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Calcium-Sensing Receptor Autoantibodies in Patients with Autoimmune Polyendocrine Syndrome Type 1: Epitopes, Specificity, Functional Affinity, IgG Subclass, and Effects on Receptor Activity

Mahmoud Habibullah,* Julie A. Porter,* Nicolas Kluger,† Annamari Ranki,‡ Kai J. E. Krohn,§ Maria L. Brandi,§ Edward M. Brown,§ Anthony P. Weetman,* and E. Helen Kemp*

A major manifestation of autoimmune polyendocrine syndrome type 1 (APS1) is hypoparathyroidism, which is suggested to result from aberrant immune responses against the parathyroid glands. The calcium-sensing receptor (CaSR), which plays a pivotal role in maintaining calcium homeostasis by sensing blood calcium levels and regulating release of parathyroid hormone (PTH), is an autoantibody target in APS1. In this study, the aim was to characterize the binding sites, specificity, functional affinity, IgG subclass, and functional effects of CaSR autoantibodies using phage-display technology, ELISA, and bioassays. The results indicated that CaSR autoantibody binding sites were at aa 41–69, 114–126, 171–195, and 260–340 in the extracellular domain of the receptor. Autoantibodies against CaSR epitopes 41–69, 114–195, and 260–340 were exclusively of the IgG1 subclass. Only autoantibodies recognizing CaSR epitopes 114–126 and 171–195 affected receptor activity; inositol-phosphate accumulation was increased significantly in HEK293-CaSR cells, and PTH secretion from PTH-C1 cells was reduced significantly when either were incubated with purified Ab and CaSR compared with CaSR alone. In conclusion, although the majority of APS1 patients do not have CaSR-stimulating autoantibodies, the hypoparathyroid state in a small minority of patients is the result of functional suppression of the parathyroid glands. The Journal of Immunology, 2018, 201: 3175–3183.

Hypoparathyroidism is a major manifestation of autoimmune polyendocrine syndrome type 1 (APS1; 240300) and is believed to result from aberrant immune responses against the parathyroid glands (1). Two parathyroid autoantigens have been defined so far in APS1: the calcium-sensing receptor (CaSR) and NACHT leucine-rich-repeat protein 5 (NALP5). CaSR autoantibodies have been identified in up to 86% of APS1 patients and have also been detected in some patients with idiopathic hypoparathyroidism (2–5). In contrast, NALP5 autoantibodies appear to be confined to patients with APS1, with a frequency of 30–40% (6, 7).

The CaSR has a central role in controlling parathyroid hormone (PTH) secretion in response to changes in extracellular calcium concentration (8). In some patients, CaSR autoantibodies that affect the functional activity of the receptor have been identified; CaSR-specific IgG from patients with PTH-dependent hypercalcemia have been shown in vitro to inhibit Ca2+-stimulated phosphorylation of ERK1/2 and accumulation of inositol phosphates and to increase PTH release from cultured parathyroid cells (9–12). Such blocking autoantibodies may inhibit CaSR signaling and cause secretion of PTH from the parathyroid even under conditions in which serum calcium is high (10). In contrast, two patients each with idiopathic hypoparathyroidism and APS1 with hypoparathyroidism have been identified with CaSR autoantibodies, which stimulate receptor activity in vitro; it is possible that such effects in vivo could result in abnormally low PTH levels, even when blood calcium levels are low (13, 14).

Although the presence of CaSR autoantibodies is compelling evidence of an autoimmune process directed against the parathyroid glands in APS1, the actual pathogenesis of hypoparathyroidism remains unclear. At least occasional patients appear to have autoantibodies with functional activity that could lead to suppression of the parathyroid gland function rather than their irreversible destruction (14). Additionally, although never analyzed, CaSR autoantibodies could destroy parathyroid cells through Ab-dependent cellular cytotoxicity or through complement fixation; Brandi et al. (15) demonstrated Ab-mediated cytotoxicity against cultured bovine parathyroid cells when exposed to serum from

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Abbreviations used in this article: APS1, autoimmune polyendocrine syndrome type 1; CaSR, calcium-sensing receptor; L.B., Luria-Bertani; PBS/BSA, PBS/1% BSA; PBS/Tween, PBS/0.05% (w/v) Tween 20; PTH, parathyroid hormone.
patients with idiopathic hypoparathyroidism, although the autoantigen responsible was not identified. To understand more fully the potential role for CaSR autoantibodies in APS1, the aim of the current study was to characterize the specificity, functional affinity, IgG subclass, and effects on CaSR activity of CaSR autoantibodies in relation to their epitopes.

