Biased agonism is a frontier field in G-protein coupled receptor (GPCR) research. Acquired hypocalciuric hypercalcemia (AHH) is a rare disease caused by calcium-sensing receptor (CaSR) autoantibodies, to date, showing either simple blocking or biased properties (i.e., stimulatory or blocking effects on different downstream signaling pathways). This emphasizes the importance of the Gi/o (pertussis toxin-sensitive G proteins, whose βγ subunits activate multiple signals including ERK1/2) in regulating PTH secretion. We here describe three patients with symptomatic AHH that shared characteristics with the two cases we previously reported as follows: [1] aged (between 74-87 years at diagnosis); [2] male; [3] unexpectedly showed no other autoimmune diseases; [4] showed spontaneously fluctuating calcium levels from approximately normal to near fatally high ranges; [5] acute exacerbations could be successfully treated with prednisolone and/or calcimimetics; [6] the presence of CaSR autoantibodies that operated as biased allosteric modulators of CaSR; and that [7] were likely to be conformational (i.e., recognizing and thereby stabilizing a unique active conformation of CaSR that activates Gq/11, activating phosphatidylinositol turnover, but not Gi/o). Our observations with these prominent commonalities may provide new insights into the phenotype and characteristics of AHH and the mechanisms by which the biased agonism of GPCRs operate.
Successful prednisolone or calcimimetic treatment of acquired hypocalciuric hypercalcemia caused by biased allosteric CaSR autoantibodies

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Abstract

Biased agonism is a frontier field in G-protein coupled receptor (GPCR) research. Acquired hypocalciuric hypercalcemia (AHH) is a rare disease caused by calcium-sensing receptor (CaSR) autoantibodies, to date, showing either simple blocking or biased properties (i.e., stimulatory or blocking effects on different downstream signaling pathways). This emphasizes the importance of the Gi/o (pertussis toxin-sensitive G proteins, whose βγ subunits activate multiple signals including ERK1/2) in regulating PTH secretion. We here describe three patients with symptomatic AHH that shared characteristics with the two cases we previously reported as follows: [1] aged (between 74-87 years at diagnosis); [2] male; [3] unexpectedly showed no other autoimmune diseases; [4] showed spontaneously fluctuating calcium levels from approximately normal to near fatally high ranges; [5] acute exacerbations could be successfully treated with prednisolone and/or calcimimetics; [6] the presence of CaSR autoantibodies that operated as biased allosteric modulators of CaSR; and that [7] were likely to be conformational (i.e., recognizing and thereby stabilizing a unique active conformation of CaSR that activates Gq/11, activating phosphatidylinositol turnover, but not Gi/o). Our observations with these prominent commonalities may provide new insights into the phenotype and characteristics of AHH and the mechanisms by which the biased agonism
of GPCRs operate.
Introduction

Biased agonism is a paradigm for explaining the selective activation of a certain and desirable signal through a G protein-coupled receptor (GPCR) that can typically mediate diverse signaling responses (1-9). The mechanisms by which selective signaling for the benefit of the cell is achieved via a “selectivity-loose” GPCR that activates multiple G protein/downstream signals remains a core question to be resolved in GPCR signaling research. An early hypothesis in this regard was that a specific combination of β and γ subunits enable a selective interaction between a specific GPCR and G protein. For example, it was shown that β3γ3 facilitates an interaction between the somatostatin SSTR2 receptor and Go2, a member of pertussis toxin (PTX)-sensitive Gi/o family of G proteins, while β1γ4 enables an interaction between muscarinic M4 receptor and Go1, another Gi/o family G protein (10, 11). An alternative theory has also been proposed in which a spatial regulation mechanism operates involving the specific co-localization of a GPCR and a G protein either at a certain site (i.e. lipid rafts or caveolae) within the plasma membrane, or at another intracellular membrane, to promote selective signal activation (12, 13).

A relatively recent model of selective activation through a GPCR is biased agonism (1-8). When a typical agonist activates a corresponding GPCR, multiple G
proteins are often then activated leading to multiple signal pathway responses. In contrast, a biased agonist (1, 2, 4, 6), or a combination of a usual (“selectivity-loose”) agonist and a biased allosteric modulator, causes a unique active conformation of the GPCR (3, 5, 7-9, 14, 15) and the subsequent activation of a specific G protein/signaling mechanism only.

Biased agonism has been sometimes described as just theoretical or often only in terms of pharmacological manipulation. Notably, however, we have now reported on the first series, to our knowledge, of autoantibodies against a calcium-sensing GPCR (CaSR), that function as endogenous biased allosteric modulators of this receptor to regulate two G protein signals, (i.e., Gq/11- and Gi/o-signals in our case), in opposite directions (14, 15), although this is a pathophysiological phenomenon.

Autoantibodies against GPCRs can result in endocrine disease, although this is a rare occurrence (16-18). It is known for example that stimulatory autoantibodies against the TSH receptor cause Graves’ disease, while gain-of-function mutations of TSH receptor cause familial hyperthyroidism. Blocking antibodies cause hypothyroidism, while loss-of-function mutations of the receptor can lead to congenital hypothyroidism (19, 20). In the case of CaSR (21-28), genetic diseases caused by its mutations and acquired diseases caused by autoantibodies directed against it have been described.

Extracellular calcium levels are tightly controlled by CaSR expressed in parathyroid
epithelial cells and at the basolateral membrane in cells of the thick ascending loop of Henle. When the extracellular calcium levels increase, increased calcium ions activate CaSR in the parathyroid gland, from which the secretion of PTH is inhibited. Inversely, when the extracellular calcium levels decrease, PTH secretion is upregulated. Gain-of-function mutations of CaSR stimulate its signaling pathways and inhibit PTH secretion from the parathyroid gland, resulting in hypocalcemia. This disorder is called autosomal dominant hypocalcemia 1 (ADH1) (27).

