Allosteric Modulation of the Calcium-Sensing Receptor Rectifies Signaling Abnormalities Associated with G-protein alpha-11 Mutations causing Hypercalcemic and Hypocalcemic Disorders

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ABSTRACT

Germline loss- and gain-of-function mutations of G-protein alpha-11 (Ga11), which couples the calcium-sensing receptor (CaSR) to intracellular calcium (Ca2+i) signaling, lead to familial hypocalciuric hypercalcemia type 2 (FHH2) and autosomal dominant hypocalcemia type 2 (ADH2), respectively, whereas somatic Ga11 mutations mediate uveal melanoma development by constitutively upregulating MAPK signaling. Cinacalcet and NPS-2143 are allosteric CaSR activators and inactivators, respectively, that ameliorate signaling disturbances associated with CaSR mutations, but their potential to modulate abnormalities of the downstream Ga11 protein is unknown. This study investigated whether cinacalcet and NPS-2143 may rectify Ca2+i alterations associated with FHH2- and ADH2-causing Ga11 mutations, and evaluated the influence of germline gain-of-function Ga11 mutations on MAPK signaling by measuring ERK phosphorylation, and assessed the effect of NPS-2143 on a uveal melanoma Ga11 mutant. WT and mutant Ga11 proteins causing FHH2, ADH2 or uveal melanoma were transfected in CaSR-expressing HEK293 cells, and Ca2+i and ERK1/2 phosphorylation responses measured by flow-cytometry and Alphascreen immunoassay following exposure to extracellular Ca2+ (Ca2+o) and allosteric modulators. Cinacalcet and NPS-2143 rectified the Ca2+i responses of FHH2- and ADH2-associated Ga11 loss- and gain-of-function mutations, respectively. ADH2-causing Ga11 mutations were demonstrated not to be constitutively activating and induced ERK phosphorylation following Ca2+o stimulation only. The increased ERK phosphorylation associated with ADH2 and uveal melanoma mutants was rectified by NPS-2143. These findings demonstrate that CaSR-targeted compounds can rectify signaling disturbances caused by germline and somatic Ga11 mutations, which respectively lead to calcium disorders and tumorigenesis; and that ADH2-causing Ga11 mutations induce non-constitutive alterations in MAPK signaling.

Guanine nucleotide-binding protein (G-protein) alpha-11 (Ga11) is a major intracellular signaling
partner of the cell-surface G-protein-coupled calcium (Ca^{2+})-sensing receptor (CaSR), which plays a pivotal role in the parathyroid and renal regulation of extracellular calcium (Ca^{2+}_o) concentrations (1,2). Ga_{11} belongs to the G_{q/11} class of G-proteins that enhance phospholipase C activity (3), thereby leading to an accumulation of inositol 1,4,5-trisphosphate and rapid increase in intracellular Ca^{2+} (Ca^{2+}_i) concentrations (2,4). These signal transduction events allow the CaSR to respond to small fluctuations in the prevailing Ca^{2+}_o concentration ([Ca^{2+}_o]) by inducing alterations in parathyroid hormone (PTH) secretion and urinary Ca^{2+} excretion (5).

The identification of germline heterozygous loss- and gain-of-function mutations of Ga_{11}, which is encoded by the GNA11 gene on chromosome 19p13.3, that lead to forms of familial hypocalciuric hypercalcemia (FHH) or autosomal dominant hypocalcemia (ADH), respectively, has demonstrated the importance of this G-protein subunit in Ca^{2+}_o homeostasis (1,6,7). FHH is a genetically heterogeneous disorder that is inherited as an autosomal dominant condition, which is characterised by lifelong elevations of serum Ca^{2+} concentrations in association with normal or mildly raised serum PTH levels and low urinary Ca^{2+} excretion (8). FHH is considered to represent a benign disorder, however some patients may develop symptomatic hypercalcemia, pancreatitis or chondrocalcinosis (8). FHH type 1 (FHH1, OMIM #145980) is caused by loss-of-function mutations of the CASR gene (9), and FHH type 2 (FHH2, OMIM #145981) is caused by loss-of-function Ga_{11} mutations, respectively, and the upregulation of ERK phosphorylation caused by a uveal melanoma-associated somatic Ga_{11} mutation. In addition, this study evaluated whether germline ADH2-causing gain-of-function Ga_{11} mutations may constitutively activate MAPK signaling and thus pose a risk for the development of uveal melanomas.

EXPERIMENTAL PROCEDURES

Cell culture and transfection - Functional studies of mutant Ga_{11} proteins were performed in HEK293 cells that stably expressed the CaSR (HEK-CaSR) (1,16,17). HEK293 cells endogenously express Ga_{11}, and co-expression of mutant Ga_{11} proteins approximately represented the heterozygous state in FHH2 and ADH2 patients (1). The HEK-CaSR cell line was cultured in high-glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% geneticin, as described (1,16,17). A high level of CaSR expression in these cells was confirmed by Western blot analysis using a mouse monoclonal antibody to human CaSR (ADD; Abcam, ab19347, 1:1,000) (1,16). WT and mutant GNA11-pBI-CMV2 constructs were transiently transfected into HEK-CaSR cells using Lipofectamine 2000.
(1,16,17). The bidirectional pBI-CMV2 cloning vector was used as it facilitated the co-expression of Gα11 and GFP (1,16,18). Expression of WT and mutant Gα11 proteins were determined by Western blot analysis using a mouse monoclonal anti-Gα11 antibody (SantaCruz, sc-390382, 1:750), and the membrane was re-probed with a polyclonal rabbit anti-α-tubulin antibody (Abcam, ab15246, 1:1000) as a loading control. Successful transfection was also confirmed by visualising GFP fluorescence using an Eclipse E400 fluorescence microscope with a Y-FL Epifluorescence attachment and a triband 4,6-diamidino-2-phenylindole-FITC-Rhodamine filter, and images captured using a DXM1200C digital camera and NIS-Elements software (Nikon) (1,16,17).

