms equivalent to the human receptor isoforms (11). The CT receptors are members of a new subfamily of seven-transmembrane domain, G protein-coupled receptors that includes receptors for parathyroid hormone/parathyroid hormone-related protein, growth hormone-releasing hormone, vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide, and glucagon-like peptide (12).

CTs have been isolated from many species and fall into three classes based on structural and biological similarities (13). These are teleost/avian CT, artiodactyl CT, and rat CT/hCT. The relative potency of representatives of each class is sCT ≥ pCT > hCT, but variations in absolute efficacies occur among receptors of different species (14). Indeed, differences in relative potency between sCT and hCT can vary from ~10-fold to >1000-fold for hCT (7, 14) and pCT receptors (14), respectively.

CD studies indicate that sCT exhibits considerable secondary structure in the presence of lipid, consistent with the generation of an amphipathic α-helix between residues 8 and 22 (15–17). However, the propensity of the less potent hCT to form this secondary structure is much weaker. Modifications of residues in the 8–22 sequence to alter the ability of sCT to form secondary structure have yielded conflicting results, in terms of hypocalcemic potency of the analogues (15, 16, 18–21). In some cases analogues with less secondary structure had correspondingly lower hypocalcemic activity (16, 20), whereas other analogues were fully active (15, 18, 19, 21), suggesting that conformational flexibility may also contribute to activity (20, 21) and that factors other than secondary structure are also critical for ligand binding and receptor activation.

The examination, in ligand-binding and bioactivity studies, of analogues with differing capacities to generate α-helical secondary structure generated early evidence for heterogeneity in CT receptor interaction and activation within the rat system (18). That work suggested that two populations of receptors existed, which were termed $CT_H$ (receptors that interacted strongly only with analogues that had high helical capacity) and $CT_L$ [receptors that interacted with both helical and nonhelical (linear) analogues]. This was consistent with the subsequent identification of two isoforms of the rat CT receptor from cDNA cloning studies (9, 10).

To explore further the structural epitopes important for CT receptor binding specificity and adenylate cyclase activation, we have examined the efficacy of sCT and the sCT/hCT chimeric peptides ACT-15 and ACT-27 (Fig. 1), together with helical and nonhelical analogues of sCT (Table 1), in cells stably expressing cloned CT receptors from rats, pigs, and humans.

## Materials and Methods

**Hormones and chemicals.** The pCT receptor cDNA clone 3J8-14-F1 was a gift from Dr. H. Lin (Renal Unit, Massachusetts General Hospital, Boston, MA). Synthetic sCT and (8–32)sCT were from Bachem (Torrance, CA). ACT-15, ACT-27, (17–32)sCT, and (17–32)hCT were synthesized and purified by high performance liquid chromatography as described previously (22). The analogues sCTA1, sCTA2, sCTA3, sCTA4, sCTA5, and sCTA6 were synthesized and purified as described previously (18, 23). G418 (Geneticin) was obtained from GIBCO (Grand Island, NY). BSA was obtained from Commonwealth Serum Laboratories (Parkville, Australia). Prostaglandin $E_2$, bacitracin, and isobutylmethylxanthine were purchased from Sigma Chemical Co. (St. Louis, MO). The anti-cAMP antibody was a gift from Dr. P. Marley (Department of Pharmacology, University of Melbourne, Melbourne, Australia). $Na^{125}I$ and $^{125}I$-cAMP were from Amersham (Sydney, Australia). Synthetic sCT was iodinated using a modification of the chloramine T method (24). Specific activity of $^{125}I$-sCT was ~700 Ci/mmol.

**Peptide secondary structure.** The conformational properties of the analogue sCTA6 were studied with CD, as described previously (17). The mean residue ellipticity at 222 nm becomes more negative as a result of increased secondary structure, particularly helical structures. Analyses were carried out in aqueous buffer (20 mM PIPES, 1 mM EDTA, 0.15 M NaCl, 0.02 mg/ml $NaN_3$, pH 7.4) and in the presence of the phospholipid dimyristoylphosphatidylglycerol. The results for the sCTA6 analogue were compared with the previously derived CD data on the other analogues (Table 1) (15, 18).

**Cell culture.** HEK-293- and BHK-derived cell lines were maintained in DME containing 5% fetal bovine serum and 200 μg/ml G418. The BHK cell line, stably transfected with the cloned T47D hCT receptor (~200,000 receptors/cell), was prepared as described previously (8, 25). The T47D form of the CT receptor does not contain the 16-amino acid insert of the original hCT receptor clone from BIN

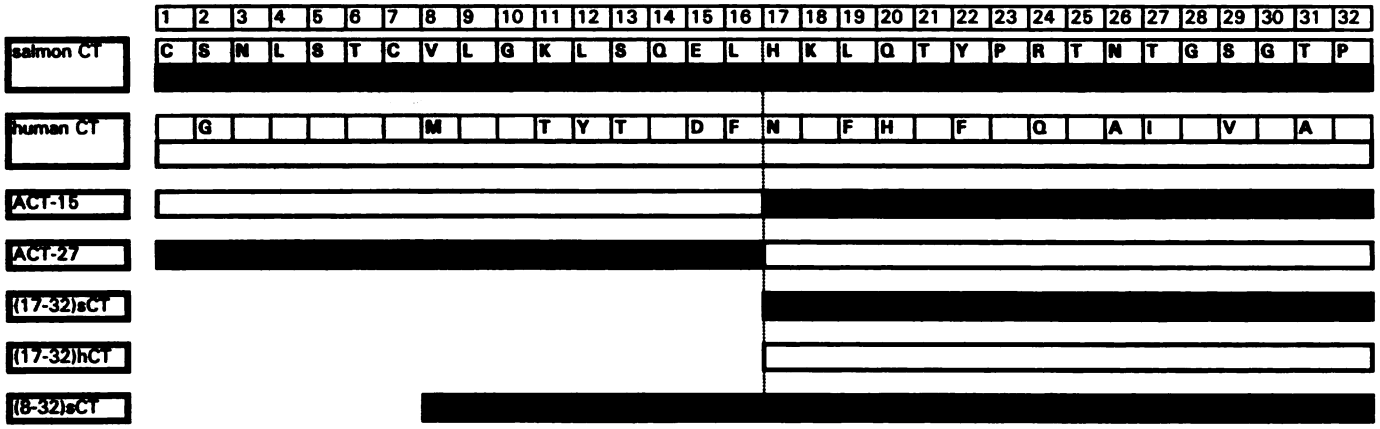


**Fig. 1.** Amino acid sequences of sCT and hCT and schematic representation of the chimeric or truncated peptides used in this study. The amino acid sequence for hCT lists only the residues unique to hCT. Residues conserved with sCT are not shown. ■, sCT equivalent residues; □, hCT equivalent residues.

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TABLE 1
**CD of CT peptides in the presence and absence of dimyristoylphosphatidylglycerol (DMPG)**

| Peptide | $-[\theta]_{222\ nm}$ | | Ref. |
|---|---|---|---|
| | No additions | DMPG | |
| | *degrees·cm²/dmol* | | |
| sCT | 4,160 | 12,350 | 15 |
| sCTA1 | 3,300 | 2,170 | 15 |
| sCTA2 | 2,260 | 1,990 | 15 |
| sCTA3 | 3,490 | 8,080 | 18 |
| sCTA4 | 2,160 | 4,070 | 18 |
| sCTA5 | 3,600 | 4,230 | 15 |
| sCTA6 | 2,600 | 2,150 | |

67 cells (7) and is the predominant form of the CT receptor expressed in humans (8).

