0026-895X/97/040658-08$3.00/0

MOLECULAR PHARMACOLOGY, **51**:658–665 (1997).

Structure/Function Relationships of Calcitonin Analogues as Agonists, Antagonists, or Inverse Agonists in a Constitutively Activated Receptor Cell System

GORDANA POZVEK, JOANNE M. HILTON, MARIBEL QUIZA, SOUHEIR HOUSSAMI, and PATRICK M. SEXTON

Neurobiology Unit, St. Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia

Received September 3, 1996; Accepted January 10, 1997

SUMMARY

The structure/function relationship of salmon calcitonin (sCT) analogues was investigated in heterologous calcitonin receptor (CTR) expression systems. sCT analogues with progressive amino-terminal truncations intermediate of sCT-(1–32) to sCT-(8–32) were examined for their ability to act as agonists, antagonists, or inverse agonists. Two CTR cell clones, B8-H10 and G12-E12, which express ~5 million and 25,000 C1b receptors/cell, respectively, were used for this study. The B8-H10 clone has an ~80-fold increase in basal levels of intracellular cAMP due to constitutive activation of the overexpressed receptor. In whole-cell competition binding studies, sCT-(1–32) was more potent than any of its amino-terminally truncated analogues in competition for ^{125}I-sCT binding. In cAMP accumulation studies, sCT-(1–32) and modified analogues sCT-(2–32) and sCT-(3–32) had agonist activities. SDZ-216–710, with an amino-terminal truncation of four amino acids, behaved as a partial agonist/antagonist, whereas amino-terminal truncations of six or seven amino acid residues produced a 16-fold reduction in basal cAMP levels and attenuated the response to the agonist sCT-(1–32) in the constitutively active CTR system. This inverse agonist effect was insensitive to pertussis toxin inhibition. In contrast, the inverse agonist activity of these peptides was not observed in the nonconstitutively active CTR system, in which sCT analogues with amino-terminal truncations of four or more amino acids behaved as neutral competitive antagonists. These results suggest that the inverse agonist activity is mediated by stabilization of the inactive state of the receptor, which does not couple to G protein, and attenuates basal signaling initiated by ligand-independent activation of the effector adenylyl cyclase.

CT is a 32-amino-acid hormone that is involved in calcium homeostasis. In mammals, CT is secreted by the thyroid gland in response to an increase in blood calcium levels. The physiological response to circulating levels of CT is an increase in calcium excretion from the kidney and a decrease in osteoclast-mediated bone resorption (1). In addition to its hypocalcemic activity, CT has distinct effects in the central nervous system, in which the exogenous administration of CT can induce diverse physiological effects such as analgesia, inhibition of appetite, and inhibition of gastric acid secretion (2). Phylogenetically, there are three main classes of CT: teleost/avian, artiodactyl, and rat/human (1). The most potent class of CT is the teleost/avian, of which sCT is the most widely used, including therapeutically in humans for the treatment of metabolic bone disorders (3, 4).

This work was supported by grants from the National Health and Medical Research Council of Australia. P.M.S. is a Research Fellow of the Australian Research Council.

The CTR is a member of the subfamily of seven-transmembrane domain GPCRs that include the receptors for secretin, parathyroid hormone, glucagon, vasoactive intestinal peptide, and growth hormone-releasing hormone (5). In the rat, two isoforms of the CTR, designated C1a and C1b, have been identified and characterized (6). These receptor isoforms arise from alternative RNA splicing of the CTR gene and differ in their tissue distribution. The C1b isoform contains a 37-amino-acid insert in the putative first extracellular loop that confers altered ligand binding characteristics (6). CTR isoforms have also been identified in the human and the mouse (7, 8). Activation of the CTR can result in coupling to several G proteins, such as $G_{s\alpha}$ and $G_{q/i\alpha}$, that activate downstream effector systems, including adenylyl cyclase and phospholipase C, respectively (9, 10). The rat C1a and C1b CTR isoforms activate both second messenger pathways (11).

A two-state model of receptor activation has been proposed

ABBREVIATIONS: CT, calcitonin; CTR, calcitonin receptor; sCT, salmon calcitonin; PBS, phosphate-buffered saline; HEK, human embryonic kidney; BSA, bovine serum albumin; PTX, pertussis toxin; R*, active conformation of receptor; R, inactive conformation of receptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; βARK, β-adrenergic receptor kinase; SDZ-212–769, [Ala^{7}]salmon calcitonin-(7–32); SDZ-216–710, isocaproyl-[Ala^{7},Aib^{10,17},Lys(For)^{11,18},Lys(fructosyl)^{24}]salmon calcitonin-(5–32) (ASC710); SDZ-218–686, [Ala^{7}]salmon calcitonin-(3–32); SDZ-219–379, Ac-[Aib^{10,17},Lys(For)^{11,18},Lys(Ip)^{24}]-salmon calcitonin-(8–32); SDZ-220–235, Ac-[Ala^{7},Aib^{17}]-salmon calcitonin-(2–32); AC512, Bolton-Hunter-[Arg^{18},Asn^{30},Tyr^{32}]salmon calcitonin-(8–32).

for GPCRs. This model predicts that receptors exist in an equilibrium between at least two allosterically different conformations, R and R*. In the R* state, the receptor is in an active conformation that can couple spontaneously to G proteins independent of ligand/receptor interaction (12). In this model, agonists have a relatively higher affinity for R* and stabilize the active state of the receptor. Inverse agonists have a relatively higher affinity for R and stabilize the inactive state of the receptor, which does not couple to G proteins. Neutral antagonists have equal affinity for both states of the receptor and do not alter the equilibrium between R and R* (13). Overexpressed receptors can move the equilibrium between these two states toward R* through effects of mass action and can result in a constitutively active receptor that is characterized by high basal levels of intracellular second messenger generation. This phenomenon has been demonstrated both *in vitro* (14) and *in vivo* (15). Constitutive activation of GPCRs may also occur as a result of mutation in the receptor protein and can result in pathophysiological disorders (14, 16, 17).

In the current study, the C1b isoform of the rat CTR was stably transfected into HEK 293 cells to examine the structure/function relationship of sCT analogues with increasing amino-terminal truncation. Two cell clones, differing in receptor density and basal cAMP levels, were used to study the relative binding affinities and efficacies of the sCT analogues with respect to their action as agonists, antagonists, or inverse agonists. Amino-terminal deletions of six or seven amino acid residues resulted in inverse agonist activity at the constitutively active CTR.