Studies involving siRNA knockdown of endogenous Gα11 were undertaken in HEK293 cells that stably expressed WT or mutant Gα11 proteins (HEK-Gα11). The HEK-Gα11 cells were generated using HEK293 T-Rex-Flp-in stable cell lines (Life Technologies), as reported (19). WT and mutant GNA11 constructs were cloned into the pcDNA5/FLP recombination target (FRT) expression vector (Life Technologies), and silent mutations introduced to render the constructs resistant to GNA11-targeted siRNA, thereby allowing investigation of the mutant Gα11 protein in the absence of endogenous WT Gα11. GNA11 constructs were transiently transfected into T-Rex-Flp-in cells, and those cells expressing the Gα11 protein selected by culturing cells in media containing Hygromycin (Gibco). The presence of the Gα11 protein, and its resistance to siRNA was confirmed by Western blot analysis. Forty-eight hours prior to measuring Ca2+ responses, HEK-Gα11 cells were transiently transfected with the reported pEGFP-CaSR construct (9) and three different commercially available GNA11-targeted siRNA constructs (Trilencer-27 siRNA kit, catalog number SR301839, Origene) or a commercially available scrambled siRNA (Trilencer-27 universal scrambled negative control siRNA duplex, catalog number SR30004, Origene), and successful transfection confirmed by fluorescence microscopy, as described for pBI-CMV2-expressing HEK-CaSR cells (1,16,17).

Measurement of Ca2+ responses - The effect of allosteric CaSR modulators on HEK-CaSR cells expressing WT or mutant Gα11 proteins was assessed by a flow cytometry-based Ca2+ assay, as reported (1,16,17). In brief, 48 hours after transfection, the cells were harvested, washed in Ca2+- and magnesium (Mg2+)-free Hank's balanced salt solution (HBSS) (Invitrogen) and loaded with 1 μg/ml indo-1-acetoxymethylester (Indo-1-AM) (Molecular Probes) for 1 hour at 37 °C (1,16,17). Transfected HEK-CaSR cells were incubated with either a 20% aqueous solution of 2-hydroxypropyl-β-cyclodextrin (Sigma) (vehicle), or positive or negative CaSR allosteric modulators, known as cinacalcet or NPS-2143, respectively, at concentrations ranging from 10-40 nM for 1 hour (15). Flow cytometry was performed with a Beckman Coulter MoFlo XDP equipped with JDSU Ycxye UV Laser and a Coherent Sapphire 488 Laser using a 550LP dichroic mirror and 580/30 bandpass filter (17). Single cells were isolated and stimulated by sequentially adding Ca2+ to the Ca2+- and Mg2+-free HBSS to increase the [Ca2+]o in a stepwise manner from 0-15 mM. The range of [Ca2+]o used to activate CaSR signaling in HEK293 cells was not representative of physiological levels of serum ionized calcium, which are homeostatically maintained between 1.1-1.3 mM (20), but use of these Ca2+ concentrations in vitro allowed a comprehensive assessment of CaSR signaling responses, which included threshold responses (1-1.5 mM Ca2+), half-maximal (EC50) responses (2-4 mM Ca2+) and near-maximal responses (>10 mM), as reported (21). The baseline fluorescence ratio was measured for 2 min, the fluorescence ratio compared to the time was recorded and data were collected for 2 min at each [Ca2+]o, as described (1,16,17). Cytomation Summit software was used to determine the peak mean fluorescence ratio of the transient response after each individual stimulus, which was expressed as a percentage normalized response (1,16,17). Concentration-response curves were generated using a 4-parameter non-linear regression curve-fit model (GraphPad Prism) to calculate the half-maximal (EC50) and area under the curve (AUC) mean ± SEM responses for each separate experiment (17).

Measurement of ERK phosphorylation - HEK-CaSR cells, transfected with WT or mutant Gα11 proteins for 24 hours, were seeded in 48-well plates and cultured overnight in high glucose DMEM containing 10% FBS, prior to being incubated for 4 hours with serum-free DMEM.
containing 0.5 mM Ca\(^{2+}\), 25 mM HEPES buffer with or without cinacalcet or NPS-2143 at 10-500 nM concentrations. Cells were stimulated for 4 min with pre-warmed serum-free DMEM that contained Ca\(^{2+}\) concentrations ranging from 0.5-10 mM, as reported (22), and lysed in SureFire lysis buffer. Alphascreen SureFire ERK phosphorylation assays were performed on whole cell lysates, as reported (23), and the fluorescence signal measured using a PHERAStar FS microplate reader (BMG Labtech) (23). ERK phosphorylation responses measured at each [Ca\(^{2+}\)]\(_o\) were normalized to the mean responses of WT expressing cells and expressed as a fold-change of responses obtained at basal (0.5 mM) [Ca\(^{2+}\)]\(_o\).

Statistical analysis - The Ca\(^{2+}\)\(_i\) and ERK phosphorylation responses of cells expressing WT or mutant Ga\(_{\alpha_{11}}\) proteins were compared from a minimum of four experiments using the F-test and Mann-Whitney U test, respectively (1). All analyses were undertaken using GraphPad Prism (GraphPad), and are presented as mean ± SEM. A value of p<0.05 was considered significant for all analyses.