**Stable transfection of HEK-293 cells.** Cells were transfected with either the pCT receptor cDNA (3J8-14-F1) or the rat C1a or C1b receptor cDNA as described (10). Forty-eight hours after transfection G418 (200 μg/ml) was added to the medium, and selection was maintained for 2 weeks. Fresh medium containing G418 was added every 2–3 days. The G418-resistant cells, which had incorporated the receptor cDNA-containing plasmids into the genome, were subcloned by two rounds of limiting dilution. Cloned cells were screened for CT receptor expression by radioligand binding assays with $^{125}$I-sCT as the radioligand. Receptor-expressing cell lines were subsequently maintained in DME containing 5% fetal bovine serum and 200 μg/ml G418. Cell lines expressing the C1a receptor (~60,000 receptors/cell) (F12), the C1b receptor (~6,000,000 receptors/cell) (B8-H10), and the pCT receptor (~600,000 receptors/cell) (A7) were mainly used in the current studies. However, because of the variation in receptor expression, two additional cell lines, expressing the C1a receptor (~500,000 receptors/cell) (D11) and the C1b receptor (~60,000 receptors/cell) (G12-E12), were also studied.

**Receptor binding assay.** For binding studies, cells in 24-well plates (Costar, Cambridge, MA) were washed once with PBS (140 mM NaCl, 2 mM KCl, 1 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$) before the addition of binding buffer (DME containing 0.1% BSA and 0.1% bacitracin). $^{125}$I-sCT (~80 pM) was added to the wells in the absence (total binding) or presence of increasing concentrations of unlabeled ligands. Nonspecific binding was defined as binding in the presence of $10^{-6}$ M unlabeled sCT. After incubation for 1 hr at 37° in 5% $CO_2$, cells were washed with PBS to remove unbound radioactivity and then solubilized with 0.5 ml of 0.5 M NaOH. Samples were counted in a Packard γ counter (70% efficiency) to determine bound radioactivity. The results shown are representative of at least two separate experiments performed in triplicate. Binding isotherms were analyzed by the iterative curve-fitting program LIGAND (26). This method of estimation was used to allow simultaneous analysis of multiple binding curves and generation of error values for the data. The predicted equilibrium dissociation constants from this method were in agreement with the calculated $IC_{50}$ values from the binding experiments. Statistical significance of affinity changes was assessed using Student's $t$ test, following $\log_n$ transformation of parameters, and $p$ values of $<0.05$ were regarded as significant. As we have reported previously (27), estimations of binding parameters based on the laws of mass action are only approximations where poor reversibility of sCT binding to its receptors occurs, which is true for all except for the rat C1b CT receptor (28).

**cAMP assay.** Cells were grown to confluence in 12- or 24-well plates (Costar) and preincubated for 20 min at 37° in 5% $CO_2$, in medium containing 0.1% BSA and 1 mM isobutylmethylxanthine. This step was omitted for C1b CT receptor-expressing cells because of the high basal levels of cAMP production. Cells were subsequently incubated for 20 min in the absence (basal) or presence of increasing ligand concentrations. The cells were then washed once with PBS and the cAMP was extracted with 0.5 ml of absolute ethanol. Levels of cAMP were assayed using a specific radioimmunoassay, as described (14). The results shown are representative of at least three separate experiments performed in triplicate.

## Results

**Structure-activity studies.** Receptor binding and activation by the sCT/hCT chimeric peptides, amino-terminally truncated CT peptides (Fig. 1), and sCT analogues with differing α-helical potentials (Table 1) were analyzed using stably transfected cell lines expressing the cloned rat C1a or C1b CT receptors, the cloned hCT receptor (from T47D cells), or the cloned pCT receptor. The rat C1a and C1b CT receptors differ only in the absence (C1a) or presence (C1b) of a 37-amino acid insert in the predicted second extracellular domain of the receptor. This results in altered specificity, between the two receptors, for different CTs in binding competition studies (10, 28).

### sCT/hCT Chimeric Peptides

**Competition for $^{125}$I-sCT binding.** In cells expressing the rat C1a CT receptor, the relative efficacies of the peptides in competition for $^{125}$I-sCT binding were sCT ≥ ACT-15 > ACT-27 > (8–32)sCT (Fig. 2A; Table 2). A similar pattern was observed with the hCT receptor, where sCT ~ ACT-15 > ACT-27 > (8–32)sCT (Fig. 2C; Table 2). In contrast, in cells transfected with the rat C1b receptor sCT was ~20-fold more potent than ACT-15, with ACT-27 and (8–32)sCT being less potent than ACT-15 (Fig. 2B; Table 2). Strikingly, in competition for $^{125}$I-sCT binding, the relative efficacies of ACT-15 and ACT-27 in pCT receptor-transfected cells were reversed, in comparison with the other CT receptors, with sCT > ACT-27 > ACT-15. (8–32)sCT was intermediate in efficacy between ACT-27 and ACT-15 (Fig. 2D; Table 2). The carboxyl-terminal peptides (17–32)sCT and (17–32)hCT had very little effect in $^{125}$I-sCT binding competition studies, although weak competition for binding at the highest concentrations ($10^{-7}$ and $10^{-6}$ M) was observed with (17–32)sCT in cells expressing the rat C1a and hCT receptors.

**Ligand-induced cAMP accumulation.** In an assay of receptor-mediated signal transduction, that of ligand-induced cAMP accumulation, the relative potencies of the peptides were again highly dependent upon the receptor species or subtype. The relative potencies of sCT and the chimeric peptides in cells transfected with the rat C1a receptor were as follows: sCT ~ ACT-15 > ACT-27. A similar specificity was observed for the rat C1b isoform, although the absolute potency of the peptides was greater with the C1a receptor than the C1b receptor (Fig. 3, A and B; Table 3). Although the relative potencies of the peptides in binding competition and cAMP accumulation assays were similar for the C1a receptor, they differed markedly for the C1b receptor. This was most striking with respect to the ACT-15 peptide, which was ~20-fold less potent than sCT in competition for $^{125}$I-sCT binding but equipotent in stimulating adenylate cyclase activity (Fig. 2, A and B, versus Fig. 3, A and B; Tables 2 and 3).