In the kidney, CaSR signaling activation in cells of the thick ascending loop of Henle results in hypercalciuria (23). Loss-of-function mutations of CaSR at one or both alleles inhibit CaSR signaling and cause increased PTH secretion, referred to as familial hypocalciuric hypercalcemia 1 (FHH1) and neonatal severe hyperparathyroidism (NSHPT), respectively (26, 27). Also in the kidney, a CaSR signaling block in cells of the thick ascending loop of Henle causes hypocalciuria.

As an acquired disease analogous to ADH1, acquired hypoparathyroidism, including immune-checkpoint inhibitor-related hypoparathyroidism, has been reported to be caused by activating autoantibodies against CaSR (29-34). Acquired hypocalciuric hypercalcemia (AHH), which is analogous to FHH1 or NSHPT, has been reported to be caused also by autoantibodies against CaSR (14, 15, 35-38). Depending on the reported
AHH cases, autoantibodies work either as pure blocking entities (35) or in a biased manner (14, 15). By investigating their mode of action, both the pathophysiological mechanisms underlying this heterologous disease from the perspective of the epitopes detected and possible effective treatments, and the physiological mechanisms by which PTH secretion is regulated in the human body, have been further elucidated (15).

In our present study, we report on three additional AHH patients who were elderly men with no evidence of any other autoimmune disorders such as chronic thyroiditis, thus differing from most autoimmune cases. This was the case also for the two AHH subjects we had previously described. These three individuals displayed symptomatic PTH-dependent hypercalcemia with hypocalciuria and were thus clinically suspected of having AHH. This diagnosis was subsequently confirmed using in vitro analysis. Their calcium levels spontaneously fluctuated from approximately normal and subfattally high ranges. In instances of acute exacerbation, all three subjects were successfully treated with prednisolone and/or calcimimetics. Their CaSR autoantibodies operated as biased allosteric modulators of this receptor and were likely to be conformational (i.e., to recognize a unique conformation of CaSR).

The three additional AHH subjects analyzed in this present study provide further mechanistic insights into this rare disease with regard to the biased agonism of CaSR (i.e.,
biased allosteric modulation of this GPCR), and raise further questions about why the
autoantibodies of some AHH patients show unique characteristics.
Results

AHH patient subjects and their clinical courses

Patient-1 (Pt-1)

The first of the AHH study subjects was a 76-year-old man with a chief complaint of appetite loss and in whom PTH-dependent hypercalcemia with hypocalciuria was detected (cCa, 13.8 mg/dL; iP, 1.9 mg/dL; Cre, 0.82 mg/dL; intact PTH, 60 pg/mL; and a fractional excretion of calcium (FECa) of 0.32%; Table 1). At more than 3 years prior to his presentation at our clinic, his serum calcium level was normal (cCa 8.6 mg/dL, iP 3.6 mg/dL). He had been treated at that time for bronchial asthma as an allergic disease. He had neither a familial history of hypercalcemia nor any personal history of autoimmune disease. He harbored no mutations in the CASR, GNA11, and AP2S1 genes. Despite undergoing a conservative treatment regimen with furosemide (10 mg/day) and zolendronic acid (4 mg/month), after an emergent therapy with hydration and etlonine, he still showed hypercalcemia (cCa, 11.8 mg/dL; FECa, 0.22%). He was then administered prednisolone (PSL) at 20 mg/day, and a diagnosis of AHH was simultaneously confirmed by in vitro analysis. One month later, his Ca level had normalized (Ca, 10.0 mg/dl; FECa, 0.67%), and the PSL treatments were tapered off gradually. Within one year however, his calcium level had increased again, and a PSL
treatment was recommenced at 5 mg per day and has been maintained at 10 mg per day thereafter (Figure 1A).

Patient-2 (Pt-2)

The next AHH case was a 75-year-old man, also with a chief complaint of appetite loss and who had PTH-dependent hypercalcemia with hypocalciuria (cCa, 13.6 mg/dL; iP, 2.2 mg/dL; Cre, 0.84 mg/dL; intact PTH, 101 pg/mL; FECa, 0.04%; Table 1). Two years prior to this, his Ca level had been normal (cCa, 10.4 mg/dL). He had undergone a prostatectomy due to prostate cancer and also had neither a family history of hypercalcemia nor personal history of autoimmune disease. He had no mutations in the CASR, GNA11, and AP2S1 genes. AHH was suspected and treatment with cinacalcet (25 mg per day) was commenced after emergent therapy with hydration, furosemide and zolendronic acid. At a higher cinacalcet treatment or 50 mg per day, his calcium levels became permissive, and the dose was thus reduced to 25 mg per day. His calcium levels then rebounded to a high concentration and AHH was subsequently confirmed by in vitro analysis. After 6 months, he showed hypocalcemia and his cinacalcet therapy was discontinued. This was followed by the onset of permissive hypercalcemia. At approximately one year after his treatment was ceased, his calcium levels flared up again.
and the cinacalcet was recommenced. He had since shown intermittent hypocalcemia followed by the cessation of his cinacalcet or evocalcet regimens, and his calcium levels have been well-controlled using these treatment approaches (Figure 1B).

Patient-3 (Pt-3)

The third AHH subject was an 87-year-old man with a disturbed consciousness in whom PTH-dependent hypercalcemia with hypocaliuria was revealed (cCa, 14.3 mg/dL; iP, 2.2 mg/dL; Cre, 0.97 mg/dL; intact PTH, 32 pg/mL; FECa, 0.18%; Table 1). He had been suspected three years previously of having primary hyperparathyroidism of unknown localization. He had a history of rectal cancer, which had been treated with a colectomy followed by irradiation for positive surgical margin six years prior to his current presentation. He had no family history of hypercalcemia nor personal history of autoimmune disease. Following treatment with intermittent hydration, eltonine and zolendronic acid with furosemide for about two years, his calcium levels normalized without further intervention for one year. His hypercalcemia eventually recurred however, and we confirmed a diagnosis of AHH by in vitro analysis. At that time, he showed no evidence of rectal cancer recurrence. He was then initially administered cinacalcet at 25 mg/day with an eventual dosage increase up to 75 mg/day after emergent therapy with
hydration, furosemide and zoledronic acid. After a maintenance therapy with cinacalcet
at 50 mg per day, he again showed hypocalcemia and this treatment was stopped. Ten
months later, he developed hypercalcemia once more, and a cinacalcet regimen was
recommenced. Despite receiving a high dosage (100 mg) of cinacalcet at that time,
however, his calcium levels were not controlled, and prednisolone was added to the
treatment for a short period. After repeated similar episodes, he passed away due to
intestinal bleeding caused by a recurrence of his rectal cancer six years after the diagnosis
of AHH (Figure 1C).