RESULTS

Effect of cinacalcet on the Ca\(^{2+}\)\(_i\) responses of FHH2-associated Ga\(_{\alpha_{11}}\) mutations - The FHH2-associated Leu135Gln and Ile199/200del Ga\(_{\alpha_{11}}\) mutations have been reported to impair the sensitivity of CaSR-expressing cells to Ca\(^{2+}\)\(_o\) (1), and we hypothesized that cinacalcet-mediated allosteric activation of the CaSR would ameliorate the loss-of-function associated with germline mutations of Ga\(_{\alpha_{11}}\), thereby rectifying the signal transduction abnormalities in cells expressing these FHH2-associated mutant Ga\(_{\alpha_{11}}\) proteins. To investigate this hypothesis, WT or mutant GNAI1-pBI-CMV2 constructs were transiently transfected into HEK-CaSR cells, and the effect of cinacalcet on the responses of Ca\(^{2+}\)\(_i\) concentrations ([Ca\(^{2+}\)]\(_i\)) to alterations in [Ca\(^{2+}\)]\(_o\) was assessed. Expression of the CaSR and Ga\(_{\alpha_{11}}\) was confirmed by fluorescence microscopy and/or Western blot analysis of whole-cell lysates (Fig. 1A and B). CaSR expression, which was normalised by comparison to \(\alpha\)-tubulin expression, did not differ between cells transfected with WT or FHH2-associated mutant GNAI1-pBI-CMV2 vectors when compared to cells transfected with empty vector, whereas the expression of Ga\(_{\alpha_{11}}\) was greater in cells transfected with WT or mutant constructs (Fig. 1B). HEK-CaSR cells transiently transfected with WT or mutant Ga\(_{\alpha_{11}}\) proteins were exposed to varying [Ca\(^{2+}\)]\(_o\), and measurement of Ca\(^{2+}\)\(_i\) responses by flow cytometry revealed the FHH2-associated Gln135 and del199/200 Ga\(_{\alpha_{11}}\) mutants to result in a rightward shift of the concentration-response curves (Fig. 1C) with a significant reduction in AUC values and increases in EC\(_{50}\) values (Gln135 = 3.54 ± 0.07 mM, del199/200 = 3.49 ± 0.04 mM) compared to WT Ga\(_{\alpha_{11}}\) (2.67 ± 0.03 mM; p<0.0001) (Fig. 1D-E), as reported (1). A dose-titration of cinacalcet in cells expressing the Gln135 Ga\(_{\alpha_{11}}\) mutant revealed this calcimimetic to act in a dose-dependent manner, with 10 and 20 nM drug concentrations significantly (p<0.0001) reducing the Gln135 mutant EC\(_{50}\) values to 2.75 ± 0.03 and 2.61 ± 0.09 mM, respectively (Fig. 1E). Indeed, 10 nM of cinacalcet induced a leftward shift of the mutant concentration-response curve, so that this was indistinguishable from that of WT-expressing cells (Fig. 1F). The addition of 10 and 20 nM cinacalcet lowered the EC\(_{50}\) values of cells expressing the del199/200 Ga\(_{\alpha_{11}}\) mutant (Fig. 1E). However, despite the del199/200 mutant having an almost identical EC\(_{50}\) value to the Gln135 Ga\(_{\alpha_{11}}\) mutant protein, these cinacalcet doses were insufficient to rectify the loss-of-function associated with the del199/200 Ga\(_{\alpha_{11}}\) mutant (Fig. 1E). Subsequently, when cinacalcet was added at a 40 nM concentration to cells expressing the del199/200 mutant, this lowered the EC\(_{50}\) value to 2.68 ± 0.04 mM (Fig. 1E), so that the del199/200 mutant concentration-response curve overlapped with that of the WT Ga\(_{\alpha_{11}}\) protein (Fig. 1G).

