Critical Cysteine Residues in Both the Calcium-Sensing Receptor and the Allosteric Activator AMG 416 Underlie the Mechanism of Action

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ABSTRACT

AMG 416 is a novel D-amino acid–containing peptide agonist of the calcium-sensing receptor (CaSR) that is being evaluated for the treatment of secondary hyperparathyroidism in chronic kidney disease patients receiving hemodialysis. The principal amino acid residues and their location in the CaSR that accommodate AMG 416 binding and mode of action have not previously been reported. Herein we establish the importance of a pair of cysteine residues, one from AMG 416 and the other from the CaSR at position 482 (Cys482), and correlate the degree of disulfide bond formation between these residues with the pharmacological activity of AMG 416. KP-2067, a form of the CaSR agonist peptide, was included to establish the role of cysteine in vivo and in disulfide exchange. Studies conducted with AMG 416 in pigs showed a complete lack of pharmacodynamic effect and provided a foundation for determining the peptide agonist interaction site within the human CaSR. Inactivity of AMG 416 on the pig CaSR resulted from a naturally occurring mutation encoding tyrosine for cysteine (Cys) at position 482 in the pig CaSR. Replacing Cys482 in the human CaSR with serine or tyrosine ablated AMG 416 activity. Decidedly, a single substitution of cysteine for tyrosine at position 482 in the native pig CaSR provided a complete gain of activity by the peptide agonist. Direct evidence for this disulfide bond formation between the peptide and receptor was demonstrated using a mass spectrometry assay. The extent of disulfide bond formation was found to correlate with the extent of receptor activation. Notwithstanding the covalent basis of this disulfide bond, the observed in vivo pharmacology of AMG 416 showed readily reversible pharmacodynamics.

Introduction

AMG 416 (Etelcalcetide, Amgen Inc., Thousand Oaks, CA) is a novel D-amino acid–containing peptide agonist of the calcium-sensing receptor (CaSR) that is currently under investigation for the treatment of secondary hyperparathyroidism (HPT) in patients receiving hemodialysis. AMG 416 acts in a fashion similar to the phenylalkylamine CaSR agonist, cinacalcet (Sensipar/Mimpara; Amgen Inc.) by decreasing circulating levels of parathyroid hormone (PTH), calcium, and phosphorus in dialysis patients with secondary HPT (Block et al., 2004; Lindberg et al., 2005; Moe et al., 2005; Martin et al., 2014b; Bell et al., 2015). However, AMG 416 differs from cinacalcet in its chemical class, intravenous route of administration, extended half-life, and thrice weekly dosing following each hemodialysis session (Martin et al., 2014b).

The CaSR, a G protein-coupled transmembrane receptor (GPCR) located on the chief cells of the parathyroid gland, plays an important role in calcium homeostasis. Increased blood calcium levels activate the CaSR, thereby suppressing PTH secretion and synthesis. In contrast, reductions in blood calcium levels reduce CaSR activity, promoting PTH synthesis and secretion. As part of a compensatory mechanism to maintain blood calcium levels within a narrow range, PTH acts on bone to increase bone resorption, and in the kidney to reduce excretion of calcium, which together modulate the amount of calcium entering the bloodstream (Rodriguez et al., 2005; Tfelt-Hansen and Brown, 2005; Goodman and Quarles, 2008). In chronic kidney disease (CKD), functional demand for PTH to rebalance calcium levels frequently leads to the development of secondary HPT (Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Work Group, 2009). Worsening secondary HPT in end-stage renal disease patients is often accompanied by parathyroid gland hyperplasia (National Kidney Foundation, 2003). Loss of sensitivity and
responsiveness to blood calcium levels in hyperplastic glands, partly as a consequence of downregulated CaSR expression (Komaba et al., 2010), results in dysregulation of PTH secretion and synthesis, and contributes to the excessive PTH levels (Felsenfeld et al., 2007) that are frequently observed in patients with secondary HPT on dialysis.

The primary pharmacodynamic (PD) effect of calcimimetics is to reduce PTH levels, either by lowering the threshold for CaSR activation in response to extracellular calcium, and/or by directly activating the CaSR itself (Nemeth et al., 1998). Reductions in calcium are a secondary PD effect of calcimimetics and are influenced by reductions in PTH (Mundy and Guise, 1999; Chen and Goodman, 2004). The first commercially available calcimimetic, cinacalcet, is an allosteric activator of the CaSR that lowers the threshold for activation by the orthosteric agonist, calcium. Cinacalcet decreases PTH, as well as calcium and phosphorus, in end-stage renal disease patients with secondary HPT (Block et al., 2004; Lindberg et al., 2005; Moe et al., 2005) Although cinacalcet is approved as a once-daily oral treatment of secondary HPT in CKD patients on dialysis, its use is limited by gastrointestinal side effects that include nausea, vomiting, and diarrhea (Sensipar Prescribing Information, Amgen Inc.) and by restricted opportunity to titrate doses owing to fixed dosage form and strength, cytochrome P450–mediated drug-drug interactions (Padhi and Harris, 2009), and diminished patient adherence (Gincherman et al., 2010). Consequently, an alternative calcimimetic therapy for secondary HPT is warranted.