Materials and Methods

Hormones and chemicals. Synthetic sCT and sCT-(8–32) were obtained from Bachem California (Torrance, CA). The analogues SDZ-212–769, SDZ-216–710, SDZ-218–686, SDZ-219–379, and SDZ-220–235 were a gift from Dr. R. Gamse (Sandoz Pharma Ltd., Basel, Switzerland). AC512 was obtained from Amylin Pharmaceuticals (San Diego, CA). The structures of these peptides are listed in Table 1. G418 (geneticin) was obtained from GIBCO (Grand Island, NY). BSA and fetal bovine serum were obtained from Commonwealth Serum Laboratories (Parkville, Australia). PTX, bacitracin, and isobutylmethylxanthine were purchased from Sigma Chemical (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 International (Buckinghamshire, UK). Synthetic sCT was iodinated using a modification of the chloramine T method (18). The specific activities of ^{125}I-sCT and ^{125}I-cAMP were ~700 and ~2000 Ci/mmol, respectively.

Heterologous expression of the rat C1b CTR. The cloned rat C1b CTR isoform was stably transfected into HEK 293 cells as previously described (6). At 48 hr after transfection, the antibiotic G418 (200 μg/ml) was included in the medium, and selection for antibiotic-resistant cells was maintained for 2 weeks. Fresh medium containing G418 was added every 2–3 days. The G418-resistant cells, which had incorporated the recombinant plasmids containing the receptor cDNA into the genome, were subcloned by two rounds of limiting dilution. Cloned cells were screened for CTR expression by radioligand binding assays with ^{125}I-sCT. Receptor-expressing cells were subsequently maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 200 μg/ml G418. Two clonal cell lines expressing the C1b receptor at ~5×10^6 (B8-H10) and ~25×10^3 (G12-E12) receptors/cell were studied with respect to their binding properties and functional response to various sCT analogues. The B8-H10 CTR clone grew ~6-fold more slowly in culture than did the G12-E12 CTR clone.

Receptor binding assay. For radioligand binding studies, B8-H10 cells were subcultured onto 24-well plates (Costar, Cambridge, MA) and G12-E12 cells were subcultured onto 12-well plates (Costar). The media was aspirated before the addition of binding buffer [Dulbecco's modified Eagle's medium (containing 0.1% BSA and 0.1% bacitracin)]. The radioligand ^{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, the cells were washed with PBS (1× = 140 mM NaCl, 2 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4) 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; Meriden, CT) to determine bound radioactivity. The results shown are representative of at least two separate experiments performed in triplicate. Binding isotherms were analyzed with the iterative curve-fitting program LIGAND (19).

cAMP bioassay. Cells were grown to confluence in 24-well plates. The media was aspirated from the wells and replaced with Dulbecco's modified Eagle's medium containing 0.1% BSA. The cells were then incubated for 20 min in the absence (basal) or presence of increasing ligand concentrations. Isobutylmethylxanthine, a phosphodiesterase inhibitor, was added to the wells at the same time as the unlabeled peptides at a final concentration of 0.1 mM. After incubation with the peptides, the cells were washed once with PBS, and the cAMP was extracted with 0.5 ml of absolute ethanol. The samples were evaporated to dryness by heating at 70° for 30 min and resuspended in buffer (50 mM sodium acetate, 1 mM theophylline). Samples were subsequently coincubated with the anti-cAMP antibody (1:24,000 final dilution) and ^{125}I-cAMP (10,000 cpm/tube) overnight at 4°. The samples were incubated with 1 ml of separation buffer (100 mM dipotassium hydrogen phosphate, 100 mM potassium dihydrogen phosphate, pH 7.4, containing 0.25% BSA and 0.2% charcoal) for 15 min at 4° to extract the unbound radioactivity. The samples were then centrifugated for 15 min at 4000 × g, and the supernatants were aspirated. The pellets were counted with the Packard γ counter. The results shown are representative of at least three separate experiments performed in triplicate. To determine the sensitivity of the cAMP response to PTX, the cells were

TABLE 1

Structure and functional activity relationships of sCT analogues in the constitutively active C1b CTR system

sCT analogue	Structure	Activity
sCT	sCT-(1-32)	Agonist
SDZ-220-235	Ac-[Ala^{7},Aib^{17}]sCT-(2-32)	Agonist
SDZ-218-686	Ala^{7}-sCT-(3-32)	Agonist
SDZ-216-710	Isocaproyl-[Ala^{7},Aib^{10,17},Lys(For)^{11,18},Lys(fructosyl)^{24}]sCT-(5-32)	Partial agonist/antagonist
SDZ-212-769	[Ala^{7}]sCT-(7-32)	Inverse agonist
sCT-(8-32)	sCT-(8-32)	Inverse agonist
SDZ-219-379	Ac-[Aib^{10,17},Lys(For)^{11,18},Lys(Ip)^{24}]sCT-(8-32)	Inverse agonist
AC512	Bolton-Hunter-[Arg^{18},Asn^{30},Tyr^{32}]sCT-(8-32)	Inverse agonist

TABLE 2

Comparison of the apparent association constants (K_a) and EC_{50} values for the sCT analogues derived from binding competition for ^{125}I-sCT and cAMP accumulation assays, respectively

SCT analogue	K_a		EC_{50}	
	B8-H10 CTR clone	G12-E12 CTR clone	B8-H10-CTR clone	G12-E12 CTR clone
	M^{-1}		M	
sCT	$1.63 \pm 0.34 \times 10^7$	$3.33 \pm 2.14 \times 10^8$	$6.67 \pm 2.73 \times 10^{-9}$	$1.49 \pm 0.52 \times 10^{-9}$
SDZ-220-235	$<1 \times 10^6$	$<1 \times 10^6$	$5.88 \pm 2.20 \times 10^{-8}$	$9.26 \pm 0.54 \times 10^{-10}$
SDZ-218-686	$<1 \times 10^6$	$<1 \times 10^6$	$7.67 \pm 2.90 \times 10^{-8}$	$7.33 \pm 0.23 \times 10^{-10}$
SDZ-216-710	$1.61 \pm 0.23 \times 10^7$	$5.55 \pm 3.85 \times 10^7$	N.A.	N.A.
SDZ-212-769	$2.95 \pm 1.80 \times 10^6$	$4.76 \pm 2.76 \times 10^6$	$\sim 1 \times 10^{-7} M^b$	N.A.
sCT-(8-32)	$<1 \times 10^6$	$<1 \times 10^6$	$\sim 1 \times 10^{-7} M^b$	N.A.
SDZ-219-379	$4.83 \pm 1.68 \times 10^6$	$7.09 \pm 3.82 \times 10^6$	$\sim 1 \times 10^{-7} M^b$	N.A.
AC512	$6.62 \pm 4.95 \times 10^6$	$4.74 \pm 2.81 \times 10^6$	$\sim 1 \times 10^{-7} M^b$	N.A.

[a] Apparent association constants were derived using the program LIGAND (19) and are mean ± standard error ($n \geq 3$).
[b] Potencies of inverse agonists to reduce constitutive activity of the CTR.
N.A. no detectable activity.

