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**AP2σ Mutations Impair Calcium-Sensing Receptor Trafficking and Signaling, and Show an Endosomal Pathway to Spatially Direct G-Protein Selectivity**

**Highlights**
- Disease-causing AP2σ mutants impair Gαq/11 and Gαi/o signaling by CaSR, a class C GPCR
- AP2σ mutants impair trafficking of the CaSR
- The CaSR can signal by a sustained endosomal pathway
- CaSR differentially uses Gαq/11 and Gαi/o for cell-surface and endosomal signaling

**In Brief**
Gorvin et al. show that the class C GPCR calcium-sensing receptor (CaSR) mediates signaling from plasma membranes using Gαq/11 and Gαi/o and from endosomes by using only Gαq/11. Adaptor protein-2 σ subunit (AP2σ) mutations impair CaSR internalization, leading to reduced sustained endosomal signaling and hypercalcemia in humans.

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**Graphical Abstract**

**NORMAL**
- Calcium-sensing receptor (CaSR), a GPCR
- Signaling from cell surface: Gαq/11 + Gαi/o
- Immediate signal: MAPK, cAMP, membrane ruffling, [Ca2+] release
- Physiological outputs: Parathyroid hormone secretion + urinary calcium excretion

**DISEASE**
- FHH3
- AP2σ mutations
- Reduced CaSR internalization, cell surface + sustained signaling
- Sustained signal from endosome: Gαq/11
- Signal outputs: MAPK, cAMP, membrane ruffling, [Ca2+] release
- Physiological outputs: Parathyroid hormone secretion + urinary calcium excretion
AP2σ Mutations Impair Calcium-Sensing Receptor Trafficking and Signaling, and Show an Endosomal Pathway to Spatially Direct G-Protein Selectivity

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SUMMARY

Spatial control of G-protein-coupled receptor (GPCR) signaling, which is used by cells to translate complex information into distinct downstream responses, is achieved by using plasma membrane (PM) and endocytic-derived signaling pathways. The roles of the endomembrane in regulating such pleiotropic signaling via multiple G-protein pathways remain unknown. Here, we investigated the effects of disease-causing mutations of the adaptor protein-2 σ subunit (AP2σ) on signaling by the class C GPCR calcium-sensing receptor (CaSR). These AP2σ mutations increase CaSR PM expression yet paradoxically reduce CaSR signaling. Hypercalcemia-associated AP2σ mutations reduced CaSR signaling via Gαq/11 and Gαi/o pathways. The mutations also delayed CaSR internalization due to prolonged residency time of CaSR in clathrin structures that impaired or abolished endosomal signaling, which was predominantly mediated by Gαq/11. Thus, compartmental bias for CaSR-mediated Gαq/11 endomembrane signaling provides a mechanistic basis for multidimensional GPCR signaling.

INTRODUCTION

The G-protein-coupled receptor (GPCR) family is the largest family of signaling receptors, and GPCRs contribute significantly to fundamental cellular functions. The archetypal model of GPCR signaling has evolved from a single, cell-surface receptor activating a specific heterotrimeric G-protein pathway to a complex network in which receptors can activate multiple pathways, exhibit signal crosstalk, and display functional selectivity (Rosebaum et al., 2009). This is illustrated by the calcium-sensing receptor (CaSR), a class C GPCR that is widely expressed and has calcitropic roles, i.e., regulation of extracellular calcium (Ca2+) by the parathyroids, kidneys, and bone, and non-calcitropic roles such as inflammation, bronchoconstriction, wound healing, gastro-pancreatic hormone secretion, hypertension, and glucose metabolism (Hofer et al., 2000; Rossol et al., 2012; Yarova et al., 2015; Zietek and Daniel, 2015). Thus, the CaSR, which like other class C GPCRs has a large extracellular domain (ECD) containing the ligand binding sites, a seven-transmembrane domain, and a large cytoplasmic C-terminal domain (Katrich et al., 2013), forms dimers and couples to multiple G-protein subtypes (e.g., Gαq/11, Gαi/o, Gα12/13, and Gαs) to induce diverse signaling pathways. For example, the CaSR, when stimulated by elevations in Ca2+, signals predominantly via Gαq/11 to activate phospholipase C (PLC), with consequent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), to the second messengers inositol 1,4,5-trisphosphate (IP3), and diacylglycerol (DAG) (Conigrave and Ward, 2013). IP3 acts upon IP3 receptors at the endoplasmic reticulum, allowing intracellular calcium mobilization into the cytosol, and DAG activates protein kinase C (PKC) signaling cascades, including mitogen-activated protein kinase (MAPK) pathways (Conigrave and Ward, 2013). CaSR has also been reported to signal via Gαq/o to inhibit adenylate cyclase (AC) and reduce cyclic AMP (cAMP) (Conigrave and Ward, 2013), Gα12/13 to initiate cytoskeletal remodeling (Davies et al., 2006; Huang et al., 2004), and Gαs, leading to elevated cAMP levels in breast cancer cell lines (Mamillapalli et al., 2008).

These CaSR signaling pathways are dependent on CaSR cell-surface expression, which is regulated by a balance between its plasma membrane (PM) insertion and removal by endocytosis (Grant et al., 2011). The PM insertion of CaSRs involves an anterograde signaling pathway, referred to as agonist-driven insertion signaling (ADIS), in which CaSRs that are continuously produced at the endoplasmic reticulum are rapidly trafficked to...
and inserted at the PM in the presence of high Ca\textsuperscript{2+} (Grant et al., 2011). Following activation, CaSRs have been reported to be endocytosed at a constant rate and targeted to the endo-lysosomal pathway for degradation (Grant et al., 2011). However, studies of patients with familial hypocalciuric hypercalcemia type-3 (FH\text{III}), an autosomal dominant calcitropic disorder that is due to mutations of the \( \sigma \) subunit of the heterotrimeric adaptor protein-2 (AP2\( \sigma \)), which has a critical role in clathrin-mediated endocytosis (Nesbit et al., 2013b), have reported that FH\text{III}-associated AP2\( \sigma \) mutations result in increased expression of the CaSR at the PM, which is paradoxically associated with reduced CaSR signaling via G\text{q/11} (Nesbit et al., 2013a). FHH3-associated AP2\( \sigma \) mutations have been found to only occur at residue R15, and these comprise one of three missense mutations, R15C, R15H, or R15L, all of which would lead to a loss or weakening of a polar contact with the dileucine-based motif within cytoplasmic regions of membrane-associated cargo proteins and thereby impair their endocytosis (Kelly et al., 2008; Nesbit et al., 2013b). In vitro studies of these FHH3-associated mutations demonstrated that these AP2\( \sigma \) mutations decreased CaSR-mediated G\text{q/11} signaling in response to elevations in Ca\textsuperscript{2+} in cells expressing the mutants, despite increased CaSR cell-surface expression (Nesbit et al., 2013b).