Even more dramatic was the difference in relative specificities of the pCT receptor in binding competition and cAMP studies, with sCT ~ ACT-15 ~ ACT-27 in the second messenger assay (Fig. 3D; Table 3). This represents approximately 25-fold and 10-fold increases in relative potency (com-

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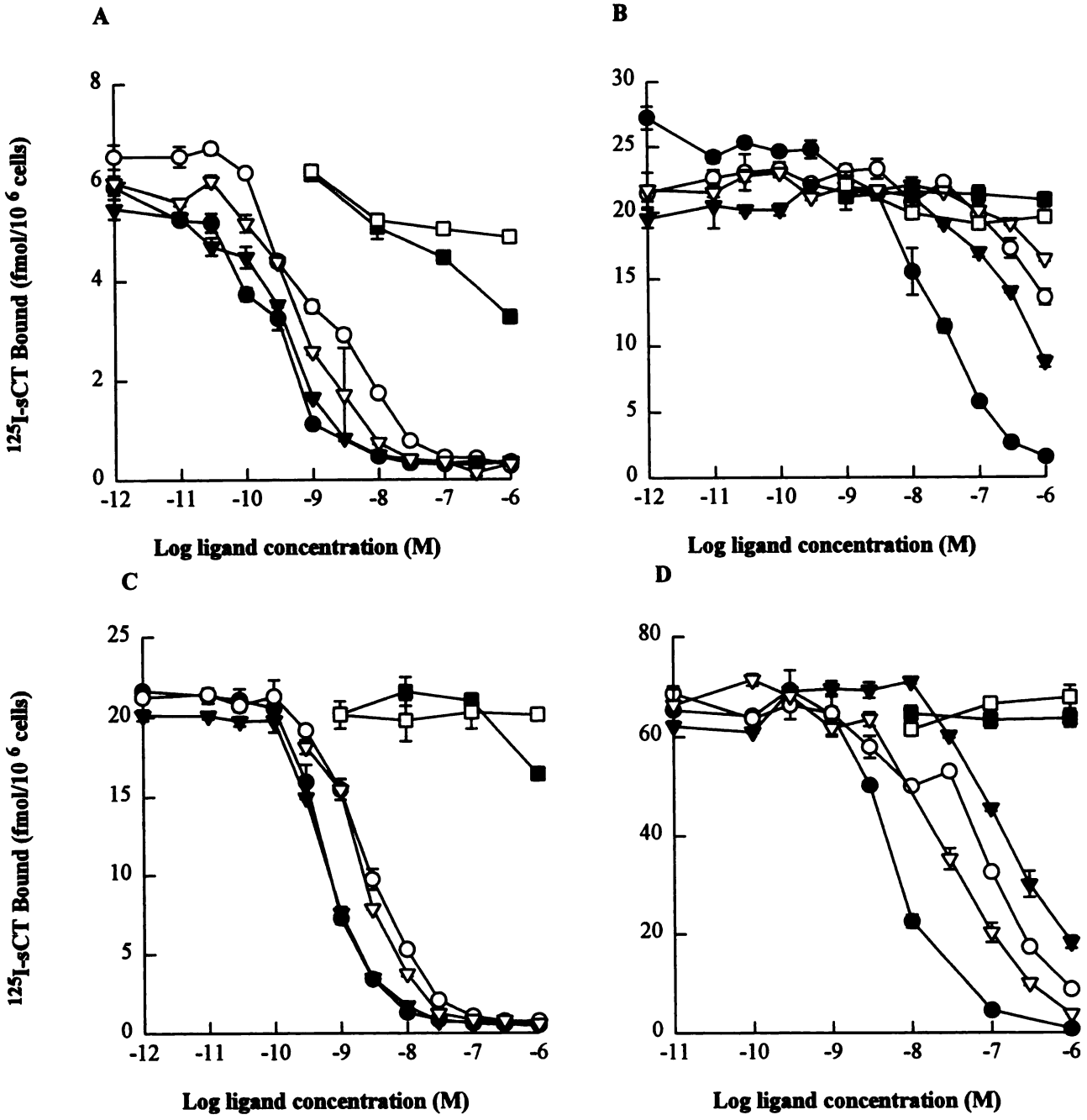


**Fig. 2.** Competition by CT peptides for $^{125}$I-sCT binding to stably expressed, cloned CT receptors. A, Rat C1a receptor; B, rat C1b receptor; C, human receptor; D, porcine receptor. ●, sCT; ▼, ACT-15; ▽, ACT-27; ○, (8–32)sCT; ■, (17–32)sCT; □, (17–32)hCT. Cells were incubated for 60 min at 37° in 5% $CO_2$ in the presence of $^{125}$I-sCT (~80 pM) and increasing concentrations of unlabeled peptide. Cells were then washed with PBS and cell-bound radioactivity was solubilized with 0.5 M NaOH. The figure illustrates results from a single representative experiment, with mean ± standard error of triplicate determinations.

pared with sCT) for ACT-15 and ACT-27, respectively, in induction of cAMP production versus competition for binding (Fig. 2D versus Fig. 3D; Table 2 versus Table 3).

In cells expressing the hCT receptor, the relative potencies of the peptides in inducing cAMP were sCT ~ ACT-15 ≥ ACT-27 (Fig. 3C; Table 3), which was similar to that seen in the binding competition assay, although there was a small apparent shift in the relative potency of ACT-27 between the two assays.

Consistent with its use as a CT receptor antagonist, (8–32)sCT was essentially without effect in inducing the second messenger response, although a very weak rise in cAMP was observed using high concentrations of the peptide in cells bearing the pCT receptor (Fig. 3). The carboxyl-terminal hCT and sCT fragments were similarly ineffective, with the only response being a small rise in cAMP mediated through the hCT or pCT receptors with $10^{-6}$ M (17–32)hCT (Fig. 3, C and D).

Assay of the peptides sCT, ACT-15, and ACT-27 in HEK-293 cells expressing 10–100-fold different levels of C1a and C1b CT receptors demonstrated that the relative specificities of the three peptides were maintained, independent of the

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receptor number, in both binding competition and cAMP accumulation studies (data not shown). With the exception of the C1b receptor-expressing (B8-H10) cells, which were assayed under different conditions, stimulation of the HEK-293 cell lines with prostaglandin $E_2$ generated a 5-fold increase (above basal) in cAMP levels, indicating that the G protein complements were similar in the different HEK-293 clones.

### Helical and Nonhelical Analogues of sCT

**Competition for $^{125}$I-sCT binding.** A summary of the sCT analogues and their secondary structures in the presence of lipid is presented in Table 1. In cells transfected with the rat C1a receptor, the relative efficacies of the analogues in competition for $^{125}$I-sCT binding were as follows: sCT = sCTA3 > sCTA5 > sCTA4 > sCTA2 ≥ sCTA1 > sCTA6 (Fig. 4, A and B; Table 4). Although sCT and sCTA3 were also equipotent in cells transfected with the C1b receptor, the C1a profile contrasted with the profile for the rat C1b receptor, where sCTA4 competed only weakly for binding, sCTA5 was again less potent, and sCTA1, sCTA2, and sCTA6 did not compete for binding at concentrations up to $10^{-6}$ M (Fig. 4, C and D; Table 4). The hCT receptor exhibited the least discrimination among the peptides, with sCT, sCTA3, and sCTA5 being equipotent in competition for $^{125}$I-sCT binding. sCTA4 and sCTA2 were approximately 3-fold less potent and sCTA1 approximately 10-fold less potent. As seen with the other CT receptors, sCTA6 was the least potent analogue, competing only at the highest concentration of $10^{-6}$ M (Fig. 4, E and F; Table 4). In cells transfected with the pCT receptor the relative order of potency of the peptides was similar to that seen in C1a receptor-expressing cells, except that sCTA2 was a relatively more potent competitor for this receptor, whereas the sCTA1 analogue did not compete for binding at concentrations up to $10^{-6}$ M (Fig. 4, G and H; Table 4).

