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Cloning and Characterization of an Abundant Subtype of the Human Calcitonin Receptor

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SUMMARY

We have cloned and characterized a second form of the human calcitonin receptor from T47D cells. It resembles the clone described by Gorn *et al.* [*J. Clin. Invest.* 90:1726–1735 (1992)] except that it lacks a 16-amino acid insert in the putative first intracellular loop. The insert-negative receptor appears to be the most abundant form, and it occurs at a relatively constant level in all expressing tissues. In contrast, the insert-positive receptor is found at low levels in most tissues but its expression levels appear to be much more variable. The insert-negative cDNA was stably expressed in baby hamster kidney cells. Like the endogenous T47D receptor, the recombinant receptor has an equally high affinity for salmon and porcine calcitonin but a 3–4-fold lower affinity for human calcitonin. High concentrations of calcitonin gene-related peptide, rat amylin, secretin, or vasoactive intestinal peptide do not significantly compete with calcitonin for binding to the recombinant receptor. Calcitonin stimulates a cAMP response in both T47D and transfected baby hamster kidney cells. Salmon calcitonin is more potent than human calcitonin for T47D cells, but the two are nearly equipotent for the transfectants. Furthermore, the ED_{50} for the cAMP response in the transfectants is 10–100-fold lower than in T47D cells. Calcitonin stimulates inositol phosphate turnover and elevates internal calcium levels in the transfectants. This response requires nonphysiological levels of calcitonin and is directly correlated with the number of receptors. Lastly, by using a human/rodent somatic cell hybrid panel and *in situ* hybridization, we localized the human calcitonin receptor gene to chromosome 7.

Calcitonin is a peptide hormone that is secreted by the C cells of the thyroid in response to a hypercalcemic signal (1). The two major targets of calcitonin are the kidney, where it stimulates the excretion of calcium and other electrolytes, and bone, where it inhibits bone resorption and hence calcium mobilization (1). Due to its antiresorptive properties, calcitonin is used clinically for the treatment of a variety of bone disorders, including Paget's disease, hypercalcemia of malignancy, and osteoporosis (1). The analgesic effect of calcitonin is well documented, and calcitonin receptors are found in the brain, primarily the hypothalamus. The calcitonin receptor is also expressed in other tissues such as stomach, placenta, and lung, but the physiological role of calcitonin in these tissues is not well understood (1).

The biological effects of calcitonin are thought to be mediated by cAMP, and perhaps by the phospholipase C pathway (2). Therefore, it was anticipated that the calcitonin receptor would be related to the muscarinic and adrenergic receptors, which have seven transmembrane domains and transmit their signals via effector G proteins (3). However, when the cDNAs encoding the porcine calcitonin receptor and the opossum parathyroid hormone/parathyroid hormone-related peptide receptor were cloned, it was found that neither had any significant homology to the classical seven-transmembrane domain receptors but they did have significant homology to each other (4, 5). Hence, the cloning of these two receptors established the existence of a new class of G protein-coupled receptors. Subsequently, other receptors have been cloned and assigned to this family. These include the receptors for secretin, glucagon, glucagon-like peptide, growth hormone-releasing hormone, and vasoactive intestinal peptide (6–9).

Recently, Gorn *et al.* (10) reported the cloning of a human calcitonin receptor cDNA from BIN-67 ovarian carcinoma cells. The predicted amino acid sequence of the human receptor is 73% identical to that of the previously cloned porcine receptor, but this human receptor contains a 16-amino acid insert in the first intracellular loop that is not found in the porcine sequence. The biological significance, if any, of this insert has not yet been defined. Two forms of the rat calcitonin receptor have been cloned, which differ by the presence or absence of a

ABBREVIATIONS: PCR, polymerase chain reaction; BHK, baby hamster kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; kb, kilobase(s).

37-amino acid insert in the second extracellular domain rather than the first cytoplasmic loop (11, 12). The larger isoform is primarily expressed in brain tissue, and it displays altered binding affinities for various calcitonins. A similar variant has also been described for the mouse calcitonin receptor (13).

In this communication, we describe the cloning and characterization of a second form of the human calcitonin receptor from T47D mammary carcinoma cells (14). This receptor is virtually identical to the human receptor described by Gorn *et al.* (10), except that it does not contain the 16-amino acid insert. We compared the tissue distribution of the two receptor isoforms by using PCR. We also prepared stable transfectants of BHK cells, which express varying numbers of the insert-negative isoform of the human calcitonin receptor, and characterized them with respect to binding and second messenger coupling. These results are compared with the behavior of the endogenous receptor expressed by T47D cells.

Materials and Methods

Cloning of the receptor cDNA. RNA was isolated from T47D cells using guanidine isothiocyanate, followed by centrifugation in CsCl (15). Poly(A)$^+$ RNA was selected twice on an oligo(dT) column (16). The Gubler-Hoffman method was used to generate double-stranded cDNA, which was then ligated into the Zem228CC expression vector (8). In this vector, the expression of the cloned cDNA is driven by a modified metallothionein promoter and the neomycin selection marker is controlled by the simian virus 40 promoter.

The Primegen program (17) was used to identify regions that are highly conserved between the porcine calcitonin, opossum parathyroid hormone, and rat secretin receptors. These sequences were then used to design low-degeneracy oligonucleotide PCR primers. The sense primer was C1 (5′-CATCCACGGCAYAARAAYATGTTYYT-3′) and the antisense primer was C2 (5′-ACTCTCCGGTTRCARAARCARTADAT-3′). The positions of these primers are shown in Fig. 1. Using these primers, a 633-base pair fragment, which was 80% identical to the porcine calcitonin receptor, was amplified from T47D cDNA. This fragment was isolated, radioactively labeled using a Stratagene Prime-It kit, and used to probe the T47D cDNA library by standard techniques. DNA sequencing was performed according to the method of Sanger *et al.* (18).