To explain this paradox, we hypothesized that the FH\text{III}-associated AP2\( \sigma \) mutations may be disrupting the contribution of endosomal sustained signaling to CaSR-dependent G-protein pathways, similar to those reported for some class A GPCRs—e.g., \( \beta_{2} \)-adrenergic receptor (\( \beta_{2} \)AR), dopamine receptor D1 (DRD1), thyroid-stimulating hormone receptor (TSHR), vasopressin receptor 2 (V2R), and luteinizing hormone receptor (LHR)—and class B GPCRs (e.g., parathyroid hormone receptor 1 receptor, PTH1R) (Calebiro et al., 2009; Feinstein et al., 2013; Ferrandon et al., 2009; Irannejad et al., 2013; Jean-Alphonse et al., 2014; Kotowski et al., 2011). These components of the endocytic pathway, which have previously been considered endpoints for signaling, are now known to provide sites for sustained GPCR signals (Feinstein et al., 2013; Ferrandon et al., 2009), although the contribution of endomembrane sustained signaling to GPCR function has only been studied in a single GPCR/G-protein pathway. However, GPCR signaling is complex, with many receptors (e.g., the CaSR) coupling to multiple G-protein-dependent and G-protein-independent pathways, and strategies to pharmacologically select for such specific pathways is increasingly recognized to be important (Rosenbaum et al., 2009). To further elucidate the role of the endocytic system in coordinating the pleiotropic activities of GPCRs, we investigated the effects of the FH\text{III}-associated AP2\( \sigma \) mutations on the different G-protein pathways activated by CaSR and discovered that impaired internalization, by clathrin-mediated endocytosis of CaSR, differentially affects G-protein pathways of CaSR.

RESULTS

Establishing AP2\( \sigma \) Mutant Stable Cell Lines

To investigate further the effects of FH\text{III}-associated AP2\( \sigma \) mutations on CaSR signaling and trafficking, HEK293 cells stably expressing AP2\( \sigma \) wild-type (WT; R15) or mutant (C15, H15, and L15) proteins were established, using appropriate pcDNA3.1-AP2\( \sigma \) constructs that also had silent mutations, which rendered them resistant to AP2\( \sigma \)-targeted small interfering RNA (siRNA), thereby allowing study of the mutant protein in the absence of endogenous protein. The presence of AP2\( \sigma \) mutant proteins or siRNA-resistant mutations did not affect expression of endogenous AP2\( \alpha \), AP2\( \beta \), or AP2\( \mu \) that with the \( \sigma \) subunit form the heterotrimeric AP2; general clathrin-mediated endocytic functions such as transferrin uptake; or internalization and signaling of another GPCR, the \( \beta_{2} \)AR (Figure S1). These stably expressing AP2\( \sigma \) cells were transiently transfected with pEGFP-CaSR-WT (AP2\( \alpha \)/CaSR-WT) cells (Figure S1). All AP2\( \sigma \) mutant/CaSR-WT cells, when compared to AP2\( \sigma \)-WT/CaSR-WT cells, had a decreased sensitivity to increases in Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}, which is mediated by G\text{q/11}, with significantly higher half-maximal effective concentration (EC\textsubscript{50}) values (Figure S2). These results, which are in agreement with our previous results from HEK293 cells transiently expressing AP2\( \sigma \) mutants (Nesbit et al., 2013b), demonstrate that these stably expressing AP2\( \sigma \) mutant cells have impaired G\text{q/11}-mediated, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}, release and that they are therefore suitable for studying the effects of FH\text{III}-associated AP2\( \sigma \) mutations on CaSR signaling pathways and trafficking.

AP2\( \sigma \) Mutations Reduce G\text{q/11} Signaling

We hypothesized that Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}, release of AP2\( \sigma \) mutant/CaSR-WT cells may be due to reduced calcium oscillations, and we assessed this by using single-cell microfluorimetry with the calcium-indicating dye Fura-2 in response to increasing concentrations (0–15 mM) of Ca\textsuperscript{2+}. CaSR-mediated Ca\textsuperscript{2+} oscillations were observed to occur from 1 to 5 mM Ca\textsuperscript{2+}, consistent with previous reports, but mutant cells were found to have reduced frequencies, with the AP2\( \sigma \)-C15 and AP2\( \sigma \)-L15 cells requiring higher Ca\textsuperscript{2+} concentrations to begin oscillating and AP2\( \sigma \)-H15 cells having oscillations with irregular amplitudes (Figures 1A and S2). Ca\textsuperscript{2+} release activates transcription factors such as nuclear factor of activated T cells (NFAT) (Chakravarti et al., 2012). Investigation of the effects of the FH\text{III}-associated AP2\( \sigma \) mutations on gene transcription, using an NFAT-response element (RE)-containing luciferase reporter construct, revealed that the AP2\( \sigma \) mutant/CaSR-WT cells had significantly reduced concentration-dependent increases in NFAT reporter activity compared to AP2\( \sigma \)-WT/CaSR-WT cells (Figure S2), thereby indicating that the PLC-IP\textsubscript{3}-DAG pathway is impaired in AP2\( \sigma \) mutant cells.