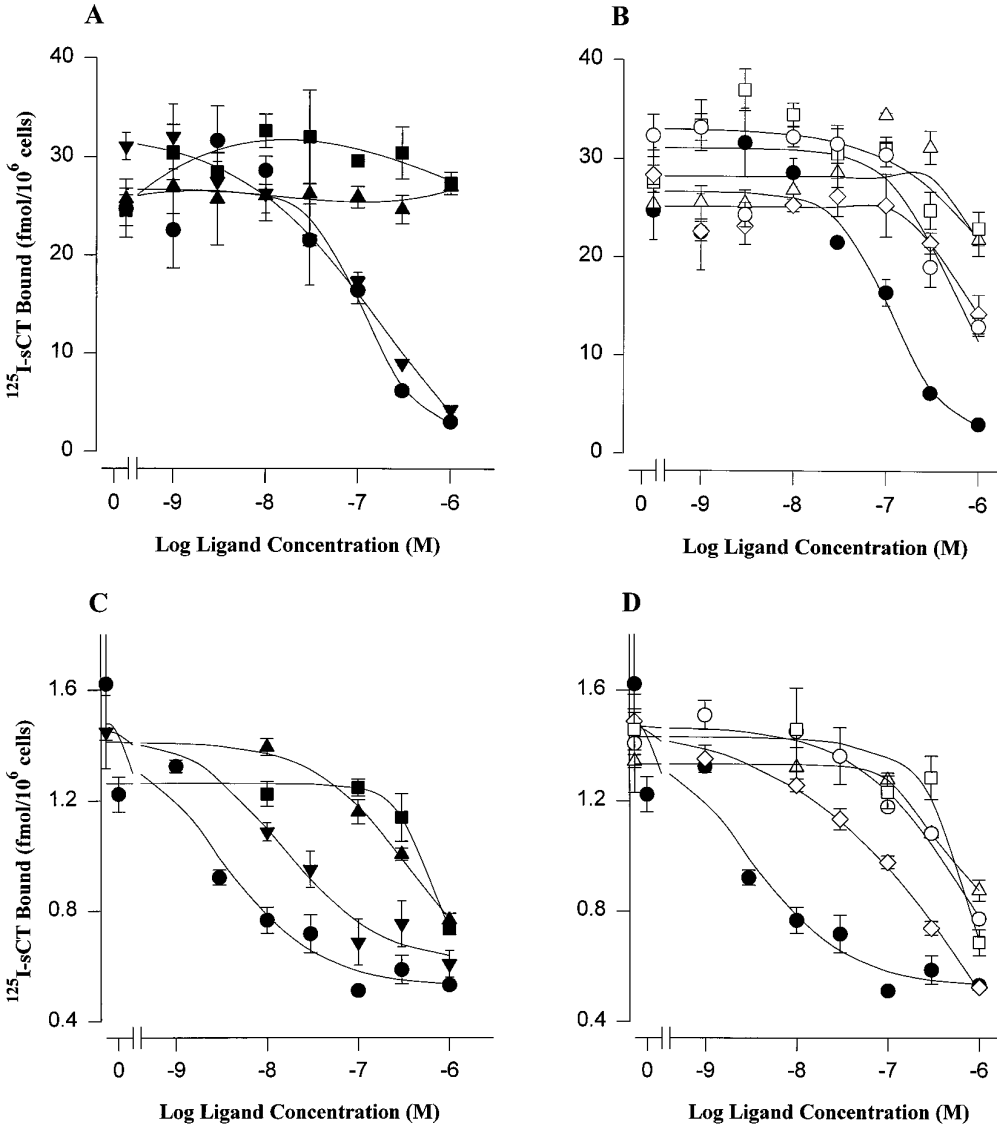

Fig. 1. Affinity of sCT and amino-terminally truncated analogues in competition for ^{125}I-sCT binding to rat C1b CTRs. A and B, B8-H10 constitutively active CTR clone expressing ~5 × 10^6 CTRs/cell. C and D, G12-E12 CTR clone expressing ~2.5 × 10^3 CTRs/cell. ●, sCT; ■, SDZ-220–235; ▲, SDZ-218–686; ▼, SDZ-216–710; □, SDZ-212–769; △, sCT-(8–32); ◇, SDZ-219–379; ○, AC512. The B8-H10 experiments were performed at passage 80. 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. The cells were then washed with PBS, and the cell-bound radioactivity was solubilized with 0.5 M NaOH. Results are from a representative experiment ($n \geq 3$) and represent mean ± standard error values of triplicate determinations.

preincubated for 16 hr at 37° with either 12.5 ng/ml PTX or fresh culture media (control).

Results

A summary of the structure/function relationships of the sCT analogues is shown in Table 1. With increasing amino-terminal truncation of these peptides, a transition occurs from agonist to antagonist or inverse agonist, with the latter being detected only in the clone expressing the constitutively active CTR (B8-H10).

The association constants (K_a) derived from ^{125}I-sCT competition binding studies and the corresponding EC_{50} values

of the peptides in stimulating cAMP production are summarized in Table 2 for both the constitutively active (B8-H10) and nonconstitutively active (G12-E12) CTR clones. In whole-cell competition binding studies, sCT was more potent than any of its amino-terminally truncated analogues in competing for ^{125}I-sCT binding to the CTR (Fig. 1, Table 2). A moderate increase in affinity for sCT and its analogues was seen in the G12-E12 clone when compared with the cell line overexpressing the receptor (Table 2), which is probably caused by the large number of receptors in the latter cell line.

The B8-H10 CTR clone, which expresses a 200-fold higher receptor number, exhibited an ~80-fold increase in basal cAMP levels compared with the G12-E12 clone (Fig. 2) or untransfected HEK-293 cells (not shown). In cAMP accumulation studies, the full-length sCT and modified analogues with amino-terminal truncations of one or two amino acids (i.e., SDZ-220–235 and SDZ-218–686) behaved as agonists (Fig. 2, A and C, Table 2). The sCT analogues with amino-terminal truncations of six or seven amino acid residues [i.e., SDZ-212–769, sCT-(8–32), SDZ-219–379, and AC512] reduced the high basal levels of cAMP by ~16-fold at peptide concentrations of 10^{-6} M in the overexpressing CTR clone (Fig. 2B). In contrast, no reduction in basal cAMP levels was observed with these peptides in the nonconstitutively active CTR clone (Fig. 2D). The activity of the modified sCT-(5–32) analogue, SDZ-216–710, was observed to change from that of a partial agonist to an antagonist when the experiments were performed using different passage numbers of the B8-H10 CTR clone. This apparent difference in ligand activity results from a rightward shift in the dose-response curve to SDZ-216–710 at high clonal passage. At passage 39, SDZ-216–710 behaved as a partial agonist (Fig. 3), whereas at passage 91, no detectable activity was observed (Fig. 2A). This effect was also observed with the agonists sCT, SDZ-220–235, and SDZ-218–686; the EC_{50} values of these peptides were ~10-fold higher at passage 91 (Fig. 2A) than at passage 39 (Fig. 3), which suggests a loss of sensitivity to agonists with time in culture. However, no change with respect to the relative efficacies of these sCT analogues was found among experiments regardless of passage number. The basal levels of intracellular cAMP also changed with respect to passage number in this clone. Compared with the basal cAMP level of the G12-E12 clone, increases in the basal level of cAMP of 20- and 80-fold were observed for passages 39 and 91 of the B8-H10 clone, respectfully. The increase in cAMP levels in