PCR analysis of various tissues. cDNAs that had been derived from various tissues known to express the calcitonin receptor were purchased from Clontech. The region containing the insert was amplified from 1 ng of cDNA by using the sense primer t1 (5′-TACCCGCATACCAAGGAGAAG-3′) and the antisense primer t2 (5′-AAGAGATAATACCACCGCAAGC-3′). Of this reaction, 1/50th was reamplified using the sense primer T1 (5′-CCATCAGAAAAGGTTACAAAAT-3′) and the antisense primer T2 (5′-CACAGAGCATCCAGAAATAGTT-3′). The positions of these primers are shown in Fig. 1. Amplifications were performed using Ampliwax PCR Gem5 (Perkin Elmer) and Vent (exo$^-$) polymerase (New England Biolabs). The conditions for the first amplification were 38 cycles of 45 sec at 94°, 45 sec at 58°, and 2 min at 72°. The conditions for the second amplification were 20 cycles of 45 sec at 94°, 45 sec at 58°, and 2 min at 72°. For both amplifications a final extension was carried out for one cycle at 72° for 7 min. The PCR products from the second reaction were transferred to a Hybond N$^+$ nylon membrane and hybridized with either (a) a 53-base pair oligonucleotide that corresponds to a portion of the second transmembrane domain and the first six amino acids of the first extracellular loop and should therefore recognize both receptor isoforms or (b) a 39-base pair oligonucleotide that corresponds exactly to the insert found between positions 174 and 175 and should hybridize only with the insert-positive form.

Insert-negative and -positive cDNAs were used as positive controls. The insert-positive cDNA was generated as follows. Amplification was performed as described above. The region of the gel that corresponded in size to the product derived from an insert-positive cDNA was purified and ligated into a pNEB193 vector. After transformation, positive clones were identified by their ability to hybridize to the insert-specific probe. An *Eco*RI/*Nsi*I fragment containing the insert was then ligated into a linearized insert-negative cDNA, and its authenticity was confirmed by sequencing. PCR amplification of varying amounts of the insert-positive and -negative constructs demonstrated that neither form underwent preferential amplification.

Cell culture and transfections. T47D human mammary carcinoma cells (HBL 133; American Type Culture Collection) were routinely cultured in RPMI 1640 medium supplemented with 0.29 mg/ml L-glutamine, 1 mM sodium pyruvate, 600 ng/ml insulin, 1 μM hydrocortisone, and 10% heat-inactivated fetal calf serum. BHK-570 cells (CRL 1632; American Type Culture Collection) were routinely grown in Dulbecco's modified Eagle's medium containing glutamine, pyruvate, and 5% heat-inactivated fetal calf serum. All cell lines were routinely passaged with trypsin-EDTA and were periodically checked for *Mycoplasma* contamination using a Geneprobe kit.

BHK cells were transfected using a modification of the calcium phosphate precipitation technique (19), and stable transfectants were selected with 500 μg/ml G418 (GIBCO). Clones were isolated using cloning rings and were maintained in the presence of G418.

Calcitonin binding. The cells were plated at 10^5 cells/24-well plate for 48 hr before the assay. The cells were rinsed with binding medium (RPMI 1640 medium containing 1 mg/ml bacitracin and 1 mg/ml bovine serum albumin) and incubated in this medium for 15 min at room temperature. The medium was then removed and replaced with binding medium containing a constant amount of either ^{125}I-labeled salmon calcitonin (800–2000 Ci/mmol; Peninsula) or ^{125}I-labeled human calcitonin (2000 Ci/mmol; Amersham) and serial dilutions of unlabeled competitor. The cells were incubated for 1.5 hr at room temperature, washed three times with phosphate-buffered saline, and solubilized with 0.25–1 N NaOH. The samples were collected from each well and counted on a Cobra-auto γ counter. Scatchard analysis was performed using the Kinetic, EBDA LIGAND, Lowry program from Biosoft (20).

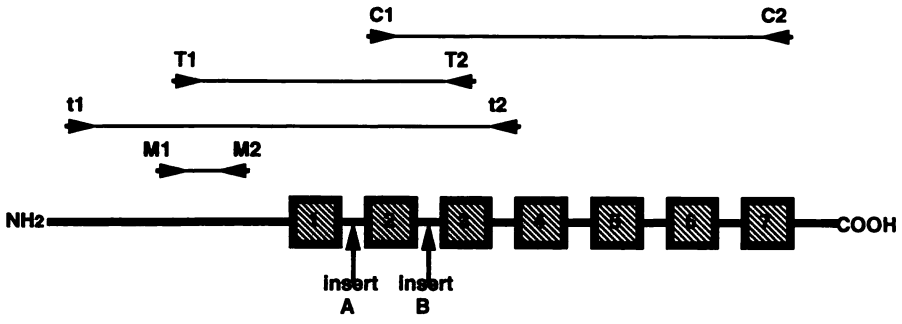

Fig. 1. Schematic diagram that shows the location of various PCR primers. *Hatched boxes*, seven transmembrane domains of the receptor. Primers C1 and C2 were used to amplify a 633-base pair fragment of the cDNA, which was then used to probe the cDNA library. A set of nested primers, t1/t2 and T1/T2, flank the 16-amino acid insert in the first cytoplasmic loop of the human calcitonin receptor. These were used to evaluate the relative frequency of the receptor subtypes in various tissues. Primers M1/M2 were used to identify the chromosomal location of the human calcitonin receptor gene. *Insert A*, location of the 16-amino acid insert in the human receptor; *insert B*, location of the 37-amino acid insert found in the mouse/rat calcitonin receptor.

cAMP measurements. To measure cAMP response, 1–3 × 10^5 cells were plated in 24-well plates for 1–2 days before the assay. Cells were exposed to varying concentrations of human or salmon calcitonin, in serum-containing medium with 10 μM isobutylmethylxanthine, for 10 min at 37°. Forskolin (25 μM) was included as a positive control. The reaction was stopped by the addition of 4 volumes of boiling water. Samples were then either frozen or assayed immediately using an Amersham Scintillation Proximity cAMP assay system. All points were assayed in triplicate.