Effect of NPS-2143 on the Ca\(^{2+}\)\(_i\) responses of ADH2-associated Ga\(_{\alpha_{11}}\) mutations - We previously reported the germline Arg181Gln and Phe341Leu Ga\(_{\alpha_{11}}\) mutations to enhance the sensitivity of CaSR-expressing cells to Ca\(^{2+}\)\(_o\) (1), thereby giving rise to the hypocalcemic disorder of ADH2. To determine whether allosteric inhibition of the CaSR can rectify the gain-of-function associated with ADH2-causing Ga\(_{\alpha_{11}}\) mutations, WT or ADH2-associated mutant GNAI1-pBI-CMV2 vectors were transiently transfected into HEK-CaSR cells, and the responses of [Ca\(^{2+}\)]\(_i\) to alterations in [Ca\(^{2+}\)]\(_o\) assayed. Expression of the CaSR and Ga\(_{\alpha_{11}}\) was demonstrated by fluorescence microscopy and/or Western blot analysis (Fig. 2A).
and B). Western blot analysis confirmed an increase in the expression of G\(\alpha_{11}\) in cells transfected with WT or ADH2-associated mutant proteins, when compared to cells transfected with empty vector alone (Fig. 2B). An assessment of the \(\text{Ca}^{2+}\) responses of HEK-CaSR cells transiently transfected with WT or ADH2-associated mutant G\(\alpha_{11}\) proteins following stimulation with \(\text{Ca}^{2+}_{o}\), demonstrated cells expressing the Gln181 or Leu341 mutants to increase in the expression of G\(\alpha_{11}\) (Fig. 2A). Western blot analysis confirmed an increase in the expression of G\(\alpha_{11}\) in HEK293 cells, siRNA knockdown of endogenous WT G\(\alpha_{11}\) was undertaken in HEK-G\(\alpha_{11}\) cells stably expressing WT, FHH2-associated Gln135, or ADH2-associated Gln181 mutant G\(\alpha_{11}\) proteins. Western blot analysis demonstrated that siRNA with a scrambled sequence did not alter endogenous WT G\(\alpha_{11}\) expression in untransfected HEK293 cells (Fig. 3A), and decreased the level of transiently expressed WT G\(\alpha_{11}\) in HEK293 cells (Fig. 3B), but did not affect the levels of stably expressed WT or mutant G\(\alpha_{11}\) proteins in HEK-G\(\alpha_{11}\) cells (Fig. 3B), which contained constructs with silent mutations that had rendered them resistant to G\(N\!A\!11\)-targeted siRNA. CaSR constructs were transiently transfected into HEK-G\(\alpha_{11}\) cells, and CaSR expression confirmed by fluorescence microscopy (Fig. 3C). The effects of cinacalcet or NPS-2143 on the \(\text{Ca}^{2+}\) responses of the FHH2- and ADH2-associated G\(\alpha_{11}\) mutants were assessed following knockdown of endogenous WT G\(\alpha_{11}\) using G\(N\!A\!11\)-targeted siRNAs (Fig. 3D-G). These studies revealed that: 10 nM of cinacalcet could rectify the rightward shift in the concentration-response curve and lower the significantly raised EC\(_{50}\) of the FHH2-associated Gln135 G\(\alpha_{11}\) mutant from a value of 3.85 ± 0.12 mM to values of 3.23 ± 0.1 mM and 3.17 ± 0.08 mM, respectively, in the presence of G\(N\!A\!11\)-targeted or scrambled siRNA (Fig. 3D and E), so that these values were not significantly different from HEK-G\(\alpha_{11}\) cells stably expressing WT G\(\alpha_{11}\) (EC\(_{50}\) = 3.33 ± 0.06 mM); and that 10 nM of NPS-2143 could normalize the leftward shift of the concentration-response curve and increased the EC\(_{50}\) of the ADH2-associated Gln181 G\(\alpha_{11}\) mutant from a value of 2.70 ± 0.07 mM to values of 3.26 ± 0.06 mM and 3.11 ± 0.08 mM, respectively, in the presence of G\(N\!A\!11\)-targeted or scrambled siRNA (Fig. 3F and G), so that these values were not significantly different from WT-expressing HEK-G\(\alpha_{11}\) cells. Thus, these results show that CaSR-targeted drugs can influence the signaling responses of downstream mutant G\(\alpha_{11}\) proteins.

**Effect of CaSR allosteric modulators on the \(\text{Ca}^{2+}\) responses in absence of endogenously expressed WT G\(\alpha_{11}\) protein** - To determine whether CaSR-targeted drugs rectify the \(\text{Ca}^{2+}\) responses of FHH2- and ADH2-mutant expressing cells by directly influencing mutant G\(\alpha_{11}\)-signaling or by indirect effects on WT G\(\alpha_{11}\) protein that is endogenously expressed in HEK293 cells, siRNA knockdown of endogenous WT G\(\alpha_{11}\) was undertaken in HEK-G\(\alpha_{11}\) cells stably expressing WT, FHH2-associated Gln135, or ADH2-associated Gln181 mutant G\(\alpha_{11}\) proteins. Western blot analysis demonstrated that siRNA with a scrambled sequence did not alter endogenous WT G\(\alpha_{11}\) expression in untransfected HEK293 cells (Fig. 3A). In contrast, G\(N\!A\!11\)-targeted siRNA reduced endogenous WT G\(\alpha_{11}\) expression in untransfected HEK293 cells (Fig. 3A), and decreased the level of transiently expressed WT G\(\alpha_{11}\) in HEK293 cells (Fig. 3B), but did not affect the levels of stably expressed WT or mutant G\(\alpha_{11}\) proteins in HEK-G\(\alpha_{11}\) cells (Fig. 3B), which contained constructs with silent mutations that had rendered them resistant to G\(N\!A\!11\)-targeted siRNA. CaSR constructs were transiently transfected into HEK-G\(\alpha_{11}\) cells, and CaSR expression confirmed by fluorescence microscopy (Fig. 3C). The effects of cinacalcet or NPS-2143 on the \(\text{Ca}^{2+}\) responses of the FHH2- and ADH2-associated G\(\alpha_{11}\) mutants were assessed following knockdown of endogenous WT G\(\alpha_{11}\) using G\(N\!A\!11\)-targeted siRNAs (Fig. 3D-G). These studies revealed that: 10 nM of cinacalcet could rectify the rightward shift in the concentration-response curve and lower the significantly raised EC\(_{50}\) of the FHH2-associated Gln135 G\(\alpha_{11}\) mutant from a value of 3.85 ± 0.12 mM to values of 3.23 ± 0.1 mM and 3.17 ± 0.08 mM, respectively, in the presence of G\(N\!A\!11\)-targeted or scrambled siRNA (Fig. 3D and E), so that these values were not significantly different from HEK-G\(\alpha_{11}\) cells stably expressing WT G\(\alpha_{11}\) (EC\(_{50}\) = 3.33 ± 0.06 mM); and that 10 nM of NPS-2143 could normalize the leftward shift of the concentration-response curve and increased the EC\(_{50}\) of the ADH2-associated Gln181 G\(\alpha_{11}\) mutant from a value of 2.70 ± 0.07 mM to values of 3.26 ± 0.06 mM and 3.11 ± 0.08 mM, respectively, in the presence of G\(N\!A\!11\)-targeted or scrambled siRNA (Fig. 3F and G), so that these values were not significantly different from WT-expressing HEK-G\(\alpha_{11}\) cells. Thus, these results show that CaSR-targeted drugs can influence the signaling responses of downstream mutant G\(\alpha_{11}\) proteins.