CaSR G\text{q/11}-mediated signaling also activates MAPK pathways (Kifor et al., 2001). Investigation of the AP2\( \sigma \) mutant/ CaSR-WT cells using AlphaScreen analyses of ERK1/2 phosphorylation (pERK1/2) in response to elevated Ca\textsuperscript{2+}...
revealed them to have significant reductions in Ca\(^{2+}\)-induced pERK1/2 responses when compared to AP2\(s\)-WT/CaSR-WT cells (Figure 1C). Moreover, pERK1/2 responses to increases in Ca\(^{2+}\) were reduced in Epstein-Barr virus (EBV)-transformed lymphoblastoid cells from FHH3 patients with the AP2\(s\)-R15C mutation (Figures 1D and S3), consistent with findings from AP2\(s\)-mutant/CaSR-WT cells. Expression of the AP2\(s\) subunit genes and proteins was similar in lymphoblastoids from FHH3 patients with the AP2\(s\)-R15C and unaffected relatives, indicating that the AP2\(s\)-R15C mutation was not affecting the stability of the AP2 complex (Figure S3). ERK1/2 activates genes containing serum response elements (SREs) (Pi et al., 2002). Use of a SRE luciferase reporter revealed the AP2\(s\)-mutant/CaSR-WT cells have reduced SRE reporter activity \((p < 0.02)\) (Figure 1E), with the more severe effects being observed in AP2\(s\)-H15 and AP2\(s\)-L15 mutant cells. Thus, these results demonstrate that the FHH3-associated AP2\(s\) mutations cause a reduction in G\(\alpha_{q/11}\) signaling via both the IP\(3\) and the DAG pathways.

**CaSR-Mediated cAMP Responses Are Altered by AP2\(\sigma\) Mutations**

CaSR activation of the G\(\alpha_{q/11}\) pathway inhibits adenylate cyclase and reduces cAMP, and we assessed the effects of the FHH3-associated AP2\(\sigma\) mutations using AlphaScreen analysis to measure Ca\(^{2+}\)-induced cAMP responses. Ca\(^{2+}\) was first confirmed to reduce cAMP responses, which were pertussis toxin (PTx) sensitive and therefore due to G\(\alpha_{i/o}\) signaling, in HEK293 cells stably expressing CaSR (HEK-CaSR) (Figure 2A). However, G\(\alpha_{i/o}\) inhibition only partially affected cAMP production, and treatment with UBO-QIC, an inhibitor of G\(\alpha_{q/11}\), revealed that the Ca\(^{2+}\)-induced reduction in cAMP was also sensitive to G\(\alpha_{q/11}\) inhibition, thereby indicating a hitherto unreported role for G\(\alpha_{q/11}\) (Figure 2B). Moreover, combined treatment of cells with both UBO-QIC and PTx halted all Ca\(^{2+}\)-induced reductions in cAMP (Figure 2B), indicating that G proteins other than G\(\alpha_{q/11}\) and G\(\alpha_{i/o}\) are unlikely to be involved in this CaSR pathway. However, UBO-QIC has been reported to inhibit G\(\beta\gamma\), in addition to G\(\alpha_{q/11}\) (Gao and Jacobson, 2016), but gallein, an inhibitor of G\(\beta\gamma\), had no effect on cAMP signaling (Figure 2C), thereby indicating that G\(\beta\gamma\) is unlikely to have a role in CaSR-mediated cAMP reductions. Increases in [Ca\(^{2+}\)]\(_e\) also led to a dose-dependent reduction in cAMP in AP2\(\sigma\)-WT/CaSR-WT cells, but not in AP2\(\sigma\)-mutant/CaSR-WT cells, with cAMP in AP2\(\sigma\)-C15/CaSR-WT cells remaining at basal levels (Figure 2D) and with AP2\(\sigma\)-H15/CaSR-WT and AP2\(\sigma\)-L15/CaSR-WT cells responding...
with reductions in cAMP (Figures 2E and 2F). Moreover, lymphoblastoid cells from FHH3 patients with the AP2s-R15C mutation, when compared to those from normal relatives, did not have Ca2+-induced cAMP responses (Figure 2G), consistent with findings from the AP2s-C15/CaSR-WT cells. Gallein did not significantly alter Ca2+-induced cAMP responses when compared to vehicle (n = 4).

(D–F) Ca2+-induced cAMP inhibition in AP2s-WT/CaSR-WT and AP2s mutant/CaSR-WT HEK293 cells. AP2s mutant cells—(D) C15, (E) H15, and (F) L15—had impaired responses when compared to WT (AP2s-R15) cells (n = 8–12).

(G) Ca2+-induced cAMP inhibition in EBV-transformed lymphoblastoid cells from FHH3 patients, with AP2s-C15 mutation, and unaffected (normal) relatives (Figure S3).

![Figure 2. AP2s-R15 Mutations Impair the Gαi/o Signaling Pathway](image)

Data are shown as mean ± SEM with *p < 0.05 and **p < 0.02 (two-way ANOVA comparing WT versus mutant in AP2s HEK293 cells and normal versus FHH3 affected in lymphoblastoid cells). (B) shows vehicle versus PTx (black asterisk), UBO (dollar signs), and combined PTx and UBO (gray asterisks).

**AP2s Mutations Reduce Membrane Ruffling**

CaSR has been reported to induce cytoskeletal changes such as membrane ruffling by both Gα12/13 and Gαq/11 signaling (Boussch et al., 2007; Huang et al., 2004; Pi et al., 2002). We therefore investigated the effects of FHH3-associated AP2s mutants on membrane ruffling, using AP2s mutant/CaSR-WT cells and phalloidin-594 as an actin marker. Elevations of Ca2+-induced membrane ruffling in AP2s-WT and mutant cells, although AP2s mutant cells had significantly reduced membrane ruffling compared to WT cells (p < 0.02) (Figures 3A and S4). Assessment of membrane ruffling-induced gene transcription (Tojkander et al., 2012) using a serum response factor (SRF)-RE reporter construct revealed AP2s mutant cells to have significantly reduced SRF activity compared to AP2s-WT cells (Figure 3B). Further investigation of SRF reporter assays in HEK293 cells transiently expressing CaSR but depleted of Gαq/11, Gα12/13, or Gαq/11/12/13 revealed SRF activity to be abolished in Gαq/11 and Gαq/11/12/13 knockout cells but to be significantly higher in...
G_{\alpha/12/13} knockout cells than in native cells (Figure 3C). Moreover, quantification of membrane ruffling in G_{\alpha/12/13} knockout cells and native HEK293 cells transiently expressing CaSR showed them to have similar levels of ruffling (Figure S4), thereby indicating the existence of G_{\alpha/12/13}-independent ruffling pathways. Overall, these results indicate that Ca^{2+}_{\text{e}}-induced membrane ruffling in HEK293 expressing CaSR is mediated by G_{\alpha/11} signaling and that FHH3-associated AP2\(\alpha\) mutations, which impair G_{\alpha/11} signaling, reduce membrane ruffling.

