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Abid, Hasnat Ali; Inoue, Asuka; Gorvin, Caroline M

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RESEARCH

Heterogeneity of G protein activation by the calcium-sensing receptor

Hasnat Ali Abid¹, Asuka Inoue² and Caroline M Gorvin^{1,3} ¹Institute of Metabolism and Systems Research and Centre for Endocrinology, Diabetes and Metabolism, University of Birmingham, Birmingham, UK²Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Miyagi, Japan³Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, UKCorrespondence should be addressed to C M Gorvin: C.Gorvin@bham.ac.uk

Abstract

The calcium-sensing receptor (CaSR) is a G protein-coupled receptor that plays a fundamental role in extracellular calcium (Ca^{2+}_e) homeostasis by regulating parathyroid hormone release and urinary calcium excretion. The CaSR has been described to activate all four G protein subfamilies ($G_{\alpha_{q/11}}$, $G_{\alpha_{i/o}}$, $G_{\alpha_{12/13}}$, G_{α_s}), and mutations in the receptor that cause hyper/hypocalcaemia, have been described to bias receptor signalling. However, many of these studies are based on measurements of second messengers or gene transcription that occurs many steps downstream of receptor activation and can represent convergence points of several signalling pathways. Therefore, to assess CaSR-mediated G protein activation directly, we took advantage of a recently described NanoBiT G protein dissociation assay system. Our studies, performed in HEK293 cells stably expressing CaSR, demonstrate that Ca^{2+}_e stimulation activates all $G_{\alpha_{q/11}}$ family and several $G_{\alpha_{i/o}}$ family proteins, although G_{α_z} was not activated. CaSR stimulated dissociation of $G_{\alpha_{12/13}}$ and G_{α_s} from $G\beta$ -subunits, but this occurred at a slower rate than that of other $G\alpha$ -subunits. Investigation of cDNA expression of G proteins in three tissues abundantly expressing CaSR, the parathyroids, kidneys and pancreas, showed $G_{\alpha_{11}}$, G_{α_z} , $G_{\alpha_{i1}}$ and $G_{\alpha_{13}}$ genes were highly expressed in parathyroid tissue, indicating CaSR most likely activates $G_{\alpha_{i1}}$ and $G_{\alpha_{i1}}$ in parathyroids. In kidney and pancreas, the majority of G proteins were similarly expressed, suggesting CaSR may activate multiple G proteins in these cells. Thus, these studies validate a single assay system that can be used to robustly assess CaSR variants and biased signalling and could be utilised in the development of new pharmacological compounds targeting CaSR.

Key Words

- ▶ calcium homeostasis
- ▶ calcium signalling
- ▶ G protein-coupled receptor
- ▶ parathyroid and bone
- ▶ signal transduction

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Introduction

The calcium-sensing receptor (CaSR) is a class C homodimeric G protein-coupled receptor (GPCR) that plays a fundamental role in extracellular calcium (Ca^{2+}_e) homeostasis by regulating parathyroid hormone (PTH) release and urinary calcium excretion. Consistent with its critical role in maintaining serum calcium concentrations,

the CaSR is highly expressed on the surface of parathyroid and kidney cells (Riccardi & Brown 2010). The CaSR is also widely expressed on other tissues, including significant expression on pancreatic islet α - and β -cells (Regard *et al.* 2008), and has been described to mediate a diverse range of functions, including inflammation, bronchoconstriction,

gastrointestinal hormone secretion and glucose metabolism (Hofer *et al.* 2000, Rossol *et al.* 2012, Yarova *et al.* 2015, Zietek & Daniel 2015).

The importance of CaSR in the regulation of Ca^{2+}_e is highlighted in patients with germline mutations of the receptor that present with disorders of calcium homeostasis. Inactivating mutations cause familial hypocalciuric hypercalcaemia type-1 (FHH1), characterised by lifelong elevated serum calcium, moderate-to-high PTH concentrations, and low renal calcium excretion, and rarely cause neonatal severe hyperparathyroidism, which can be fatal if untreated (Pollak *et al.* 1993). Activating CaSR mutations cause autosomal dominant hypocalcaemia type-1 (ADH1), characterised by mild-to-moderate hypocalcaemia, with inappropriately low-to-normal serum PTH (Pearce *et al.* 1996). In addition, inactivating mutations in the G protein- α_{11} ($\text{G}\alpha_{11}$), by which the CaSR signals and the adaptor protein-2 σ -subunit ($\text{AP}2\sigma$), which regulates endocytosis, cause FHH2 and FHH3, respectively; whereas activating $\text{G}\alpha_{11}$ mutations cause ADH2 (Mannstadt *et al.* 2013, Nesbit *et al.* 2013a,b).