**Effect of ADH2-associated G\(\alpha_{11}\) mutants on MAPK signaling** - To investigate whether the germline Arg181Gln and Phe341Leu ADH2-associated mutant G\(\alpha_{11}\) proteins may lead to constitutive upregulation of MAPK signaling, WT and mutant G\(N\!A\!11\)-pBI-CMV2 vectors were transiently transfected into HEK-CaSR cells and fold-change ERK phosphorylation (phospho-ERK) responses assessed following exposure to varying \([\text{Ca}^{2+}]_{o}\). The effects of the ADH2-associated mutants on phospho-ERK responses were compared to the uveal melanoma-associated Gln209Leu G\(\alpha_{11}\) mutation (11). Following stimulation with \([\text{Ca}^{2+}]_{o}\), the germline Gln181 and
Leu341 mutants were revealed to have significantly (p<0.001) increased maximal phospho-ERK fold-change responses (Gln181 = 18.1 ± 1.1, Leu341 = 18.3 ± 0.9) compared to WT Ga11 (14.7 ± 0.3), consistent with a gain-of-function (Fig. 4A). However, in the absence of Ca2+o stimulation, the basal phospho-ERK responses of the ADH2 mutants were demonstrated to not differ from WT Ga11 (Fig. 4A and B), and thus these mutants are not constitutively activating. In contrast, the tumor-associated somatic Gln209Leu Ga11 mutation led to both significantly (p<0.0001) increased basal and maximal phospho-ERK fold-change responses when compared to the ADH2 mutants or WT Ga11, consistent with a constitutive upregulation of MAPK signaling (Fig. 4A and B). The effect of NPS-2143 on the phospho-ERK responses of HEK-CaSR cells expressing the ADH2-associated Gln181 or Leu341 mutants, or the uveal melanoma-associated Leu209 mutant, was also assessed. NPS-2143 was added at 10 and 30 nM concentrations to cells expressing the Gln181 and Leu341 mutants, respectively, as these doses had rectified the Ca2+ responses of the Ga11 mutants (Fig. 2F and G). The addition of 10 and 30 nM NPS-2143 significantly lowered the maximal fold-change responses of the Gln181 and Leu341 mutants to 14.0 ± 0.5 and 14.9 ± 0.4, respectively, so that these values did not differ from the phospho-ERK responses of cells expressing WT Ga11 (Fig. 4C and D). However, cells expressing the uveal melanoma-associated Leu209 mutant required NPS-2143 at a higher dose of 500 nM to successfully rectify increases in phospho-ERK responses (Fig. 4E).

**DISCUSSION**

Our studies demonstrate that cinacalcet and NPS-2143, which are allosteric CaSR activators and inactivators, respectively, can successfully rectify the loss-of-function associated with FHH2-causing Ga11 mutations and the gain-of-function associated with Ga11 mutations that lead to ADH2 or uveal melanoma (1,11). Cinacalcet and NPS-2143 are allosteric modulators that are predicted to bind to the CaSR transmembrane domain (24) and influence receptor activity by altering its conformational status. These compounds have been reported to rectify the activity of FHH1- and ADH1-associated mutant CaSR proteins *in vitro* (15,25-27). However, the ability of these agents to normalise CaSR sensitivity in the presence of an abnormality downstream of the CaSR remained unknown. The *in vitro* findings of our study indicate allosteric modulation at the level of the receptor can rectify such loss- and gain-of-function associated with mutations of the intracellular Ga11 protein. Indeed, these studies demonstrate that pharmacological GPCR modulation may directly overcome abnormalities affecting the downstream effector G-protein rather than by indirect effects on endogenously expressed WT G-proteins.

However, the Ga11 mutations showed differences in their responsiveness to allosteric CaSR modulators. For example, our study of the FHH2 mutants revealed that a 4-fold increase in the cinacalcet dose was required to normalise the loss-of-function associated with Ile199/200del compared to the Leu135Gln mutation, despite both mutations having similar EC50 values. Similarly, a 3-fold increase in the NPS-2143 dosage was required to rectify the gain-of-function due to the ADH2-associated Phe341Leu mutation when compared with the gain-of-function Arg181Gln mutation, despite both mutations having similar EC50 values. Thus, the Ile199/200del and Phe341Leu mutations showed diminished sensitivity to cinacalcet and NPS-2143, respectively, and these differences in the sensitivities of the mutants to CaSR-targeted drugs may be explained by a reported crystallography study, which showed residues homologous to Ile199 and Phe341, in the related Gas protein to be located at the interface between GPCR and Gα-subunit (28). Thus, Ga11 mutations located at the GPCR-Gα interface may potentially influence the efficacy of CaSR allosteric modulators.

Cells expressing loss- and gain-of-function Ga11 mutants responded to nanomolar concentrations of cinacalcet (10-40 nM, which is equivalent to 3.6-14.3 ng/mL) and NPS-2143 (10-30 nM, which is equivalent to 4.4-13.3 ng/mL), respectively. However, previous *in vitro* studies of CaSR mutations leading to FHH and ADH have indicated that micromolar concentrations of these drugs may be required to rectify associated signal transduction abnormalities (25-27), and *in vivo* studies in WT rats have reported that the plasma drug concentrations of cinacalcet and NPS-2143
required to alter PTH secretion are ≥20 ng/mL and >100 ng/mL, respectively (29,30). The responsiveness of Ga11 mutants to low doses of CaSR-targeted drugs may be explained by the finding that these mutants induced only minor disturbances of CaSR signal transduction. Indeed, the FHH2 and ADH2 mutants were associated with up to a 30% shift in the EC50 values of HEK-CaSR cells used in this study, whereas CaSR mutations leading to FHH1 and ADH1 generally cause a >50% shift in the EC50 value (9,21,31,32). However, it remains to be established whether such low concentrations of calcimimetic and calcilytic drugs will be able to rectify in vivo the alterations in mineral homeostasis in FHH2 and ADH2 patients.

