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Discovery of novel peptide/receptor interactions: identification of PHM-27 as a potent agonist of the human calcitonin receptor

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Abstract

Many naturally occurring peptides exhibit a high degree of promiscuity across G-protein coupled receptor subtypes. The degree to which this phenomenon occurs, and its physiological significance is not well characterized. In addition, many 'orphan' peptides exist for which there are no known receptors. Therefore, to identify novel interactions between biologically active peptides and G-protein coupled receptors, a library of nearly 200 peptides was screened against the human calcitonin (hCTr), human Parathyroid Hormone (PTH1R), human Corticotropin Releasing Factor (CRF1), and the human Glucagon-like peptide (GLP1) receptors using a cell-based functional assay (Receptor Selection and Amplification Technology). Functional profiling revealed that the 'orphan peptide' PHM-27 selectively activated the hCTr; no activity was observed at the PTH1, CRF1, or GLP1 receptors. PHM-27 was a potent agonist at the hCTr, with similar efficacy as human calcitonin, and a potency of 11 nM. These results were confirmed in cyclic AMP assays. Responses to calcitonin and PHM-27 could be suppressed by the antagonist salmon calcitonin (8–32). In competition binding studies, salmon calcitonin (8–32), calcitonin, and PHM-27 were each able to inhibit ^{125}I-calcitonin from cell membranes containing transiently expressed hCTr. These results indicate that the orphan peptide PHM-27 is a potent agonist at the hCTr.

Keywords: Calcitonin; High-throughput screen; G-protein coupled receptor; R-SAT; Orphan

1. Introduction

Many naturally occurring G-protein coupled receptor (GPCR) ligands exhibit a high degree of promiscuity, both among immediate receptor family members, and also more distantly related receptors. For example, monoamines such as dopamine, norepinephrine, serotonin and their metabolic precursors activate trace amine receptors as well as their preferred receptors [1]. For peptide receptors, ligand/receptor promiscuity is probably more common and more physiologically significant, but less well characterized. Besides systems where one ligand activates a closely related family of receptors, groups of related peptide ligands can activate multiple receptors. For example, CRF, Sauvagine and Urocortinin activate all the CRF receptors [2]. Similarly, the calcitonin gene family is another example where multiple homologous ligands and receptors have overlapping, but distinct biological actions [3]. Peptides representing splice variants or alternatively processed versions of the same gene product, such as the kinin [4] or enkephalin [5] gene products further increase the potential complexity of peptide-receptor interactions. In addition, many 'orphan' peptides exist for which there are no known receptors.

hCTr, hPTH1R and CRF1, GLP1 receptors are members of the type B superfamily of GPCRs that also includes the receptors for secretin, glucagon, GIP, GHRH, CGRP, and VIP/PACAP [3,6]. These receptors share some structural features, including a large N-terminal extracellular domain that contains six well-conserved cysteine residues, and all increase intracellular cAMP upon binding their cognate agonist.

The PTH/PTHrP receptors have the highest predicted amino acid sequence similarity to the CTR (~32% identity and overall ~56% similarity, [6–8]), and share a common

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Abbreviations: CT, calcitonin; PTH, parathyroid hormone; GLP, glucagon-like peptide; CRF, corticotropin releasing factor; GPCR, G-protein coupled receptor; R-SAT, receptor selection and amplification technology.

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mechanism of ligand binding with the calcitonin receptor [9,10]. To explore potential novel peptide interactions at the CT, PTH1, CRF1 and GLP1 receptors, we utilized a high throughput functional screen to evaluate a library of bioactive peptides for agonist activity. Included in the peptide library were peptides having either defined or unknown pharmacological targets, i.e. 'orphan peptides'. As hypothesized, we identified many previously unrecognized ligand-receptor interactions. Based on this analysis we demonstrate that an 'orphan peptide', PHM-27/PHI, has potent agonist activity at hCTr.