**Ligand-induced cAMP accumulation.** In contrast to the competition binding studies, there was little difference in the potency of sCT, sCTA3, sCTA5, and sCTA2 in inducing accumulation of cAMP in cells transfected with the rat C1a receptor, and sCTA1 was ~10-fold less potent than sCT in this assay system (compared with >100-fold in binding competition assays) (Fig. 5, A and B). However, sCTA4, which is substituted with glycine in the residues encompassed by the amino-terminal, disulfide-bridged loop of sCT, was essentially without activity in stimulating adenylate cyclase (Fig. 5A). sCTA6 was only very weak in stimulating cAMP production (Fig. 5B), which is consistent with its poor efficacy in binding competition studies.

The relative potencies of the analogues in inducing cAMP production in cells transfected with the rat C1b receptor were similar to their efficacies in competing for $^{125}$I-sCT binding, with sCT being equipotent with sCTA3 and sCTA5 being approximately 2 orders of magnitude less potent (Fig. 5C). The analogues sCTA2, SCTA1, and sCTA6, which did not compete for $^{125}$I-sCT binding at the concentrations used, were able to stimulate cAMP production through the C1b receptor only at the highest concentrations studied (Fig. 5D). In contrast to its complete lack of activity in cells expressing the C1a receptor, sCTA4 was able to stimulate cAMP accumulation through the C1b receptor, albeit only at the highest concentration studied (Fig. 5C).

In cells transfected with the hCT receptor there was no observable difference in the potencies of sCT, sCTA1, sCTA2,

TABLE 2

**Apparent association constants, determined by competition for $^{125}$I-sCT binding, by the CT analogues in specificity studies with cloned CT receptors**

Apparent association constants were determined using the program LIGAND (26).

| | Association constant | | | | |
|---|---|---|---|---|---|
| | sCT | ACT-15 | ACT-27 | (8–32)sCT | hCT |
| | $M^{-1}$ | | | | |
| Rat C1a receptor | $2.24 \pm 0.34 \times 10^{9}$ | $9.10 \pm 1.84 \times 10^{8a}$ (41%)[b] | $4.92 \pm 0.97 \times 10^{8}$ (22%) | $3.84 \pm 0.99 \times 10^{8a}$ (17%) | $7.37 \pm 0.87 \times 10^{8a}$ (0.33%) |
| Rat C1b receptor | $8.09 \pm 0.65 \times 10^{7}$ | $5.18 \pm 1.08 \times 10^{8a}$ (6.5%) | $2.13 \pm 0.98 \times 10^{8a}$ (2.6%) | $1.49 \pm 0.29 \times 10^{8a}$ (1.8%) | $<1 \times 10^{6}$ ND[c] |
| pCT receptor | $2.50 \pm 0.26 \times 10^{8}$ | $8.92 \pm 1.69 \times 10^{8a}$ (3.6%) | $3.42 \pm 0.70 \times 10^{7a}$ (13.7%) | $1.56 \pm 0.21 \times 10^{7a}$ (6.2%) | $<1 \times 10^{6}$ ND |
| hCT receptor | $4.07 \pm 1.23 \times 10^{9}$ | $3.24 \pm 0.79 \times 10^{9}$ (80%) | $8.73 \pm 2.42 \times 10^{8a}$ (21%) | $6.86 \pm 0.98 \times 10^{8a}$ (16.9%) | $6.14 \pm 1.31 \times 10^{7a}$ (1.6%) |

[a] $p < 0.05$, versus the value for sCT.
[b] Values in parentheses, efficacy of the analogue expressed as a percentage of the value for sCT.
[c] ND, not determined.

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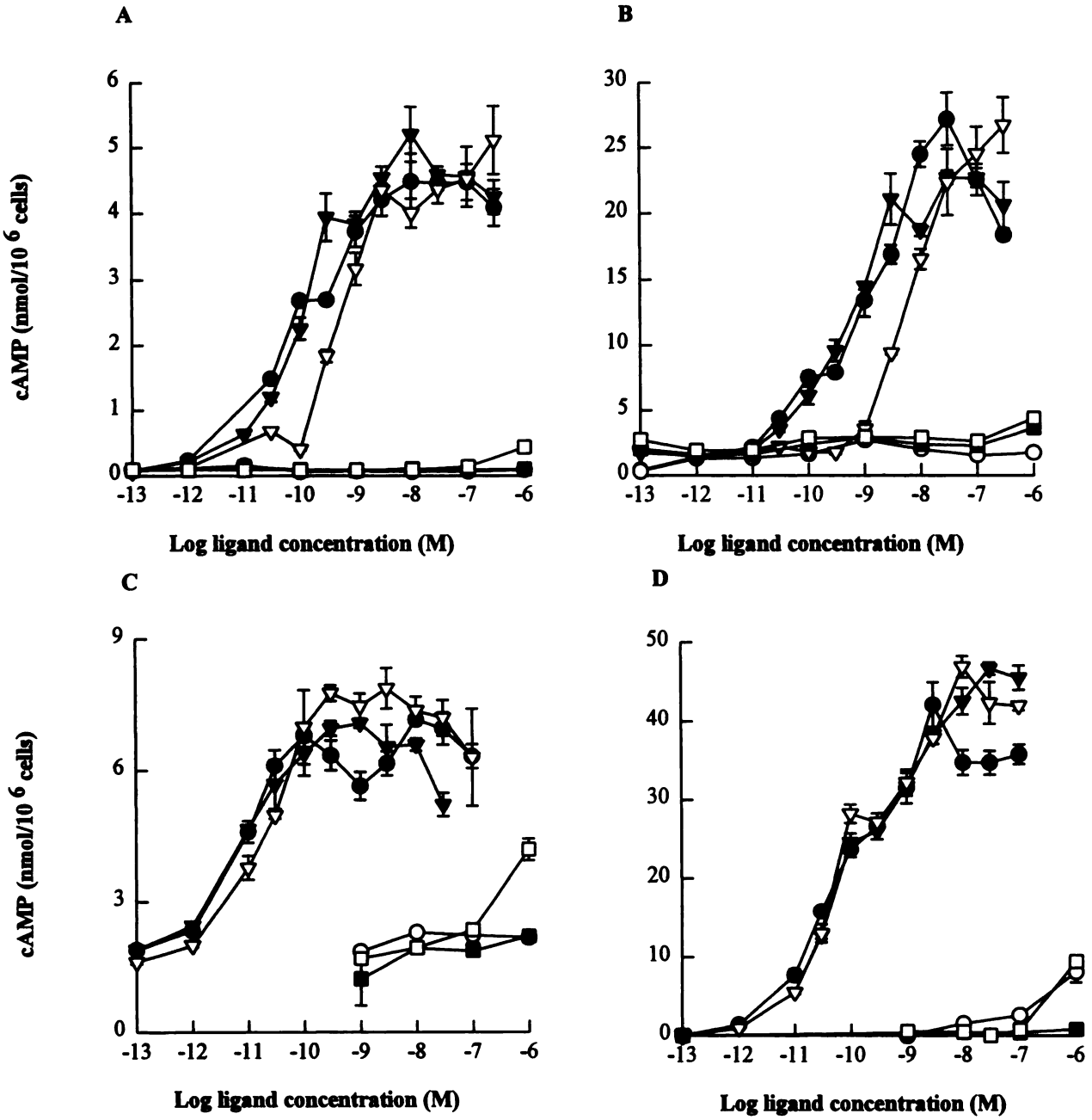