Somatic gain-of-function Ga11 mutations that induce constitutive MAPK activation have been reported in uveal melanoma and are associated with an increased likelihood of metastases (11). We therefore assessed the effects of germline ADH2-associated Arg181Gln and Phe341Leu gain-of-function Ga11 mutations on MAPK signaling by measuring phospho-ERK responses. Our studies demonstrated that the ADH2 Ga11 mutants induced a milder increase in ERK phosphorylation when compared to the uveal melanoma Gln209Leu Ga11 mutant. Moreover, up-regulation of ERK phosphorylation by the ADH2-associated Ga11 mutants only occurred in the presence of Ca2+ stimulation, and therefore these Arg181Gln and Phe341Leu Ga11 mutants do not harbor constitutive activity. These findings are consistent with a recent report of an ADH2-associated Arg60Leu Ga11 mutation, which also enhanced MAPK activation in a non-constitutive manner (7). The finding that ADH2-associated mutations are not constitutively activating can be explained by their locations within the GTPase domain of the Ga subunit. Thus, the Gln209 residue, which is mutated in uveal melanomas (11), is required to spatially orientate the terminal phosphate group of Ga-bound GTP (33), thereby facilitating its hydrolysis and the conversion of GTP to GDP. Mutations affecting the Gln209 residue have been shown to abolish GTP hydrolysis, thereby leaving the Ga subunit in a permanent GTP-bound state of activation (34). In contrast, the Arg181 and Phe341 Ga11 residues, which are mutated in ADH2, are not located near to the terminal phosphate of GTP, and likely induce more indirect and subtle effects on GTP hydrolysis (1). The ADH2-associated Ga11 mutations represent the first reports of non-constitutively activating G-protein mutations (1,6,7), and the milder nature of these mutations is consistent with post-natal survival, in contrast to the constitutively activating Gln209Leu mutation, which has been shown to be cytotoxic when expressed at high levels (35), and is likely to be embryonically lethal. The occurrence of non-constitutively activating Ga11 mutations that are tolerated in humans and heritable, highlights the potential for such germline mutations to affect other G-proteins and be associated with disease-related phenotypes, and this possibility remains to be explored.

In summary, our studies have revealed that germline gain-of-function Ga11 mutations induce non-constitutive alterations in MAPK signaling, and that CaSR-targeted compounds may rectify signaling disturbances caused by germline and somatic Ga11 mutations, which are associated with calcium disorders and tumorigenesis, respectively. These findings indicate that allosteric modulation at the level of the receptor may influence signaling disturbances associated with mutations of the downstream G-protein.

Conflicts of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: F.M.H., M.A.N., A.C.H. and R.V.T. designed the experiments. V.N.B., S.A.H. and N.R. performed the Ca2+ measurement experiments. C.M.G. performed the siRNA knockdown experiments. V.N.B. performed the ERK phosphorylation experiments. J.H. and A.M.S. prepared and supplied the NPS-2143 compound. F.M.H., V.N.B., C.M.G. and R.V.T. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.
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FOOTNOTES
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The abbreviations used are: ADH, autosomal dominant hypocalcemia; ADH1, ADH type 1; ADH2, ADH type 2; AUC, area under the curve; Ca\(^{2+}\), calcium; Ca\(^{2+}\)_i, intracellular calcium; Ca\(^{2+}\)_o, extracellular calcium; CaSR, calcium-sensing receptor; EC\(_{50}\), half-maximal effective concentration; FBS, fetal bovine serum; FHH, familial hypocalciuric hypercalcemia; FHH1, FHH type 1; FHH2, FHH type 2; FRT, FLP recombination target; G\(\alpha_{11}\), G-protein alpha-11; Gas, G-protein alpha-s; GPCR, G-protein-coupled-receptor; HBSS, Hank's balanced salt solution; HEK-CaSR, HEK293-CaSR; HEK293-G\(\alpha_{11}\), HEK-G\(\alpha_{11}\); Indo-1-AM, indo-1-acetoxymethylester; m, mutant; Mg\(^{2+}\), magnesium; PTH, parathyroid hormone.

FIGURE LEGENDS

FIGURE 1. Effect of cinacalcet on the Ca\(^{2+}\)_i responses of FHH2-associated G\(\alpha_{11}\) mutations. A. Fluorescence microscopy of HEK293 cells stably expressing CaSR (HEK-CaSR) and transiently transfected with WT or FHH2-associated (Gln135 and del199/200) mutant (m) G\(\alpha_{11}\)-pBl-CMV2-GFP constructs, or with vector only. GFP expression in these cells indicates successful transfection and expression by these constructs. Bar indicates 20\(\mu\)m. B. Western blot analysis of whole cell lysates using antibodies to CaSR, \(\alpha\)-tubulin and G\(\alpha_{11}\). Transient transfection of WT or FHH2-associated mutant constructs resulted in over-expression of G\(\alpha_{11}\) when normalised to \(\alpha\)-tubulin expression. C-D. Ca\(^{2+}\)_i response to changes in [Ca\(^{2+}\)]\(_o\) of HEK-CaSR cells transfected with WT or FHH2-associated G\(\alpha_{11}\) mutants. The Ca\(^{2+}\)_i responses to changes in [Ca\(^{2+}\)]\(_o\) are expressed as a percentage of the maximum normalized responses and shown as the mean ± SEM of 6-16 assays from 2-4 independent transfections. The FHH2-associated G\(\alpha_{11}\) mutants (Gln135 and del199/200) led to a rightwards shift of the concentration-response curves (blue) with significantly reduced AUC values when compared with WT G\(\alpha_{11}\) (black), which harbors Leu and Ile residues at codons 135 and 199/200, respectively. E. The FHH2-associated Gln135 and del199/200 mutants (blue bars) are associated with significantly increased EC\(_{50}\) values compared to cells expressing WT G\(\alpha_{11}\) (open bar). The addition of 10 and 20 nM cinacalcet (Cin) decreased the EC\(_{50}\) of cells expressing Gln135 to values that were not significantly different from WT,
whereas 40 nM cinacalcet was required to rectify the increased EC$_{50}$ value of cells expressing the del199/200 mutant. F-G. The addition of cinacalcet at 10 and 40 nM concentrations rectified the rightward shift in the concentration-response curves of the Gln135 and del199/200 mutant Ga$_{11}$ proteins, respectively. *p<0.05, ***p<0.0001.