2. Materials

NIH-3T3 cells were from ATCC CRL 1658. HEK293 cells were from ATCC CRL 1573. *o*-Nitrophenyl-β-D-galactopyranoside and nonidet P-40 were from Sigma. Tissue culture media used was Dulbecco's modified Eagles medium (DMEM) (Gibco-BRL) supplemented with 4500 mg/L glucose, 4 nM L-glutamine, 50 U/mL penicillin G, 50 U/mL streptomycin (A.B.I.) and 10% calf serum (Sigma) or 2% cyto-SF3 synthetic supplement (Kemp Laboratories). 96-well, 6-well, and 15 cm^2 tissue culture dishes were from Falcon. Hanks balanced salt solution without magnesium chloride, magnesium sulfate, and calcium chloride, Trypsin-EDTA was all from Gibco-BRL. The biologically active peptide collection was from Synpep. All peptides were solubolized in water containing 0.1% bovine serum albumin to prevent sticking to the storage tubes or assay plates.

3. Procedures

3.1. Cell culture

NIH-3T3 cells were incubated at 37° in a humidified atmosphere (5% CO_2) in supplemented DMEM. HEK293 cells were cultured similarly except 10% fetal calf serum was substituted for 10% calf serum.

3.2. Constructs

The receptors used in this study were cloned by polymerase chain reaction using Pfu Turbo (Stratagene). The human calcitonin receptor used was the 3rd isoform lacking the 16 amino acid insert (see Ref. [8]). Ras/rap1B(AA) was constructed by ligating PCR amplified coding sequence for residues 1–60 of c-Ha-Ras and residues 61–184 (including the termination codon) of rap1B. Quick-change mutagenesis (Stratagene) was used to change amino acids 179 and 180 from serines to alanines in ras/rap1B. All clones were sequenced before use. The adenylyl cyclase Type II (AC2) clone used in these studies has been described ([11], generous gift of Dr. P. Ram).

3.3. Functional assays

Receptor selection and amplification technology (R-SAT™) assays were performed as described [12] with the following modifications. Briefly, cells were plated one day before transfection using 7×10^3 cells in 0.1 mL of media per well of a 96-well plate. Cells were transiently transfected with 5 ng/well of receptor DNA, 20 ng/well ras/rap1B(AA), 2 ng/well adenylyl cyclase Type II (AC2) and 30 ng/well pSI-β-galactosidase (Promega) per well of a 96-well plate using Superfect (Qiagen) according to the manufacturers instructions. The use of ras/rap1B(AA) and AC2 was found to improve responses of GPCRs in this functional assay. One day after transfection media was changed and cells were combined with ligands in DMEM supplemented with 2% cyto-SF3 synthetic supplement (Kemp Laboratories) instead of calf serum to a final volume of 200 uL/well. After five days in culture β-galactosidase levels were measured essentially as described [12]. The media were aspirated from the wells and the cells rinsed with PBS, pH 7.4. 200 uL of PBS with 3.5 mM *o*-nitrophenyl-β-D-galactopyranoside and 0.5% nonidet P-40 (both Sigma) was added to each well and the 96-well plates were incubated at room temperature. After 3 hr the plates were read at 420 nm on a plate-reader (Bio-Tek EL 310 or Molecular Devices). Dose response data from R-SAT assays were fitted using the equation:

$$R = \frac{D + (A - D)}{1 + (x/c)}$$

where A is the minimum response, D is the maximum response and c is EC_{50} (R is the response, x is the concentration of ligand).

3.4. Intracellular cyclic AMP assay

HEK293 were plated one day before transfection using 1×10^5 in 0.1 mL of media per well of a 96-well plate and transiently transfected as described above with hCTr DNA (20 ng/well). At 18–20 hr post-transfection, the medium was removed and the cells were incubated overnight with 200 μL/well of 2.0% cyto-SF3/0.5% fetal serum/1% PSG/DMEM. Approximately 40–44 hr post-transfection, medium was replaced with serum-free DMEM/0.1% BSA containing 0.45 mM IBMX for 30 min (0.1 mL/well), then ligands were added for an additional 15 min. The cells were lysed and the plates processed immediately using the cAMP enzyme immunoassay (EIA) assay kit (Amersham Pharmacia Biotech) according to the manufacturers instructions.