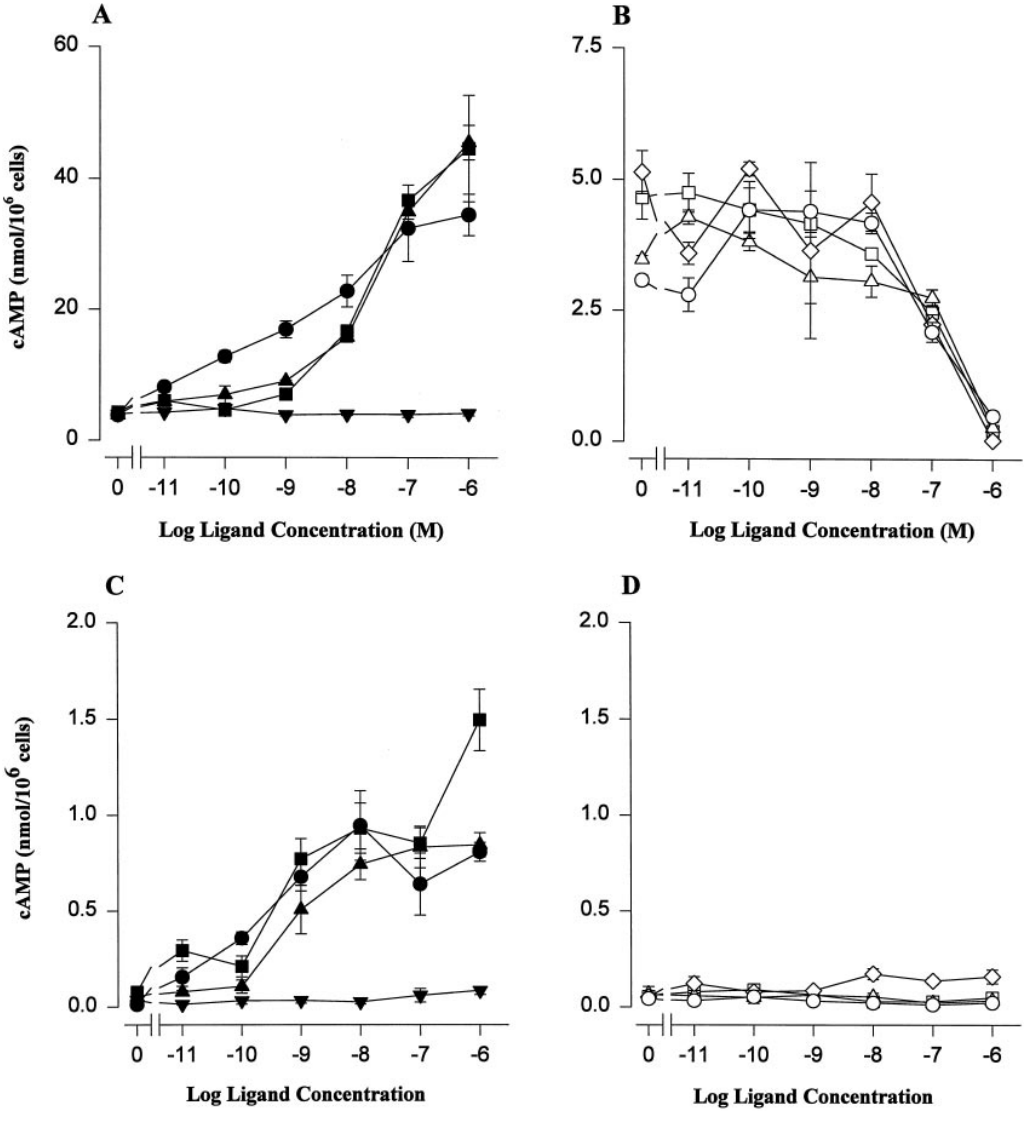

Fig. 2. Effect of sCT amino-terminal truncation on ligand-induced cAMP responses in C1b CTR expressing cells. A and B, B8-H10 CTR clone; C and D, G12-E12 CTR clone. ● sCT; ■ SDZ-220–235; ▲ SDZ-218–686; ▼ SDZ-216–710; □ SDZ-212–769; △ sCT-(8–32); ◇ SDZ-219–379; ○ AC512. The B8-H10 experiments were performed at passage 91. Cells were incubated for 20 min at 37° in 5% CO_2 in the presence of increasing concentrations of sCT analogues. The cells were washed with PBS, and intracellular cAMP was extracted with absolute ethanol. Quantification of cAMP levels was performed using a cAMP radioimmunoassay. Results from a representative experiment ($n \geq 3$) and represent mean ± standard error values of triplicate determinations.

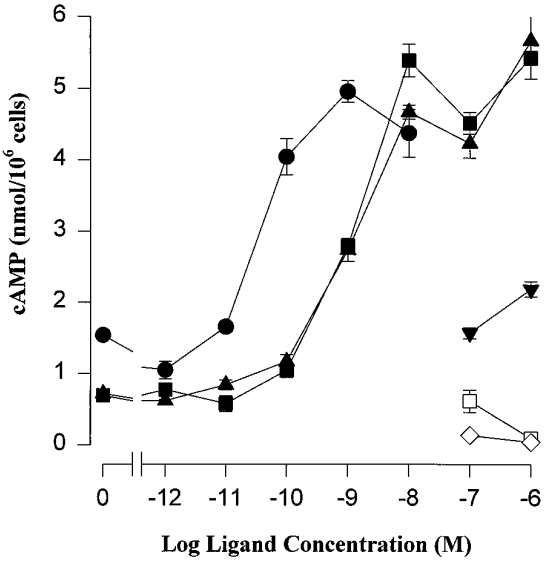

Fig. 3. cAMP accumulation in response to ligand in the B8-H10 CTR clone at passage 39. ● sCT; ■ SDZ-220–235; ▲ SDZ-218–686; ▼ SDZ-216–710; □ SDZ-212–769; ◇ SDZ-219–379. Cells were incubated for 20 min at 37° in 5% CO_2 in the presence of increasing concentrations of sCT analogues. The cells were washed with PBS and intracellular cAMP was extracted with absolute ethanol. Quantitation of cAMP levels was performed using a cAMP radioimmunoassay. Results are from a representative experiment (n = 3) and represent mean ± standard error values of triplicate determinations.

response to agonist was also greater at the higher passage number (10- versus 5-fold). The inverse agonist activities of SDZ-212–769, sCT-(8–32), SDZ-219–379, and AC512 were amplified when assayed at a higher passage (Fig. 2B versus Fig. 3), suggesting that this system was more sensitive to the detection of inverse agonism than assay at lower passage.