**Fig. 3.** Ligand-induced accumulation of cAMP in cells stably expressing cloned CT receptors. A, Rat C1a receptor; B, rat C1b receptor; C, human receptor; D, porcine receptor. ●, sCT; ▼, ACT-15; ▽, ACT-27; ○, (8–32)sCT; ■, (17–32)sCT; □, (17–32)hCT. With the exception of the C1b receptor-expressing cells (B8-H10), the cells were preincubated for 20 min at 37° in 5% $CO_2$ in assay medium. Cells were then incubated for 20 min at 37° in 5% $CO_2$ in the presence of increasing concentrations of peptide before washing with PBS and extraction of intracellular cAMP with ethanol. The figure illustrates results from a single representative experiment, with mean ± standard error of triplicate determinations.

sCTA3, and sCTA5 in inducing cAMP production (Fig. 5, E and F). As with the other CT receptors, the sCTA6 analogue was very weak in stimulating cAMP accumulation, whereas sCTA4 was 2–3 orders of magnitude less potent than sCT in this assay system (Fig. 5, E and F), despite there being only a 3-fold difference in efficacy in binding competition assays, again supporting a role for the amino-terminal loop region of CTs in activation of CT receptors. In cells expressing the pCT receptor, sCT, sCTA3, sCTA5, and sCTA2 were equipotent in stimulating cAMP production, whereas sCTA1 was approximately 10-fold less potent than these analogues (Fig. 5, G and H). This represents 10- to >100-fold increases in the relative efficacy of the sCTA5, sCTA2, and sCTA1 analogues in the cAMP assay, compared with their efficacy in competition for $^{125}$I-sCT binding (Fig. 5, G and H, versus Fig. 4, G and H). sCTA4 was a weak stimulator of cAMP accumulation through the pCT receptor, whereas sCTA6 was again the least potent of the analogues (Fig. 5, G and H).

## Discussion

**Structure-activity relationships of CT analogues.** The CTs are 32-amino acid peptides that share a number of common structural features, including an amino-terminal,

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TABLE 3

**Estimated $EC_{50}$ values of peptide-induced production of cAMP for the CT analogues in specificity studies with cloned CT receptors**

| | $EC_{50}$ | | |
|---|---|---|---|
| | sCT | ACT-15 | ACT-27 |
| | | M | |
| Rat C1a receptor | $1 \times 10^{-10}$ | $1 \times 10^{-10}$ (100%)[a] | $5 \times 10^{-9}$ (20%) |
| Rat C1b receptor | $7 \times 10^{-10}$ | $7 \times 10^{-10}$ (100%) | $6 \times 10^{-9}$ (12%) |
| pCT receptor | $1 \times 10^{-10}$ | $1 \times 10^{-10}$ (100%) | $1 \times 10^{-10}$ (100%) |
| hCT receptor | $1 \times 10^{-11}$ | $1 \times 10^{-11}$ (100%) | $2 \times 10^{-11}$ (50%) |

[a] Values in parentheses, potency of the analogue expressed as a percentage of the value for sCT.

cysteine-bridged loop from residue 1 to residue 7, which is important for agonist activity (29), a region of predicted amphipathic $\alpha$-helix between residues 8 and 22, and an invariant carboxyl-terminal proline-amide, which is essential for full biological activity (16, 30–32). There are, however, significant differences in amino acid sequence among species (5, 13). This is notable between sCT and hCT (Fig. 1), which share only 50% amino acid identity. These differences in amino acid sequence result in altered biological activity for the two peptides, with sCT being always more potent than hCT. As documented previously, the difference in relative efficacies of these two peptides varies between different CT receptors, with hCT being 10–50-fold less potent than sCT at hCT receptors (7, 14, 24) and >1000-fold less potent at sheep brain membrane receptors (33) and cloned pCT receptors (14). In this study we have used chimeric peptides of sCT and hCT, as well as analogues of sCT with differing $\alpha$-helical secondary structure, to analyze the basis of the increased potency of sCT in binding competition and cAMP accumulation studies.

ACT-15 was more potent (equipotent with sCT) than was the reverse chimera ACT-27 in inhibiting binding to the rat C1a and hCT receptors, indicating that specific sCT residues in the carboxyl terminus are required for the higher affinity binding of sCT at these receptors but that the different residues in the amino terminus (in the presence of carboxyl-terminal sCT residues) do not have a significant impact on binding affinity. The same is true for stimulation of adenylate cyclase through these receptors, although the carboxyl-terminal sCT residues appear less important for this functional response through the hCT receptor. The importance of carboxyl-terminal residues of sCT has also been noted for CT action in mouse osteoclast-like cell preparations (34). For the rat C1b receptor, sCT residues in both halves of the molecule were required for full potency in binding competition studies. However, in this case also, replacement of carboxyl-terminal hCT residues with those of sCT caused greater increases in apparent affinity than did substitution of amino-terminal residues. The specificity of peptides for adenylate cyclase activation, however, was similar to that of the C1a receptor. This discrepancy between binding competition and functional specificity is discussed below.

Unlike the case for other CT receptors, amino-terminal sCT residues were more important in determining the efficacy of binding to the pCT receptor. CD studies have indicated that sCT has a significantly greater likelihood of forming an amphipathic $\alpha$-helix in the residue 8–22 region than does hCT (16), and the ability to form this secondary structure, in the presence of lipids, has been linked to bioactivity of the peptides in some systems (16, 18). Six of the seven variant amino acids in the amino-terminal half of the two CTs occur in the residue 8–16 sequence (Fig. 1), suggesting that the ability to form the $\alpha$-helix may have a greater impact on binding to the pCT receptor than do specific residues of the carboxyl terminus of sCT. Indeed, sCT analogues with reduced $\alpha$-helix-forming potential had reduced potency in competition for $^{125}$I-sCT binding, supporting the supposition that the capacity to form $\alpha$-helical secondary structure contributes significantly to binding affinity. However, substitution of hCT residues with sCT residues in either the amino- or carboxyl-terminal half of the molecule was sufficient to recover full agonist potency at this receptor, as indexed by cAMP production. Similarly, the potency of the sCT analogues did not appear to be dependent upon the helical capacity of the analogues, suggesting that agonist potency at the pig receptor is independent of this structural feature.

It is notable that the sCT/hCT chimeric peptides were more similar to sCT at all receptors, in terms of binding inhibitory potency, than was hCT (Table 2), despite having either the amino- or carboxyl-terminal half of the amino acid sequence identical to the sequence of hCT. Thus, residues of sCT in both halves of the molecule are able to significantly improve binding efficacy. Because the reduction or loss of inducible secondary structure appeared to have greater impact on the competition for $^{125}$I-sCT binding and less impact on the potency of the analogues in stimulating cAMP production, it is possible that improvements in helical secondary structure arising from the sCT half of the chimeric peptides may contribute to their improved efficacies in binding competition studies.