3.5. Receptor binding studies

Binding studies were carried out with ^{125}I-Calcitonin (human) (2000 Ci/mmol, Amersham Pharmacia Biotech) using membranes of HEK293 cells transiently transfected

as described above with hCTr DNA (10 μg/15 cm dish) and prepared as described previously [12] using increasing concentrations of radiolabeled ligand for saturation binding experiments and 12.5 pM radioligand for competition binding assays. Binding reactions were terminated by filtration through Type B glass fiber filters (Millipore, MultiScreen Harvest plates) presoaked for 30 min in 0.1% polyethyleneimine. Nonspecific binding was determined using 1 μM sCT (8–32).

4. Results

In order to identify novel receptor-peptide interactions, a high-throughput, cell-based functional assay (R-SAT™, [12]) was used to screen the hCTr, PTH1R, CRF1, and the GLP1 receptors against a collection of nearly 200 biologically active peptides. The peptide library was comprised of peptides identified as ligands for GPCRs and peptides whose receptor targets remain undefined, i.e. 'orphan peptides'. The primary screen was conducted using 1 μM concentration of each peptide. Expected interactions, such as calcitonin, beta-CGRP and adrenomedullin at the hCTr receptor, PTH (1–34) and PTHrP (1–34) at PTH1R receptor, CRF and Sauvagine at the CRF1 receptor, and GLP at the GLP1 receptor were readily detected (not shown). Surprisingly, the 'orphan' peptide PHM-27 [13] was active at the hCTr. This interaction was characterized further.

As shown in Fig. 1, PHM-27 displayed full efficacy at the hCTr compared with calcitonin (see Fig. 1A). In contrast, PHM-27 had no activity at the PTH1, GLP1, or CRF1 receptors (Fig. 1B–D). The pEC_{50} value for PHM-27 was approximately 8.0, considerably lower than calcitonin, but comparable to the reported affinity of PHM-27 for the VIP receptors [14,15].

To characterize the interactions of these peptides with hCTr further, we tested whether or not the calcitonin receptor antagonist sCT (8–32) could suppress ligand-induced responses at hCTr. As expected, sCT (8–32) antagonized the calcitonin-induced response (Fig. 2A). sCT (8–32) also suppressed responses to PHM-27 confirming that PHM-27 mediates functional responses through the hCTr (Fig. 2B).

The CTr couples strongly to Gs to mediate production of cyclic AMP [8]. We therefore analyzed the activities of