AMG 416 is a synthetic eight–amino acid peptide calcimimetic that activates the CaSR in the presence of extracellular calcium in vitro but also demonstrates lower activity when extracellular calcium is absent (Walter et al., 2013). AMG 416 administration leads to dose-dependent suppression of PTH in normal and uremic rats (Walter et al., 2013), healthy adults (Martin et al., 2014a), and patients with secondary HPT on hemodialysis (Martin et al., 2014b). AMG 416 also reduces serum phosphorus and calcium levels in patients with secondary HPT on hemodialysis (Martin et al., 2014b).

The current study was undertaken to examine the mechanism of action of AMG 416. Early studies showed reductions in PTH and calcium following exposure to AMG 416 in rats, dogs, and humans, but similar PD effects were not observed in pigs. We hypothesized that one or more key amino acids in the CaSR from responsive species govern AMG 416 activity and are mutated in the pig. Through studies involving determination of the pig CaSR sequence, comparisons to CaSR sequences from responsive species, receptor activation measures with mutated forms of the receptor, and mass spectrometry methods, we have identified a critical interaction between AMG 416 and the CaSR. We will discuss the relevance of this interaction for observed pharmacological activities of AMG 416.

Materials and Methods

All animal studies were performed according to Institutional Animal Care and Use Committee guidelines and approved by each institution that conducted the studies.

Peptide Synthesis and Sequences

Peptides AMG 416 (Ac-c[c]arrr-ar-NH₂; 1048 Da), KP-2067 (Ac-carrar-ar-NH₂; 929 Da), and KP-2140 (Ac-arrrar-NH₂; 826 Da) were synthesized using the methods described previously (Walter et al., 2013). Lower case single letters in sequences represent D-amino acids and upper case is an L-amino acid.

Pharmacology Studies in Normal Rats

Male Sprague-Dawley rats with weights averaging 300 g (Charles River Laboratories International, Wilmington, MA) were administered either 3.0 mg/kg KP-2140, 3.0 mg/kg KP-2067, 0.5 mg/kg KP-2067, or saline via intravenous bolus. Blood samples were collected at predose and 1, 2, 3, and 4 hours postdose and plasma PTH was quantified using a Rat Bioactive Intact PTH ELISA (Immutopics International, San Clemente, CA) in accordance with the manufacturer’s protocol. Two-way analysis of variance (ANOVA) was used to determine statistical significance with Bonferroni adjustment for multiple comparison (GraphPad Prism v6.03; GraphPad, La Jolla, CA).

Pharmacology Studies in Normal Dogs

Single Intravenous Bolus. This study was conducted by Charles River Laboratories, (Reno, NV). Male beagle dogs aged 12 months with weights ranging from 10.5 to 11.9 kg were administered 1.5 mg/kg AMG 416 via intravenous bolus. Blood samples were collected predose and every 3 hours postdose administration for up to 48 hours to determine plasma AMG 416, PTH, and total calcium concentrations. AMG 416 was not quantified in predose samples. Plasma concentrations of AMG 416 were quantified following an ion-exchange solid-phase extraction procedure by liquid chromatography–tandem mass spectrometry (LC-MS/MS). LC separations were performed by reverse-phase high-performance liquid chromatography (Beta Basic C18 column; 2.1 mm × 50 mm; Thermo Fisher Scientific, Inc., Walkham, MA) using 0.07% trifluoroacetic acid (TFA)/0.1% formic acid and acetonitrile as mobile phase. Analyte detection was performed with a multiple reaction monitoring (MRM) method in the positive ion mode on a triple quadrupole mass spectrometer (API; AB Sciex, Framingham, MA). Plasma PTH was determined using the manufacturer’s protocol with a Canine Intact PTH ELISA kit (cat. no. 60-3800; Immutopics International). Total calcium was quantified using a Beckman SYNCHRON CX7 Automated Chemistry Analyzer. Statistical significance was determined by two-way ANOVA with Bonferroni adjustment for multiple comparison (GraphPad Prism v6.03).

Twenty-Four Hour intravenous Infusion. The study was conducted by ITR Laboratories Canada Inc., (Baie d’Urfé, Québec, Canada). A single group of male beagle dogs aged 14–16 months with weights ranging from 7.6 to 10.9 kg were sequentially administered vehicle (20 mM succinate with 0.81% sodium chloride; pH 4.5–4.6), AMG 416 at 0.192 mg/kg (8 µg/kg per hour), and then AMG 416 at 0.48 mg/kg (20 µg/kg per hour) via 24-hour intravenous infusion, with a 1-week washout period between dose groups. The same dogs were used throughout the study to minimize the use of animals and to reduce variability between treatment groups. Blood samples were collected predose and at 2, 4, 8, 12, 16, 20, and 24 hours after the start of infusion, and at 1, 3, 6, 12, 18, and 24 hours after the end of infusion to determine total calcium, plasma PTH, and AMG 416 plasma concentrations. AMG 416 was not quantified in predose samples. Statistical analysis and plasma concentrations of AMG 416 and PTH were determined as described in the dog single intravenous bolus study. A Roche/Hitachi 912 Biochemistry Analyzer using o-cresolphthalein complexone was used to quantify total calcium.

Pharmacology Studies in Normal Pigs

The study was conducted by Avanza Laboratories (Gaithersburg, MD). Male Gottingen minipigs with weights ranging from 8.9 to 11.2 kg were administered either 0.3 mg/kg, 1 mg/kg, or 5 mg/kg AMG 416 via intravenous bolus. Blood samples were collected predose and at 0.08, 2, 4, 8, 12, 18, 24, 36, and 48 hours postdose administration to determine plasma AMG 416, PTH, and total calcium concentrations. AMG 416 was not quantified in predose samples. Statistical analysis and plasma concentrations of AMG 416 and PTH were determined as described in the dog single intravenous bolus study.
determine total calcium and AMG 416 plasma concentrations. Plasma concentrations of AMG 416 were measured as described for the dog studies. Two-way ANOVA was used to determine statistical significance with Bonferroni adjustment for multiple comparisons.