Serum reactivity in the three AHH subjects to COS-7 or HEK293 cells expressing CaSR

Immunofluorescence analyses of the sera from all three AHH patients using
three-dimensioned confocal microscopy (Figure 1, A-C, Analysis-1) indicated reactivity
to COS-7 cells transiently expressing FLAG-tagged CaSR without permeabilization. This
immunoreactivity of the patient serum samples to COS-7 cells was found to be almost
completely co-localized with that of an anti-FLAG antibody (Figure 2A). These serum
samples also reacted to HEK293 cells stably expressing CaSR (HEK293-CaSR cells)
without permeabilization (Figure 2B). This reactivity was significantly reduced after
prednisolone treatment in Pt-1 (Figure 1A, Analysis-2) but not after discontinuing
cinacalcet treatment in Pt-2 (Figure 1B, Analysis-2) or commencing cinacalcet treatment
in Pt-3 (Figure 1C, Analysis-2). These trends were confirmed by cell surface ELISA
(Figure 2C).

Effects of AHH patient IgG on Ca-stimulated IP1 accumulation and ERK1/2 phosphorylation

We investigated functional changes in our AHH patient IgG at two timepoints
using HEK293-CaSR cells, in the same manner described in our previous study (Figure 3). We previously reported using HEK293 cells expressing CaSR that CaSR-induced
inositol phosphate (IP) accumulation was mostly dependent on Gq/11 because an
exogenously expressed RGS domain of GRK2 (14, 39), which captures activated Gqα,
inhibited this signal (14, 15). We also reported that this signal was mostly independent of
Gi/o, the pertussis toxin (PTX)-sensitive G proteins, because treatment with PTX, which
inhibits Gi/o signals, did not inhibit this signal (14, 15). At a Ca level of 2.0 mM, IP1
accumulation was found to be augmented by the co-administration of each AHH patient’s
IgG at the time of Analysis-1 (Figure 3A), which was similar to the findings in the two
previous cases we reported (14, 15). After PSL treatment, Ca-stimulated IP accumulation
decreased (Figure 3A) in Pt-1 (Figure 1A, Analysis-2). After discontinuing cinacalcet treatment (Figure 1B, Analysis-2) or commencing this treatment (Figure 1C, Analysis-2), Ca-stimulated IP accumulation continued to be higher than the control IgG in both Pt-2 and Pt-3 (Figure 3A). Although not significantly, it seemed to slightly decrease in Pt-3 (Figure 3A), possibly reflecting a fluctuation of autoimmunity in this case.

In contrast to the aforementioned findings, ERK1/2 phosphorylation at the Ca concentration of 2.0 mM was suppressed by the co-administration of each AHH patient’s IgG at the timing of Analysis 1 (Figure 3, B and C, Supplementary Figure 1). After PSL treatment, this suppression was recovered at least partially in Pt-1 (Figure 1A, Analysis-2). After ceasing the cinacalcet treatment in Pt-2 (Figure 1B, Analysis-2), or after commencement of this therapy in Pt-3 (Figure 1C, Analysis-2), the suppression remained in both cases although appeared to have slightly recovered in Pt-3, consistent with the notion that cinacalcet does not affect autoimmunity.

Ca-stimulated ERK1/2 phosphorylation and the effects of PTX and Gq inhibitors in HEK293 cells expressing CaSR

We have also previously reported that, at a Ca level of up to 2.5 mM, ERK1/2 phosphorylation is largely dependent on the Gi/o proteins and their βγ subunits because
both PTX treatment and exogenously expressed Gtα, which captures free βγ subunits, largely inhibited CaSR-dependent ERK1/2 phosphorylation in HEK293 cells expressing CaSR stably (HEK293-CaSR cells) (14). We next confirmed that ERK1/2 phosphorylation was largely dependent on the Gi/o proteins in HEK293-CaSR cells, based on the effect of PTX, which was consistent with our previously reported findings (14) (Figure 4, A and B and 5, A and B). Recently, however, it has been reported that some Giβγ-induced signals leading to calcium mobilization also depend on Gq (40). We therefore queried whether CaSR-induced ERK1/2 phosphorylation was also dependent on Gq/11 using an exogenously expressed RGS domain of GRK2 (RGS) (14, 39) and also the YM-254890 (YM) (41), both of which have been reported to specifically inhibit Gq/11 signal. Unexpectedly, both RGS and YM largely inhibited Ca-dependent ERK1/2 phosphorylation at a concentration of less than 2.5 mM (Figure 4, A and B and 5, A and B). However, the specificity of YM remains a matter of dispute (42). In the system we used to investigate Gi activation through the Ca-dependent inhibition of cAMP accumulation stimulated by forskolin, PTX treatment almost completely inhibited the Gi-dependent inhibition of cAMP (Figure 6A). A 10 nM concentration of YM also inhibited the Gi-dependent inhibition of cAMP, at least in part, especially at a non-saturated concentration of Ca (Figure 6A). YM inhibited IP1 accumulation at the Ca concentration
of less than 3.0 mM (Figure 6B). The inhibitory effects of YM upon Gi were found to be
dose dependent (Figure 6C). Unfortunately, however, the inhibition of Gi signal using
the patient’s IgG could not be evaluated in this cAMP assay so far because IgG appeared
to have non-specific effects and because the amount of our patient IgG is limited.