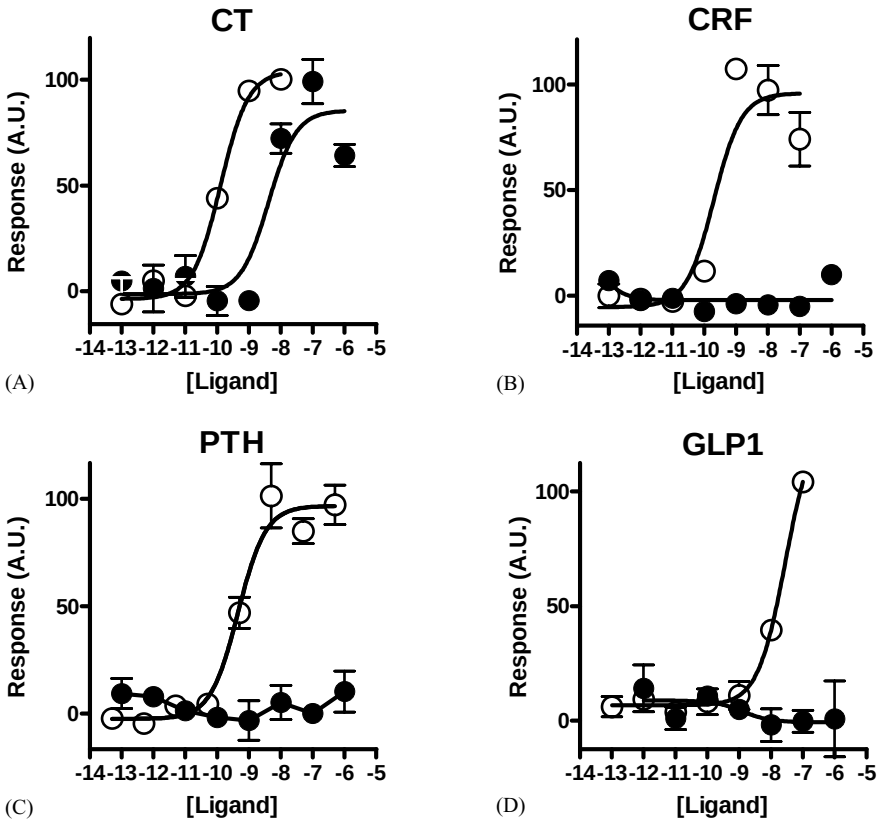

Fig. 1. Pharmacological profile of PHM-27. Receptors were transiently expressed in NIH-3T3 cells using 5 ng receptor DNA/well of a 96-well plate and functionally assayed with the indicated concentrations of ligand using receptor selection and amplification technology (R-SAT™) which measures stimulation of cellular proliferation as a functional determinant of receptor activation (see Section 3). (A) Human calcitonin receptor. Filled circles, PHM-27; open circles, calcitonin. (B) Human corticotropin releasing factor 1 receptor. Filled circles, PHM-27; open circles, CRF. (C) Human parathyroid hormone receptor 1. Filled circles, PHM-27; open circles, PTH. (D) Human glucagon-like peptide receptor 1. Filled circles, PHM-27; open circles, GLP. Data are normalized to the maximum response of the reference agonist, which is 100% and represents a 3- to 5-fold response over basal activity in each case.

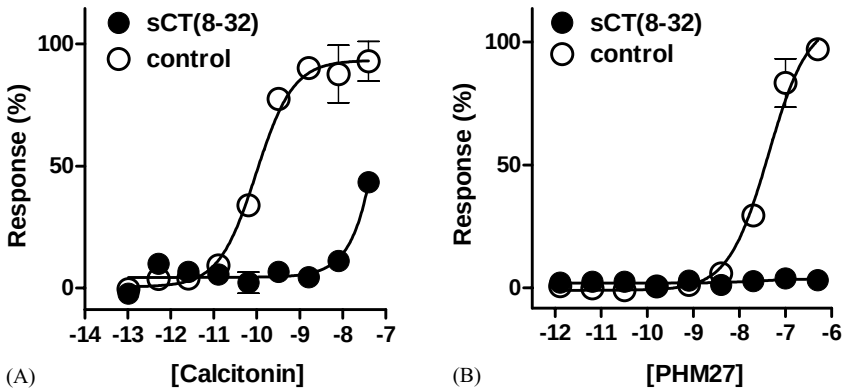

Fig. 2. Functional antagonism of calcitonin receptor responses. The indicated concentrations of ligands were tested for activity against the human calcitonin receptor in the R-SAT functional assay using NIH-3T3 cells transiently transfected with the hCTr (5 ng/well) as described in Section 3 in either the presence or absence of 1 uM salmon calcitonin (8–32). (A) Open circles, calcitonin; filled circles, calcitonin plus 1 uM sCT (8–32). (B) Open circles, PHM-27; filled circles, PHM-27 plus 1 uM sCT (8–32). Data were normalized to the maximum response of calcitonin alone, which is 100% and represents a 4-fold response over basal activity.