Materials and Methods

Participants
The Medical Ethics Committee of Helsinki University Central Hospital approved the study, which was performed in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants or their parents prior to inclusion in the study. The 16 APS1 patients were unrelated and were positive for autoantibodies against the CaSR (7). The demographic, clinical, AIRE gene mutations, and autoantibody profile of the patients are summarized in Supplemental Table 1. Sixteen healthy blood donors to the Finnish Red Cross Blood Service, who had no clinical signs or a family history of APS1 or of any other autoimmune disease, served as controls (7). Serum samples obtained from participants were stored at −80°C.

IgG purification
IgG was isolated from sera using protein G Sepharose 4 Fast Flow (Amersham Biosciences, Uppsala, Sweden) affinity chromatography, according to the manufacturer’s instructions. IgG was eluted using 0.2 M glycine hydrochloride (pH 3), and the collected 1-mL fractions were neutralized with 50 µl of 1 M Tris base (pH 9). Fractions containing IgG, as determined by photometry at 280 nm, were analyzed against PBS (pH 7.4) and concentrated using an Amicon Concentrator (Amicon, Beverly, MA). IgG samples were sterilized with a Millex Filter Unit (MilliporeSigma, Bedford, MA) and stored at 10 mg/mL at −20°C.

Phage-display panning experiments
The preparation of the CaSR peptide phage-display library has been described elsewhere (16). For panning experiments, a 10−µl aliquot of patient or control IgG was applied to the well of a 96-well microtiter plate in 50 µl of buffer containing 1.5 mM sodium carbonate and 3.5 mM sodium hydroxide carbonate. Plates were incubated at room temperature for 2 h before washing with PBS/0.05% (w/v) Tween 20 (PBS/Tween). To block any nonspecific phage binding, 400 µl of 2% BSA in PBS was added to the wells, and incubation at room temperature continued for 2 h. The wells were rinsed again with PBS/Tween before the addition of a 100-µl sample of phage-display library containing an estimated 1 × 1011 phages. Plates were incubated overnight at 4°C to allow the interaction of CaSR Abs with peptides displayed on the surface of the phage particles. The wells were washed extensively with PBS/Tween to remove unbound phage. Bound phage were then eluted with 150 µl of 100 mM hydrochloric acid (adjusted to pH 2.2 with glycine) and then neutralized with 9 µl of 2 M Tris base. The phage suspension was subsequently used to infect 2 ml of exponentially growing Escherichia coli XL1 Blue MRF (Agilent Technologies, Wokingham, U.K.) for 15 min at room temperature. Aliquots of the infected cells were then plated onto Luria–Bertani (LB) agar containing 10 µg/mL tetracycline and 100 µg/mL ampicillin, and the plates were incubated overnight at 37°C. This allowed the assessment of the total number of eluted phage in each round of panning.

To generate a phage-display library for the next round of panning, the E. coli XL1 Blue MRF culture infected with eluted phage was made up to 100 ml with LB broth and incubated for 1 h with shaking at 37°C before superinfection with 1 × 1011 PFUs of VCSM13 helper phage (Agilent Technologies). Following incubation at room temperature for 30 min, the cells were transferred to 100 ml of LB medium containing 10 µg/mL tetracycline, 100 µg/mL ampicillin, and 10 µg/mL kanamycin and grown overnight with shaking at 37°C. The 100-ml culture was centrifuged at 2000 × g for 15 min. Phage particles were precipitated from the supernatant by addition of polyethylene glycol 4000 to 6% and sodium chloride to 750 mM. Following overnight incubation at 4°C, phage were harvested by centrifugation at 2000 × g for 30 min, and the pellet was resuspended in 1–2 ml of PBS before storage at −80°C. This first round phage-display library was used in a second round of panning. In all, five rounds of panning were undertaken for each IgG sample.