Taken together, we found from these analyses that, at least at less than 2.5 mM
Ca, CaSR-dependent ERK1/2 phosphorylation is likely to be dependent on both Gi/o and
Gq/11. In addition, a potentially greater suppression of ERK phosphorylation by YM may
be due to its inhibition of not only Gq/11 but also Gi/o proteins, at least in part. Based on
these results, it was confirmed that AHH patient IgG likely inhibits Gi/o and stimulates
Gq/11, as we previously reported (14, 15).

**AHH patient serum reactivity against the extracellular domain of CaSR and its mutants**

** harboring alanine substitutions at amino acids 214-235**

Immunofluorescent analysis and cell surface ELISA using HEK293-CaSR cells
confirmed that the sera of our three current AHH patient subjects reacted against the
extracellular domain (ECD) of CaSR. We previously reported that the autoantibodies of
two AHH subjects recognized the conformation of amino acids 214-235 within the ECD
of CaSR (15). We thus investigated whether the sera of our current AHH cases might
recognize this same sequence of residues (Figure 7, A and B). The serum of Pt-2 reacted to the full-length ECD of the CaSR (CaSR-ECD), and this reactivity decreased against an ECD mutant in which residues 214-235 were substituted for alanine (CaSR-polyA). This result indicated that these sera likely recognize this region of the ECD, at least in part. No particular band was detected against CaSR-ECD in Pt-1, while this reaction was detected although not significantly, in Pt-3 (Figure 7, A and B). These findings were compatible with our earlier immunofluorescence and cell surface ELISA results (Figure 2, A-C), showing that the reactivities of Pt-1 and Pt-3 sera against COS7 cells and HEK293 cells expressing CaSR were lower than that of Pt-2.

AHH patient serum reactivity against peptides covering the CaSR extracellular domain

There have been some prior reports that the serum from patients with AHH or autoimmune hypoparathyroidism reacts to certain peptides that match segments of the CaSR-ECD (29, 31, 33-36, 38). In addition to those peptides, we prepared others to cover the entire portion of the ECD. We also prepared peptides constituting the extracellular loop of the CaSR (Figure 8, A and B). Our current AHH patient sera did not react to any of these peptides however, including those covering amino acids 214-235 (Figure 8A), suggesting that the autoantibodies in these cases may only recognize a specific
conformation of the CaSR-ECD.
Discussion

We here report on three cases of symptomatic AHH that harbor CaSR autoantibodies, and that showed similar characteristics to two prior patients that we described (14, 15) i.e., [1] elderly (ranging from 74 to 87 years old at diagnosis), [2] male, [3] unexpectedly showing no evidence of other autoimmune diseases, which is atypical for this disorder (29, 36, 37), [4] spontaneously fluctuating serum calcium levels between a permissive to normal and high near-fatal range, [5] had been successfully treated with either PSL and/or cinacalcet when needed, [6] harbored CaSR autoantibodies operating as biased allosteric modulators of CaSR, and [7] these autoantibodies were likely to be conformational (i.e., to recognize and thereby stabilize a unique active conformation of CaSR that activates Gq/11 but not Gi/o).

AHH patients were originally reported as familial females showing asymptomatic hypercalcemia with relative hypocalciuria, a condition which is very similar to typical familial hypocalciuric hypercalcemia (FHH) (35). FHH caused by a heterologous loss of function mutation of CaSR is usually an asymptomatic disease which does not require treatment. In contrast, neonatal severe hyperparathyroidism (NSPHT), caused by a homozygous loss of function mutation of CaSR, is a very severe disorder which requires resection of all parathyroid glands immediately after birth (23). Based on
its terminological analogy with FHH, AHH may be misunderstood as being similar to
FHH. However, the severity of AHH depends on the titer of the autoantibodies to CaSR,
such that it can range from asymptomatic like FHH to severely symptomatic like NSPHT
(15). Moreover, during the clinical course of AHH, the disease status can fluctuate
depending on the autoantibody titer of the patient (15). Although severe and symptomatic
instances of AHH need to be treated, only a few such cases have been reported to date
(15, 36).

High levels of extracellular calcium inhibit the secretion of PTH via the CaSR
that activates at least Gq/11 and also Gi/o proteins. However, the molecular mechanism
of this remains to be fully understood. It has been postulated that CaSR classically
functions via Gq/11 (14, 21, 43). In support of this contention, a loss-of-function mutation
of G11 causes FHH2 (27, 44), which is similar as FHH1, due to a disruption of CaSR
activity. Conversely, a gain-of-function mutation of G11 causes ADH2 (27, 44, 45), which
is similar to ADH1 due to a gain-of-function mutation of CaSR. In contrast, we (14, 15)
and others (21) have proposed that not only Gq/11 but also Gi/o factors may play an
additional important role in inhibiting PTH secretion. In our model system using HEK293
cells expressing CaSR, we have disclosed that CaSR autoantibodies work as biased
allosteric modulators that stimulate Gq/11-PI turnover and inhibit Gi/o-ERK1/2
phosphorylation (14, 15). We observed similar results in the current three AHH cases.