FIGURE 2. Effect of NPS-2143 on the Ca$^{2+}$$_i$ responses of ADH2-associated Ga$_{11}$ mutations. A. Fluorescence microscopy of HEK293 cells stably expressing CaSR (HEK-CaSR) and transiently transfected with vector, WT or ADH2-associated (Gln181 and Leu341) mutant (m) constructs. GFP expression in these cells indicates successful transfection and expression by these constructs. Bar indicates 20µm. B. Western blot analysis of whole cell lysates using antibodies to CaSR, α-tubulin and Ga$_{11}$. Transient transfection of WT or ADH2-associated mutant constructs resulted in over-expression of Ga$_{11}$ when normalised to α-tubulin expression. C-D. Ca$^{2+}$$_i$ response to changes in [Ca$^{2+}$]$_o$ of HEK-CaSR cells transfected with WT or ADH2-associated Ga$_{11}$ mutants. The Ca$^{2+}$$_i$ responses to changes in [Ca$^{2+}$]$_o$ are expressed as a percentage of the maximum normalized responses and shown as the mean ± SEM of 6-16 assays from 2-4 independent transfections. The ADH2-associated Ga$_{11}$ mutants (Gln181 and Leu341) led to a leftwards shift of the concentration-response curves (red) with significantly increased AUC values when compared with WT Ga$_{11}$ (black), which harbors Arg and Phe residues at codons 181 and 341, respectively. E. The ADH2-associated Gln181 and Leu341 mutants (red bars) are associated with significantly reduced EC$_{50}$ values compared to cells expressing WT Ga$_{11}$ (open bars). The addition of 10 nM NPS-2143 (2143) increased the EC$_{50}$ value of cells expressing Gln135 so that this was not significantly different from WT, whereas 30 nM of NPS-2143 was required to rectify the reduced EC$_{50}$ value of cells expressing the Leu341 mutant. F-G. The addition of NPS-2143 at 10 and 30 nM concentrations rectified the leftward shift in the concentration-response curves of the Gln181 and Leu341 mutant Ga$_{11}$ proteins, respectively. *p<0.05, **p<0.01, ***p<0.0001.

FIGURE 3. Effect of cinacalcet and NPS-2143 on the Ca$^{2+}$$_i$ responses of FHH2- and ADH2-associated Ga$_{11}$ mutations following siRNA knockdown of endogenously expressed WT Ga$_{11}$. A. Western blot analysis of untransfected HEK293 cells, which express endogenous WT Ga$_{11}$ only, and have been treated with either scrambled siRNA, or three different GNA11-targeted siRNAs (siRNAs 1-3), either alone or all together (siRNAs 1+2+3), and compared to untreated HEK293 cells. All three GNA11-targeted siRNAs, but not scrambled siRNA, reduced endogenous WT Ga$_{11}$ expression. B. Western blot analysis showing combined effects of the three GNA11-targeted siRNAs on Ga$_{11}$ protein expression in HEK293 cells transiently expressing WT Ga$_{11}$ proteins and in HEK-Ga$_{11}$ cells, which stably express WT or mutant Ga$_{11}$ proteins. Use of GNA11-targeted siRNAs reduced Ga$_{11}$ expression in HEK293 cells, but not in HEK-Ga$_{11}$ cells, which are resistant to GNA11-targeted siRNA. C. Fluorescence microscopy confirming transfection of untreated and siRNA-treated (scrambled or combined siRNAs 1+2+3) HEK-Ga$_{11}$ cells with the pEGFP-CaSR construct. Bar indicates 50µm. D-E. Ca$^{2+}$$_i$ response to changes in [Ca$^{2+}$]$_o$ of FHH2-associated mutant Gln135 HEK-Ga$_{11}$ cells following siRNA knockdown of endogenous WT Ga$_{11}$. The Ca$^{2+}$$_i$ responses to changes in [Ca$^{2+}$]$_o$ are expressed as a percentage of the maximum normalized responses and shown as the mean ± SEM of 4-5 independent transfections (i.e. biological replicates). The FHH2-associated Ga$_{11}$ mutant (Gln135) led to a rightward shift of the concentration-response curve (blue), with a significant increase in EC$_{50}$ value compared to WT Ga$_{11}$ (black). The addition of 10 nM cinacalcet (Cin) normalized the EC$_{50}$ value of cells in the presence of scrambled (grey) or GNA11-targeted siRNAs (siRNAs 1+2+3) (red). F-G. Ca$^{2+}$$_i$ response to changes in [Ca$^{2+}$]$_o$ of ADH2-associated mutant Gln181 HEK-Ga$_{11}$ cells following siRNA knockdown of endogenous WT Ga$_{11}$. The ADH2-associated Ga$_{11}$ mutant (Gln181) led to a leftward shift of the concentration-response curve (blue), with a significant decrease in EC$_{50}$ value compared to WT Ga$_{11}$ (black). The addition of 10 nM NPS-2143 (2143) normalized the EC$_{50}$ value of cells in the presence of scrambled (grey) or GNA11-targeted siRNAs (siRNAs 1+2+3) (red). **p<0.0001; -, nil; scram, scrambled.
FIGURE 4. Phospho-ERK responses of Gα11 mutations associated with ADH2 or uveal melanoma. A. The phospho-ERK response to changes in [Ca$^{2+}$]o was measured by quantitative immunoassay (Alphascreen) in HEK-CaSR cells transiently transfected with WT or ADH2-associated Gα11 mutants (Gln181 and Leu341), or the uveal melanoma (UV)-associated Leu209 Gα11 mutant protein. Phospho-ERK responses at each [Ca$^{2+}$]o are expressed as a fold-change of the response of cells stimulated with basal (0.5 mM) [Ca$^{2+}$]o, and are shown as the mean ± SEM of 9-24 assays from 3-8 independent transfections. The uveal melanoma-associated Leu209 Gα11 mutant is associated with significantly increased maximal phospho-ERK fold-change responses compared to WT (Qln209) and the ADH2 mutant Gα11 proteins. The Gln181 and Leu341 mutants also induce significant increases in maximal phospho-ERK fold-change responses compared to WT Gα11, which harbors Arg and Phe residues at codons 181 and 341, respectively. B. Quantification of the basal phospho-ERK responses shown in A. Values are expressed as a percentage of the WT basal phospho-ERK response. The uveal melanoma-associated Leu209 Gα11 mutant induces a significant phospho-ERK elevation when exposed to basal 0.5 mM [Ca$^{2+}$]o, whereas the basal phospho-ERK responses of the ADH2-associated Gα11 mutants are not significantly different compared to WT Gα11. C-E. The addition of NPS-2143 (2143) at 10, 30 and 500 nM concentrations significantly decreased the phospho-ERK responses of the ADH2-associated Gln181 and Leu341 Gα11 mutants, and the UV-associated Leu209 mutant Gα11 protein, respectively, to values that were not significantly different from WT Gα11. ***p<0.0001.
Figure 1