**Pig CaSR DNA Sequence Determination**

Complete sequencing of the pig CaSR DNA was performed by Agilent Inc. (San Diego, CA) and is further detailed in Supplemental Fig. 1. Pig CaSR sequence has been deposited in GenBank with accession number KT309043.

**DNA Constructs for Wild-Type and Mutant CaSRs**

Native and mutant CaSR expression constructs were synthesized as open reading frames (ORFs) and subcloned into a mammalian expression vector (vector pJ609; DNA 2.0, Inc.; Menlo Park, CA). Each ORF contained an amino-terminal leader sequence, followed by a FLAG epitope tag, linked to the first amino acid (tyrosine) normally found in the mature native CaSR. All amino acid position designations (e.g., 482) were with respect to the native (nontagged) human calcium-sensing receptor (hCaSR) sequence. Only a single residue substitution was made relative to the native amino acid sequence for each mutant construct. Constructs expressed and analyzed in these studies were: 1) native hCaSR, which encodes a cysteine at position 482 (C482); 2) mutant hCaSR (C→Y482), a tyrosine substitution for the wild-type cysteine at position 482; 3) mutant hCaSR (C→S482), a serine substitution for the wild-type cysteine at position 482; 4) native pig CaSR (pCaSR), which encodes a tyrosine at position 482 (Y482); and 5) mutant pCaSR (Y→C482), a cysteine substitution for the wild-type tyrosine at position 482 in pig CaSR. All CaSR constructs were completely sequenced and confirmed by DNA 2.0, Inc. (Menlo Park, CA).

**Expression and Analysis of DNA Constructs**

Human embryonic kidney cells (HEK293T) were transiently transfected with Lipofectamine (Life Technologies, Carlsbad, CA) and one CaSR construct per transfection. For any given transfection session each of the CaSR constructs listed in either Fig. 3 or 4 was run in parallel. A separate transfection was run on a different date for each of the CaSR constructs listed in either Fig. 3 or 4, with four (unless otherwise noted) replicates. Following transfection, cells were replated at 32–34 hours in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine and assayed for inositol 1-phosphate (IP-1) production at 48 hours.

**Analysis of KP-2067 and KP-2140 in a Stable Cell Line Expressing hCaSR**

Dose-response curves for KP-2067, KP-2140, and calcium were measured in a clonal cell line of HEK293T stably transfected with the human CaSR (Multispan, Hayward, CA) and one CaSR construct per transfection. For any given transfection session each of the CaSR constructs listed in either Fig. 3 or 4 was run in parallel. A separate transfection was run on a different date for each of the CaSR constructs listed in either Fig. 3 or 4, with four (unless otherwise noted) replicates. Following transfection, cells were replated at 32–34 hours in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine and assayed for inositol 1-phosphate (IP-1) production at 48 hours.

**IP-1 Production in HEK293T Cells Expressing CaSR**

CaSR activation was measured in vitro by quantifying the accumulation of IP-1, a surrogate of inositol (1,4,5)-triphosphate production and Gq stimulation, as previously described (Walter et al., 2013) with the following modification: 500 μM 2-mercaptoethanol was added to the base stimulation buffer for AMG 416 but not for calcium, KP-2067, or KP-2140. This concentration of 2-mercaptoethanol was chosen on the basis of previous studies that provided optimal AMG 416 activity, yet had no measurable effect on stimulation of the hCaSR by either calcium or KP-2067.

Each replicate is a mean value of IP-1 measured independently in four separate wells. Mean IP-1 values from each replicate were entered individually in GraphPad Prism v6.03, and means ± S.E.M. were plotted.

**Determination of Fractional Occupancy of CaSR by Agonist Peptide**

**Ligand Binding to the CaSR.** A clonal cell line of HEK293T stably transfected with the human CaSR (Multispan) was grown to 90% confluence in glutathione-free RPMI-1640 media (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 1 μg/ml puromycin. Cells were washed with Ca2+/Mg2+-free PBS, dislodged with 500 μM EDTA, and following a wash with Ca2+/Mg2+-free PBS and complete aspiration resuspended in base stimulation buffer (Walter et al., 2013) containing 1.2 mM Ca2+. Cell suspensions were incubated with vehicle (saline) or KP-2067 at 8 μM (EC50), 25 μM (EC80), or 80 μM (EC90) for 30 minutes at 37°C with constant inversion. Cells were washed with cold base stimulation buffer, aspirated completely and then lysed with 20 mM phosphate-citrate buffer (pH 6) containing 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, and 1× Roche Complete Mini Cocktail (cat. no. 04 693 124 001; Roche Diagnostics, Indianapolis, IN). Lysates were centrifuged twice at 4°C for 10 minutes at 14,000g and the pellet was harvested.