**AP2\(\alpha\) Mutations Impair CaSR Internalization and Differentially Affect CaSR Cell-Surface Expression, which Both Require G_{\alpha/11}**

FHH3-associated AP2\(\alpha\) mutations have been reported to result in increased CaSR cell-surface expression, which represents the net balance between its PM insertion by ADIS and removal by endocytosis (Grant et al., 2011). We therefore simultaneously measured the effects of the FHH3-associated AP2\(\alpha\) mutations on ADIS and endocytosis by transfecting AP2\(\alpha\)-WT and AP2\(\alpha\) mutant cells with a plasmid construct containing full-length CaSR, with an N-terminal modification that in tandem comprised a minimal \(\alpha\)-bungarotoxin (BTX)-binding site to monitor endocytosis and superscribed pHluorin (SEP) to monitor total cell-surface CaSR, referred to as BSEP-CaSR (Figure 4A) (Grant et al., 2011). Total internal reflection fluorescence (TIRF) microscopy was used to assess CaSR cell-surface expression under basal (0.1 mM Ca^{2+}_{\text{e}}) conditions or following exposure to 5 or 10 mM Ca^{2+}_{\text{e}}. Immediately before TIRF microscopy continuous recordings, cells were exposed to BTx with a fluorescent tag (BTx-594). AP2\(\alpha\)-WT and mutant cells expressed CaSR at the cell surface (Figures 4B and 4C), and both 5 and 10 mM Ca^{2+}_{\text{e}} induced elevations in SEP fluorescence and reductions in BTx-594. These were greater at 10 mM Ca^{2+}_{\text{e}} which was used for subsequent imaging experiments (Figures 4B, 4C, and S5). Thus, elevations in Ca^{2+}_{\text{e}} increased CaSR PM insertion (Figures 4B and 4C), and returning Ca^{2+}_{\text{e}} to basal conditions induced a reduction in cell surface CaSR, observed by a decline in SEP fluorescence (Figure 4C). Maximal SEP fluorescence in AP2\(\alpha\)-C15 cells was similar to WT, but AP2\(\alpha\) mutant L15 cells had reduced SEP fluorescence and H15 cells had significantly higher CaSR PM expression (p < 0.01, F test) (Figures 4B and 4C). All AP2\(\alpha\) mutant cells had slower declines in BTx-594 PM fluorescence when compared to AP2\(\alpha\)-WT cells, thereby indicating delayed internalization (Figure 4D). The time to internalize 75% of the BTx-594 at the PM was significantly increased from 268 s in AP2\(\alpha\)-WT to 346, 741, and 350 s in AP2\(\alpha\)-C15, AP2\(\alpha\)-H15, and AP2\(\alpha\)-L15 mutant cells, respectively (p < 0.05 to p < 0.02) (Figure 4E). This was greatest in the AP2\(\alpha\)-H15 cells, which may partly account for the very high CaSR PM expression in these cells (Figure 4C). Moreover, TIRF microscopy analysis of G_{\alpha/11} knockout cells transfected with BSEP-CaSR showed that the Ca^{2+}_{\text{e}}-induced increase in SEP fluorescence (i.e., increased CaSR PM expression via ADIS) was lost and that CaSR internalization measured by BTx-594 fluorescence was severely impaired (Figures 4F and 4G). These findings indicate that G_{\alpha/11} signaling is required for ADIS responses and that CaSR endocytosis requires a signal within the G_{\alpha/11} pathway for its maintenance.

**CaSR Delayed Internalization Is Due to Prolonged CaSR-Clathrin Colocalization in AP2\(\alpha\) Mutant Cells**

AP2\(\alpha\) mutants impair but do not abolish CaSR internalization (Figure 4), indicating that AP2 and clathrin are still recruited to the forming endocytic pit but that CaSR internalization occurs at a slower rate. We therefore predicted that the duration of colocalization between CaSR and clathrin may be prolonged, reflecting this slower internalization rate. We investigated this by transfecting AP2\(\alpha\) mutant and AP2\(\alpha\)-WT cells with BSEP-CaSR and dsRed-Clathrin and analyzed colocalization by TIRF microscopy. Clathrin fluorescence increased in the AP2\(\alpha\)-WT and AP2\(\alpha\) mutant cells during the TIRF microscopy recording, with AP2\(\alpha\) mutant cells exhibiting a prolonged clathrin-CaSR association that was not observed in AP2\(\alpha\)-WT cells (Figure 5A). Quantification of positive Pearson’s correlation coefficients (r) for vesicles containing both clathrin and CaSR revealed that the increase in clathrin recruitment to the PM was significantly greater in AP2\(\alpha\)-WT than in AP2\(\alpha\) mutant cells (p < 0.02) (Figure 5A). Vesicles containing both clathrin and CaSR were analyzed for motility, because higher motility is associated with increased
likelihood of viable endocytic events (Rappoport and Simon, 2003). Vesicles that had both CaSR and clathrin were highly motile in AP2s-WT cells, which had a greater proportion of highly motile CaSR-clathrin-containing vesicles than AP2s-H15 and AP2s-L15 cells; instead, these AP2s mutant cells had a significantly greater number of non-motile CaSR-clathrin-containing vesicles (p < 0.02) (Figures 5B and 5C). The reduced motility of the CaSR-clathrin-containing positive vesicles in AP2s mutant cells would delay vesicle internalization and thereby likely prolong the colocalization of CaSR and clathrin in clathrin-coated pits. Assessment of the duration of CaSR-clathrin colocalization in individual vesicles revealed that all AP2s mutant cells, when compared to AP2s-WT cells, had prolonged CaSR-clathrin associations (Figure 5D). However, motile vesicles in AP2s-WT and AP2s-C15 cells had a significantly shorter duration of colocalization when compared to non-motile vesicles, indicating that these motile vesicles are likely resulting in endocytic events, although there was no significant difference between motile and non-motile vesicles in H15 and L15 cells (Figure 5D). These results indicate that CaSR internalization is impaired in AP2s mutant cells at distinct stages of endocytosis by prolonged residency time at clathrin-coated pits and/or vesicles.