(A) Representative images of CaSR expression in cells transfected with different constructs: Vector, WT, Gln135, and del199/200 mutants.

(B) Western blot analysis of CaSR and α-tubulin expression in FHH2 mutants. CaSR and α-tubulin levels are shown for Vector, WT, Gln135, and del199/200 mutants.

(C) Dose-response curves for intracellular calcium concentration (%) normalized to extracellular calcium concentration (mM) for WT, Gln135, and del199/200 mutants. The EC50 values are indicated with asterisks: *** for WT, **** for Gln135, and ** for del199/200.

(D) Bar graph showing AUC values (%.mM) for different Cin concentrations (0-40 nM) in WT and FHH2 mutants. The AUC values are statistically significant compared to WT.

(E) Bar graph showing EC50 values (mM) for different Cin concentrations (0-40 nM) in WT and FHH2 mutants. The EC50 values are significantly different between WT and mutants.

(F) Dose-response curves for intracellular calcium concentration (%) normalized to extracellular calcium concentration (mM) for WT and Gln135 mutants with 10nM Cin.

(G) Dose-response curves for intracellular calcium concentration (%) normalized to extracellular calcium concentration (mM) for WT and del199/200 mutants with 40nM Cin.
Figure 2

A

Vector WT Gln181 (m) Leu341 (m)

B

ADH2 mutants (m)

CaSR α-tubulin Gα11

C

Extracellular calcium concentration (mM)

Normalized response of intracellular calcium concentration (%)

WT Gln181 (m) Leu341 (m)

D

AUC (%.mM)

2143 (nM): 0 10 20 0 20 30

Gln181 Leu341

WT ADH2 mutants (m)

E

EC50 (mM)

2143 (nM): 0 10 20 0 20 30

Gln181 Leu341

WT ADH2 mutants (m)

F

Normalized response of intracellular calcium concentration (%)

Extracellular calcium concentration (mM)

WT Gln181 + 10nM 2143

G

Normalized response of intracellular calcium concentration (%)

Extracellular calcium concentration (mM)

WT Leu341 + 30nM 2143
Figure 3

A

|          | Untreated | Scrambled siRNA | siRNA (1+2+3) | siRNA 1 | siRNA 2 | siRNA 3 |
|----------|-----------|-----------------|---------------|---------|---------|---------|
| Gα11     |           |                 |               |         |         |         |
| α-tubulin|           |                 |               |         |         |         |

B

|          | Untreated | GNA11-siRNA (1+2+3) |
|----------|-----------|---------------------|
| Transiently-expressed Gα11 | WT | WT |
| Stably-expressed Gα11 | Gln135 | Gln181 |

|          | WT | WT | WT |
|----------|----|----|----|
| Gα11     |    |    |    |
| α-tubulin|    |    |    |

C

|          | WT | Gln135 FHH2 mutant | Gln181 ADH2 mutant |
|----------|----|-------------------|-------------------|
| Untreated|    |                   |                   |
|          |    |                   |                   |
|          |    |                   |                   |

D

Normalized response of intracellular calcium concentration (%)

Extracellular calcium concentration (mM)

E

EC₅₀ (mM)

Cin (nM):
- Scram
- 10
- 10

G

Normalized response of intracellular calcium concentration (%)

Extracellular calcium concentration (mM)