The pellet was reconstituted in a digestion buffer containing 4 M urea, 20 mM ammonium carbonate stock (pH 6), and ProteinMax (no. V92072; Promega, Madison, WI). Free sulfhydryl groups were modified by the addition of acrylamide (0.1 M) followed by a 30-minute incubation at room temperature. Samples were protelyzed by the addition of sequencing grade chymotrypsin (cat. no. 11418467001; Roche). The digest reaction was quenched by acidification with 1% formic acid and insoluble material was removed by centrifugation. This chymotryptic digested mixture was analyzed by LC-MS/MS.

**Mass Spectrometric Analysis of Ligand Binding to the hCaSR.** Ligand-receptor adducts were monitored by LC-MS/MS. Peptide standards representing the putative cysteine-containing chymotryptic peptides of the CaSR were synthesized and reacted with either KP-2067 to represent the disulfide pair with ligand, or with acrylamide to represent the unmodified free sulfhydryl form of CaSR. The resulting synthetic conjugates were infused into the triple quadrupole mass spectrometer (TSQ Quantum Ultra; Thermo Fisher Scientific, San Jose, CA) and electrospray ionization was performed in the positive ion mode. The resulting ions were subjected to collision-induced dissociation, and secondary spectra tandem mass spectrometry (MS/MS) were acquired to identify the fragmentation pattern for each conjugate. Representative MS/MS spectra of acrylamide-modified and KP-2067/CaSR (DECGDL) peptide conjugates are shown in Fig. 5. The fragmentation data (precursor ion m/z, collision energy, and product ion m/z) from these standards were used to identify the corresponding species from MRM assay of peptide digest samples from cellular assays.

Chromatographic separation of the chymotrypsin digests was performed by reverse-phase high-performance liquid chromatography on an Agilent 1100 system. A reverse-phase column (Bio-Rad C18, 5 μM, 2.1 × 50 mm; Thermo Fisher Scientific) maintained at 40°C was employed. The mobile phase flowing at 400 μl/min was composed of 0.1% TFA in water (solvent A), and 0.1% TFA in acetonitrile (solvent B). The solvent gradient was as follows: 0–1 minute, 100% A; 1–5 minutes, 100–20% A; 5–6 minutes, 20% A; 6–7 minutes, 20%–100% A; 7–10 minutes, 100% A. The total run time was 10 minutes.

Analyte detection was performed in the positive ion mode employing a MRM method on a triple quadrupole mass spectrometer (TSQ Quantum Ultra). The monitored mass transitions were as follows: acrylamide-modified DECGDL, m/z 722.2 → m/z 446.8 and 346.8 and DECGDL-KP-2067 conjugate, m/z 789.9 → m/z 448.2 and 464.2. Under these conditions, the acrylamide-modified CaSR peptide eluted at 4.88 minutes and the KP-2067/CaSR peptide conjugate eluted at 5.02 minutes. All data were acquired and processed using Xcalibur software (Thermo Fisher Scientific) and peak areas were computed from the MRM chromatograms.
The mass spectrometry responses for the CaSR peptide bound to both acrylamide and KP-2067 were assumed to be the same. The fractional occupancy of KP-2067 was calculated as follows:

\[
\text{Fractional occupancy (\%) = 100 \times \frac{\text{Peak Area}_{\text{KP-2067}}}{\text{Peak Area}_{\text{acrylamide}} + \text{Peak Area}_{\text{KP-2067}}}}
\]

The subscripts acrylamide and KP-2067 represent the species conjugated to the CaSR peptide.

**Results**

**The Role of Cysteine in the Peptide Agonist for Activation of the CaSR**

**In Vitro hCaSR Studies.** Three synthetic peptides were selected to help elucidate the critical components required for hCaSR activation (Fig. 1A). AMG 416 is a D-amino acid peptide containing an N-terminal cysteine residue disulfide linked to L-cysteine (sequence structure = Ac-c[C]arrrar-NH2). KP-2067 is a structurally related peptide (Ac-carrarar-NH2) in which the N-terminal D-cysteine residue is unconjugated and carries a free sulfhydryl group. KP-2140 (Ac-arrarr-NH2) does not have a cysteine at the N-terminus. Each peptide contained the identical arginine-alanine composition and spacing that is found in the D-peptide backbone of AMG 416. A clonal cell line of HEK293T overexpressing the hCaSR was used to examine the responses to the CaSR orthosteric ligand, calcium, and the peptides KP-2067 and KP-2140 (Fig. 1A). The peptide dose-response curves were each run in the presence of 1.2 mM calcium. The EC50 for calcium was calculated to be 4.6 ± 0.04 mM, within the range of published values (Breitwieser and Gama, 2001; Conigrave et al., 2004; Wei et al., 2014). The measured EC50 for KP-2067 was 18.4 ± 0.07 μM, and the calculated Hill coefficient was 1.5 ± 0.33 similar to the reported values of 25 μM and 1.1, respectively, for AMG 416. These Hill coefficients are consistent with a single binding site for agonist peptide on the CaSR. The dose-response curve for calcium was steeper than that for KP-2067, with a Hill coefficient of 3 to 4, consistent with the presence of multiple binding sites for calcium on the CaSR (Wei et al., 2014). In contrast, IP-1 levels remained at or near baseline value when incubated with KP-2140. The mean maximal IP-1 induction for KP-2067 was 29-fold higher than the baseline value.