**CaSR Is Able to Induce Sustained Signaling from a Cytoplasmic Location**

The FHH3-associated AP2s mutations resulted in impaired CaSR-induced signaling (Figures 1, 2, and 3), despite increased CaSR cell-surface expression (Figure 4) due to delayed internalization. This led us to hypothesize that CaSR signaling may...
require, or be enhanced, by receptor internalization that would contribute to sustained (i.e., non-canonical) signaling. To test this hypothesis, we treated HEK293-CaSR cells with the dynamin-blocking agent Dyngo, which would abolish endocytosis and prevent endosomal signaling, and assessed their MAPK signaling responses by measurement of pERK1/2 to a 5 min pulse of 5 mM Ca$^{2+}$. pERK1/2 accumulated in Dyngo-treated and control DMSO-treated cells from 2 to 5 min and then rapidly decreased in Dyngo-treated cells, but not DMSO-treated cells; in the latter, pERK1/2 remained significantly increased at 30 min, indicating a potential sustained signaling response (Figures 6A, 6B, and S5). Loss of this sustained response in Dyngo-treated cells was not due to increased apoptosis, decreased proliferation, or inhibition of CaSR protein synthesis, because the sustained rise in pERK1/2 was not blocked by tunicamycin (Figure S5). The effects of this sustained pERK1/2 signaling in Dyngo-treated cells was not due to increased apoptosis, decreased proliferation, or inhibition of CaSR protein synthesis, because the sustained rise in pERK1/2 was not blocked by tunicamycin (Figure S5).

Figure 5. Impairments in CaSR Internalization Are due to Prolonged CaSR-Clathrin Co-localization

TIRF microscopy analyses of colocalized CaSR (BSEP-CaSR) and Clathrin (dsRed-Clathrin) performed in AP2a-CaSR (R15) or mutant (C15, H15, or L15) cells. (A) Quantification of clathrin fluorescence with changes in [Ca$^{2+}$]$_{in}$ (shown above). Data are normalized to the fluorescence in the first frame of each movie (set at 100%). Data shown as mean ± SEM. (B) Images of CaSR and clathrin expression in single vesicles (yellow arrow). (C and D) Proportion of motile (M) versus non-motile (NM) CaSR and clathrin-containing vesicles (C), and duration of colocalization between CaSR and Clathrin in individual (motile, M, filled box, and non-motile, NM, open box) vesicles (D).

Data from 95 to 200 vesicles (n = 14–16 recordings) are expressed as mean ± SEM with *p < 0.05 and **p < 0.02 (two-way ANOVA) illustrated by black and red asterisks for WT motile versus mutant motile vesicles and C15 motile versus non-motile vesicles, respectively.

with Dyngo abolished the second peaked response in HEK-CaSR cells given a 5 min pulse of 5 mM Ca$^{2+}$ (Figure 6E), thereby indicating that the sustained signaling response was likely originating from endosomes. An endosomal origin of this sustained response was further investigated by measuring pERK1/2 responses at 5 and 30 min in HEK-CaSR cells overexpressing the early endosome guanosine triphosphatase (GTPase) Rab5; a dominant-negative (DN) S34N guanosine diphosphate (GDP)-bound form, which delays endocytosis by retaining cargo in clathrin-coated pits (CCPs); and a constitutively active (CA) Q79L form, which enhances endocytic processes (Galperin and Sorkin, 2003; Stenmark et al., 1994). Rab5 was shown to be overexpressed by these constructs, and confocal microscopy showed that FLAG-CaSR-WT internalized over time in response to 5 mM Ca$^{2+}$ and partially colocalized with Rab5-WT-containing structures (Figure S6). Expression of Rab5-DN did not affect CaSR internalization, while the Rab5-DN protein delayed and reduced receptor internalization (Figure S6). In addition, HEK-CaSR cells expressing Rab5-CA when compared to Rab5-WT had enhanced pERK1/2 signals at 5 and 30 min, while Rab5-DN had reduced pERK1/2 signals at 30 min (Figures 6F and 6G). Furthermore, investigation of SRE reporter responses showed that the Rab5-DN reduced overall CaSR-driven SRE reporter activity (Figure 6H), which was due to loss of the sustained signal at 9 hr rather than reduction in immediate signaling (Figure 6I). MAPK signaling can be activated via G$_{q/11}$ and G$_{i/o}$ pathways (Figure S5) (Holstein et al., 2004). To assess the contribution of G$_{q/11}$ and G$_{i/o}$ signaling to sustained endosomal signaling, we measured SRE reporter activity in HEK-CaSR cells treated with UBO-QIC, an inhibitor of G$_{q/11}$, or PTx, a specific inhibitor
Figure 6. Second Signal of CaSR Is from the Rab5-Endosomal Internalization Pathway

(A) Effects of dynamin inhibitor Dyngo on MAPK signaling by western blot analyses of pERK1/2 responses in HEK-CaSR cells treated with Dyngo (+) or DMSO (–), given a 5 min pulse of 5 mM Ca\(^{2+}\), and then incubated in 0.1 mM Ca\(^{2+}\).

(B) Densitometry analysis showing data from blots (n = 8). Black and blue asterisks indicate p values of response versus response at 0 min for DMSO and Dyngo treated, respectively; green asterisks indicate DMSO versus Dyngo responses.

(C) SRE luciferase reporter responses to treatment of either 0.1 or 5 mM Ca\(^{2+}\) over 12 hr in HEK-CaSR cells. Asterisks indicate p values of response versus response to 0.1 mM (n = 4).

(D) SRE luciferase reporter activity in response to 5 min pulses of 0–10 mM Ca\(^{2+}\) in HEK-CaSR cells. Asterisks indicate p values of 0.1 mM responses versus 2.5 mM (red), 5 mM (green), 7.5 mM (blue), and 10 mM (yellow) (two-way ANOVA) (n = 4). Both initial and sustained peaks were enhanced by increasing concentrations of Ca\(^{2+}\), which plateaued at 7.5 mM. Subsequent experiments were performed at Ca\(^{2+}\) = 5 mM.