Of the fifth round of panning, plasmid DNA was prepared from randomly selected bacterial clones using a Wizard Plus SV Miniprep DNA Purification System (Promega, Southampton, U.K.). Sequencing using a pComb3-specific primer pc3prim1 (5’-GGTGGGCGCCGCAGATT-3’) (Eurofins Genetic Services, London, U.K.) with a BigDye Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and an ABI 3730 capillary sequencer (Applied Biosystems) was used to identify cloned CaSR DNA fragments. The latter were analyzed and translated using Lasergene Core Suite sequence analysis software (DNASTAR, Madison, WI). The EMBOSS Pairwise Sequence Alignment tool provided as part of the European Bioinformatics Institute–European Molecular Biology Laboratory bioinformatics services (www.ebi.ac.uk) (Cambridge, U.K.) was used to locate the CaSR peptide sequences on the full-length receptor.

CaSR peptide ELISAs
CaSR peptide ELISAs were used to verify the binding of CaSR autoantibodies to identified CaSR epitopes, as detailed previously (16). The synthetic peptides (Cambridge Peptides, Birmingham, U.K.) used are listed in Supplemental Table 2. For ELISAs, the required peptide was diluted in PBS to 200 ng/ml, and 100-µl samples were used to coat the wells of a 96-well microtiter plate. The plates were then incubated overnight at 4°C. Excess peptide was removed by decanting, and the wells were blocked with blocking buffer (PBS containing 0.1% [w/v] Tween 20 and 5% [w/v] BSA) for 30 min at 37°C. Plates were washed four times with washing buffer (PBS containing 0.1% [v/v] Tween 20). Duplicate 100-µl samples of sera at a 1:100 dilution in blocking buffer were added to the wells. PBS was applied as a control to measure any nonspecific binding of ELISA reagents in the absence of sera. The plates were incubated at room temperature for 1 h and then washed four times with PBS (pH 7.4) and then incubated at room temperature for 1 hour. After washing five times with washing buffer, 100 µl of alkaline phosphatase substrate SIGMAFAST p-Nitrophenyl phosphate (Sigma-Aldrich, Poole, U.K.) diluted 1:2000 in blocking buffer was added to each well for 1 h at room temperature. After washing five times with washing buffer, 100 µl of alkaline phosphatase substrate SIGMAFAST p-Nitrophenyl phosphate (Sigma-Aldrich) was applied to each well and plates were incubated at room temperature. A LabSystems Integrated ELISA Management System spectrophotometer (Life Sciences, Basingstoke, U.K.) was used to read absorption of the wells at 405 nm. All sera were tested in duplicate, and the mean OD405 value was calculated. Each serum was tested in three experiments. For each ELISA, the upper limit of normal was calculated as the mean OD405 + 3 SD of 16 healthy individuals. Sera with an Ab index above the upper limit of normal were designated as positive for CaSR autoantibodies against the specific CaSR peptide. For estimating CaSR autoantibody titers, patient sera were analyzed at dilutions ranging from 1:100 to 1:10,000. Titers were defined as the serum dilution at which Ab binding could still be detected above the upper limit of normal for the CaSR peptide ELISA.

CaSR autoantibody functional affinity
Autoantibodies against specific CaSR epitopes were purified using peptide affinity chromatography. The required CaSR peptides (2 mg) were coupled to CarboxyLink Columns according to a CarboxyLink Immobilization Kit (Thermo Fisher Scientific, Waltham, MA). Patient IgG samples were applied to peptide affinity columns in PBS and eluted in IgG Elution Buffer (Thermo Fisher Scientific). Samples were dialyzed, concentrated, and stored as detailed earlier.

Absorption experiments
Absorption experiments were used to evaluate CaSR autoantibody specificity. Purified CaSR autoantibodies were preabsorbed at a nonsaturating dilution with a 200× M excess of the required CaSR peptide and an unrelated control MCHR1 peptide (Alpha Diagnostics, San Antonio, TX). After incubation at 4°C for 2 h, samples were centrifuged at 13,000 × g for 15 min, and the preabsorbed Ab was analyzed in duplicate in CaSR peptide ELISAs alongside nonabsorbed Ab. Ab binding after preabsorption was expressed as a percentage of Ab binding without preabsorption: 100 × [OD405 in CaSR peptide ELISA after preabsorption of Ab/OD405 in CaSR peptide ELISA without preabsorption of Ab]. All Ab samples were analyzed in three separate experiments.