On stimulation by elevations in Ca^{2+}_e , the CaSR activates diverse signalling pathways. In most cell types, CaSR is understood to signal via $\text{G}\alpha_{q/11}$ to activate phospholipase C (PLC)-mediated increases in intracellular calcium and mitogen-activated protein kinase (MAPK), and $\text{G}\alpha_{i/o}$ to inhibit adenylate cyclase and reduce cAMP (Kifor *et al.* 1997, Brown & MacLeod 2001, Hofer & Brown 2003). Additionally, CaSR has been described to activate cytoskeletal remodelling, attributed to $\text{G}\alpha_{12/13}$ pathways in some cell types (e.g. MDCK cell-lines) (Huang *et al.* 2004) and $\text{G}\alpha_{q/11}$ in others (Pi *et al.* 2002). Finally, in breast cancer and AtT20 pituitary tumour cells, CaSR activates elevations in cAMP by switching of CaSR coupling from $\text{G}\alpha_{q/11}$ and $\text{G}\alpha_{i/o}$ to $\text{G}\alpha_s$ (Mamillapalli *et al.* 2008, Mamillapalli & Wysolmerski 2010).

The specific coupling of CaSR to individual G protein pathways was investigated in early studies by radio-ligand measurements of phospholipases; while subsequent studies have measured a range of second messengers in cells depleted of G α proteins by chemical (e.g. YM-254890 or pertussis toxin that inhibit $\text{G}_{q/11}$ and $\text{G}_{i/o}$, respectively) or genetic manipulation (by siRNA knockdown or CRISPR-Cas9) (Kifor *et al.* 1997, Pi *et al.* 2002, Gorvin *et al.* 2018d). Although these studies have successfully identified many components of the CaSR signalling pathway, these methodologies are associated with several disadvantages, including variable sensitivity and complexity of assays; poor targeting of individual G proteins by chemical inhibitors and siRNAs, and the available CRISPR-generated

cell-lines have deletions of whole G protein subfamilies rather than individual G proteins (Takasaki *et al.* 2004, Devost *et al.* 2017, Gundry *et al.* 2017). These limitations, combined with the distinct activation kinetics of second messengers, and recognition that multiple G proteins converge on the same signal proteins or genes (Goldsmith & Dhanasekaran 2007), impair the researcher's ability to compare GPCR stimulation of different signalling pathways using many assay platforms.

The ability to assess GPCR activation of individual G proteins is increasingly important as it is recognised that receptors may couple preferentially to different signalling pathways in diverse tissues, and disease-causing mutations may confer alternative receptor conformations that couple differentially to intracellular signalling pathways (Wingler & Lefkowitz 2020). Such tissue- and mutation-specific bias has been described for the CaSR indicating that assessment of multiple signalling pathways may be required to assess pathogenicity of receptor variants identified in patient samples (Leach *et al.* 2012, Gorvin *et al.* 2018c). Moreover, allosteric modulators of the CaSR exhibit signalling bias, indicating it may be possible to develop additional pharmacological compounds that convey unique functional selectivity on individual G proteins to separate desirable from adverse (i.e. off-target) effects (Hauser *et al.* 2017). Therefore, a direct measure of G protein coupling to CaSR would be beneficial to aid in future drug design, in assigning decisions regarding the pathogenicity of receptor variants, and in identifying biased signalling by receptor mutants.

Recently, a NanoBiT G protein dissociation assay system that assesses activation of multiple G α subunits was described (Inoue *et al.* 2019). This assay utilises a split nano-luciferase (NanoLuc), in which the large part of NanoLuc (LgBiT) is tagged to the G α -subunits and the small part (SmBiT) is tagged to the G β -subunit. Thus G protein activation can be monitored by agonist-induced reductions in luciferase due to separation of the G α - and G $\beta\gamma$ -subunits following receptor stimulation. The development of this assay afforded an opportunity to interrogate the promiscuity of CaSR coupling to G proteins without the problem of signal pathway cross-talk. These studies, combined with gene expression analyses, indicate that the physiological effects of CaSR are likely mediated by a combination of G protein activation and expression of individual G α -proteins in different tissues. This simple assay system is easily scalable, measures in real-time and could be used for drug screening and assessment of pathogenicity and signalling bias of receptor variants by multiple CaSR signalling pathways.