Pallais et al. have reported some CaSR autoantibodies only inhibit ERK1/2 phosphorylation in AHH (37). However, additionally and unexpectedly, our current analyses revealed that ERK1/2 phosphorylation via CaSR at a Ca concentration of 2.0 mM is largely inhibited not only by PTX that inhibits GPCR dependent activation of Gi/o, but also by Gq inhibitors, i.e., the RGS domain of GRK2 or YM-254890. ERK1/2 phosphorylation (at 2.0 mM Ca) via CaSR might therefore be dependent on both the Gi/o and Gq/11. Recently it has been reported that Giβγ-induced calcium mobilization also depends on Gq/11 (40). It has also been demonstrated that Gqα and Giβγ synergistically activate PLCβ3 and Ca mobilization (46). These results suggest, but do not prove yet, that Gq/11 signal and Gi/o signal may synergistically stimulate ERK1/2 phosphorylation in our HEK293-CaSR cells. Because AHH autoantibodies augment Gq/11 signaling, we speculate that an inhibition of ERK1/2 phosphorylation by AHH autoantibodies may be caused by the suppression of Gi/o signaling, overcoming the potentiated Gq/11 signaling. Unfortunately, however, the inhibition of Gi (but not Go) signal by AHH autoantibodies could not be evaluated in the cAMP assay so far partly because the amount of our patient IgG is limited. A more direct demonstration of the effects of autoantibodies on Gi/o signaling will be desirable in the future. Furthermore, these results tempt us to speculate...
that, in parathyroid cells as well, Gq/11 signal and Gi/o signal may act synergistically to
decrease PTH secretion. If our speculation is correct, inhibition of Gq/11 signal or Gi/o
signal may increase PTH secretion in parathyroid cells, resulting in the phenotype of
AHH/FHH. And in our cases with CaSR autoantibodies working as its biased allosteric
modulators, we speculate that the inhibition of Gi/o signal may have overcome the
potentiated Gq/11 signal, overall resulting in our AHH phenotype.

Two prior AHH cases have been described in which PSL treatments were
attempted. One of these patients showed improvement following this therapy (36), but no
benefit was observed in the other case (37). In the report on the PSL-responsive case,
however, the function of the autoantibodies was not analyzed. Our current study is thus
the first to describe the successful treatment of a conclusively diagnosed AHH case with
PSL. Our analyses indicated that both the titer and the function of the CaSR
autoantibodies had improved, mostly in parallel, in Pt-1. The autoantibody titer was found
in this case to have decreased by immunofluorescent staining and ELISA after PSL
treatment (Figures 2, B and C). With regard to the function of these CaSR autoantibodies,
we further found that the inhibition of Ca-stimulated ERK1/2 phosphorylation using
patient IgG was significantly recovered by PSL treatment (Figures 3, B and C). Notably,
as Gi/o-dependent signaling is a key component of the regulation of PTH secretion (14,
this functional change is consistent with the observed clinical course in our present AHH cases. Conversely, the augmentation of Ca-stimulated IP accumulation was found in our current analyses to be decreased by PSL treatment (Figures 3A).

In terms of the impacts of calcimimetic treatment, we have recently described the first AHH case who was successfully treated with cinacalcet over a long period (15). We analyzed two additional cases undergoing this treatment in our current study. Both patients showed fluctuation of their Ca levels with intermittent hypocalcemia, followed by a discontinuation of the cinacalcet therapy. Overall, the Ca levels in these two cases were well controlled. In Pt-2, we anticipated that in vitro analyses using serum sampled during the cinacalcet treatment (Figure 1B, Analysis-1), and then after this treatment had ceased (Figure 1B, Analysis-2), would reflect the observed clinical course. However, no particular differences were evident between these sets of assay data (Figures 3, A-C).

During the period after the cinacalcet therapy had been discontinued, this patient showed slight hypercalcemia, indicating that CaSR autoantibodies continued to be produced. We speculated therefore that a slight change in the autoantibody titer might not be detectable by our analysis.

The autoantibodies detected in our current patients were found to function as biased allosteric modulators, in a similar manner to our previously reported cases (14, 15),
and differing from other cases (29). The observed mode of action in our current AHH patient subjects represents additional unique evidence that biased agonism operates endogenously in the human body and is not just a pharmacological phenomenon. In addition, the biased effects of our patients’ autoantibodies in relation to regulate two G protein signals in opposite direction further supports the importance of the Gi/o proteins in regulating PTH secretion.

Some of the significant questions arising from our present observations include where and how these autoantibodies react against CaSR. In the patients with AHH or autoimmune hypoparathyroidism reported by other researchers, the patient sera were found to be reactive against several peptides that can be found in the ECD of CaSR [41-69, 114-126, 171-195, 214-236, 344-358 and 374-391] (29, 31, 33-36, 38). The 114-126 peptide is particularly noteworthy in this regard as both activating and blocking antibodies have been reported (31, 38). In contrast, the sera of our present AHH cases did not show any reactivity against these ECD peptides, including the 214-238 peptide. This suggests that the autoantibodies produced in our current cases may be conformational (i.e., they recognize a unique conformation of CaSR). Monoclonal antibodies derived from AHH patients will play a key role in the future in elucidating where and how these autoantibodies react. A functional assessment of how a biased monoclonal antibody might
stabilize a unique CaSR structure will likely provide insights into the mechanism of the specific GPCR signaling activation in AHH and assist with the development of drugs that will not have undesirable side effects.

It is uncertain why all of the published research to date on CaSR autoantibodies that work as biased allosteric modulators has been limited almost exclusively to Japan. It is possible that this unique and somewhat complex signaling mechanism has simply been overlooked in some other studies. Alternatively, a causative genetic background that is unique to Japanese AHH patients may be involved. This latter hypothesis is also compatible with our observation that autoantibodies in our AHH patients developed without other autoimmune diseases. Further investigation of this phenomenon represents an important future research question. Clarification of a possible underlying genetic background for our AHH cases may provide novel insights into the molecular mechanism of AHH itself and of the biased agonism of GPCRs in general.

In conclusion, we report here on three Japanese AHH patients that shared unique clinical and in vitro properties. Our observations may help to foster new insights into the phenotypes and characteristics of AHH, including the underlying genetic background and mechanisms of how the biased agonism of GPCRs operate.
Methods

Sample Preparation

Serum samples were collected and stored at −80° until just before use. Purified IgG was isolated using a Montage antibody purification kit (Millipore, Billerica, MA), followed by concentration using Amicon Ultra 15 (Millipore) for immediate assay (14, 15). Experiments using patient were performed under approval of the Institutional Review Board of the University of Tokyo.