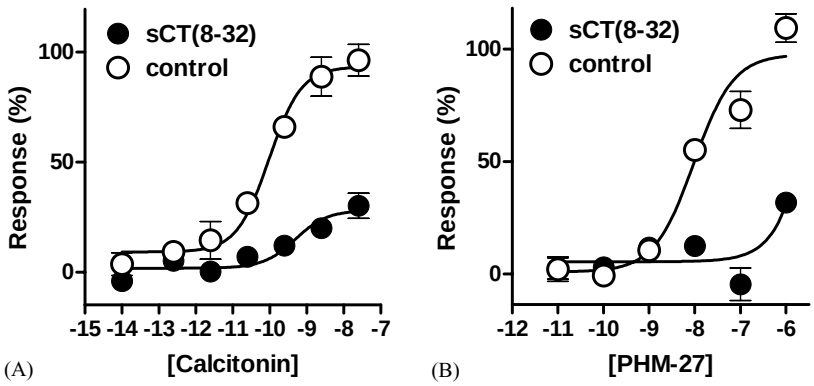

Fig. 3. PHM-27 stimulates cyclic AMP responses through the calcitonin receptor. Receptors were transiently expressed in HEK293 cells using 20 ng of receptor DNA/well of a 96-well plate and assayed for cyclic AMP production with the indicated concentrations of ligand using cyclic AMP EIA kits as described in Section 3. (A) Open circles, calcitonin; filled circles, calcitonin plus 1 uM sCT (8–32). (B) Open circles, PHM-27; filled circles, PHM-27 plus 1 uM sCT (8–32). Data were normalized to the maximum response of calcitonin alone, which is 100% and represents a 6-fold response over basal activity.

PHM-27 and CT in cyclic AMP assays. As shown in Fig. 3 and Table 1, PHM-27 displayed full efficacy at hCTr compared with calcitonin in agreement with the data obtained using R-SAT. As in the R-SAT functional assay, the responses to both calcitonin and PHM-27 could be suppressed by the calcitonin antagonist sCT (8–32) (Fig. 3B). PHM-27 did not show activity in cyclic AMP assays against cells transfected with the PTH1 receptor (not shown).

Table 1
Pharmacological profile of PHM-27

	R-SAT		cAMP	
	pEC_{50}	Efficacy (%)	pEC_{50}	Efficacy (%)
hCalcitonin	10.1	100	10.3	100
PHM-27	8.0	99	7.8	113

The indicated compounds were tested at the hCTr in the R-SAT functional assay or in cyclic AMP assays as described in Section 3. Results are the means of two or more experiments. Data were normalized to the maximum response of calcitonin alone, which is 100% and represents a 4-fold response over basal activity. Potency values are given as $-\log(EC_{50})$ or pEC_{50}.

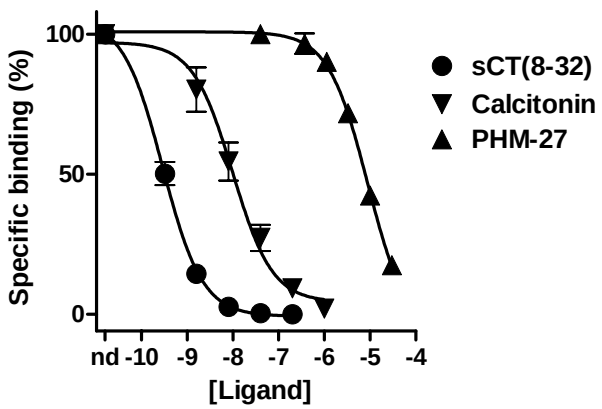

Fig. 4. ^{125}I-CT binding to the human calcitonin receptor. Membrane preparations of HEK293 cells transiently expressing the hCTr at 8.5 pmol/mg (determined by saturation binding, see Section 3) were used for competition binding experiments as described in Section 3. For competition binding assays, the membranes were pre-incubated with the indicated concentrations of peptide ligands for 1 hr on ice, and then mixed with 12.5 pM radioligand for an additional 2 hr-incubation at RT. Reactions were terminated by filter binding and bound ^{125}I-CT quantified as described in Section 3. Circles, salmon calcitonin (8–32); inverted triangles, calcitonin; diamonds, PTH; triangles, PHM-27.

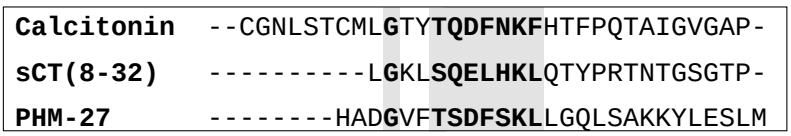

Fig. 5. Structural homology of calcitonin receptor agonists. Alignments were done using the Decypher ClustalW multiple sequence alignment.