(E) SRE luciferase reporter responses to a 5 min pulse of 0.1 or 5 mM Ca\(^{2+}\) with DMSO (–) or Dyngo (+) in HEK-CaSR cells. DMSO (blue)-treated cells and Dyngo (red)-treated cells had a peak at 4 hr, while the second peak at 9 hr was abolished by treatment with Dyngo. Asterisks indicate p values of 0.1 mM Ca\(^{2+}\) versus DMSO (blue) or Dyngo (red) and DMSO versus Dyngo (green) (two-way ANOVA).

(F) Western blot analysis of pERK1/2 responses in HEK-CaSR cells exposed for 5 or 30 min to 5 mM Ca\(^{2+}\). Cells were transiently transfected with the Rab5 WT (S34/Q79) or the constitutively active (CA; L79) or dominant-negative (DN; N34) Rab5 mutants.

(G) Densitometric analyses of pERK1/2 in western blots (n = 4). Asterisks indicate p values of mutants compared to WT responses at each time point (two-way ANOVA). Rab5-CA had higher expression of pERK1/2 after 5 and 30 min of treatment, while Rab5-DN had lower pERK1/2 responses after 30 min.

(H) SRE luciferase reporter responses to treatment of 0.1 or 5 mM Ca\(^{2+}\) over 12 hr in HEK-CaSR cells transiently transfected with Rab5 WT (S34/Q79) or the constitutively active (CA; L79) or dominant-negative (DN; N34) Rab5 mutants.

(I) SRE luciferase reporter responses to treatment of 0.1 or 5 mM Ca\(^{2+}\) over 12 hr in HEK-CaSR cells transiently transfected with Rab5-WT or Rab5-DN mutant (n = 8).

(J) SRE luciferase reporter responses to treatment of 0.1 or 5 mM Ca\(^{2+}\) over 12 hr in HEK-CaSR cells treated with DMSO or the G\(_a\)q/11 inhibitor UBO-QIC (UBO) (n = 4).

(K) SRE luciferase reporter responses to 5 min pulses of 0.1 or 5 mM Ca\(^{2+}\) in HEK-CaSR cells transiently transfected with Rab5-WT or Rab5-DN mutant (n = 8).

(L) SRE luciferase reporter responses to treatment of 0.1 or 5 mM Ca\(^{2+}\) over 12 hr in HEK-CaSR cells treated with vehicle (Veh) or PTx, a G\(_a\)i/o inhibitor (n = 8).

(M) SRE luciferase reporter responses to treatment of 0.1 or 5 mM Ca\(^{2+}\) over 12 hr in HEK-CaSR cells treated with Veh or PTx (n = 8).

Rab5-DN, UBO, and PTx all reduced constant Ca\(^{2+}\) responses. In (H)–(M), asterisks show basal 0.1 mM Ca\(^{2+}\) responses versus 5 mM Ca\(^{2+}\) responses in Rab5-WT, DMSO-, or Veh-treated cells (black); basal 0.1 mM Ca\(^{2+}\) responses versus 5 mM Ca\(^{2+}\) responses in Rab5-DN-, UBO-, or PTx-treated cells (blue); and Rab5-WT versus Rab5-DN, DMSO versus UBO, or Veh versus PTx (green) (two-way ANOVA). *p < 0.02, **p < 0.05. Rab5-DN and UBO reduced the sustained MAPK signal, while PTx had no effect on the sustained signal.
of Gαq/11 (Figures 6J–6M). In the presence of constant 5 mM Ca2+-e, SRE reporter activity was reduced in UBO-QIC- and PTx-treated cells compared to vehicle-treated cells (Figures 6J and 6L). However, in cells treated with a 5 min pulse of 5 mM Ca2+-e, UBO-QIC and PTx similarly impaired the early SRE response (Figures 6K and 6M), but only UBO-QIC reduced the sustained signal, which was not affected by PTx (Figures 6K and 6M). Thus, these findings indicate that Gαq does not contribute to the sustained MAPK response from endosomes, which solely involves Gαq/11. The presence of Gαq/11 signaling pathway components in endosomes containing internalized CaSR was confirmed by using HEK293 cells transfected with FLAG-tagged CaSR and either Gαq-Venus or a known GFP-tagged biosensor of PIP2 (the lipid catalyzed by PLC), which contains the pleckstrin homology domain of PLC-delta (PH-PLC) (Stauff et al., 1998). Before addition of 5 mM Ca2+-e, colocalization of CaSR with either Gαq or PH-PLC was observed only at the PM; however, following treatment with 5 mM Ca2+-e for 10 and 30 min, a subpopulation of CaSR-containing endosomes that colocalized with Gαq or PH-PLC was detected, thereby indicating that internalized CaSR endosomes have Gαq/11 signaling components (Pearson’s correlation coefficients = 0.658 ± 0.027 for CaSR/Gαq and 0.652 ± 0.024 for CaSR/PH-PLC at 10 min and 0.693 ± 0.049 for CaSR/Gαq and 0.743 ± 0.059 for CaSR/PH-PLC at 30 min; n = 8–15) (Figure S6). To further assess the role of PLC in sustained signaling, we measured the effect of inhibitors of the PLC-DAG-IP3 pathway (Figure S7) on pERK1/2 responses. HEK-CaSR cells were pulsed with 5 mM Ca2+-e and then treated with DMSO or with U73122, GF-109203X (GFX), or 2-aminoethoxydiphenyl borate (2-APB), which inhibits PLC, PKC, or the IP3 receptor (IP3R), respectively (Figure S7). pERK1/2 accumulation was detected in all cells from 2 to 5 min, and sustained responses were observed in DMSO-treated cells but were significantly reduced in U73122, GFX, and 2-APB-treated cells (Figure S7), thereby confirming the requirement of this Gαq/11 effector for sustained signaling. Finally, we assessed the effects of the scaffold proteins βarrestin-1 and βarrestin-2, which are important for endosomal signaling of GPCRs such as V2R and PTH1R (Feinstein et al., 2013; Wehbi et al., 2013), on the sustained signaling in HEK-CaSR cells and HEK293 cells that had deletions of βarrestin-1 and βarrestin-2, which were generated by CRISPR-Cas and stably overexpressed CaSR (Figure S7). The pERK1/2 and SRE reporter responses to a 5 min pulse of Ca2+-e in these cells lacking βarrestin-1 and βarrestin-2 showed no difference in responses when compared to WT cells, thereby indicating that βarrestin-1 and βarrestin-2 are not required for the CaSR sustained signal (Figure S7).