The inverse agonists SDZ-212–769, sCT-(8–32), and SDZ-219–379 were only weakly effective in antagonizing sCT-mediated rises in cAMP in the constitutively active CTR system (Fig. 4, B–E). In contrast, the partial agonist/antagonist SDZ-216–710 induced a much more marked attenuation in the sCT-mediated cAMP response (Fig. 4A). The inverse agonist activities of SDZ-219–379, AC512, and sCT-(8–32) were not altered by preincubation of cells with PTX (Fig. 5), which suggests that alternative coupling of the receptor to $G_{i\alpha}$ protein does not mediate the reduction in basal cAMP levels observed in the constitutively active CTR clone. Nevertheless, pretreatment with PTX potentiated the response of high concentrations of sCT, indicating that activation of $G_{i\alpha}$ can occur with high concentrations of agonist.

Discussion

Constitutively active GPCRs can arise from receptor overexpression (15, 20, 21) or through mutations in the receptor protein that allow it to spontaneously relax into the R* state (22–26). This phenomenon has been observed both *in vitro* (12, 22–24) and *in vivo* (14–17, 26). Several studies have reported mutations in human GPCRs that result in a constitutively active receptor phenotype associated with pathophysiological disorders (14, 16, 17). A constitutively active receptor phenotype is characterized by elevated basal levels of second messengers. Where high affinity binding is G protein dependent, this can lead to an increased affinity for agonist (12, 13). Higher levels of receptor expression increase basal effector activity as more copies of R* are stochastically present at any given time. In the R* state, the receptor can couple spontaneously to G protein independently of agonist binding and therefore propagate downstream signaling cascades (16, 17, 27).

In this study, overexpression of the rat C1b receptor in HEK 293 cells (~5 × 10^6 receptors/cell) resulted in 10–80-fold increases in the basal production of cAMP compared with receptor naive cells or cells expressing relatively low receptor levels (~25,000 receptors/cell). At other characterized CTRs, deletion of the amino-terminal loop structure of sCT in sCT-(8–32) leads to an antagonist peptide (28). In the C1b receptor-overexpressing cell line, sCT-(8–32) reduced the basal production of cAMP, indicating that the raised cAMP response was derived from constitutive activation of the rC1b CTR. This is consistent with the extended ternary complex model for GPCR activation (12).

Late passage number cells exhibited higher basal cAMP levels than early passage numbers from the same cell line (80- versus 10–20-fold). The increased basal production of cAMP in the late passage cells provided an improved window for the study of inverse agonist efficacy; consequently, late passage cells were used for the majority of experiments of the current study. However, concomitant with the raised basal cAMP was a 10–100-fold increase in EC_{50} values for agonist peptides (Fig. 3) (29). One consequence of this was a shift in phenotype of SDZ-216–710, which exhibited partial agonism in early passage cells but behaved as a neutral antagonist in late passage cells.

The loss of sensitivity to agonist peptides is consistent with a decrease in G protein-coupling efficiency. Mechanistically, this may arise from desensitization of the receptor, presumably through the action of GRKs on the R* substrate (23, 30). In many receptors, the carboxyl-terminal cytoplasmic domain of GPCRs serves as a target for desensitization functions that negatively regulate receptor activity (25). Studies with human CTR indicate that the receptor carboxyl terminus is the primary target for phosphorylation and that this may be mediated by both second messenger-dependent and -independent kinases (31). Thus, phosphorylation of serine and threonine residues in this region may be involved in initiating and/or maintaining receptor desensitization. Constitutively active GPCRs have also been observed to be constitutively phosphorylated and desensitized, as the R* state (with or without bound ligand) is a substrate for GRKs (23, 24). Constitutively active α_2-adrenergic receptors are substrates for βARK-mediated phosphorylation, even in the absence of agonist (24). Constitutively active β_2-adrenergic receptors are also constitutively desensitized by βARK-mediated phosphorylation (23). Inverse agonists reduce βARK-mediated phosphorylation of the β_2-adrenergic receptor, presumably by reduction in the levels of the R* substrate (30), and a similar mechanism may underlie the improved efficacy of inverse agonist peptides in late passage cells in the current study. However, the reduced efficacies of agonists in this system could also be due to a change in cellular phenotype with higher passage numbers. The increase in basal cAMP production and magnitude of agonist-induced responses is consistent with up-regulation of signaling components downstream of the receptor, such as G_s or adenylate cyclase. Several studies have implicated constitutively active