IgG subclass ELISAs
To determine the IgG subclass of purified CaSR autoantibodies, anti-human IgG subclass-specific monoclonal antibodies (mAb) (IgG1, IgG2, and IgG3, IgM, and IgA: AbD24240, AbD24241, and AbD24242; Biolegend, San Diego, CA), and an anti-human IgG (IgG1–3, IgM, and IgA: Sigma-Aldrich, Poole, U.K.) were used to detect the subclass of CaSR autoantibodies. The bound subclass-specific mAb was detected with a peroxidase-conjugated subclass-specific mAb followed by substrate solution (100 µm p-nitroaniline). Optical density (OD405) was measured at 30 sec intervals and stopped after 2 min.

CaSR autoantibody functional affinity
To determine functional affinities, purified CaSR autoantibodies were incubated at nonsaturating dilutions with a range of concentrations (0–1000 nM).
of the required CaSR peptide for 30 min before analysis in the appropriate CaSR peptide ELISA. Functional affinity was expressed as the concentration of peptide that blocked 50% of Ab binding by reducing the OD405nm by 50%. All Abs were analyzed in three separate experiments.

Inositol-1-phosphate ELISAs

As detailed previously (14), human embryonic kidney cells expressing the CaSR (HEK293-CaSR) were plated at 4 × 10^5 cells per well in 24-well plates (Nalge Nunc International, Rochester, NY) in 500 µl of DMEM containing 4.5 g/l glucose, 110 mg/ml sodium pyruvate, 10% FCS, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 2 mM l-glutamine (all from Life Technologies, Paisley, U.K.). Cells were cultured at 37°C overnight in a 95% humidified atmosphere of 5% CO2. Cell monolayers were washed first with serum-free medium and then with stimulation buffer containing 146 mM sodium chloride, 2.5 mM potassium chloride, 50 mM lithium chloride, 0.2 mM magnesium chloride, and 2.8 mM glucose, and then the HEPES buffer (pH 7.4). Subsequently, HEK293-CaSR cells were incubated with a 1:100 dilution of purified CaSR autoantibody or control IgG sample in 100 µl of stimulation buffer or with 100 µl of stimulation buffer alone. After 10 min at 37°C, a further 100 µl of stimulation buffer containing 0, 1.0, 3.0, 6.0, 12.0, or 18.0 mM of calcium chloride was applied to the cells, and incubation continued for 1 h at 37°C.

Following incubation, cells were lysed for 30 min at 37°C with 50 µl of 2.5% IP-One ELISA Kit Lysis Reagent (Cisbio, Gif-sur-Yvette, France). The accumulation of intracellular IP1 was measured according to an IP-One ELISA Kit (Cisbio), an immun assay based on competition between free IP1 and IP1–HRP conjugate for binding to an anti-IP1 MAb. The results for IP1 accumulation were expressed as percentage inhibition of IP1–HRP binding = [1 – IP1–HRP binding in stimulated cells/IP1–HRP binding in unstimulated cells] × 100. Increasing IP1 accumulation in the HEK293-CaSR cells was reflected by an increase in the percentage inhibition of IP1–HRP binding. All Ab samples were analyzed in six separate experiments.

PTH ELISAs

The rat parathyroid epithelial cell line PTH-C1 has been described elsewhere (17). Cells were seeded into 24-well plates in Ham’s F-12 Coon’s Modification Medium (Sigma-Aldrich) containing 10% FCS, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate. Once confluent, the cells were incubated with a 1:100 dilution of purified Ab sample in 500 µl of 1:1 DMEM/Ham’s F-12 Nutrient Mixture (Sigma-Aldrich) or with 500 µl of medium only. After 10 min at 37°C, a further 500 µl of medium containing 3.0 mM of calcium chloride was applied to the cells, and incubation continued for 4 h at 37°C. The cell culture supernatants were collected and stored at −80°C. PTH levels in supernatants were measured in duplicate with a Rat Intact PTH ELISA Kit (Immutopics, San Clemente, CA) detailed in the manufacturer’s protocol, and expressed in picograms per milliliter. All Ab samples were analyzed in six separate experiments.