**AP2α-C15 Mutations Impair Sustained Endosomal Signaling**

FHH3-associated AP2α mutations impair CaSR signaling and internalization. We hypothesized that these AP2α mutations were inhibiting sustained endosomal CaSR signaling and tested this by measuring the non-canonical SRE reporter responses in AP2α-WT/CaSR-WT and AP2α mutant/CaSR-WT cells treated with Dyno, or overexpressing DN Rab5 (Figures 7A and S6). In the presence of constant 5 mM Ca2+-e, SRE reporter responses were significantly higher in AP2α-WT than in mutant cells, with peak expression occurring between 3 and 5 hr, in all cell lines (Figure 7A). Measurements of SRE reporter activity following a 5 min pulse of 5 mM Ca2+-e showed that the second Dyno-sensitive peak was significantly reduced in C15 cells and abolished in H15 and L15 cells compared to WT cells (Figure 7B), thereby revealing that the FHH3-associated AP2α mutations impaired early and sustained endosomal signaling. Moreover, the reduced sustained signaling in AP2α-C15 cells was abolished by Rab5-DN, further demonstrating the endosomal origin of the sustained signaling (Figure S6). In summary, our results show that CaSR can induce sustained MAPK signaling from Rab5 endosomes and that FHH3-associated AP2α mutations (C15, H15, and L15) impair Ca2+-mediated signaling, MAPK responses, cAMP reductions, and membrane ruffling and impair or abolish sustained signaling from the endosome.

**DISCUSSION**

Our study, which demonstrates that CaSR sustained signaling can occur by a non-canonical endosomal pathway, in addition to the established canonical PM pathway (Figure 7C), provides an explanation for the observed reduction in CaSR signaling that is paradoxically associated with increased CaSR PM expression because of FHH3-associated AP2α mutations (Figures 1, 2, 3, and 4) (Nesbit et al., 2013b). Thus, in normal cells, total CaSR signaling comprises the output from the PM immediate and endosomal sustained pathways (Figure 7C); however, in cells with FHH3-associated AP2α mutations, which impair CaSR internalization (Figure 4), the contribution from the endosomal pathway is lost or markedly reduced, with the remaining CaSR signaling occurring from the PM pathway (Figure 7C). Thus, CaSR endosomal signaling, which is sensitive to the dynamin-blocking agent Dyno (Figure 6) and to DN mutants of the early endosomal protein Rab5 (Figure 6), occurs via Gαq/11 (Figures 5 and 6). Gαq/11 mediates alterations in Ca2+ (Figure 1), cAMP (Figure 2), membrane ruffling (Figure 2), and MAPK responses (Figure 1), all of which are impaired in cells expressing FHH3-associated mutations of AP2α (Figures 1 and 2) that forms part of the heterotetrameric AP2 that plays a critical role in clathrin-mediated endocytosis. This CaSR sustained signaling is also not affected by tunicamycin (Figure S5), indicating a lack of requirement for newly synthesized CaSRs (Grant et al., 2011).

The three FHH3-associated AP2α-R15 mutants, which all affected CaSR internalization—but not uptake of other clathrin-mediated endocytic cargos, such as transferrin or another GPCR, the β2AR (Figure S1)—had different effects on CaSR endocytosis and consequently different effects on signaling. Critically, these AP2α mutations revealed that Gαq/11 signaling was more sensitive to alterations in CaSR endocytosis than the Gαq pathway. Thus, the AP2α-C15 mutant delayed CaSR internalization at the CCP (Dyno sensitive) stage, whereas the AP2α-H15 and AP2α-L15 mutants inhibited CaSR internalization at the clathrin-coated vesicle (CCV) (Rab5-DN sensitive) stage. These milder effects of the AP2α-C15 mutant on CaSR internalization still reduced Gαq/11 signaling, thereby indicating a possible threshold requirement for receptor occupancy within endosomes for activation of this G-protein pathway. In addition,
the AP2s-C15 mutant, but not AP2s-L15 or AP2s-H15, significantly affected Gαi/o signaling at high [Ca2+]i, i.e., 10 mM (Figure 2), thereby suggesting that CaSR-mediated Gαi/o signaling at high [Ca2+]i is regulated at the CCPs, as opposed to Rab5 endosomes. Furthermore, Gαq/11, which can enhance MAPK signaling and to reduce cAMP, and increase membrane ruffling and Ca2+i release, using Gαq/11. Following activation, CaSR is clustered into CCPs, before vesicle scission and internalization in clathrin-coated vesicles, and then into endosomes. Our results show that CaSR can induce sustained MAPK signaling (blue) from Rab5 endosomes and that FHH3-associated AP2s mutations (C15, H15, and L15) impair all immediate signaling pathways (red) and impair or abolish sustained Gαq/11 signaling from the endosome, with responses of MAPK shown as a solid blue line (Figures 6 and 7) and other likely responses shown as a broken blue line and in parentheses. Pit invagination can be blocked by Dyngo, and maturation to Rab5-positive vesicles can be blocked by DN Rab5 mutant.
et al., 2013), and the class B PTH1R, which has sustained signals for PTH but rapid signals for PTH-related peptide (Ferrandon et al., 2009). Such spatial control of GPCR signaling has emerged as an important mechanism by which cells translate complex information into distinct cellular responses using a finite number of signal proteins. This is particularly the case for the CaSR, which has wide-ranging functions in diverse cell types, is able to couple to multiple G proteins, and responds to a variety of ligands. Thus, the ability to use immediate and sustained signaling pathways could account for some tissue- and cell-specific functions of the CaSR. For example, an immediate signaling pathway would likely facilitate the CaSR to rapidly respond to changes in [Ca\textsuperscript{2+}]\textsubscript{i} to restore calcium homeostasis by parathyroid and renal cells. In contrast, the role of CaSR in fetal development and bone mineralization (Goltzman and Hendy, 2015; Riccardi et al., 2013), which may require long-acting signals, may be facilitated by a sustained signaling pathway, providing a mechanism for the functional diversity of the CaSR.

In conclusion, our studies have demonstrated that the CaSR, a class C GPCR, mediates a sustained signal from an internal location that is likely to be the endosomes. In addition, our systematic characterization of CaSR signaling by such non-canonical, internalization-dependent (e.g., endosomal) pathways provides a paradigm for understanding how pleiotropic signaling pathways activated by a single GPCR can be resolved via spatially directed G-protein selectivity.