**Rat Pharmacodynamic Studies with Peptides**

Intravenous bolus administration of KP-2067 at 3 mg/kg and 0.5 mg/kg significantly reduced plasma PTH to below 5% of predose values at 1 hour after dosing (Fig. 1B). At 4 hours postdose, the plasma PTH levels in rats administered 3 mg/kg and 0.5 mg/kg KP-2067 were 4.6 ± 0.9% (P < 0.05) and 36.6 ± 15.3% (P < 0.001) of predose values, respectively. Mean plasma PTH levels for rats dosed with 3 mg/kg KP-2067 were significantly different (P < 0.001) from the saline control group at all measured time points. KP-2140 dosed at 3 mg/kg resulted in no significant change from predose plasma PTH values throughout the 4-hour study, and did not differ significantly from saline control.

**Dog and Pig Pharmacokinetic/Pharmacodynamic Studies with AMG 416**

Preliminary pharmacokinetic/pharmacodynamic relationships were established in dogs following administration of a single 1.5 mg/kg AMG 416 intravenous bolus dose. Plasma concentrations of AMG 416 were highest at the first measured time point postdose (3 hours) and decreased gradually over 48 hours (Fig. 2A). Total serum calcium decreased to 68 ± 1.6% of predose values (P < 0.001) at 24 hours after dosing, and returned to 89.7 ± 0.5% of predose values (P < 0.001) at 48 hours after dosing (Fig. 2B). PTH also declined in dogs dosed with AMG 416 (Supplemental Fig. 2). AMG 416 was detected in all plasma samples from pigs dosed with AMG 416. Plasma concentrations were consistent within dose groups with minimal interanimal variation (Fig. 2C). AMG 416 dose-pharmacokinetic exposure relationships were similar between the dog and pig (compare Fig. 2, A and C). Serum calcium levels did not change significantly over the sampling period, thus showing an absence of AMG 416 PD effect in pigs (Fig. 2D). This absence of an AMG 416 effect in pigs (even at a very high dose of 5.0 mg/kg) contrasts with results observed for normal dogs, rats, and humans. Data for dogs (Fig. 2B) at 9 hours following a dose of AMG 416 at 1.5 mg/kg show reduction in total blood calcium of 19.0 ± 1.4% (mean ± S.E.M.) (P < 0.001; paired Student’s t test) relative to predose values. Data from rats (Walter et al., 2013) describe significant reductions in total blood calcium at 8 hours relative to predose (mean ± S.E.M.) values of 6.2 ± 0.8%, 12.3 ± 0.6%, 25.2 ± 1.3% (all P < 0.0001; paired Student’s t test) for 0.3 mg/kg, 1 mg/kg, and 3 mg/kg dose groups, respectively. Lastly, data from healthy young male human subjects (Martin et al., 2014a) show significant reductions in blood ionized calcium levels of 16.3 ± 1.0% (mean ± S.E.M.; P < 0.0001;
paired Student's t test) at 9 hours following an intravenous dose of 5 mg AMG 416 relative to predose values. The absence of a PD response in pigs, in the presence of a normally effective exposure level of AMG 416, led to further investigations into the cause.

The Role of Cysteine 482 in the CaSR for Activation by AMG 416 Sequence and Topology of the Pig CaSR

The pCaSR was completely sequenced to explore whether the lack of PD response to AMG 416 in pigs was associated with receptor composition. The entire 3240-nucleotide open reading frame (ORF) of the pCaSR encodes 1079 amino acids and a stop codon (Supplemental Fig. 1). This sequence has been deposited into GenBank with accession number KT309043.

As is typical of Class C GPCRs (Conigrave and Hampson, 2010), the mature (surface-expressed) pCaSR contains three major domains: the large extracellular domain (ECD; 587 amino acids), the heptahelical or transmembrane domain (245 amino acids), and the cytosolic or intracellular domain (228 amino acids). Two of the three CaSR domains are highly conserved (> 90%) across the four species receiving AMG 416 (human, rat, dog, pig) with amino acid identities of 94.7% in the ECD and 97.7% in the heptahelical domain, but a lesser 73.8% conservation in the cytosolic domain. The ECD further comprises two subdomains: the amino terminal Venus fly trap (VFT) containing 509 amino acids and the carboxy-terminal cysteine-rich region (CRR) containing 78 amino acids.

Candidate Ligand Binding Residues: Amino Acids Not Found in the Pig

Table 1 shows all nine amino acid residues unique to the pCaSR ECD that are not found in the CaSR ECD from human, dog, or rat. All of these residues are located within the VFT

**TABLE 1**

Unique pig residues within the VFT subdomain of the pCaSR ECD

<table>
<thead>
<tr>
<th>Amino Acid Residues</th>
<th>Human</th>
<th>Dog</th>
<th>Rat</th>
<th>Pig</th>
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<td>Aspartate</td>
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subdomain. Of particular note was the tyrosine at position 482 in the pCaSR (numbering per hCaSR) instead of the equivalent cysteine in the CaSR of each of the species which respond to AMG 416. There are 19 orthologous cysteines in the CaSR ECD of all responsive species (10 in the VFT and 9 in the CRR). Owing to a tyrosine at position 482, pigs have only 18 of the otherwise orthologous cysteines. Cysteine in the CaSR is a logical binding partner to the critically important cysteine in the agonist peptide. Therefore, investigations focused on cysteine 482 (Cys482).