To directly examine whether or not PHM-27 interacted with the hCTr, binding studies were performed. The hCTr was expressed in HEK293 cells at 8.5 pmol/mg and bound ^{125}I-hCalcitonin with an apparent affinity of 95 pM. As shown in Fig. 4, sCT (8–32), calcitonin itself, as well as PHM-27 were each able to displace ^{125}I-hCalcitonin with pIC_{50} values of 9.5 for sCT (8–32), 8.0 for calcitonin, and 5.1 for PHM-27.

5. Discussion

We have employed a high-throughput cell-based functional assay to screen a library of biologically active peptides against a pool of four GPCRs including hCTr, hPTH1R, CRF1, and GLP1 receptors. Surprisingly, the 'orphan peptide' PHM-27 was shown to be a potent, full agonist at the calcitonin receptor. To date, little information is available about physiological actions of PHM-27 other than it being expressed and secreted with VIP and PACAP in certain neuroblastoma and neuroendocrine tumors [16]. PHM-27 has been shown to bind heterologously expressed VIP receptors with high affinity [14,15]. Recently a receptor was cloned and proposed to be the receptor for PHM-27 [17]. We showed that PHM-27 is a full agonist at the hCTr with potency that is comparable to its activities at its other proposed targets *in vivo* [14,15,17,18].

Receptors belonging to family B GPCRs are structurally related and appear to share common mechanisms of ligand binding, utilizing at least two separable ligand/receptor binding sites [9,10,19]. To examine the possible structural basis for the cross-reactivity of PHM-27 with the hCTr, PHM-27 along with calcitonin and sCT (8–32) were aligned using the Decypher ClustalW multiple sequence alignment algorithm (see Fig. 5). Although there was limited direct sequence homology, a cluster of conserved residues was identified in the central portion of these peptides. It logically seems possible that these residues mediate the common actions of these ligands at the hCTr. In general, the N-terminus of peptide ligands for the family B (secretin-like) receptors is required for full agonist activity, and removal of the extreme N-terminal loop structure generates potent antagonists, such as sCT (8–32), CGRP (8–37), and PTH (7–32) [20–22]. However our results showing PHM-27 is a full agonist indicate that ligand residues distally located to the extreme N-terminus also mediate agonist activity at the hCTr. It would be of interest to mutate the residues conserved between human calcitonin and PHM-27 to determine which residues are critical for mediating agonist activity. Cross-reactivity can be mediated through relatively few conserved residues as has been shown for peptide agonists at the PACAP receptor [23]. Therefore it is not surprising to find additional, previously unrecognized ligand/receptor interactions within this family given the structural similarities throughout this ligand/receptor superfamily.

Calcitonin is a 32 amino acid peptide that primarily regulates calcium homeostasis. Calcitonin is secreted by the thyroid gland in response to increased levels of blood calcium and acts to reduce serum calcium levels by inhibiting osteoclast-induced bone resorption and increasing renal excretion of calcium [26]. Due to its ability to inhibit bone resorption, calcitonin has been used therapeutically to treat disorders of bone metabolism such as osteoporosis and Paget's disease. A small molecule surrogate ligand for calcitonin would be preferred due to the disadvantages of using peptide drugs. High-throughput functional screens of small molecule chemical libraries may eventually yield such leads.

The results presented above and in previous other studies underscore the advantages of using functional screens to discover novel agonists. It is unlikely that the activity of PHM-27 at the CTr would have been discovered using binding assays alone as screens. Consistently we have observed that agonist affinity measured in competition binding studies is not a reliable predictor of agonist potency in functional assays. This is consistent with observations that structural changes in calcitonin analogues can have considerable effects on potency in competition binding assays while having significantly lesser effects in functional assays [24]. Likewise, a small molecule agonist for the calcitonin receptor has been described which does not displace ^{125}I-hCalcitonin [25]. We have observed similar discrepancies in other systems between data derived from binding studies and functional studies for agonists that bind receptors in ectopic sites [12]. Ligands that bind through ectopic sites offer potentially greater selectivity since they utilize non-conserved regions of receptors, and therefore represent valuable drug leads. Future drug-discovery efforts aimed at development of non-peptide compounds for receptors that utilize peptide ligands *in vivo* will also benefit from the approaches described above.

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