Inositol phosphate measurement. Cells (2 × 10^5) were plated in a 24-well plate for 24 hr and then labeled overnight with 4 μCi/ml *myo*-[2-^{3}H]inositol (specific activity, 20 Ci/mmol; Amersham) in inositol-free Dulbecco's modified Eagle's medium containing glutamine, pyruvate, and 10% dialyzed serum. The cells were then washed with medium buffered with 20 mM HEPES, pH 7.0, containing 10 mM lithium chloride. The wash medium was replaced by the same medium containing increasing concentrations of human or salmon calcitonin. Cells were incubated for 30 min at 37°. The reaction was terminated by the addition of cold Dulbecco's modified Eagle's medium/10% perchloric acid (1:1). EDTA was added to the lysates to a final concentration of 2 mM, and the samples were neutralized with 1.5 M KOH in 60 mM HEPES buffer. The labeled inositol phosphates were isolated on an Amersham AMPREP column according to the manufacturer's instructions and were counted in a Beckman S1800 scintillation counter. All points were assayed in triplicate.

Calcium analysis. The quantitation of internal calcium levels was performed as described previously (21). The cells were plated at a density of 3 × 10^4 cells/ml in uncoated, coverglass-well, two-chamber slides (Nunc) and were incubated for 48 hr. The slides were then rinsed with imaging buffer (140 mM NaCl, 10 mM glucose, 5 mM KCl, 0.5–1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, pH 7.4). The cells were loaded with fura-2/acetoxymethyl ester (10 μg/ml in imaging buffer) for 30 min at room temperature and then rinsed three times. Slides were mounted on a Nikon Epiphot inverted microscope, and the ratio of fura-2 emission at excitation wavelengths of 340 and 380 nm was recorded every 5 sec. The data were analyzed on a Sun computer using an Inovision software system designed for ratio imaging. Fura-2 340:380 ratios (R) were converted to calcium concentrations, after calibration, using the following formula: calcium (nM) = 2240 $[(R - 0.3)/(20 - R)]$. Typical basal values of internal calcium levels ranged from 80 to 150 nM.

Chromosome mapping. The Human Genetic Mutant Cell Repository Human/Rodent Somatic Cell Hybrid Panel 2 (National Institute of General Medical Sciences, Coriell Institute for Medical Research, Camden, NJ) was used in PCR amplification to identify the somatic hybrid that contains the human calcitonin receptor gene. The sense primer, M1, corresponds to a 21-base pair stretch of an adjacent intron (5′-GGTTGGGTATGACTGGTGTAG-3′). This intron sequence was obtained from a 6.5-kb *Hind*III fragment of the calcitonin receptor gene that had been identified by its ability to hybridize to the cDNA insert.[1] The locations of both the sense primer and the antisense primer, M2 (5′-CTGGGCAGAACTGATAGGACA-3′), are shown in Fig. 1. The PCR amplification was performed with 100 ng of each DNA sample. The conditions were 30 cycles of 1 min at 94°, 1 min at 62°, and 1 min at 72°. This was followed by a 7-min final extension at 72°. The predicted product is 198 base pairs.

In situ hybridization was performed on human metaphase chromosome spreads, which were prepared from primary human lung cells as described previously (22). A genomic probe for the human calcitonin receptor, DMPC-HFF#1–1301(11D) (series A), was obtained by screening a human P1 library (Genome Systems, Inc.). A centromeric probe specific for chromosome 7 (D7Z1) was obtained from Oncor. The probes were labeled by nick-translation with biotin-11-dUTP (Sigma). The unincorporated nucleotides were removed using a Mini-Spin G-50 column (Worthington), and the size of the labeled probe was verified on a 0.7% agarose gel. The hybridization was performed as described previously (23), except that hybridization with the calcitonin receptor gene probe was carried out in 50% (v/v) formamide, 10% dextran sulfate, 300 mM NaCl, and 30 mM Nacitrate (2× standard saline citrate) at 37° for 6 hr before the addition of the centromeric probe. At that point the formamide concentration was raised to 65% (v/v), and the hybridization was continued overnight. Preparations were viewed and photographed using an Olympus BH-2 microscope with a BH-2 RFCA fluorescence attachment and Ektachrome 400 film.

[1] R. E. Kuestner, and E. E. Moore, unpublished observations.

Results

Cloning of an alternative form of the human calcitonin receptor. An expression cDNA library was prepared from T47D cells, a human mammary carcinoma cell line known to express the calcitonin receptor (14). A 633-base pair fragment of the human calcitonin receptor was amplified from this cDNA by PCR using degenerate primers that correspond to regions that are highly conserved between the porcine calcitonin, opossum parathyroid hormone, and rat secretin receptors. This fragment was then used to probe the cDNA library. A single full-length clone was found among 400,000 clones. This clone contains a 3.3-kb insert, of which 1422 base pairs constitute an open reading frame. There is a long 3′ untranslated region (1871 base pairs) and a short 5′ sequence (37 base pairs) that does not extend to the point where Gorn *et al.* (10) found a second in-frame AUG start site. The coding sequence encodes a 474-amino acid protein that is 70% identical to the porcine calcitonin receptor sequence and is virtually identical to the human calcitonin receptor cDNA isolated by Gorn *et al.* (10), with the following exceptions. Most notably, our clone does not contain an insert of 16 amino acids between positions 174 and 175 and in this regard is more similar to the porcine receptor. The absence of this insert in our clone indicates that at least two subtypes of the human calcitonin receptor exist. In addition, there is a single nucleotide difference at position 1377, which results in the substitution of leucine for proline in our clone. This difference may reflect individual variation in the source of the cloned DNA, or it may represent a cloning artifact. Fig. 2 shows an alignment of our clone with other known homologues of this receptor subfamily.