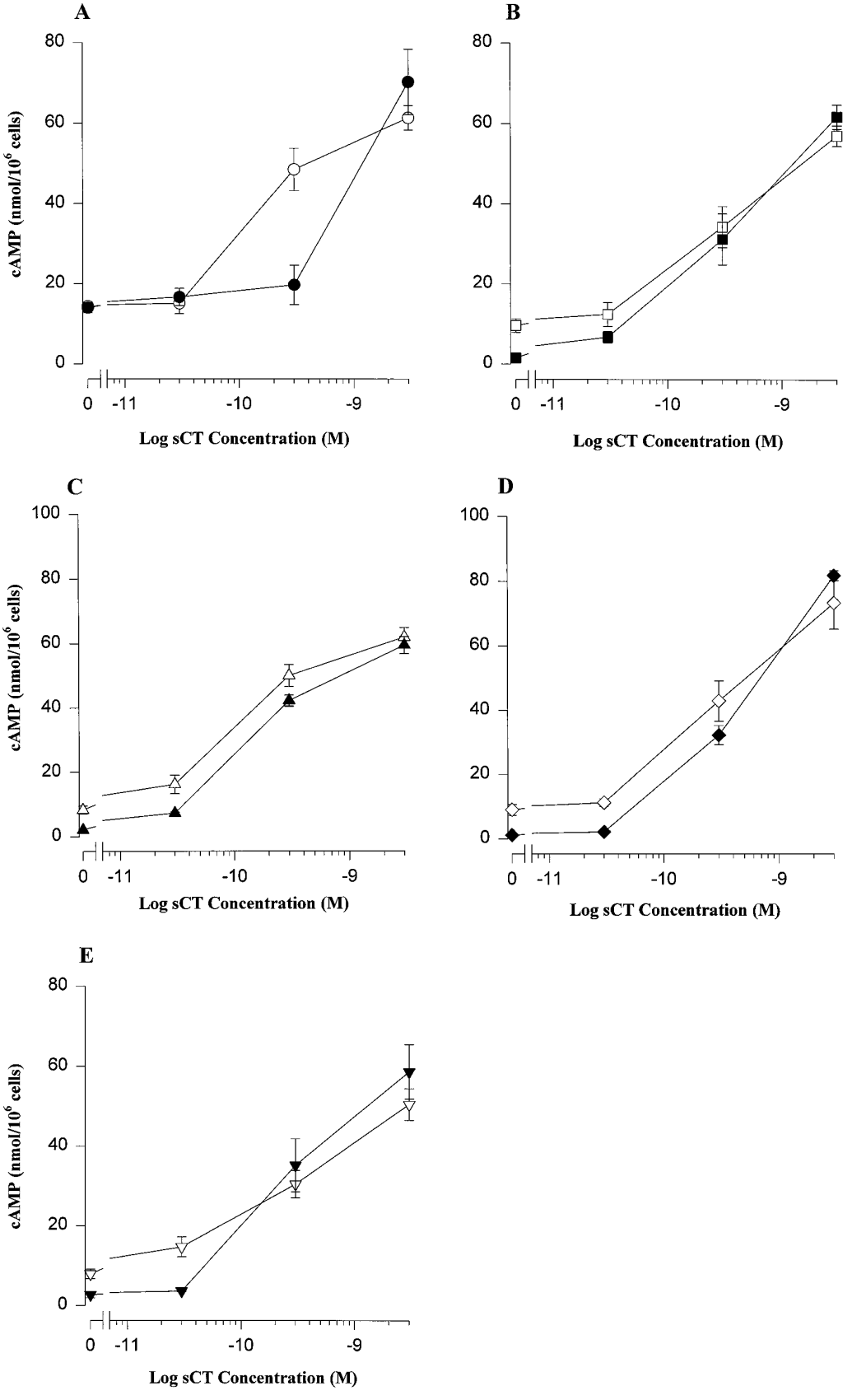

Fig. 4. Effect on the sCT dose-response curve of coincubation with amino-terminally truncated sCT analogues in the B8-H10 CTR clone: A: ○, sCT; ●, sCT and SDZ-216–710 (10^{-6} M). B: □, sCT; ■, sCT and SDZ-212–769 (10^{-6} M). C: △, sCT; ▲, sCT and sCT-(8–32) (10^{-6} M). D: ◇, sCT; ◆, sCT and SDZ-219–379 (10^{-6} M). E: ▽, sCT; ▼, sCT and AC512 (10^{-6} M). The B8-H10 experiments were performed at passage 68. Cells were incubated for 20 min at 37° in 5% CO_2 in the presence of increasing concentrations of sCT analogues. The cells were washed with PBS, and intracellular cAMP was extracted with absolute ethanol. Quantitation of cAMP levels was performed using a cAMP radioimmunoassay. Results are from a single representative experiment (three separate experiments) and represent mean ± standard error values of triplicate determinations.

GPCR as having latent oncogenic potential (17, 32). The transforming effects of high cAMP levels have been observed both *in vivo* and *in vitro*, with a constitutively active thyroid-stimulating hormone receptor as the causative agent in producing thyroid adenomas (17). However, whether inverse agonists developed for constitutively active hormone recep-

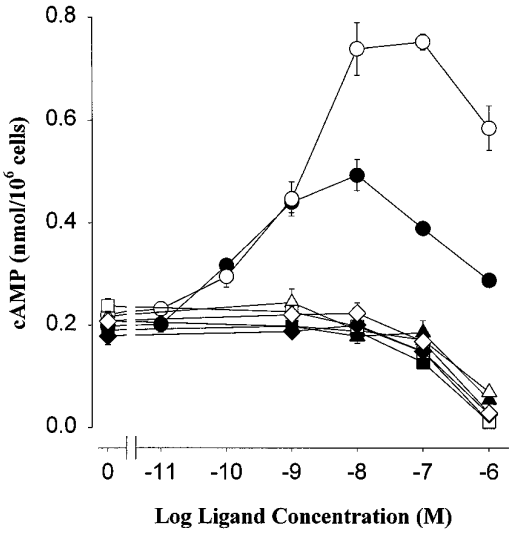

Fig. 5. Effect of PTX preincubation on the activities of the sCT analogues in the constitutively active B8-H10 CTR clone. Cells were preincubated for 16 hr with either fresh culture media (closed symbols) or 12.5 ng/ml PTX (open symbols). ○ and ●, sCT; △ and ▲, sCT-(8–32); □ and ■, SDZ-219–379; ◇ and ◆, AC512. The B8-H10 experiments were performed at passage 34. After pretreatment with PTX, the cells were incubated for 20 min at 37° in 5% CO_2 in the presence of increasing concentrations of sCT analogues. The cells were washed with PBS, and intracellular cAMP was extracted with absolute ethanol. Quantification of cAMP levels was performed using a cAMP radioimmunoassay. Results are from a single representative experiment ($n = 4$) and represent mean ± standard error values of triplicate determinations.

tors are of possible therapeutic benefit as anticancer drugs requires further investigation.

The constitutively active CTR clone grew ~6-fold more slowly in culture than the nonconstitutively active CTR clone. A decrease in cellular proliferation associated with high cAMP levels has been observed (33, 34) and is due to the convergence of the cAMP pathway into the mitogenic signaling pathway. The cAMP-mediated activation of protein kinase A inhibits downstream signaling by Ras, thus preventing Ras-dependent activation of the protein kinase Raf-1 (33) and subsequent activation of mitogen-activated protein kinase. Further studies of inverse agonists may reveal whether these drugs can also influence cell signaling pathways relating to proliferation and receptor cross-talk between the GPCR and receptor tyrosine kinase pathways.

With increasing truncation from the amino terminus, there was a transition of peptides from full agonist to partial agonist/antagonist to inverse agonist. Peptides with amino-terminal truncations of one or two residues retained full agonist activity, whereas a four-residue deletion (i.e., SDZ-216–710) produced a peptide with partial agonist/antagonist activity, and peptides with six (i.e., SDZ-212–769) or seven deleted residues [i.e., sCT-(8–32), SDZ-219–379, AC512] behaved as inverse agonists in the constitutively active CTR system. A previous report of sCT analogues with sequential amino-terminal truncations suggested that progressive deletions of one to seven residues resulted in peptides with decreasing *in vivo* and *in vitro* bioactivities, with antagonism observed for deletions of seven or more residues (28).

The related peptides CGRP and amylin share with the CTs a disulfide bonded amino-terminal loop structure with six or seven amino acids. In these peptides also, deletion of the loop structure (residues 1–7) generates antagonist peptides (35–37), implying that this region of the peptides is crucial for agonist binding and receptor activation.