Chemicals

We purchased all chemicals except those we referred from, including YM-254890 and pertussis toxin, from Wako Pure Chemical Industries (Fujifilm), Osaka, Japan.

Expression Constructs, Cell culture and Transfection.

Plasmid expressing only extracellular domain of CaSR (1-603) (CaSR-ECD) in pcDNA3.1 was made by subcloning PCR product using FLAG-tagged human CaSR in pcDNA3.1 as a template. Mutagenesis of CaSR-ECD replaced amino acids of 214-235 to alanines (CaSR-polyA) was performed by KOD-Plus Mutagenesis kit (TOYOBO, Japan)
Plasmid expressing RGS domain of GRK2 was gifted by Tohru Kozasa (Department of Biochemistry, Yokohama University of Pharmacy, Yokohama, Japan). HEK293 cells or COS7 cells (both from Henry Bourne lab, Department of Cellular and Molecular Pharmacology, UCSF, CA, USA), maintained in DMEM containing 10% FBS, were transfected with constructs encoding CaSR, RGS domain of GRK2, pGloSensor-22F cAMP plasmid (Promega Corporation, Madison, WI, USA.), CaSR-ECD or CaSR-polyA, using lipofectamine 2000 (Thermo Fisher Scientific) (14, 47, 48). The transiently transfected cells were used for immunoprecipitation study, immunostaining study, cAMP assay and ERK1/2 phosphorylation assay after 48 h in culture. Otherwise, we used HEK293 cells stably expressing human CaSR, selected and cloned in medium containing 0.8 mg/ml G418 as described previously (14, 49-52).

Immunofluorescence imaging

Immunofluorescence imaging were performed using patient or control sera (1:100 dilution) (Fig. 2A, B). Briefly, HEK293 cells stably expressing human CaSR or COS7 cells transiently expressing FLAG-tagged human CaSR were incubated with patient or control sera (1:100) and/or monoclonal anti-FLAG antibody (1:2,000) (F3165, 47, 48).
Sigma-Aldrich) for one hour at 37°C and fixed at 4°C for 15 min in 4% paraformaldehyde/PBS. Bound IgG were detected by using goat anti-human IgG tagged with Alexa 488 (green) (A-11013, Molecular Probes, Eugene, OR) and anti-mouse IgG tagged with Alexa 568 (red) (A-11004, Molecular Probes, Eugene, OR) (14, 53). Fluorescence images were collected by using confocal microscope AX or ECLIPSE E600 microscope (NIKON, Tokyo, Japan).

Cell surface ELISA

Immunoreactivity was quantified by cell surface ELISA. Briefly, HEK293 cells expressing human CaSR stably were plated onto 48-well plates. Next day, the cells were incubated with patient or control sera (1:500) for 1h at 37°C. After washing, the cells were fixed at 4°C for 15 min in 4% formaldehyde/PBS (without permeabilization). The cells were then incubated in HRP-conjugated anti-human IgG (074-1002, Kirkegaard and Perry laboratories, Gaithurburg, MD) (1:5,000) at room temperature for 1h. After washing, the cells were treated with substrates (o-phenylenediamine dihydrochloride, Sigma) for 5 min at room temperature. This reaction was stopped by the addition of an equivalent volume of 2.5 N HCl and the absorption levels were read at 492 nm using a plate reader.
**Measurement of CaSR-Stimulated IP1 Accumulation**

IP1 was measured by modified manufactory protocol using IP one-HRT kit (Cisbio). Briefly, HEK293 cells expressing the human CaSR were applied onto 96-well plate on the day before assay, preincubated with or without 10 nM YM-254890 for 30 minutes or PTX for 4 h, and incubated with various concentrations of calcium or purified IgG at Ca 2.0 mM with 50mM LiCl for 1h at 37°C. Then reaction was stopped by replacing and extracted by Hank’s Balanced Salt Solution (HBSS) containing 1% Triton X, and IP1 was measured based on manufactory procedure using TRF technology. Calculated IP1 values based on standard curve were fitted to a four parameter sigmoidal concentration-response curve using the Prism 8 software (GraphPad Prism) and the values for EC50 were calculated from the curve.

**Measurement of cAMP-dependent luminescent signal**

HEK293-CaSR cells transfected with pGloSensor-22F cAMP plasmid one day
before with or without PTX treatment for 4 h were detached, centrifuged, and suspended with Hank’s Balanced Salt Solution (HBSS) containing 5 mM HEPES (pH 7.4), 0.01% (w/v) bovine serum albumin, 0.5 mM IBMX (Sigma-Aldrich) and 2 mM D-luciferin. Cells (600/μl) were seeded in a 384-well white microplate at a volume of 15 μl per well and incubated at room temperature for 2 h in dark with or without 5μl of 5x YM-254890 for 30 minutes. 5 μl of 5x calcium diluted in 15 μM forskolin were manually added to the cell and incubated at room temperature for 20 min. Measured luminescent signals were fitted to a four parameter sigmoidal concentration-response curve using the Prism 8 software (GraphPad Prism) and the values for IC50 were calculated from the curve.

**Measurement of ERK1/2 phosphorylation**

For determining ERK1/2 phosphorylation, the cells were plated and starved for 12-16 hours in DMEM with 0.3 mM CaCl₂. The cells were then stimulated with various concentrations of calcium with or without YM-254890 or purified IgG at Ca 2.0 mM for 2 h 37°C, and the reactions were terminated. Protein extracts were then analyzed on western blotting using anti-phospho ERK1/2 antibody (1:2,000) (E10) (#9106, Cell Signaling). Each membrane was stripped by stripping buffer (0.2 M Glycine with 0.1%
SDS and 1% Tween 20, pH 2.2) washed by PBS and PBS-0.1% Tween twice respectively, blocked and incubated by anti-ERK1/2 (1:2,000) (Enzymology). Intensity of phosphorylated ERK1/2 and ERK1/2 was analyzed by Image-J software, and each ratio against control ratio was calculated, respectively. Calculated signals were fitted to a four parameter sigmoidal concentration-response curve using the Prism 8 software (GraphPad Prism) and the values for EC50 were calculated from the curve.