Tissue distribution of receptor subtypes. Our results indicate that at least two alternative forms of the human calcitonin receptor exist. We used a PCR approach to determine whether they are expressed in a tissue-specific fashion. Although this approach is not strictly quantitative, we demonstrated that the insert-positive and -negative receptor constructs are amplified equally well under our PCR conditions (data not shown). Therefore, it seemed reasonable to use this approach to assess the relative distribution of these two subtypes in a variety of tissues. We obtained cDNAs derived from a variety of tissues known to express the calcitonin receptor. These included kidney, hypothalamus, stomach, fetal brain, cerebral cortex, mammary gland, cerebellum, placenta, ovary, lung, and bone marrow. PCR primers that flank the region of the insert containing 16 amino acids were used to amplify this region of the calcitonin receptor from each of the cDNA sources. As a positive control, we also amplified cDNAs representing both the insert-positive and -negative forms of the receptor. The PCR products were separated on a 2.5% agarose gel, transferred to a nylon membrane, and hybridized either with a probe that should interact with both forms of the receptor or with an oligonucleotide that should hybridize only to the insert-

Fig. 2. Alignment of human calcitonin receptor (*HU_CTR*), porcine calcitonin receptor (*PORCTR*), human, opossum, and rat parathyroid hormone receptors (*HUPTHR*, *OPPTHR*, and *RAPTHR*, respectively), rat glucagon-like peptide 1 receptor (*RAGLPR*), rat secretin receptor (*RASECR*), rat vasoactive intestinal peptide receptor (*RAVIPR*), human and rat glucagon receptors (*HU_GLR* and *RA_GLR*, respectively), and human, rat, and mouse growth hormone-releasing hormone receptors (*HUGRFR*, *RAGRFR*, and *MUGRFR*, respectively). The putative transmembrane domains are *underscored* (*###*) and *numbered*. *, Cysteine residues in the extracellular domains. All proteins are *numbered* (*right margin*) from the initiating methionine. @, Location of the insertion in the ovarian carcinoma cDNA that corresponds to the location of an intron in the human glucagon receptor gene. This version of the human calcitonin receptor sequence has been deposited in GenBank and EMBL sequence databanks (accession number X69920).

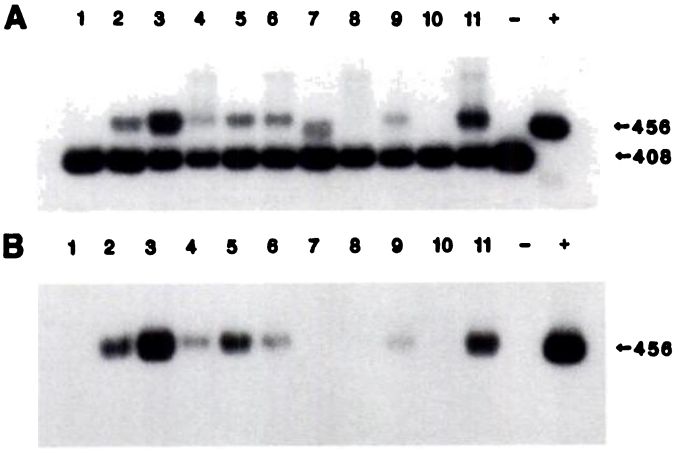

Fig. 3. cDNAs obtained from various tissues were PCR amplified using primers that flank the region containing the insert. Controls included both insert-negative (−) and -positive (+) cDNA. A, Southern blot hybridized with a probe that recognizes both forms of the receptor; B, Southern blot hybridized with a probe that is specific for the insert. *Lane 1*, stomach; *lane 2*, lung; *lane 3*, ovary; *lane 4*, kidney; *lane 5*, bone marrow; *lane 6*, mammary gland; *lane 7*, whole fetal brain; *lane 8*, hypothalamus; *lane 9*, cerebellum; *lane 10*, cerebral cortex; *lane 11*, placenta. The anticipated size for the product amplified from the insert-negative form is 408 base pairs and that for the product amplified from the insert-positive form is 456 base pairs.

positive form (Fig. 3). The insert-negative form of the receptor was expressed at a fairly constant level in all tissues examined and appeared to be the most abundant form of the receptor. In contrast, the expression of the insert-positive receptor was apparently much more variable. It was found at low levels in most tissues, but its level of expression ranged from being undetectable in stomach, fetal brain, and cerebral cortex to constituting almost half of the transcripts in ovary and placenta.

Interestingly, these PCR primers should also amplify the region that encodes the second extracellular domain, where a 37-amino acid insert has been found in the rat and mouse calcitonin receptors (11–13). If an equivalent isoform exists for the human receptor, the predicted size of the PCR product is 519 base pairs. Faint bands in this region of the gel were observed for kidney, mammary gland, hypothalamus, and placenta, and this may indicate that this subtype is expressed at low levels by these tissues. In contrast to the rodent system, we did not observe a preferential high level of expression of this subtype in any of our brain samples. However, in fetal brain a PCR product was produced that is intermediate in size, compared with the insert-positive and -negative forms. Additional studies are underway to characterize this product and to verify that a human calcitonin receptor subtype exists that is analogous to the rat brain isoform.

Binding. To assess the biological activity of the insert-negative form of the receptor, its cDNA was transfected into BHK cells, and multiple stable transfectants were isolated. Two of these, Hx1 and Hx2, were characterized in detail, and their behavior was compared with that of T47D cells. The recombinant receptor expressed by both transfectants bound ^{125}I-labeled salmon calcitonin in a specific fashion; the level of nonspecific binding was <10% of the total counts bound. BHK cells transfected with vector only showed no specific binding of calcitonin (data not shown). The recombinant receptor was highly specific for calcitonin, because even very high levels (1 μM) of closely related ligands such as calcitonin gene-related peptide, amylin, vasoactive intestinal peptide, and secretin had little or no effect on calcitonin binding (Fig. 4). It was previously reported that the calcitonin receptor expressed by T47D cells binds porcine calcitonin with affinity similar to that for salmon calcitonin but has a significantly lower affinity for human calcitonin (24). We confirmed this observation and showed that the recombinant receptor behaves in the same fashion (Fig. 4).

Scatchard analysis was used to calculate the K_d of the recombinant and endogenous receptors for both human and salmon calcitonin. A sample Scatchard plot is shown in Fig. 5, and the results are summarized in Table 1. Similar K_d values were observed for the recombinant and endogenous receptors, and both receptors had a 3–4-fold higher affinity for salmon calcitonin, compared with human calcitonin. This observation indicates that the affinity for calcitonin is an inherent property of the receptor itself and is not significantly affected by expression levels or by association with a different array of G proteins.