DNA Construct Studies—Cys482 and CaSR Activation

Replacing cysteine at position 482 in the hCaSR with either tyrosine or serine eliminated activation by peptide agonists but did not affect the response to the orthosteric ligand, calcium (Fig. 3A). The CaSR responses to peptide agonists KP-2067 (Fig. 3B) and AMG 416 (Fig. 3C) differed among the three receptor constructs. A cysteine at position 482 was required for peptide agonist activation of the hCaSR. Peptide agonists were not active when cysteine 482 was replaced by either serine (conservative substitution) or tyrosine (the native residue in pigs). In contrast, Fig. 3A shows that responses to calcium were similar among transfectants for each of three CaSR constructs: native human CaSR (Cys482), one mutant human CaSR (Tyr482), and a second mutant human CaSR (Ser482). Expression analysis using immunostaining of the FLAG epitope (Sigma) and flow cytometry analysis corroborated the calcium activation data and showed similar levels of expression for each construct (Supplemental Fig. 3).

To test whether cysteine at position 482 is sufficient to provide a gain of activation of the pCaSR to agonist peptide, the full pig CaSR was studied with either a native (tyrosine) or substituted (cysteine) residue at position 482. All of the other amino acid residues in the native pCaSR ORF were maintained. Native human CaSR was used as a positive control for each transfection experiment. Figure 4A shows that calcium responses in each of the two pCaSR constructs were largely indistinguishable from that of the hCaSR, and that either tyrosine or cysteine at position 482 in pCaSR led to a normal response to the orthosteric ligand, calcium. Expression analysis using FLAG confirmed similar levels of expression for each construct. (Supplemental Fig. 4). Despite the normal in vitro response to calcium, native pCaSR (with tyrosine) was not activated by KP-2067 (Fig. 4B) or AMG 416 (Fig. 4C). However, complete activation of the pCaSR by both AMG 416 and KP-2067 was gained by simply substituting a cysteine at position 482 in the native pig sequence. The degree of mutant pCaSR (with Cys482) activation by agonist peptide was indistinguishable from activation seen with the hCaSR (Fig. 4, B and C).

Identifying the Presence of a Disulfide Linkage in the Human CaSR Peptide Conjugate

Agonist peptide products quantified by MS/MS in these studies are consistent with the presence of a disulfide bond

Fig. 3. Response to activation of native and point mutant hCaSR by the orthosteric agonist calcium and agonist peptides. IP-1, a measure of Gq activation, was quantified. Transiently transfected HEK293T cells [(■) native human (Cys482) CaSR cells (n = 4); (○, ○) mutant human Ser482 CaSR cells (n = 4); and (▲, ▲) mutant human Tyr482 CaSR cells (n = 4)] were stimulated (A) with calcium (closed symbols) or without calcium added (open symbols). Mean ± S.E.M. shown: (B) in the presence of 1.2 mM calcium either with (closed symbols) or without (open symbols) agonist peptide KP-2067 (n = 4; mean ± S.E.M.); (C) in the presence of 1.2 mM calcium either with (closed symbols) or without (open symbols) agonist peptide AMG 416 (n = 2; mean ± S.E.M.).
between the peptide and the CaSR. The proposed structures of the CaSR products and the dissociation mechanisms of the D-peptide as a disulfide conjugate with a L-cysteinyl peptide (or L-cysteine) are shown in Fig. 5B with a representative mass spectrum. Under MS/MS conditions the protonated peptide conjugate yields major products resulting from sulfur-carbon (S–C) and sulfur-sulfur (S–S) bond cleavages. The major product ion for the KP-2067/CaSR peptide conjugate was \( m/z \) 448.33; the ion was formed upon loss of sulfur and led to formation of a dehydroalanine analog of KP-2067. The product ion seen with \( m/z \) 464 was the result of S–S bond cleavage in the disulfide conjugate, whereas the product ion at \( m/z \) 481 was formed from S–C cleavage in the DECGDL peptide from the CaSR (chymotryptic peptide 480–485). The DECGDL peptide which is unmodified by agonist peptide was characterized following modification of the cysteine with acrylamide. This peptide is shown in Fig. 5A with a representative spectrum.

**Occupancy of hCaSR Cysteine 482 is Proportional to Concentrations of Peptide Ligand**

HEK293 cells expressing the hCaSR were incubated with KP-2067 at concentrations roughly reflecting the EC\(_{20}\) (8 \( \mu \)M), EC\(_{50}\) (25 \( \mu \)M), and EC\(_{80}\) (80 \( \mu \)M) (cross-reference Fig. 1A). Binding of KP-2067 to cysteine 482 was monitored by using MRM mass spectrometry. The disulfide nature of the linkage was confirmed by the formation of the dehydroalanine ligand product after collisional activation of peptide conjugates in the mass spectrometer. Fractional occupancy of Cys482 in the CaSR (% bound) was determined by comparing the relative abundance of disulfide linked cysteine 482 to the sum of both acrylamide-modified (unbound) cysteine 482 and bound (agonist peptide disulfide–linked) fragments liberated after chymotryptic digestion. Both peptide conjugates were analyzed simultaneously by LC-MS/MS from a single sample. The results from this analysis are summarized in Table 2.

Other cysteine residues at positions 101, 358, 395, 541, 545, and 598 in the ECD of the hCaSR exhibited only low, variable levels of modification by KP-2067. For all of the other cysteines, modification by agonist peptide corresponded to fractional occupancy less than 1% at the EC\(_{80}\) peptide concentrations, was not seen in all replicate experiments, and no binding was observed at EC\(_{20}\) or EC\(_{50}\) levels. This degree of modification is consistent with nonspecific binding of agonist peptide.