**Immunoprecipitation**

The COS7 cells transiently transfected CaSR-ECD or CaSR-polyA 48 hours before were washed with ice-cold PBS and lysed in ice-cold lysis buffer (40 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2mM MgCl$_2$, 1% Triton X, 100 mM EGTA and protease inhibitors) on ice by gently shaking each for 5 minutes. After 60 minutes, cell lysates were collected in tube and centrifuged at 20,000 g for 10 minutes at 4°C. Supernatants were incubated with 2 μg monoclonal anti-FLAG antibody (F3165, Sigma-Aldrich) for 2 hours at 4°C and absorbed to protein G plus Sepharose (sc-2002, Santa Cruz) for 1 hour at 4°C. Bound complexes were washed 3 times with immunoprecipitation buffer. Proteins were then analyzed on western blotting using monoclonal anti-FLAG antibody (1:5,000).
Peptide ELISA assay

BSA/PBS-Tween 0.1% at room temperature for 1 h. Following 3 times wash with PBS-Tween 0.05%, cells were incubated by anti-human antibody (074-1002, Kirkegaard and Perry laboratories, Gaithurburg, MD) (1:5,000) for wells reacted by sera, or anti-mouse antibody (074-1804, Kirkegaard and Perry laboratories, Gaithurburg, MD) (1:5,000) for wells reacted by anti-CaSR at room temperature for 1 h. After 3 times wash with PBS-Tween 0.05%, the cells were treated with substrate (o-phenylenediamine dihydrochloride, Sigma) for 5 min at room temperature. This reaction was stopped by the addition of an equivalent volume of 2.5 N HCl and the absorption levels were read at 492 nm using a plate reader (EnSpire, Perkin Elmer).

**Statistics.**

Two-sided Student’s \(t\) test (for two comparisons) and two-sided Tukey’s multiple comparison procedures (for multiple comparisons) were used for statistical analysis. All analyses were performed using Prism 8 software. Averaged data from more than three independent experiments are shown, and error bars represent the SEM unless otherwise stated. A value of \(P < 0.05\) was considered significant for all analyses. \(*P < 0.05; **P < 0.01; ***P < 0.001\)
Study approval

This study was performed under approval of the Institutional Review Board of the University of Tokyo (approved number: G1067) and written informed consent was received from our AHH patient prior to inclusion in this study.

Author Contributions

NM, YH, HH and JS performed the experiments; KA, T Ito, HY and AM diagnosed the patients as AHH clinically and followed patients; MN, KM, NM, and T Iiri analyzed the data; MN and T Iiri wrote the paper; all authors discussed the manuscript.

Acknowledgments

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Table 1. Characteristics of the three AHH study patients

<table>
<thead>
<tr>
<th></th>
<th>CCa</th>
<th>iP</th>
<th>Mg</th>
<th>Cre</th>
<th>eGFR (mL/min/1.73m²)</th>
<th>Intact PTH (pg/mL)</th>
<th>FECa (%)</th>
<th>Reactivity to ECD</th>
<th>Gq/11</th>
<th>Gi/o</th>
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<td>12.1</td>
<td>2.8</td>
<td>3.0</td>
<td>0.88</td>
<td>64.6</td>
<td>64.6</td>
<td>0.03</td>
<td>+</td>
<td>↑</td>
<td>↓</td>
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<tr>
<td>Posi-2</td>
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<td>1.4</td>
<td>3.3*</td>
<td>0.70</td>
<td>68.4</td>
<td>83</td>
<td>0.04</td>
<td>+</td>
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<td>↓</td>
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<tr>
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<td>69.5</td>
<td>60</td>
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<td>↓</td>
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<tr>
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<td>101</td>
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<tr>
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<td>3.4</td>
<td>0.97</td>
<td>55.7</td>
<td>32</td>
<td>0.18</td>
<td>+</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

Posi-1 and Posi-2 are AHH patients we previously reported.
Pt-1, patient-1; Pt-2, patient-2; Pt-3, patient-3
FECa, fractional excretion of calcium;
ECD, extracellular domain of calcium-sensing receptor;
Cina, cinacalcet; PSL, prednisolone; Evo, evocalcet;
* The Mg level of Posi-2 was checked on another day.
Figure 1. Temporal profile of the corrected serum Ca levels in the three AHH study patients
(A) Pt-1 (B) Pt-2 (C) Pt-3
Black filled circles denote the corrected calcium levels in (A-C).
Gray bar indicates the reference interval of calcium level.
Filled and open arrowheads indicate the timing of the in vitro analysis
(Analysis-1 and Analysis-2).
Zol, zolendronic acid; PSL, prednisolone;
Figure 2. Immunofluorescence and cell surface ELISA using the AHH patients’ serum against COS7 cells and HEK293 cells expressing human CaSR

(A) Immunofluorescence staining of COS7 cells transiently expressing FLAG-tagged human CaSR with the AHH patient/control sera (1:100) and anti-FLAG antibody (1:2,000). Scale bar: negative c., 7.57 μm; Pt-1, 4.04 μm; Pt-2, 5.8 μm; Pt-3, 5.05 μm (B) Immunofluorescence staining of HEK293 cells expressing human CaSR with the AHH patient/control sera (1:100). Scale bar: 50 μm. (C) Cell surface ELISA against HEK293 cells expressing human CaSR with the AHH patient/control sera (1:500). Statistical analysis was performed using a two-sided Student’s t test. Values represent the mean ± SEM of triplicate determinations. Each set of results is representative of at least two additional experiments. ***,P < 0.001, n.s., not significant.