In the current study, the removal of even one residue in SDZ-220–235 decreased efficacy of the peptide at the constitutively active C1b receptor and essentially abolished binding in competition binding assays. In whole-cell binding assays, in which GTP is generally in excess, it is likely that potency in competition for binding predominantly reflects affinities for the inactive (G protein-uncoupled) state of the receptor (29, 38). The dramatic decrease in binding affinity for peptides in the current study is in contrast with the C1a isoform of the receptor, in which little change is seen in affinity in relation to sCT.[1]

Ligand/receptor interaction at the C1b receptor isoform has a greater requirement for peptides to form α-helical secondary structure than that at the C1a receptor (29). Thus, the loss of efficacy for SDZ-220–235 and SDZ-218–686 may arise from alteration in secondary structural potential of the peptide. At the C1a receptor, the dissociation kinetics of peptides is dependent on the capacity to form α-helical secondary structure. At this receptor, the truncated analogues, with the exception of SDZ-216–710, exhibited increased dissociation rates, which would be consistent with a progressive decrease in α-helix formation.[1] It has been postulated that residues in the amino-terminal loop structure act to stabilize the amphipathic α-helix predicted to occur between residues 8–22 of the molecule (39), and therefore removal of loop residues in the current study may lead to less stable secondary structure.

In contrast to the other truncated peptides, the modified sCT-(5–32) analogue, SDZ-216–710, exhibited a relatively high affinity for the C1b receptor in competition binding studies. It is possible that the amino-terminal isocaproyl group of this analogue serves to stabilize α-helix formation in this peptide. Consistent with this hypothesis, SDZ-216–710 also displays a relatively slow rate of dissociation from the rat C1a receptor.[1] However, because the modified sCT-(8–32) analogue SDZ-219–739, which has similar modifications to SDZ-216–710 in the mid-carboxyl-terminal portions of the peptide, had improved binding affinity relative to unmodified sCT-(8–32), it is possible that the additional carboxyl-terminal peptide modifications also contribute to the increased potency of SDZ-216–710.

Consistent with the relatively high affinity of SDZ-216–710, this peptide was the most potent in antagonizing sCT-induced rises in cAMP. That the peptide did not act as an inverse agonist but was a neutral antagonist or partial agonist of this system may imply that it also has high affinity for active-state receptor. In contrast, the inverse agonist peptides were relatively weak competitive antagonists. This is in accord with the relative weak competitive binding affinity of the peptides but suggests that they have much greater affinity for the inactive- than the active-state receptor (40).

Intriguingly, in the pig renal carcinoma cell line LLC-PK1, the sCT-A[7](7–32) analogue, which is an inverse agonist of the constitutively active C1b receptor, exhibits weak partial agonist activity (28). This suggests that the requirements for

[1] J. M. Hilton, M. Dowton, S. Houssami, and P. M. Sexton. Ability to form an amphipathic α-helix is a critical factor in the irreversibility of salmon calcitonin binding to calcitonin receptors. Submitted for publication.

agonist versus antagonist binding may differ across species for CTRs.

The study of novel pharmacological drugs with inverse agonist activity is of potential therapeutic importance in the treatment of pathophysiological disorders caused by constitutively active GPCRs. The physiological effects of inverse agonists have been demonstrated in transgenic mice with myocardium-specific overexpression of the β_2-adrenoceptor (15). Further studies are required to investigate the effects of chronic inverse agonist administration on receptor number, desensitization by GRK and receptor metabolism, and the pharmacokinetic properties of inverse agonists. These issues are important in determining the efficacies of inverse agonists *in vivo* because these drugs may be more therapeutically useful than classic antagonists in certain disease conditions associated with constitutively active GPCRs.