We also used Scatchard analysis to calculate the number of receptors present on T47D cells and the transfectants (Table 1). The number of receptors in the T47D cells was highly variable and decreased with prolonged culture. In contrast, the phenotype of the two transfectants was relatively stable and both expressed large numbers of receptors. Hx1 cells expressed approximately 8-fold more receptors than did Hx2 cells, and Hx2 cells expressed 2.5–10-fold more receptors than did T47D cells.

cAMP response. Calcitonin elicits a rapid rise in intracellular cAMP levels in many cell types, including T47D cells and osteoclasts. Calcitonin also stimulated cAMP production in the Hx1 and Hx2 transfectants, but this response was different from that observed in T47D cells in several respects. First, the magnitude of the maximum response was much lower in the transfectants than in T47D cells (5–10-fold versus 2000-fold). However, in both cases, the maximum response induced by calcitonin was equivalent to the maximum response induced by forskolin, a direct stimulator of adenylate cyclase. Therefore, this difference can probably be attributed to differences in the cAMP machinery of the two cell types, rather than the receptor itself.

For T47D cells, the ED_{50} values for the cAMP response were roughly equivalent to the K_d values (Fig. 6; Tables 1 and 2) and therefore salmon calcitonin is more potent than human calcitonin. This is not the case for the transfectants. The ED_{50} values were significantly lower than the K_d values and salmon and human calcitonin are nearly equipotent at stimulating cAMP production, despite their difference in binding affinities.

Calcium response. Calcitonin is known to elevate intracellular calcium levels in a variety of cell types including osteoclasts and kidney cells (25, 26). Furthermore, the recombinant porcine calcitonin receptor, expressed either transiently in human embryonic kidney 293 cells or stably in MC3T3 cells, has been reported to stimulate a rise in intracellular calcium levels via the phospholipase C pathway (27, 28). We obtained similar results with our human recombinant calcitonin receptor expressed in BHK cells.

The effect of 20 nM salmon calcitonin on intracellular calcium levels was monitored in fura-2-loaded Hx1 transfectants (Fig. 7). Calcitonin elicited a rapid but transient rise in intracellular calcium levels, of approximately 3-fold, in 80–100% of the cells. The same experiment performed in the presence of

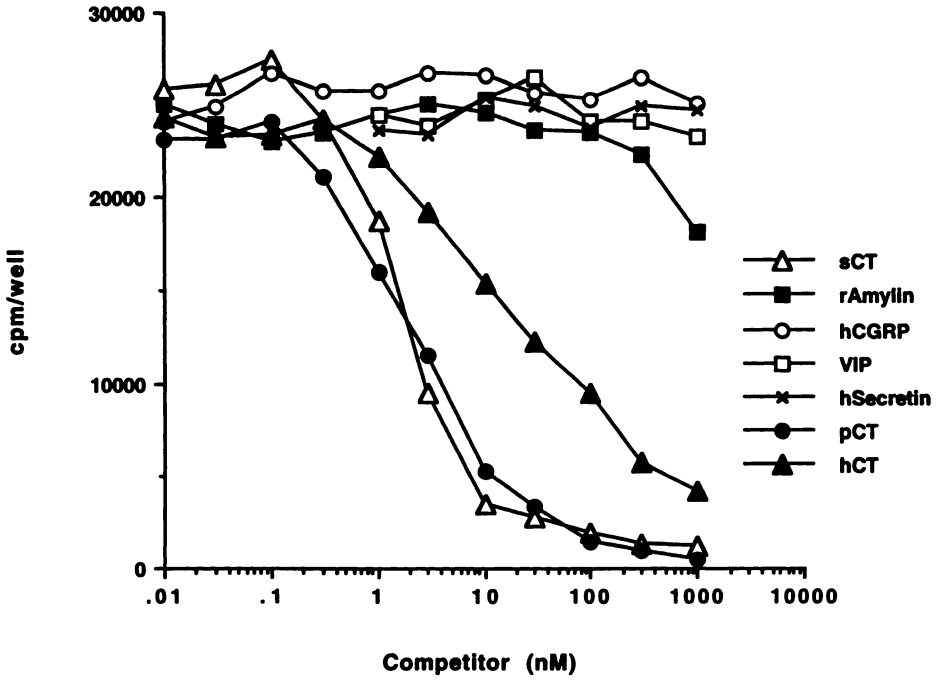

Fig. 4. Competition with binding of ^{125}I-labeled salmon calcitonin to Hx1 cells by increasing concentrations of unlabeled salmon calcitonin (*sCT*), porcine calcitonin (*pCT*), human calctionin (*hCT*), rat amylin (*rAmylin*), human calcitonin gene-related peptide (*hCGRP*), vasoactive intestinal peptide (*VIP*), and human secretin (*hSecretin*). Maximum binding in the absence of competitor ranged from 22,000 to 27,000 cpm/well.

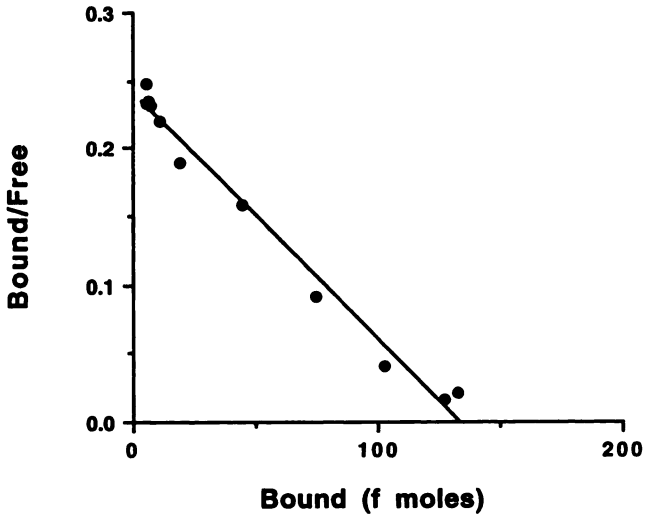

Fig. 5. Scatchard analysis of binding of human calcitonin to Hx1 cells, performed as described in Materials and Methods.