Flow cytometry

Flow cytometry was used for the detection of Ab binding to HEK293 CaSR-GPI cell-surface expressing cells, exactly as previously detailed (4). Briefly, cells transfected with pcDNAs3-CaSR-GPI (4) and untransfected HEK293 cells (as a control) were washed in PBS and treated with Cell Dissociation Solution (Life Technologies). Cells were then washed with PBS/1% BSA (PBS/BSA), centrifuged for 5 min at 4°C, and resuspended in PBS/BSA at a density of 1 × 10^6 cells/ml. Aliquots of the cell suspension (1 × 10^6 cells) were transferred to LP4 tubes (Becton Dickinson, Oxford, U.K.) and then incubated on ice for 15 min before purified CaSR autoantibodies were added in duplicate to a 1:50 dilution. CaSR autoantibodies preabsorbed with the relevant CaSR peptide were included as controls. Following incubation on ice for 30 min, cells were washed with 3 ml of cold PBS/BSA and centrifuged for 5 min at 4°C. A 5-µl aliquot of FITC-conjugated anti-human Fab-specific IgG (Sigma-Aldrich) was added to the cells, and the samples were incubated on ice for 30 min. Cells were then washed twice and centrifuged before resuspension in 100 µl of PBS/BSA. The mean fluorescence intensity of each sample was measured using a FACScan Flow Cytometer and CellQuest software (Becton Dickinson).

Statistical analysis

Statistical analysis was performed by one-way ANOVA with posttest using GraphPad Prism software (GraphPad, La Jolla, CA); p values < 0.05 were considered significant.

Results

CaSR autoantibody binding sites are located in the extracellular domain

Autoantibodies against the CaSR were demonstrated previously in 16 APS1 patients whose details are summarized in Supplemental Table I. To identify the receptor binding sites of patient CaSR autoantibodies, a phage-display methodology was used that employed a CaSR peptide phage-display library in rounds of selective enrichment (panning) against individual patient IgG. The number of eluted phage increased with each panning round, indicating enrichment of IgG-binding CaSR peptides. Following the fifth round of panning, plasmid DNA was prepared from 50 bacterial clones and subjected to DNA sequencing to identify the cloned CaSR DNA fragments and thus the encoded CaSR peptides.

Subsequent analysis of the encoded CaSR sequences indicated that for each patient there was at least one immunodominant epitope enriched during the panning procedure. For example, following alignment, 90% (45/50) of the peptides enriched by IgG from patient 1 contained the CaSR aa 26–71, and 84% (42/50) by patient 2 IgG included residues 41–71 (Supplemental Table III). Comparing the immunodominant peptides from this region of the receptor between all 16 cases identified one potential common immunodominant epitope at amino acid residues 41–69 (Supplemental Table III). Similar analysis demonstrated two other common immunodominant epitopes between amino acid residues 114–126 in five cases and between 171–195 in six (Supplemental Table III). A fourth potential epitope between residues 260–340 was only found in a panning experiment with one patient IgG (patient 4) (Supplemental Table III). In all cases, the immunodominant peptide sequences were in-frame with respect to both the PelB leader peptide and the gene III coat protein of the pComb3 phage-display vector (16). This is imperative for the correct phage-surface expression of the encoded CaSR peptides.

In panning experiments with IgG from two healthy controls and two APS1 patients without CaSR autoantibodies, eluted phage numbers did not increase and no in-frame CaSR immunodominant peptides were enriched.

To verify further the potential epitopes at amino acid residues 41–69, 114–126, 171–195, and 260–340, sera from patients and 16 healthy controls were analyzed for Ab binding reactivity in ELISAs using the relevant CaSR peptides (Supplemental Table II) as the target Ag. The results (Fig. 1) indicated that the prevalence of CaSR Ab positivity in the patient group was 100% (16/16) against CaSR peptide 41–69, 31% (5/16) against 114–126, 38% (6/16) against 171–191, and 44% (7/16) against 260–340 (Table I). Overall, 5/16 (31%), 4/16 (25%), and 7/16 (44%) of the patient sera showed immunoreactivity against one, two, and three different epitopes, respectively (Table I). All identified epitopes resided within the extracellular domain of the CaSR (Supplemental Fig. 1).

CaSR peptide ELISAs were used to determine CaSR autoantibodies titers against each relevant epitope. The results are summarized in Table I. Titers ranged from 1:100–1:1000 for autoantibodies against epitope 41–69, 1:500–1:1000 for epitope 114–126, 1:200–1:1000 for epitope 171–195, and 1:500–1:2000 for epitope 260–340.