EXPERIMENTAL PROCEDURES

Detailed methods and information on constructs, oligonucleotides, and antibodies can be found in the Supplemental Experimental Procedures.

Ethics Statement

Informed consent was obtained from individuals using protocols approved by local and national ethics committees, London, UK (MREC/02/2/93).

Cell Culture

HEK-CaSR have been described (Nesbit et al., 2013b). HEK293 cells stably expressing AP2\textsubscript{c} WT or mutant proteins were generated using a pcDNA3.1 construct (Invitrogen) containing full-length AP2\textsubscript{c} cDNA with silent mutations to protect against AP2\textsubscript{c} siRNA (Santa Cruz Biotechnology). Clonal cells were generated as described (Nesbit et al., 2013b), and cells with deletion of G\textsubscript{na}, G\textsubscript{ni1}, G\textsubscript{ni2}, L\textsubscript{arrestin-1}, and L\textsubscript{arrestin-2} by CRISPR-Cas have been described (Devost et al., 2017). Epstein-Barr virus-transformed lymphoblastoid cells were generated from members of the FH/H3 kindred as described (Parkinson and Thakker, 1992). Transfections were performed with Lipofectamine 2000 (Invitrogen). Mutations within constructs were introduced by site-directed mutagenesis using Quikchange Lightning XL or Multi kits (Agilent)

Western Blot

For sustained signaling studies, cells were stimulated with 5 mM CaCl\textsubscript{2} for 5 min, followed by incubation in 0 mM CaCl\textsubscript{2} for 0–60 min. For studies with 30 \textmu M Dyno-4a (Abcam) (Jean-Alphonse et al., 2014), cells were pre-incubated for 30 min. For studies with 5 \textmu M U73122 (Sigma), 1 \textmu M GFX (Sigma), 100 \textmu M 2-APB (Sigma), or 5 \mu g/mL tunicamycin (Sigma), compounds were added to the media and cells were incubated after calcium stimulation. For studies of Rab5 contribution to sustained signaling, 100 ng/mL mCh-Rab5-WT (Addgene plasmid 49201), mCh-Rab5 dominant negative (DN; S34N) or mCh-Rab5 CA (G79L), were transfected 48 hr before western blot analysis. Western blots for pERK1/2 were then performed as described (Gorvin et al., 2017).

Functional Assays

Transferrin assays were performed as described (Gorvin et al., 2013). Ip\textsubscript{3} assays were performed according to manufacturer’s instructions. For pERK1/2 AlphaScreen assays, cells were transfected with pEGFP-CaSR and treated with 0–10 mM CaCl\textsubscript{2} for 5 min. For cAMP assays, cells were pre-treated with forskolin for 30 min. For inhibitor studies, cells were pre-treated with 300 ng/mL PTX or vehicle (ethanol) for 6 hr, 1 \textmu M UBO-QIC or vehicle (DMSO) for 2 hr, or 15 \textmu M gallein or vehicle (DMSO) for 15 min (Grant et al., 2011). AlphaScreen assays were performed as previously described (Gorvin et al., 2017). Apoptosis and proliferation were assessed using Caspase-Glo 3/7 and CellTiter Blue kits, respectively (Promega). For luciferase reporter assays, cells were transfected with pEGFP-CaSR, a reporter construct (pGL4-NFAT, pGL4-SRE, or pGL4-SRF), and a renilla construct (pRL) as described (Gorvin et al., 2017). Cells were treated with 0–10 mM CaCl\textsubscript{2} for 4 hr. For sustained signaling studies, HEK-CaSR cells were transfected with luciferase construct and pRL and given one of four treatments: (1) 0.1 mM CaCl\textsubscript{2}, (2) 5 mM CaCl\textsubscript{2} for the whole experiment (constant), (3) 5 min pulse of 5 mM CaCl\textsubscript{2} followed by 0.1 mM CaCl\textsubscript{2} with vehicle (DMSO) for the duration of the experiment, or (4) 5 min pulse of 5 mM CaCl\textsubscript{2} followed by 0.1 mM CaCl\textsubscript{2} with 30 \textmu M Dyno-4a for the duration of the experiment. Cells were pre-incubated with 1 \textmu M UBO-QIC or DMSO for 2 hr or 10 \textmu M forskolin (MP Biomedicacls) and 300 ng/mL PTX (Sigma) or vehicle (ethanol diluent) for 6 hr (Avlanji et al., 2013). Luciferase assays and Caspase-Glo 3/7 were measured on a Veritas luminometer (Promega), and CellTiter Blue was measured on a CytoFluor microplate reader (PerSeptive Biosystems).

Fluorescent Imaging

For membrane ruffling, cells were transfected with pEGFP-CaSR, and actin was visualized with Phalloidin-594 (Molecular Probes) following treatment with 0, 5, and 10 mM Ca\textsuperscript{2+}. Cells were imaged on a Nikon Eclipse E400 wide-field microscope using standard methods. UBO-QIC was visualized with M 2-APB (Sigma), or 5 \mu M tunicamycin (Sigma), compounds were added to the media and cells were incubated after calcium stimulation. Membrane ruffling was visualized with Phalloidin-594 (Molecular Probes) following treatment with 0, 5, and 10 mM Ca\textsuperscript{2+}.

Western Blot

For sustained signaling studies, cells were stimulated with 5 mM CaCl\textsubscript{2} for 5 min, followed by incubation in 0 mM CaCl\textsubscript{2} for 0–60 min. For studies with 30 \textmu M Dyno-4a (Abcam) (Jean-Alphonse et al., 2014), cells were pre-incubated for 30 min. For studies with 5 \textmu M U73122 (Sigma), 1 \textmu M GFX (Sigma), 100 \textmu M 2-APB (Sigma), or 5 \mu g/mL tunicamycin (Sigma), compounds were added to the media and cells were incubated after calcium stimulation. For studies of Rab5 contribution to sustained signaling, 100 ng/mL mCh-Rab5-WT (Addgene plasmid 49201), mCh-Rab5 dominant negative (DN; S34N) or mCh-Rab5 CA (G79L), were transfected 48 hr before western blot analysis. Western blots for pERK1/2 were then performed as described (Gorvin et al., 2017).
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