TABLE 1
Binding characteristics of T47D, Hx1, and Hx2 cells

	T47D	Hx2	Hx1
Receptors/cell	7–40,000	100,000	800,000
K_d			
Human calcitonin	2.1 ± 0.3	1.7 ± 0.1	4 ± 2.5
Salmon calcitonin	0.6 ± 0.5	0.3 ± 0.01	0.5 ± 0.2

EGTA, a calcium chelator, demonstrated that the primary response is due to the mobilization of internal calcium stores. However, the decay of the response was more rapid in the absence of external calcium, suggesting that a second component of the response may be mediated by a calcium channel.

Although calcium responses were also seen in Hx2 transfectants and T47D cells, only 30–60% of the Hx2 and 10–20% of the T47D cells showed a response. A similar reduction in amplitude was seen.

Inositol phosphate production. To determine whether the increased mobilization of internal calcium stores is mediated by inositol triphosphate, we examined the effect of calcitonin on inositol phosphate turnover. We found that calcitonin induced a significant increase in inositol phosphate turnover in Hx1 cells (Fig. 8). Forksolin (25 μM) did not induce this response, which indicates that it is not an indirect consequence of elevated cAMP levels (data not shown). Table 3 is a summary of the ED_{50} values, which were averaged from several experiments. As observed for the cAMP response, the ED_{50} values were identical for salmon and human calcitonin. However, approximately 100-fold higher levels of calcitonin were required to trigger an inositol phosphate response, compared with a cAMP response.

Paralleling the results with the calcium studies, we found that the inositol phosphate response in Hx2 cells was lower than that in Hx1 cells and that no response could be detected in T47D cells. The inositol phosphate assay is probably not sensitive enough to detect the lower percentage of T47D cells that show a calcium response when the cells are monitored at a single-cell level.

Our results suggest that the inositol phosphate response may occur only in cells that express a sufficiently large number of receptors. We therefore characterized several other BHK transfectants, which express different levels of calcitonin receptors, for their inositol phosphate response. Fig. 9 shows that, in this series of transfectants, the magnitude of the inositol phosphate response appears to be a direct function of the number of receptors.

We also attempted to obtain T47D transfectants that would express a larger number of recombinant receptors, to determine whether there would be an accompanying increase in their inositol phosphate response. Unfortunately, the expression levels of the transfected receptor in T47D cells were too low for us to make this assessment.

Mapping. To identify the chromosomal location of the human calcitonin receptor, we used PCR primers corresponding

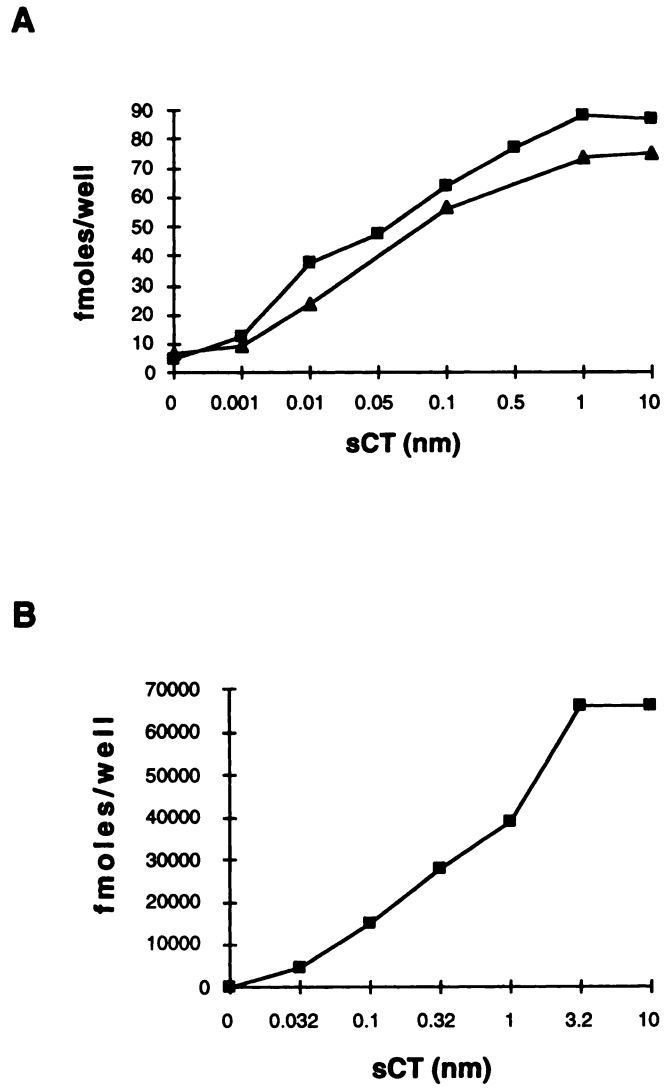

Fig. 6. Effect of increasing concentrations of salmon calcitonin (*sCT*) on cAMP accumulation in T47D, Hx1, and Hx2 cells. The results are presented as fmol of cAMP/24-well plate. A, Hx1 (▲) and Hx2 (■); B, T47D.

TABLE 2
ED_{50} for cAMP response

Cell line	ED_{50}	
	Human calcitonin	Salmon calcitonin
	nM	
T47D	2.7 ± 1.2	0.45 ± 0.2
Hx1	0.08 ± 0.01	0.06 ± 0.02
Hx2	0.07 ± 0.01	0.04 ± 0.01

to the calcitonin receptor gene to amplify DNA samples isolated from a series of human/rodent somatic cell hybrids that contain only one or two human chromosomes. The results, shown in Fig. 10, show that a band of the expected size was obtained only with the control human DNA and with DNA isolated from the somatic cell hybrid that contains human chromosome 7. This indicates that the calcitonin receptor gene resides on chromosome 7.