CaSR autoantibodies are epitope specific

The specificity of patient CaSR autoantibodies was analyzed by preabsorption of purified Abs with CaSR peptides representing identified epitopes and an unrelated MCHR1 peptide. Any effects upon CaSR autoantibody binding were then assessed in CaSR peptide ELISAs. Ab binding was only statistically significantly
reduced ($p < 0.0001$) by preabsorption with the CaSR peptide recognized as the Ab epitope (Fig. 2A–D), indicating there was no detectable cross-reactivity between CaSR autoantibodies that recognized different epitopes.

**CaSR epitopes are surface expressed**

HEK293 cells were transfected with plasmid pcDNA3-CaSR-GPI and the transfected cells used in flow cytometry analysis with CaSR autoantibodies purified from APS1 patients. Untransfected HEK293 cells were used as a control. Fluorescence histograms (Fig. 3A–D) showed a clear increase in the number of fluorescing cells when CaSR autoantibodies were incubated with HEK293 cells expressing the CaSR-GPI protein compared with incubation of the same samples with untransfected cells and compared with preabsorbed Ab. The results indicated that the recognized epitopes were surface expressed.

![FIGURE 1. CaSR autoantibody binding sites are located in the extracellular domain. Sera from 16 APS1 patients and 16 healthy controls were evaluated at a dilution of 1:100 in ELISAs for autoantibodies against CaSR peptides 41–69, 114–126, 171–195, and 260–340. The OD$_{450}$ value is shown for each APS1 patient, and control serum and is the mean of three separate experiments.](http://www.jimmunol.org/)

Table I. APS1 patient CaSR Ab epitopes, titers, IgG subclass, and functional affinity

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<th>APS1 Patient</th>
<th>CaSR Abs against Epitope 41–69</th>
<th>Functional Affinity (M)$^b$</th>
<th>CaSR Abs against Epitope 114–126</th>
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CaSR Abs against Epitope 171–195

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<th>Ab Titer$^a$</th>
<th>IgG Subclass</th>
<th>Functional Affinity (M)$^b$</th>
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CaSR Abs against Epitope 260–340

<table>
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<th>Ab Reactivity</th>
<th>Ab Titer$^a$</th>
<th>IgG Subclass</th>
<th>Functional Affinity (M)$^b$</th>
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<td>IgG1</td>
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</table>

$^a$CaSR Ab titers are defined as the APS1 patient serum dilution at which Ab binding could still be detected above the upper limits of normal for the CaSR peptide ELISAs.

$^b$Ab functional affinity is given as the concentration of the relevant CaSR peptide that blocked 50% of CaSR Ab binding in CaSR peptide ELISAs.

- , no CaSR Abs to be tested; +, positive for CaSR Ab reactivity in ELISAs; −, negative for CaSR Ab reactivity in ELISAs.
CaSR autoantibodies are mainly of the IgG1 subclass

CaSR peptide ELISAs were used to analyze the IgG subclass of patient CaSR autoantibodies. The results indicated that autoantibodies against the CaSR epitopes 41–69, 171–195, and 260–340 were restricted to the IgG1 subclass (Table I). Ab responses against CaSR epitope 114–126 were of the IgG1 and IgG3 subclasses in two (patients 5 and 6) and three (patients 7, 10, and 16) patients, respectively (Table I).

CaSR autoantibodies' functional affinities

CaSR peptide ELISAs were used to analyze the functional affinities of purified CaSR autoantibodies. The results are summarized in Table I and show that functional affinities ranged from $10^{-8}$ to $10^{-7}$ M, representing the concentration of CaSR peptide that was required to inhibit Ab binding in CaSR peptide ELISAs by 50%.

CaSR autoantibodies can stimulate the CaSR

Peptide affinity chromatography was used to purify CaSR autoantibodies with different CaSR binding sites from the relevant APS1 patient sera. The effect of CaSR autoantibodies against epitopes 41–69, 114–126, 171–195, and 260–340 on Ca$^{2+}$ stimulation of the CaSR was then analyzed by preincubating human embryonic kidney cells expressing the CaSR (HEK293-CaSR cells) with Ab samples prior to stimulating with Ca$^{2+}$ at a range of concentrations (0.5 to 9.0 mM) (14). Cells without preincubation with Ab and with preincubation with control IgG were also included. Intra-cellular IP1 accumulation was measured as the indicator of CaSR stimulation.