The greatest divergence in receptor responses to the sCT analogues occurred for the rat C1a and C1b receptor isoforms and was most apparent in comparison of the ligand-induced stimulation of cAMP production. In C1a receptor-expressing cells, the helical analogues sCT and sCTA3 were essentially equipotent (in stimulating cAMP production) with sCTA5 and sCTA2, analogues that have reduced or absent helical potential, respectively (Table 1). sCTA1, which also lacks helical secondary structure in the presence of lipids, was only ~10-fold less potent than sCT. In contrast, in C1b receptor-expressing cells the nonhelical analogues were approximately 1000-fold less potent than sCT and sCTA3, whereas sCTA5, which retains a reduced ability to form $\alpha$-helical secondary structure (Table 5), was 100-fold less potent. The analogues used in the present study previously enabled the delineation of two potential subtypes of CT receptor in rat brain, termed $CT_L$ and $CT_H$ (18). The current data are strongly supportive of the hypothesis that the cloned C1a and C1b rat receptor isoforms (9, 10) correspond to the $CT_L$ and $CT_H$ receptor subtypes, respectively. Although there is a discrepancy between the relative potencies of sCTA4 and sCT in competition for binding to C1a receptors in the current study and in competition for $^{125}$I-sCTA1 ($CT_L$-specific) binding to brain membranes (18), this is likely due to the weak interaction of sCTA4 with the C1b receptor in the mixed receptor membrane assay. In support of this, the greatest divergence between $IC_{50}$ values of analogues in competition for $^{125}$I-sCT, compared with $^{125}$I-sCTA1, binding in brain

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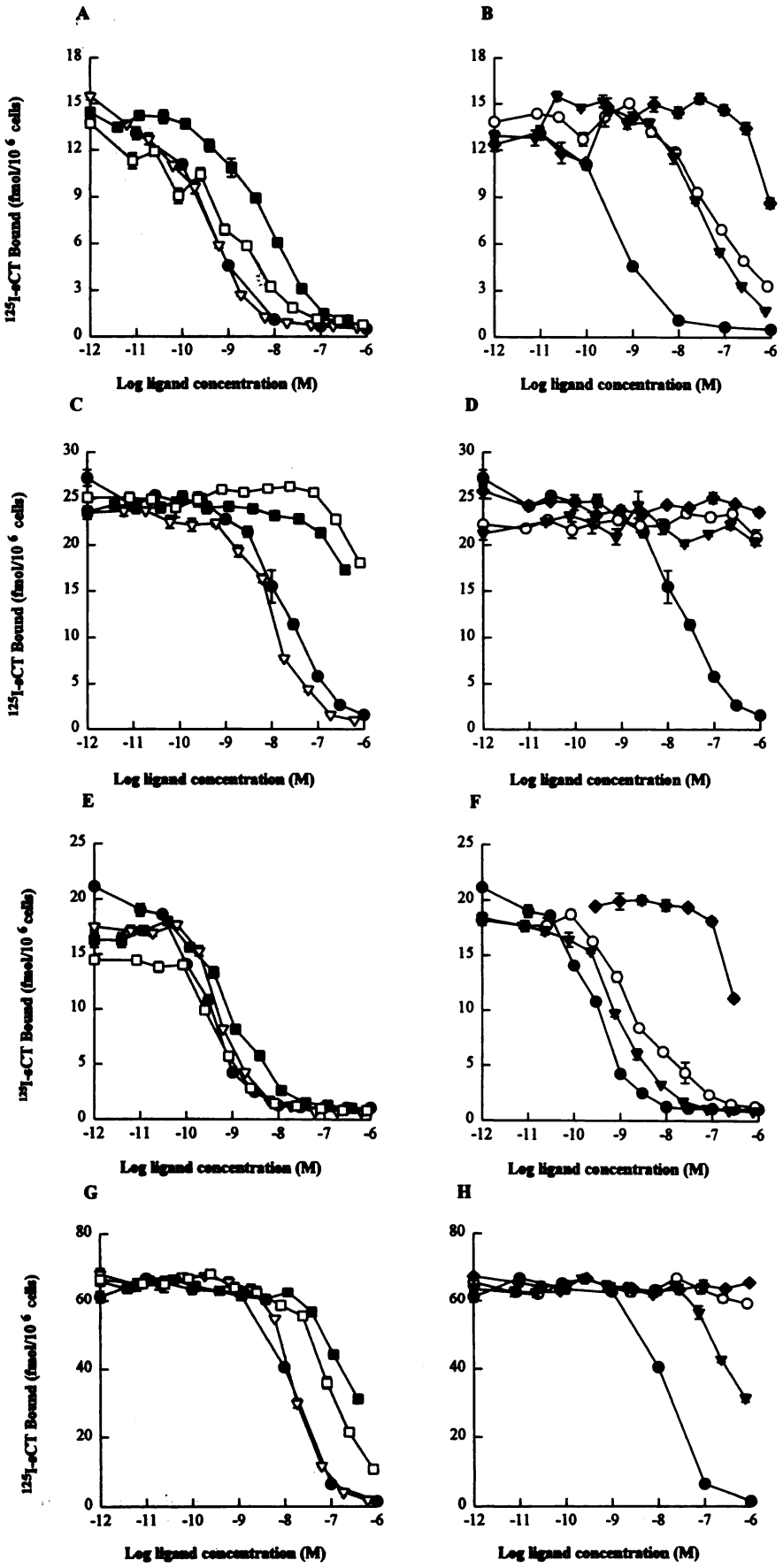


**Fig. 4.** Competition by sCT analogues for $^{125}$I-sCT binding to cell lines stably expressing the cloned CT receptors. A and B, Rat C1a receptor; C and D, rat C1b receptor; E and F, human receptor; G and H, porcine receptor. A, C, E, and G, ●, sCT; ▽, sCTA3; ■, sCTA4; □, sCTA5; B, D, F, and H, ●, sCT; ○, sCTA1; ▼, sCTA2; ♦, sCTA6. Cells were incubated for 60 min at 37° in 5% $CO_2$ in the presence of $^{125}$I-sCT (~80 pM) and increasing concentrations of unlabeled peptide. Cells were then washed with PBS and cell-bound radioactivity was solubilized with 0.5 M NaOH. The figure illustrates results from a single representative experiment, with mean ± standard error of triplicate determinations.