This location was confirmed by fluorescent *in situ* hybridization (Fig. 11). Our probes were a genomic fragment (70–100

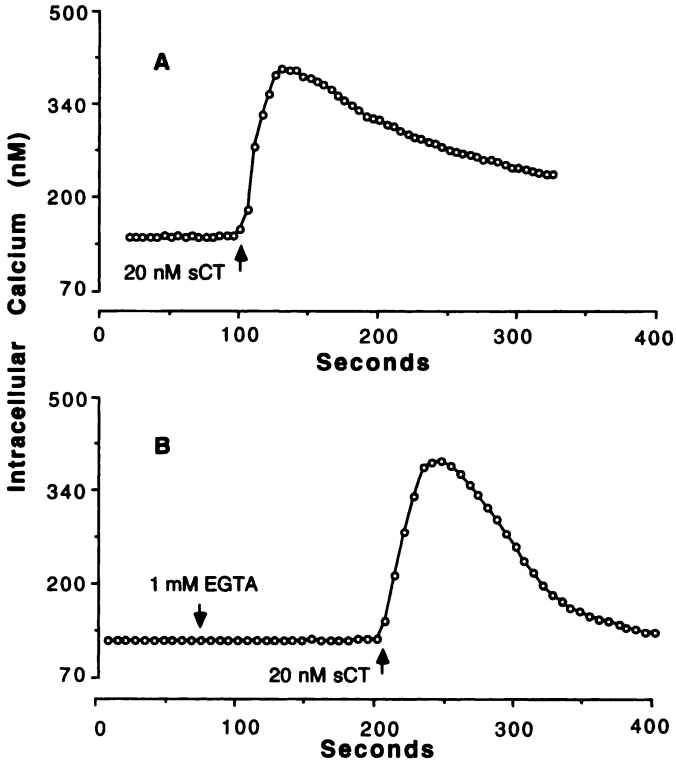

Fig. 7. Effect of 20 nM salmon calcitonin (*sCT*) on intracellular calcium levels in Hx1 cells. A, Results obtained in buffer containing 1.8 mM calcium; B, results obtained in buffer containing 0.5 mM calcium and 1 mM EGTA.

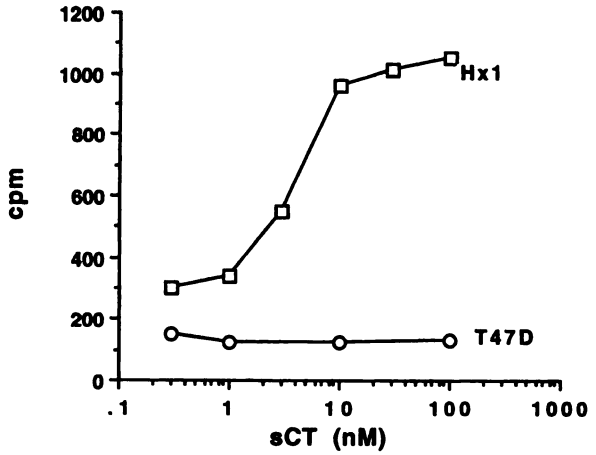

Fig. 8. Effect of increasing concentrations of salmon calcitonin (*sCT*) on the production of inositol phosphates in Hx1 and T47D cells. The average background level for Hx1 was 278 cpm and for T47D was 135 cpm.

TABLE 3
ED_{50} for inositol phosphate response in Hx1 cells

Agent	ED_{50}
	nM
Human calcitonin	9.5 ± 0.7
Salmon calcitonin	6 ± 1.4

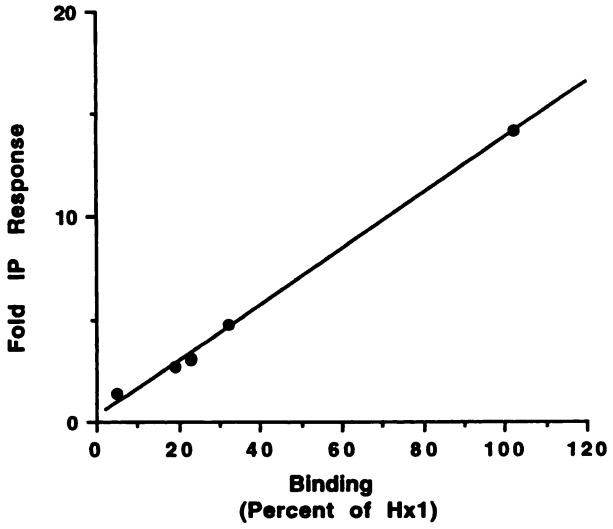

Fig. 9. Maximum inositol phosphate (*IP*) responses to salmon calcitonin determined in a series of transfectants that express varying levels of the calcitonin receptor. The binding capacity of each transfectant was determined in the presence of saturating amounts of ^{125}I-human calcitonin. This value was normalized to 10^6 cells and is expressed as the percentage of binding displayed by Hx1 cells.

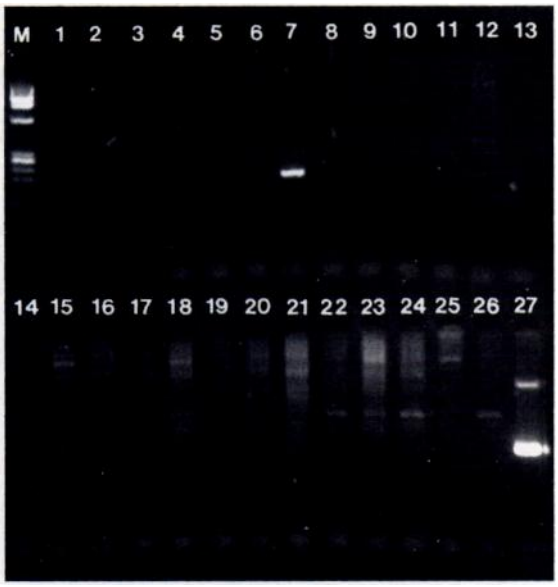

Fig. 10. PCR amplification of DNA obtained from a panel of human/rodent somatic cell hybrids that each contain one or two human chromosomes. Each lane corresponds to a different hybrid; *lanes 25, 26,* and *27*, mouse, Chinese hamster, and human DNA, respectively. *Lane 7*, a hybrid that contains only human chromosome 7.

kb) containing a portion of the human calcitonin receptor gene and a centromeric probe specific for chromosome 7.