Figure 2
Figure 3. Ca-stimulated IP1 accumulation and ERK1/2 phosphorylation following the co-administration of AHH patient IgG at two timepoints
HEK293 cells expressing human CaSR stably were stimulated by 2.0 mM of Ca with 2 mg/dL of IgG. (A) IP1 accumulation was shown. (B) The phosphorylation of ERK1/2 was detected by immunoblotting, and each ERK1/2 was detected by reblotting of the same membrane. (C) Intensity of pERK and ERK was analyzed by Image-J software, and each intensity ratio (%) against the ratio at 2.0 mM Ca with control IgG was calculated, respectively. Statistical analysis was performed using two-sided Student’s t test (A) or Tukey’s multiple comparison test (C). Values represent the mean ± SEM of triplicate determinations. Each set of results is representative of at least two additional experiments. **P < 0.01, ***P < 0.001, n.s., not significant.
Figure 4. Ca-dependent phosphorylation of ERK1/2 (pERK) and the effects of pertussis toxin (PTX), YM-254890 (YM) and RGS domain of GRK2 (RGS) in HEK293 cells transiently expressing CaSR and RGS or a mock control. (A) Ca-dependent pERK and the effects of RGS, 500 ng/mL PTX for 4 h or 10 nM YM were detected by immunoblotting, and each ERK1/2 was detected by reblotting of the same membrane. (B) Intensity of pERK and ERK was analyzed by Image-J software, and each ratio against the ratio at 0.3 mM Ca was calculated, respectively. Statistical analysis was performed using a Tukey’s multiple comparison test. Values represent the mean ± SEM of triplicate determinations. Each set of results is representative of at least two additional experiments. ***P < 0.001, n.s., not significant.
Figure 5. Ca-dependent phosphorylation of ERK1/2 (pERK) and the effects of pertussis toxin (PTX) and YM-254890 (YM) in HEK293 cells stably expressing CaSR. (A) Ca-dependent pERK and the effects of pretreatment of 500 ng/mL PTX for 4 h or 10 nM YM were detected by immunoblotting, and each ERK1/2 was detected by reblootting of the same membrane. (B) Intensities of pERK and ERK were analyzed using Image-J software, and each ratio against the ratio at 1.5 mM Ca was calculated, respectively. Each point reflects a Ca-dependent ratio (blue closed circle; EC50, 2.06 ± 0.01 mM and Emax, 13.8, calculated except ratio at 5 mM Ca) under exposure to PTX (red closed square), and YM (green closed triangle). Statistical analysis was performed using a Tukey’s multiple comparison test. Values represent the mean ± SEM of triplicate determinations. Each set of results is representative of at least two additional experiments. **P < 0.01, ***P < 0.001, n.s., not significant.
Figure 6. Ca-dependent Gi/o-cAMP inhibition and Gq/11-IP1 accumulation with the effects of pertussis toxin (PTX) and YM-254890 (YM) in HEK293 cells stably expressing CaSR. (A) A Ca-dependent luminescent count stimulated by 3 μM forskolin (blue closed circle; IC50, 1.52 ± 0.09 mM) with the effects of 500 ng/mL PTX for 4 h (red closed square) and 10 nM YM (green closed triangle; IC50, 1.88 ± 0.03 mM). (B) Ca-dependent IP1 accumulation (blue closed circle; EC50, 2.37 ± 0.02 mM; Emax, 644 nM) with the effects of 500 ng/mL PTX for 4 h (red closed square) and 10 nM YM (green closed triangle). (C) Luminescent count stimulated by 2.0 mM Ca and 3 μM forskolin without (blue closed circle), or with 1 nM (red closed triangle), 10 nM (green closed triangle) or 100 nM (square closed purple) YM. Statistical analysis was performed using a two-sided Student’s t test (A, B) or Tukey’s multiple comparison tests (C). Values represent the mean ± SEM of triplicate determinations. Each set of results is representative of at least two additional experiments. *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant.
Figure 7. Immunoblotting analysis of AHH study patient serum reactivity against the extracellular domain of CaSR (ECD) or an ECD mutant containing alanine substitutions at amino acids 214-235 (polyA)

(A) Immunoprecipitated ECD or polyA was resolved by SDS-PAGE and immunoblotted against anti-FLAG (1:5,000) or sera from a negative control and the three AHH patients (1:200). (B) Bands were analyzed by Image-J software. Statistical analysis was performed using a two-sided Student’s t test. Values represent the mean ± SEM of triplicate determinations. Each set of results is representative of at least two additional experiments. **P <0.01, n.s., not significant. Purple closed circles, ECD; orange closed triangles, polyA.

Figure 7
Figure 8. Peptide ELISA with patient/control sera against peptides covering all the extracellular domain of CaSR and a 3D representation of dimerized CaSR drawn using PyMOL.

(A) Immunoreactivity against peptides covering the extracellular domain or extracellular loop of CaSR. Each number reflects each peptide; 1; 1-20, 2; 21-31, 3; 32-43, 4; 44-58, 5; 56-70, 6; 70-79, 7; 79-95, 8; 96-110, 9; 108-122, 10; 123-137, 11; 138-150, 12; 151-164, 13; 165-179, 14; 177-191, 15; 187-203, 16; 204-213, 17; 214-238, 18; 239-255, 19; 256-266, 20; 267-281, 21; 282-292, 22; 293-307, 23; 308-314, 24; 314-323, 25; 322-329, 26; 330-343, 27; 344-358, 28; 359-373, 29; 374-391, 30; 392-405, 31; 406-420, 32; 419-428, 33; 428-440, 34; 441-455, 35; 456-470, 36; 471-483, 37; 484-493, 38; 494-508, 39; 506-520, 40; 519-532, 41; 533-546, 42; 547-561, 43; 559-573, 44; 571-585, 45; 586-603, 46; 667-686, 47; 724-773, and 48; 824-841. Values represent the mean ± SEM of triplicate determinations. Each set of results is representative of at least two additional experiments. (B) 3D depiction of dimerized CaSR based on 7DTW in the protein data bank (PDB) drawn using PyMOL 2.0.7. Green, regions covered by the peptides cover; Magenta, 214-235 residues of CaSR,