**AMG 416 Exhibits Readily Reversible Pharmacodynamics**

Dogs were infused for 24 hours with vehicle or AMG 416 at either 0.192 mg/kg (8 \( \mu \)g/kg per hour; \( n = 4 \)) or 0.480 mg/kg (20 \( \mu \)g/kg per hour; \( n = 4 \)). Steady state levels of AMG 416 were reached by the end of infusion for both doses and declined thereafter (Fig. 6A). Plasma concentration of AMG 416 was dose-dependent, as indicated by an average of 2.5 ± 0.1-fold.
difference between doses over the course of the infusion. Plasma PTH levels were inversely correlated with AMG 416 plasma concentrations. Although at times plasma PTH levels showed substantial interanimal variability in the vehicle control, AMG 416 still produced significant reductions in PTH compared with vehicle after the start of infusion: 0.480 mg/kg dose, at 2, 8, 12, and 16 hours (P < 0.05), 0.192 mg/kg dose, at 12 hours (P < 0.05). Plasma PTH began to return to preinfusion levels immediately following the end of infusion, and was inversely correlated with AMG 416 plasma levels (Fig. 6B).

At 12 hours postinitiation of infusion, total calcium decreased by approximately 3.8% and 9.8% (P < 0.01) with 0.192 mg/kg and 0.480 mg/kg AMG 416 doses, respectively, relative to preinfusion values (Fig. 6C). Maximal AMG 416-induced calcium reductions relative to preinfusion values were 12.9% (P < 0.001) with 0.192 mg/kg at 25 hours and 23.4% (P < 0.001) with 0.480 mg/kg at 24 hours. Total
calcium for the 0.480 mg/kg AMG 416-dosed group differed significantly ($P < 0.05$) from that of the vehicle-dosed group at every time point evaluated after the start of infusion. Significant differences ($P < 0.05$) in total calcium between the 0.192 mg/kg AMG 416-dosed group and vehicle-group were observed at 12 hours after the start of infusion through 36 hours.

**CaSR Cysteine 482 and Other Related GPCRs**

**GPCR Alignment.** There are 23 independent Class C GPCRs, some with known functions [two receptors that are activated by calcium (CaSR, GPRC6a), eight metabotropic glutamate receptors (mGluR1–8), three taste receptors [TAS1R1-3], and a mouse pheromone receptor (V2R) that does not appear to have a human counterpart], and others with orphan status (GPR156, GPR158, GPR179, GPR5A/RAIG1, GPR5B/RAIG2, GPR5C/RAIG3, GPR5D/RAIG4). Two other gene products considered members of the Class C GPCR family are the GABA B1 and GABA B2 subunits, which together form the heterodimeric GABA B receptor. All but the orphan members of the Class C GPCR family have large VFT domains, and all but the GABA B1 and B2 subunits have additional CRRs located between the amino terminal VFT domain and the heptahelic transmembrane domain (IUPHAR/BPS, 2014; Pawson et al., 2014). A regional view of an amino acid alignment from all human GPCRs having a VFT was completed with a focus on the third interlobe hinge strand, and cysteine 482 of the CaSR (Fig. 7). First, using full-length human ORFs, a global alignment grouped the VFT and CRR, as well as the heptahelic and intracellular domains of every GPCR with a known VFT in the extracellular domain. A more refined alignment of the third strand in the hinge of the VFT was subsequently generated using three main refinement criteria: 1) Each VFT is in strict register with the downstream CRR, with good alignment of the nine cysteines within those subdomains (alignment view not shown). Exception: GABA B subunits do not have a CRR; 2) Structural information for four independent members of the Class C GPCRs demonstrated identical positioning of landmarks in three-dimensional space within the alignment (see boxes within columns 1, 3, 5, and 7); 3) Alignment of highly conserved residues was observed within the third hinge strand, which included two entirely conserved phenylalanines (columns 2 and 4) and a largely conserved glycine-anion dipeptide (column 6).

**TABLE 2**

<table>
<thead>
<tr>
<th>KP-2067 (μM)</th>
<th>Mean Percent Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>25</td>
<td>5.3 ± 3.2 a</td>
</tr>
<tr>
<td>80</td>
<td>12.1 ± 4.4 a</td>
</tr>
</tbody>
</table>

ND, Not detected.

*S.E.M.

**Fig. 6.** Twenty-four hour intravenous infusion of AMG 416 in the dog. Dogs were infused with AMG 416 at (□), 0.192 mg/kg ($n = 4$) or (■), 0.480 mg/kg ($n = 4$), or with (▲), vehicle. (A) Log plasma concentration of AMG 416 (ng/ml), (B) plasma PTH (pg/ml), and (C) total calcium (mg/dl) were measured over 48 hours. Mean ± S.E.M. shown.
Cysteine 482 is within the apex of a second turn in the third hinge strand commonly found in all four Class C GPCR structures. This hinge region is also very near the proposed location for the high affinity calcium binding site in the CaSR (Huang et al., 2009). Examination of all other Class C GPCRs with VFTs indicates that none of them contains a cysteine in this same hinge region.

To support the location of Cys 482 on the basis of the alignment-based model, an independent structural model of the hCaSR was developed. This model also places Cys482 in the third hinge strand of the human CaSR (Fig. 8).