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TABLE 4

**Apparent association constants ($K_a$) for the CT analogues derived from competition for $^{125}$I-sCT binding to cell lines stably expressing cloned CT receptors**

Values were derived using the program LIGAND (26) and are means ± standard errors.

| | $K_a$ | | | |
|---|---|---|---|---|
| | Rat C1a receptor | Rat C1b receptor | pCT receptor | hCT receptor |
| | $M^{-1}$ | | | |
| sCT | 2.24 ± 0.34 ×10$^9$ | 8.09 ± 0.65 ×10$^7$ | 2.50 ± 0.26 ×10$^8$ | 4.07 ± 1.23 ×10$^9$ |
| sCTA1 | 1.93 ± 0.35 ×10$^{7a}$ | ND[b] | ND | 2.69 ± 0.38 ×10$^{8a}$ |
| sCTA2 | 4.17 ± 0.78 ×10$^{7a}$ | ND | 8.10 ± 1.70 ×10$^{6a}$ | 8.18 ± 1.80 ×10$^{8a}$ |
| sCTA3 | 4.35 ± 1.56 ×10$^9$ | 1.60 ± 0.17 ×10$^{8a}$ | 1.17 ± 0.16 ×10$^8$ | 4.79 ± 1.04 ×10$^9$ |
| sCTA4 | 1.65 ± 0.10 ×10$^{8a}$ | ND | 1.04 ± 0.20 ×10$^{7a}$ | 7.79 ± 0.82 ×10$^{8a}$ |
| sCTA5 | 5.95 ± 0.83 ×10$^{8a}$ | ND | 2.99 ± 0.28 ×10$^{7a}$ | 3.41 ± 0.83 ×10$^9$ |

[a] $p < 0.05$, versus the value for sCT.
[b] ND, not determined.

TABLE 5

**Comparison of the secondary structure potential of the sCT analogues [expressed as a ratio of helical secondary structure in the presence and absence of dimyristoylphosphatidylglycerol (DMPG)] and their efficacy in binding competition and cAMP assays (expressed as percentage change in apparent $K_d$ or $EC_{50}$, relative to that of sCT) with the hypocalcemic activity of the peptides**

| Peptide | $-[\theta]_{222\ nm}$ ratio, DMPG/no addition | Binding affinity | | cAMP response | | Hypocalcemic activity | Ref. |
|---|---|---|---|---|---|---|---|
| | | C1a | C1b | C1a | C1b | | |
| | | % of sCT | | % of sCT | | IU/mg | |
| sCT | 2.97 | | | | | 4,500 | 15 |
| sCTA3 | 2.32 | 196 | 199 | 100 | 100 | 4,300 | 18 |
| sCTA4 | 1.88 | 7.4 | ND[a] | ND | 0.2 | 23 | 18 |
| sCTA5 | 1.18 | 26.8 | ND | 100 | 2 | 11,000 | 15 |
| sCTA2 | 0.88 | 1.9 | ND | 50 | 0.33 | 5,400 | 15 |
| sCTA1 | 0.66 | 0.9 | ND | 10 | 0.06 | 2,500 | 15 |

[a] ND, not determined.

membranes occurred for sCT and sCTA4, analogues that compete for $^{125}$I-sCT binding to cloned C1b receptors (18). The C1b receptor data also support the original supposition of Nakamuta *et al.* (18) that binding to $CT_H$ (C1b) receptors is dependent upon helix-forming potential. Indeed, with the exception of sCTA4, which is discussed below, the activity of the analogues in cAMP stimulation at the C1b receptor is paralleled by their helix-forming capacity in lipids (Table 5). Because the hypocalcemic activity of the analogues correlates with the bioactivity of the peptides at the C1a receptor (Table 5), this indicates that the hypocalcemic action of CT in rats *in vivo* is predominately mediated by the C1a CT receptor isoform. This is in accord with the finding that the predominant receptor isoform in rat osteoclasts is C1a (35).

The hCT receptor was the least affected in its interaction with and activation by the modified CTs. The human receptors, whether expressed endogenously or expressed through transfection of cloned receptors, are also the least discriminatory among different classes of CTs (7, 8, 14, 24, 36). The human receptor has 78% identity with the rat C1a receptor and 73% identity with the pCT receptor, and it is likely that these differences in primary receptor structure alter the ability of the different receptors to recognize CT ligands. However, an alternative possibility for differences between responses observed with the hCT receptor and the other CT receptors is the different cell background for expression of these receptors, i.e., BHK for the hCT receptor and HEK-293 for the other receptors. However, similar specificity profiles for different CTs occur with the pCT receptor transfected into three different cell lines (HEK-293, COS, and UMR 106–01),[1] indicating that cell background has little impact upon CT receptor specificity.

sCTA6, the D-Arg$^{24}$ isomer of sCTA5, was the least potent analogue at all CT receptors studied and was also of low potency in rat hypocalcemic assays (23). The L/D-Arg$^{24}$ shift by itself does not appear to be sufficient to interfere with the bioactivity of the peptide, as assessed in rat hypocalcemic bioassays, but this bioactivity is 50% reduced in combination with the Gly$^8$ modification (21). This indicates that the D-isomer conformation, in combination with the other modifications, removes critical contacts between the ligand and the receptors, either directly, through a shift in position of key residues, or indirectly, through loss of intramolecular interactions that constrain the molecule in an active conformation, as has been proposed previously (21). Because sCTA6 did not interact significantly with either C1a or C1b rat CT receptors, it is possible that the previously reported actions of this peptide on rat neuronal membrane sensitivity (23) are due to a nonspecific interaction of the peptide with the membrane, rather than receptor-specific actions.

**Divergence in CT specificity for different transition states of its receptors.** According to the extended ternary complex model for G protein-coupled receptors, the receptors may exist in at least two potential transition states, an active isoform (R*, able to couple to G protein) and an inactive isoform (R, unable to couple to G protein) (37–39). Ligands may bind to either form of the receptor, with compounds with

[1] S. Houssami, P. M. Sexton, and D. M. Findlay, unpublished observations.

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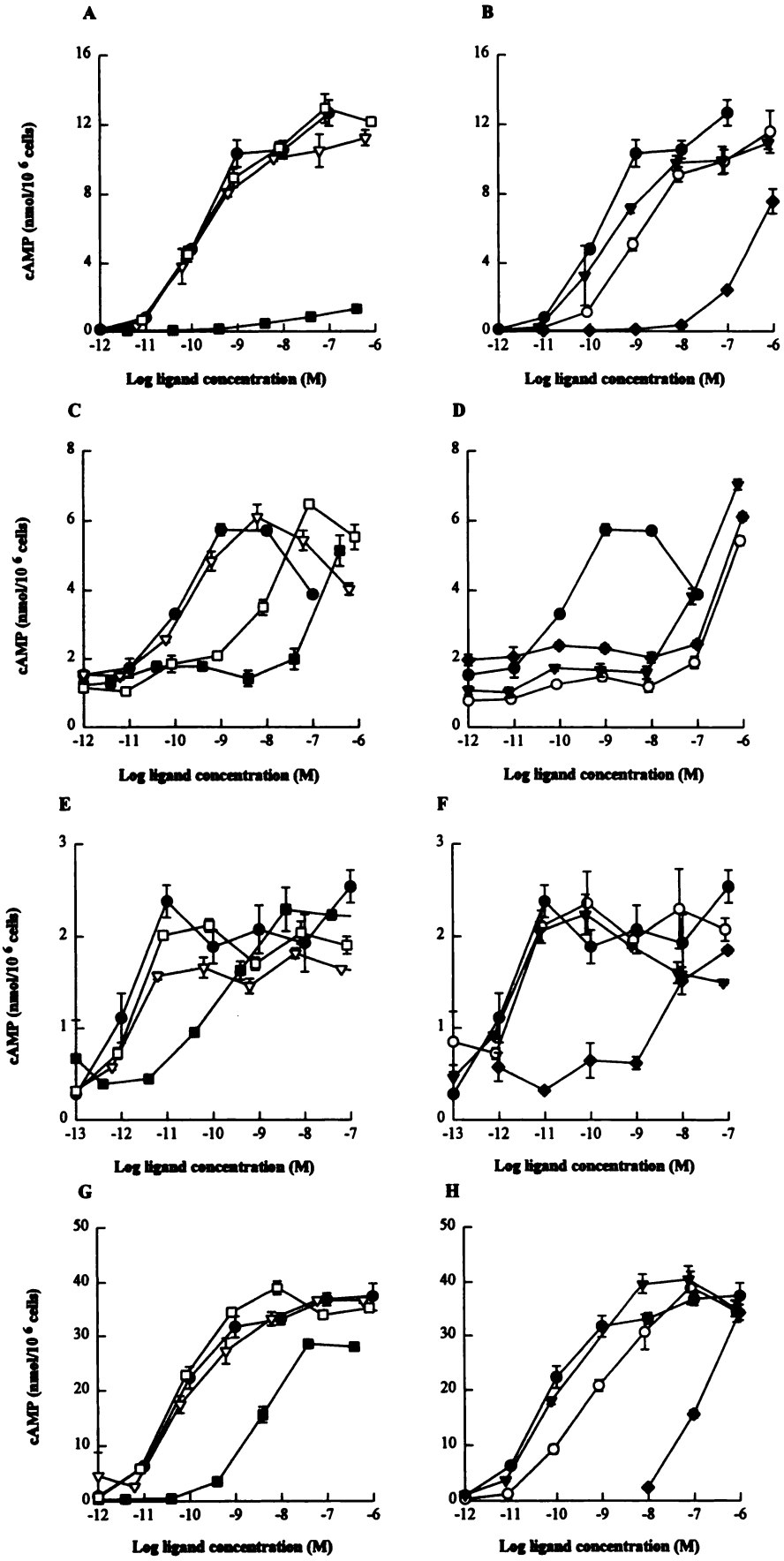