Discussion

Our results demonstrate that there are two isoforms of the human calcitonin receptor, which differ by the presence or absence of a 16-amino acid insert in the first intracellular loop. Subtypes of other G protein-coupled receptors that are expressed in a tissue-specific fashion and have different pharmacological properties have been reported (3). After confirming that both forms of the receptor underwent equivalent amplification using the PCR, we used this technique to examine the relative frequency of these two receptor subtypes in a variety of tissues that express the calcitonin receptor. These included kidney, whole fetal brain, hypothalamus, mammary gland, cerebral cortex, cerebellum, lung, stomach, placenta, ovary, and bone marrow. The insert-negative form of the receptor appears to be the most abundant form of the receptor and is expressed at a relatively constant level in all tissues that we examined. In contrast, the levels of expression of the insert-positive receptor ranged from being undetectable in stomach, fetal brain, and cerebral cortex to constituting almost half of the transcripts found in ovary and placenta. This observation suggests that the expression of this receptor isoform may be regulated in a tissue-specific manner or may be affected by various physiological factors.

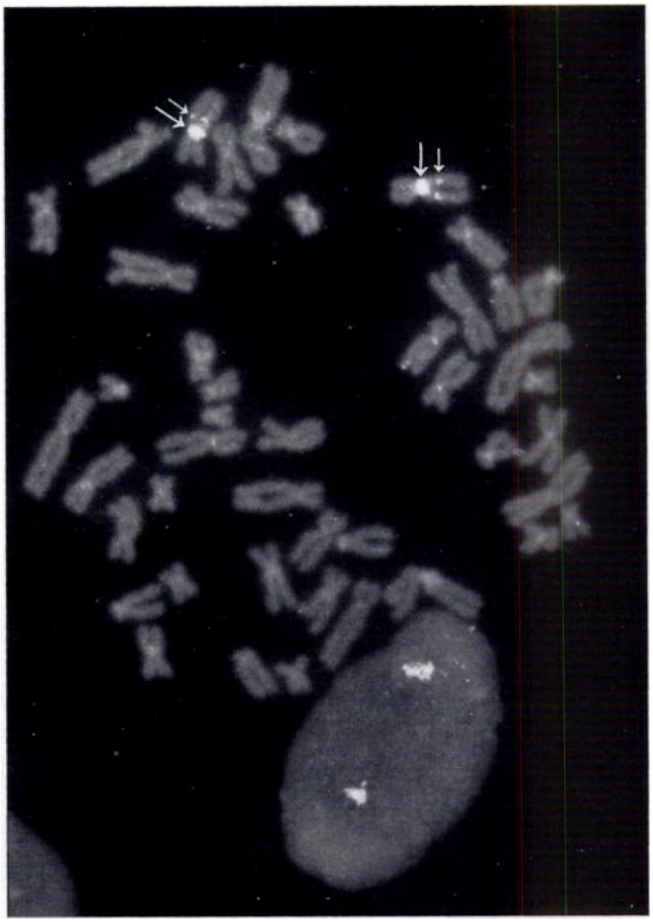

Fig. 11. Human metaphase chromosome spread hybridized with a genomic fragment of the human calcitonin receptor gene and with a centromeric probe specific for chromosome 7. *Large arrows*, centromeric labeling of chromosome 7; *small arrows*, location of the calcitonin receptor gene on the q arm.

We expressed the insert-negative form of the receptor in BHK cells and characterized the transfectants with respect to binding and second messenger coupling. The binding affinities for human calcitonin and salmon calcitonin are the same as those of the endogenous receptor in T47D cells and are very similar to the values reported for the insert-positive form of the receptor (10). Therefore, the binding properties of the receptor are apparently not affected by the presence or absence of the insert.

We found that the transfected insert-negative form of the

receptor couples to both the cAMP and phospholipase C pathways. A similar observation has been made for the porcine calcitonin receptor (27, 28). Unfortunately, a comparison with the insert-positive form of the human receptor is difficult to make. Gorn *et al.* (10) reported that the insert-positive receptor couples to the cAMP pathway at saturating amounts of salmon calcitonin, but those authors did not look for phospholipase C-mediated events.

We found that 10–100-fold lower levels of calcitonin are required to stimulate a cAMP response in the transfectants, compared with T47D cells. This augmented cAMP response may be due to overexpression of the receptors in the transfectants. However, the ED_{50} values are identical for Hx1 and Hx2, despite an 8-fold difference in their receptor numbers. Furthermore, Force *et al.* (27) found that the ED_{50} for the recombinant porcine calcitonin receptor is 40-fold lower than the ED_{50} for the endogenous receptor expressed by LLC-PK1 cells. In that case, both the transfectant and LLC-PK1 cells express 300,000 receptors/cell, so the difference in the ED_{50} values is not due to a difference in receptor numbers. It is possible that the augmented cAMP response mediated by the transfected receptor occurs because the down-regulation or desensitization of the recombinant receptor is impaired in BHK cells. This situation occurs in yeast cells, which express a desensitization-resistant form of the α factor receptor and are hyperresponsive to pheromone action (29). Furthermore, a recombinant thyrotropin receptor expressed in Chinese hamster ovary cells does not undergo homologous desensitization as it does in thyroid cells, suggesting that a tissue-specific factor is required for this process (30). A study of the down-regulation and desensitization of the human recombinant calcitonin receptor in BHK cells is underway.

We also found that stimulation of the phospholipase C pathway in the transfectants appears to be directly correlated with the receptor number and requires approximately 100 times more calcitonin than the cAMP response. The level of calcitonin required for a phospholipase C response (ED_{50} = 5 nM) is significantly higher than normal plasma levels (2–10 pM) and even exceeds the peak plasma levels obtained after calcitonin injection therapy (100 pM) (1). These observations may indicate that the phospholipase C pathway does not play a physiological role in calcitonin signaling. However, the second messenger coupling of the calcitonin receptor can be modulated by a variety of factors. For example, in LLC-PK1 kidney cells calcitonin preferentially stimulates the cAMP pathway during the G_2 phase of the cell cycle, whereas protein kinase C is activated during S phase (2). Furthermore, the ability of calcitonin to stimulate internal calcium levels in osteoclasts can be significantly augmented by elevating extracellular calcium levels from 2 mM to 6 mM (26).

In conclusion, our work shows that there are subtypes of the human calcitonin receptor. We are currently investigating whether these subtypes are generated by alternative splicing or are products of two independent genes. The physiological significance of these subtypes remains to be established.

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