References

1. Zaidi, M., B. S. Moonga, P. J. R. Bevis, A. S. M. Towhidul Alam, S. Legon, S. Wimalawansa, I. MacIntyre, and L. H. Breimer. Expression and function of calcitonin gene products. *Vitamins Hormones* **46:**87–164 (1991).
2. Sexton, P. M. Central nervous system binding sites for calcitonin and calcitonin gene-related peptide. *Mol. Neurobiol.* **5:**251–273 (1991).
3. Wisneski, L. A. Review of calcitonin: future perspectives and new opportunities in therapy. *Bone Mineral.* **16:**213–216 (1992).
4. Chesnut, C. H., III. Review of calcitonin-present: current status of calcitonin as a therapeutic agent. *Bone Mineral.* **16:**211–212 (1992).
5. Peroutka, S. J. *Handbook of Receptors and Channels*. CRC Press, Boca Raton, FL (1994).
6. Sexton, P. M., S. Houssami, J. M. Hilton, L. M. O'Keeffe, R. J. Center, M. T. Gillespie, P. Darcy, and D. M. Findlay. Identification of brain isoforms of the rat calcitonin receptor. *Mol. Endocrinol.* **7:**815–821 (1993).
7. Moore, E. E., R. E. Keustner, S. D. Stroop, F. J. Grant, S. L. Matthewes, C. L. Brady, P. M. Sexton, and D. M. Findlay. Functionally different isoforms of the human calcitonin receptor result from alternative splicing of the gene transcript. *Mol. Endocrinol.* **9:**959–968 (1995).
8. Yamin, M., A. H. Gorn, M. R. Flannery, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, D. R. Tapp, S. M. Krane, and S. R. Goldring. Cloning and characterisation of a mouse brain calcitonin receptor complementary deoxyribonucleotide acid and mapping of the calcitonin receptor gene. *Endocrinology* **135:**2635–2643 (1994).
9. Horne, W. C., J.-F. Shyu, M. Chakraborty, and R. Baron. Signal transduction by calcitonin: multiple ligands, receptors, and signalling pathways. *Trends Endocrinol. Metab.* **5:**395–401 (1994).
10. Chakraborty, M., D. Chatterjee, S. Kellokumpu, H. Rasmussen, and R. Baron. Cell cycle-dependent coupling of the calcitonin receptor to different G proteins. *Science (Washington D. C.)* **251:**1078–1082 (1991).
11. Houssami, S., D. M. Findlay, C. L. Brady, D. E. Myers, T. J. Martin, and P. M. Sexton. Isoforms of the rat calcitonin receptor: consequences for ligand binding and signal transduction. *Endocrinology* **135:**183–190 (1994).
12. Samama, P., S. Cotecchia, T. Costa, and R. J. Lefkowitz. A mutation-induced activated state of the β_2-adrenergic receptor: extending the ternary complex model. *J. Biol. Chem.* **268:**4625–4636 (1993).
13. Milligan, G., R. A. Bond, and M. Lee. Inverse agonism: pharmacological curiosity or potential therapeutic strategy. *Trends. Pharmacol. Sci.* **16:** 10–13 (1995).
14. Rao, V. R., G. B. Cohen, and D. D. Oprian. Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature (Lond.).* **367:**639–642 (1994).
15. Bond, R. A., P. Leff, T. D. Johnson, C. A. Milano, H. A. Rockman, T. R. McMinn, S. Apparsundaram, M. F. Hyek, and R. J. Lefkowitz. Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β_2-adrenoceptor. *Nature (Lond.).* **374:**272–276 (1995).
16. Shenker, A., L. Laue, S. Kosugi, J. J. Merendino, Jr., T Minegishi, and G. B. Cutler, Jr. A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature (Lond.).* **365:** 652–654 (1993).
17. Parma, J., L. Duprez, J. Van Sande, P. Cochaux, C. Gervy, J. Mockel, J. Dumont, and G. Vassart. Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature (Lond.).* **365:**649–651 (1993).
18. Findlay, M,. M. deLuise, V. P. Michelangeli, M. Ellison, and T. J. Martin. Properties of a calcitonin receptor and adenylate cyclase in a human cancer cell line (BEN cells). *Cancer Res.* **40:**1311–1317 (1980).
19. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterisation of ligand-binding systems. *Anal. Biochem.* **107:**220–239 (1980).
20. MacEwan, D. J., G. D. Kim, and G. Milligan. Analysis of the role of receptor number in defining the intrinsic activity and potency of partial agonists in neuroblastoma × glioma hybrid NG108–15 cells transfected to express differing levels of the human β_2-adrenoceptor. *Mol. Pharmacol.* **48:**316–325 (1995).
21. Seeman, P, H.-C. Guan, and H. H. M. Van Tol. Dopamine D4 receptors elevated in schizophrenia. *Nature (Lond.)* **365:**441–445 (1993).
22. Kosugi, S., F. Okajima, T. Ban, A. Hidaka, A. Shenker, and L. D. Kohn. Substitutions of different regions of the third cytoplasmic loop of the thyrotropin (TSH) receptor have selective effects on constitutive, TSH-, and TSH receptor autoantibody-stimulated phosphoinositide and 3′,5′-cyclic adenosine monophosphate signal generation. *Mol. Endocrinol.* **7:**1009–1020 (1993).
23. Pei, G., P. Samama, M. Lohse, M. Wang, J. Codina, and R. J. Lefkowitz. A constitutively active mutant β_2-adrenergic receptor is constitutively desensitised and phosphorylated. *Proc. Natl. Acad. Sci. USA* **91:**2699–2702 (1994).
24. Ren, Q., H. Kurose, R. J. Lefkowitz, and S. Cotecchia. Constitutively active mutants of the α_2-adrenergic receptor. *J. Biol. Chem.* **22:**16483–16487 (1993).
25. Boone, C., N. G. Davis, and G. F. Sprague, Jr. Mutations that alter the third cytoplasmic loop of the a-factor receptor lead to a constitutive and hypersensitive phenotype. *Proc. Natl. Acad. Sci. USA* **90:**9921–9925 (1993).
26. Robbins, L. S., J. H. Nadeau, K. R. Johnson, M. A. Kelly, L. Roselli-Rehfuss, E. Baack, K. G. Mountjoy, and R. D. Cone. Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell.* **72:**827–834 (1993).
27. Lefkowitz, R. J. Turned on to ill effect. *Nature (Lond.)* **365:**603–604 (1993).
28. Feyen, J. H. M., F. Cardinaux, R. Gamse, C. Bruns, M. Azria, and U. Trechsel. N-terminal truncation of salmon calcitonin leads to calcitonin antagonists. *Biochem. Biophys. Res. Commun.* **187:**8–13 (1992).
29. Houssami, S., D. M. Findlay, C. L. Brady, T. J. Martin, R. M. Epand, E. E. Moore, E. Murayama, T. Tamura, R. C. Orlowski, and P. M. Sexton. Divergent structural requirements exist for calcitonin receptor binding specificity and adenylate cyclase activation. *Mol. Pharmacol.* **47:**798–809 (1995).
30. Samama, P., G. Pei, T. Costa, S. Cotecchia, and R. J. Lefkowitz. Negative antagonists promote an inactive conformation of the β_2-adrenergic receptor. *Mol. Pharmacol.* **45:**390–394 (1993).
31. Nygaard, S. C., R. E. Kuestner, E. E. Moore, and S. D. Stroop. Phosphorylation of the calcitonin receptor localized at the C-terminus. *J. Bone Miner. Res.* **10** (Suppl. 1)**:**5141 (1995).
32. Allen, L. F., R. J. Lefkowitz, M. G. Caron, and S. Cotecchia. G-protein-coupled receptor genes as protooncogenes: constitutively activating mutation of the α1B-adrenergic receptor enhances mitogenesis and tumorigenicity. *Proc. Natl. Acad. Sci. USA* **88:**11354–11358 (1991).
33. Cook, S. J., and F. McCormick. Inhibition by cAMP of Ras-dependent activation of Raf. *Science (Washington D. C.)* **262:**1069–1072 (1993).
34. Jwu, J., P. Dent, T. Jelinek, A. Wolfman, M. J. Weber, and T. W. Sturgill. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3′,5′-monophosphate. *Science (Washington D. C.)* **262:**1065–1069 (1993).
35. Aiyar, N., E. Baker, J. Martin, A. Patel, J. M. Stadel, R. N. Willette, and F. C. Barone. Differential calcitonin gene-related peptide (CGRP) and amylin binding sites in nucleus accumbens and lung: potential models for studying CGRP/amylin receptor subtypes. *J. Neurochem.* **65:**1131–1138 (1995).
36. Sexton, P. M., S. Houssami, C. L. Brady, D. E. Myers, and D. M. Findlay. Amylin is an agonist of the renal porcine calcitonin receptor. *Endocrinology.* **143:**2103–2107 (1994).
37. Chiba, T., A. Yamaguchi, T. Yamatani, A. Nakamura, T. Morishita, T. Inui, M. Fukase, T. Noda, and T. Fujita. Calcitonin gene-related peptide receptor antagonist human CGRP-(8–37). *Am. J. Physiol.* **256:**E331–E335 (1989).
38. Kenakin, T. *Pharmacological Analysis of Drug-Receptor Interaction.* Raven Press, New York (1993).
39. Meadows, R. P., E. P. Nikonowicz, C. R. Jones, J. W. Bastian, and D. G. Gorenstein. Two-dimensional NMR and structure determination of salmon calcitonin in methanol. *Biochemistry* **30:**1247–1254 (1991).
40. Chidiac, P., T. E. Herbert, M. Valiquette, M. Dennis, and M. Bouvier. Inverse agonist activity of β-adrenergic antagonists. *Mol. Pharmacol.* **45:** 490–499 (1994).

Send reprint requests to: Dr. Patrick M. Sexton, Neurobiology Unit, St. Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy 3065, Victoria, Australia. E-mail: pms@rubens.its.unimelb.edu.au