The results are shown in Fig. 4A–E. Preincubation of HEK293-CaSR cells with CaSR 114–126 autoantibodies from three patients (patients 5, 7, and 16) and with CaSR 171–195 autoantibodies from one patient (patient 9) resulted in a statistically significant increase in IP1 accumulation upon Ca$^{2+}$ stimulation (at 0.5 and 1.5 mM) compared with Ca$^{2+}$ stimulation alone; all $p$ values were <0.0001 (Fig. 4C, 4D). In contrast, preincubation with autoantibodies against CaSR epitopes 41–69 and 260–340 had no detectable effect upon IP1 accumulation in Ca$^{2+}$-stimulated HEK293-CaSR cells; all $p$ values were >0.05 (Fig. 4A, 4B, 4E).
CaSR autoantibodies can reduce PTH secretion

With respect to epitopes 41–69 and 114–126, the rat CaSR shows 100% amino acid homology with the human CaSR. In the case of epitope 171–195, 24 of the 25 aa residues are identical in the rat and human receptors. Therefore, to test whether CaSR autoantibodies against epitopes 41–69, 114–126, 171–195 could affect PTH secretion, rat parathyroid PTH-C1 cells were preincubated with Ab samples prior to calcium stimulation. Cells without preincubation with Ab were also included as a control. Secreted PTH was then measured in cell supernatants.

The results showed that preincubation of PTH-C1 cells with CaSR 114–126 autoantibodies purified from three patients (patients 5, 7, and 16) reduced PTH secretion by 72, 70, and 66%, respectively, when compared with PTH secretion from PTH-C1 cells not preincubated with Ab (Fig. 5A). Likewise, pretreatment with CaSR 171–195 autoantibodies from one patient (patient 9) reduced PTH secretion by 69% (Fig. 5B). In these four cases, the reduction in PTH secretion was statistically significant; all $p$ values were <0.0001 (Fig. 5A, 5B). In contrast, preincubation with any of the patient autoantibodies against CaSR epitope 41–69 did not reduce the secretion of PTH; all $p$ values were >0.05 (Fig. 5A, 5B).

Discussion

To understand more fully the potential role for CaSR autoantibodies in APS1, the aim of the current study was to characterize specificity, functional affinity, IgG subclass, and effects on CaSR activity of CaSR autoantibodies identified in relation to their epitopes. Initially, CaSR autoepitopes were identified at amino acid residues 41–69 (all patients), 114–126, 171–195 could affect PTH secretion, rat parathyroid PTH-C1 cells were preincubated with Ab samples prior to calcium stimulation. Cells without preincubation with Ab were also included as a control. Secreted PTH was then measured in cell supernatants.

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Discussion

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CaSR autoantibodies can stimulate the CaSR. HEK293-CaSR cells were preincubated with purified Ab against CaSR epitopes 41–69, 114–126, 171–195, and 260–340 or control IgG at a 1:100 dilution prior to stimulation with Ca²⁺ at 0.0, 0.5, 1.5, 3.0, 6.0, and 9.0 mM. Cells without Ab preincubation and with preincubation with control IgG were also included. Inositol-1-phosphate (IP1) accumulation in the cell lysates was measured in duplicate in an IP-One ELISA, and IP1 accumulation was expressed as percentage inhibition of IP1–HRP binding. All Ab samples were analyzed in six separate experiments. The mean IP1 accumulation ±SD is shown for (A) CaSR autoantibodies against epitope 41–69, (B) CaSR autoantibodies against epitope 114–126, (C) CaSR autoantibodies against epitope 171–195, and (D) CaSR autoantibodies against epitope 260–340. Preincubation of HEK293-CaSR cells with CaSR 114–126 autoantibodies from three patients (patients 5, 7, and 16), and CaSR 171–195 autoantibodies from one patient (patient 9) resulted in a significant increase (p < 0.0001) in IP1 accumulation upon Ca²⁺ stimulation (at 0.5 and 1.5 mM) compared with Ca²⁺ stimulation alone. IC₅₀ values were 1.05–1.09 and 1.10 mM for the CaSR 114–126 and 171–195 autoantibodies, respectively.
Gravis (19). In the case of the CaSR, autoantibody-induced activation could result in low levels of PTH and the resultant hypoparathyroidism that is associated with APS1. To date, only a small number of patients with hypoparathyroidism has been identified who also have CaSR-activating autoantibodies that could potentially reduce PTH secretion. First, sera from two patients with idiopathic hypoparathyroidism inhibited PTH release from human parathyroid cells (13). Second, IgG from two patients with APS1, both of whom had hypoparathyroidism, activated the CaSR expressed in HEK293 cells (14). In the current study, 4 of 16 (25%) patients had CaSR autoantibodies that stimulated the receptor expressed in HEK293-CaSR cells and suppressed PTH secretion from PTH-C1 cells. In three cases, the CaSR autoantibodies recognized epitope 114–126 and, in one case, the 171–195 Ab binding site. There were no apparent differences in the clinical details of patients with or without functional autoantibodies to either of the two determinants on the CaSR. However, there is potential for heterogeneity within the autoantibody populations that recognize these two epitopes, and this may distinguish functional autoantibodies that are associated with particular clinical characteristics.