**Discussion**

Stimulation of the CaSR by AMG 416 requires cysteines in both the agonist peptide and the CaSR. The requirement for a cysteine in the agonist peptide was independently determined from head-to-head comparisons with KP-2067 and...
Many naturally occurring mutations have been described in the ECD of the CaSR that either increase or decrease the potential for receptor activation (Hu and Spiegel, 2007). In the VFT domain alone over 20 independent inactivating mutations lead to decreased CaSR activation. Most of these mutations are to amino acid residues distinct from those believed to bind directly to calcium or other natural CaSR agonists (Zhang et al., 2002; Huang et al., 2007; Huang et al., 2009; Khan and Conigrave, 2010; Zhang et al., 2014). With the CaSR demonstrating a tendency for a high degree of inactivating mutations not directly involved in ligand binding, it is possible that substitutions for cysteine 482 would also fall within this class of mutations. To differentiate between direct or indirect influences of cysteine 482 on activity of AMG 416, we quantified a direct interaction between the agonist peptide and cysteine 482 by disulfide bond formation and determined that the extent of this interaction correlated with pharmacological activity. Under conditions of increasing ligand exposure and elevated CaSR activity, agonist peptide demonstrated increased fractional occupancy of cysteine 482. We conclude that the importance of cysteines in both agonist peptide and CaSR is attributable to a disulfide linkage between the two that is required for receptor activation by AMG 416.

Cysteine 482 has a nonessential role in normal CaSR function. Earlier studies using scanning mutagenesis of cysteines within the CaSR did not demonstrate activity dependent on cysteine 482 (Ray et al., 1999; Hu et al., 2001; Zhang et al., 2001). Native pCaSR is fully responsive to calcium and it is presumed that pigs are able to satisfy PTH control and other physiologic demands with a CaSR that does not have cysteine at position 482.

Cysteine 482 is predicted to reside in a region of the CaSR that is believed to be intimately involved in receptor activation for Class C GPCRs. Most, but not all, of the known agonist interaction sites for the CaSR are mapped to the large ECD, and principally within the VFT domain (Khan and Conigrave, 2010; Zhang et al., 2014). Of exception is cinacalcet, a member of the phenylalkylamine class of calcimimetics that is believed to bind within the heptahelical domain, and not within the ECD of the CaSR (Rodriguez et al., 2005). The VFT domain is characterized by two large lobes hinged together by three strands crossing between the lobes (Fig. 8). X-ray crystallography of mGlurR1 (Kunishima et al., 2000; Tsuchiya et al., 2002) and mGlurR3 (Muto et al., 2007) and mutational analysis of TASSR1 and TASSR3 (Zhang et al., 2008; Li, 2009; Zhang et al., 2010) support a high affinity binding site for orthosteric ligands between the lobes and near the hinge of the VFT domain. Upon occupancy of Class C GPCR by orthosteric agonists, the hinge provides a flex point by which closure of the lobes is able to direct open and closed conformations in the protomeric receptor complex (Kunishima et al., 2000; Bessis et al., 2002; Tsuchiya et al., 2002). Closed conformations of the dimeric complex lead to receptor activation and initiation of signal transduction. Cysteine 482 is predicted to reside in the middle of the third hinge strand, in the interface between the two CaSR protomers. Whereas there is a cysteine in this location for the CaSR of most species (e.g., human, dog, mouse, rat, and rabbit), no other Class C GPCR have cysteines in this location. Owing to the importance of cysteine 482 for AMG 416 activity, it is not expected that AMG 416 will have activity against other members of this GPCR family. In support of this, AMG 416 is inactive against the rat mGlurR1 when overexpressed in HEK293T cells (data on file, Amgen).

The observed in vivo pharmacology of AMG 416 supports rapid and readily reversible pharmacodynamics, suggesting that the covalent disulfide bond between agonist peptide and
the CaSR is labile and does not provide slow off rates. Following a 24-hour infusion of two different doses of AMG 416 in dogs, plasma PTH began to return to preinfusion levels immediately upon cessation of infusion. The time to recovery of PTH levels toward baseline was inversely and principally correlated with AMG 416 plasma exposure levels, and there were no unusual delays in PTH recovery. This recovery behavior is expected for drugs that are not tightly associated with their targets, and which includes a drug that exhibits a readily dissociable covalent bond. Similar readily reversible PD behavior for AMG 416 has been shown in two other responsive species, human (Martin et al., 2014a, b) and rat (Walter et al., 2013, 2014). The data support the concept of an environment at the parathyroid gland cell surface that is balanced for reductive and oxidative modification of the cysteines in AMG 416 and position 482 of the CaSR.

In conclusion, the calcimimetic actions of AMG 416 are mediated through direct binding to the CaSR, which leads to reductions in circulating levels of PTH as well as calcium. In responsive species, agonist peptide binding to the CaSR is mediated, in part, by a covalent disulfide bond between the D-cysteine in AMG 416 and cysteine 482 of the CaSR, which appears to be labile in vivo as evidenced by readily reversible PDs. The AMG 416 binding site on the CaSR determined by cysteine 482 is separate from the binding sites for the orthosteric agonist calcium. Thus, binding of AMG 416 is topographically distinct from calcium, and AMG 416 is an allosteric activator of the CaSR.

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Conducted experiments: Alexander, Hunter, Dong, Subramanian, Tomlinson.

Contributed new reagents or analytic tools: Hunter, Tomlinson.

Performed data analysis: Alexander, Hunter, Walter, Subramanian, Tomlinson.

Wrote or contributed to the writing of the manuscript: Alexander, Hunter, Walter, Maclean, Baruch, Raju, Tomlinson.

References


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