**Fig. 5.** Induction of cAMP production by sCT analogues in cell lines stably expressing cloned CT receptors. A and B, Rat C1a receptor; C and D, rat C1b receptor; E and F, human receptor; G and H, porcine receptor. A, C, E, and G, ●, sCT; ▽, sCTA3; ■, sCTA4; □, sCTA5; B, D, F, and H, ●, sCT; ○, sCTA1; ▼, sCTA2; ◆, sCTA6. With the exception of the C1b receptor-expressing cells, the cells were preincubated for 20 min at 37° in 5% $CO_2$ in assay medium. Cells were then incubated for 20 min at 37° in 5% $CO_2$ in the presence of increasing concentrations of peptide before washing with PBS and extraction of intracellular cAMP with ethanol. The figure illustrates results from a single representative experiment, with mean ± standard error of triplicate determinations.

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higher binding affinity for R* behaving as agonists and compounds with higher affinity for R behaving as antagonists or inverse agonists.

The divergence between the relative specificities of the CT peptides in competition for radioligand binding and their potencies in cAMP production suggests that significant differences in the relative affinities of the peptides for the different transition states of the receptor can occur. Because the R* state is required for G protein coupling, the potency of the peptides in cAMP accumulation studies likely reflects the relative affinity of the peptides for this state of the receptor. Although agonists may stabilize the R* state long enough to interact with the G protein, in the absence of continued occupation of this complex by the G protein, such as may occur if GTP is limiting, this complex is inherently unstable. Consequently, under conditions where GTP is in excess (as is likely in the whole-cell assays used in the current study), radioligand binding predominately reflects affinity of the competing peptides for the inactive (R) state of the receptor (40). A recent study with receptor chimeras between the hCT receptor and the glucagon receptor, in which the amino-terminal extracellular domain (proximal to transmembrane domain 1) was switched between the receptors, suggests that the predominant epitopes involved in binding to R and R* may be located in different parts of the receptor (41). In that study, the receptor expressing the amino-terminal extracellular domain of the CT receptor was able to bind $^{125}$I-sCT, albeit with 40-fold decreased affinity, but sCT did not stimulate adenylate cyclase. However, sCT was able to stimulate adenylate cyclase through the mutant receptor containing the amino-terminal region of the glucagon receptor (carboxyl-terminal CT receptor), with an $EC_{50}$ only 10-fold less than that for the wild-type hCT receptor (41). Despite the relatively high potency of sCT in stimulating adenylate cylase, binding of $^{125}$I-sCT to this receptor could not be detected, supporting the supposition that the R* state of the CT receptor, in whole-cell binding assays, is insufficiently stable to contribute to the observed radioligand binding. The current observation of equivalent relative potencies of peptides, in binding and adenylate cyclase assays, in cells expressing 100-fold differences in the levels of C1b receptors is consistent with the level of GTP in the cells being in excess, because the increase in R* in the cell line expressing high levels of receptors (as indexed by the increased basal cAMP level) did not influence binding affinity.

The amino-terminal, residue 1–7, disulfide-bridged loop of CT is implicated in interactions of the peptide with the active state of the CT receptor, with removal of this region generating high affinity antagonist peptides (29). The residue 1–7 region is highly conserved between species and, indeed, is also highly conserved with respect to the related peptides CT gene-related peptide and amylin (42). The residue 1–7 region is similarly implicated in signal transduction at the CT gene-related peptide receptor (43). Substitution of the naturally occuring residues 2–6 with glycine in sCTA4 decreased the efficacy of the analogue in competition for $^{125}$I-sCT binding at all receptors. Comparison of the $\alpha$-helix-forming potential of the analogues indicates that the loss of binding affinity of sCTA4 is not due solely to the loss of secondary structure potential. Thus, it is possible that there are contacts formed by amino-terminal residues that contribute to efficacy in binding competition. This is supported by the decrease in binding affinity of (8–32)sCT for the CT receptors. However, constraint of a preferred secondary structure through intramolecular interactions with the amino-terminal domain cannot be excluded. Unlike the other analogues, which had greater potency (relative to sCT) in induction of cAMP production than in binding competition, sCTA4 had reduced or negligible efficacy in stimulating cAMP accumulation. This confirms that specific residues in the amino-terminal loop domain are required for full agonist activity of sCT at CT receptors. The pCT and hCT receptors exhibited the least disparity between binding affinity and agonist potency of sCTA4, suggesting that interaction with the active state of these receptors also involves significant interaction of other parts of the CT molecule with the receptor. In contrast, sCTA4 did not stimulate cAMP production through the rat C1a receptor even at the highest concentrations used, indicating that specific residues of the amino terminus are critical for interaction with the active state of this receptor. However, the disulfide bridge by itself does not appear to be essential for activation of the receptor, as indicated by the full bioactivity of the sCTA2 analogue at the rat C1a, pCT, and hCT receptors. This is consistent with the observations of other investigators (17, 18, 44).

In conclusion, these results confirm that considerable differences in binding and activation properties occur among different CT receptors, and they demonstrate that the specific requirements for competition for radioligand binding and activation of second messengers may also differ markedly within a single cloned receptor. There is pharmacodynamic heterogeneity of responses for CT receptors both within species and among different species. Taken together with the fact that there are multiple isoforms of the CT receptors within species, it is clear that predictions of physiological responses need to be based upon carefully selected assays.

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