The epitope constituting aa 114–126 overlaps with the loop 2 domain of the CaSR (20). Point mutations in or deletions of this part of the receptor increase the sensitivity of the CaSR to Ca$^{2+}$ and result in autosomal dominant hypoparathyroidism (20, 21). From these observations, it has been suggested that loop 2 may have an important role in maintaining the inactive state of the CaSR involving disulfide linkage between cysteine residues C129 and C131. Indeed, mutations involving either of these amino acid residues results in autosomal dominant hypoparathyroidism (www.casrdh.mcgill.ca). Ab binding to epitopes that are found within loop 2 could either inhibit or activate the CaSR, depending on whether Ab binding favored the active or inactive state of the receptor, respectively. The Ab binding site at aa 171–195 is of particular interest because site-directed mutagenesis and molecular models have indicated that some of these residues (S147, S170, and D190) form part of a binding site for Ca$^{2+}$ that is found in the crevice between the two lobes of each receptor monomer (20, 22, 23). Therefore, binding of an Ab to this area of the CaSR could disrupt the interaction of Ca$^{2+}$ with this Ca$^{2+}$ binding site.

Similar to previous studies on IgG subclass determination in autoimmune disease (24, 25), CaSR autoantibodies against all identified epitopes were mainly of the IgG1 subclass. In a minority of APS1 patients, autoantibodies against epitope 114–126 were of the IgG3 subclass. CaSR autoantibodies of the IgG2 and IgG4 subclasses were not identified. This is the first report, to our knowledge, regarding IgG subclass for autoantibodies against the CaSR in patients with hypoparathyroidism in the context of APS1, although CaSR autoantibodies exclusively of the IgG4 subtype have been found in a patient with autoimmune hypocalciuric hypercalcemia (11). Notably, both IgG1 and IgG3 subclass Abs can induce cell-mediated effector mechanisms and have the capacity to activate complement (26, 27). However, these pathomechanisms require investigation in relation to autoantibodies against the CaSR.

In patients with CaSR-stimulating autoantibodies, it could be that their hypoparathyroidism is caused by autoantibody activity against the CaSR instead of parathyroid gland destruction. This would be analogous to the action of pathogenic autoantibodies against the thyroid-stimulating hormone receptor in Graves disease (19). However, there were no data on whether the parathyroid of the APS1 patients with CaSR autoantibodies did or did not have lymphocyte infiltration. An important implication of the present results is that although the majority of APS1 patients do not have CaSR-stimulating Abs, there may be a small but substantial minority of patients in whom the hypoparathyroid state is the result of functional suppression of the parathyroid glands rather than their irreversible destruction. The documentation of the inactivating CaSR autoantibodies in autoimmune hypocalciuric hypercalcaemia proves that the presence of CaSR autoantibodies need not produce destruction of the parathyroid glands (9). In addition, our earlier demonstration of the presence of activating autoantibodies to the CaSR in one patient with transient hypoparathyroidism as well as in a second hypoparathyroid patient with a morphologically intact parathyroid gland despite many years of hypoparathyroidism proves that activating Abs also need not irreversibly destroy the parathyroids (13).

In such cases, it is possible that a calcilytic CaSR inhibitor might be of diagnostic and/or therapeutic utility. Because such agents...
References