Methods

Cell culture and transfection

Adherent HEK293 (AdHEK) cells were purchased from Agilent Technologies. HEK293 cells with deletion of the $G\alpha_{q/11}$, $G\alpha_{i/o}$, $G\alpha_{12/13}$ or $G\alpha_{s/1}$ family of G proteins (G protein knockout cells) were described previously (Inoue *et al.* 2019). AdHEK cells and G protein knockout cells were maintained in DMEM-Glutamax media (Sigma) with 10% foetal bovine serum (FBS, Sigma) at 37°C, 5% CO₂. The full-length CaSR cDNA with an N-terminal FLAG tag was PCR amplified (reagents from Promega) from total human kidney cDNA (Ambion) and cloned into a pcDNA3.1 expression vector (Life Technologies). The full-length somatostatin receptor-5 (SSTR5) was PCR amplified from SSTR5-Tango (a gift from Bryan Roth (Addgene plasmid # 66506; RRID:Addgene_66506) (Kroeze *et al.* 2015)) and cloned into pcDNA3.1. Expression constructs were sequence-verified by Source Bioscience (Nottingham, UK). NanoBiT G protein dissociation and the IP3 biosensor constructs were described in detail previously (Inoue *et al.* 2019). The pGloSensor-20F plasmid was purchased from Promega. Transfections were performed using Lipofectamine 2000 (LifeTechnologies), according to the manufacturer's instructions. To generate AdHEK-CaSR, cells stably transfecting pcDNA3.1-FLAG-CaSR cells were plated in six-well plates, transfected with 1 µg DNA, then single clones selected using cell culture media containing 500 µg/mL geneticin (Gibco).

Western blot analysis

Western blot analysis was performed as previously described (Gorvin *et al.* 2018d). Endogenous calnexin was used as a loading control. Lysates were resuspended in Laemmli buffer, boiled and separated on 6% SDS-PAGE gels. Following transfer to polyvinylidene difluoride membrane (ThermoFisher), blots were blocked in 5% marvel/TBS-T, then probed with anti-CaSR (ADD, Abcam), anti-Gα11 (SantaCruz Biotechnology), anti-Gα12 (SantaCruz), and anti-calnexin (Millipore) antibodies. Blots were visualised using the Immuno-Star WesternC kit (BioRadK) on a BioRad Chemidoc XRS+ system. For cell surface expression of CaSR, plasma membrane fractions were extracted using the Plasma Membrane Protein Extraction kit (Abcam). Plasma membrane calcium adenosine triphosphatase (PMCA1) (Abcam) was used as a housekeeping protein for the plasma membrane fraction.

cAMP GloSensor assays

Cells were plated in six-well plates and transfected with 50 ng pGloSensor-20F plasmid. Twenty-four hours later cells were re-plated in 96-well plates in FluoroBrite DMEM media (ThermoScientific) containing 10% FBS. On the following day cells were incubated with 100 µL of equilibration media consisting of Hank's buffered saline solution (HBSS, calcium and magnesium-free) containing 2% (v/v) dilution of the GloSensor cAMP Reagent stock solution according to manufacturer's instructions. Cells were incubated for 2 h at 37°C. Basal luminescence was read on a PheraStar FSX (BMGLabtech) for 8 min, then agonist added with 10 µM forskolin (Sigma), and plates read for a further 30 min.

NanoBiT G protein dissociation and IP3 assays

Cells were plated in six-well plates and transfected 24 h later with: 250 ng LgBiT-Gα plasmid, 500 ng SmBiT-Gβ plasmid, 500 ng untagged Gγ₂ plasmid. For studies shown in Supplementary Figs 1, 2 and 3 (see section on [supplementary materials](#) given at the end of this article), AdHEK were also transiently transfected with 250 ng of FLAG-CaSR, while studies of SSTR5 were transiently transfected with pcDNA3.1-SSTR5. For reactions with LgBiT-Gα₁₁ cells were co-transfected with RIC8A, as previously described (Inoue *et al.* 2019). Following 24 h, cells were harvested in DMEM-fluorobrite (ThermoScientific) with 10% FCS and 4mM L-glutamine and seeded in 8 wells of a 96-well plate. NanoBiT assays were performed the following day using NanoGlo reagent (Promega). Media was changed to HBSS without antibiotics (containing 0.1 mM CaCl₂) on the morning of the assay. Approximately 4 h later, each well was loaded with 40 µL substrate and baseline signal read on a Pherastar FSX plate reader (BMGLabtech, Aylesbury, UK) at 37°C for four cycles (equivalent to 8 min). Vehicle and agonists were prepared in Hank's Balanced Saline Solution (HBSS, Sigma) at 10× concentration and added to wells once baselines were stable and responses recorded immediately following agonist addition for up to 25 min. All responses were normalised to that treated with vehicle at 0 min. For SSTR5 studies cells were exposed to either vehicle (DMSO) or 50 nM somatostatin (purchased from Sigma). The consequent fold-change values were fitted to a four-parameter sigmoidal concentration-response using GraphPad Prism. IP3 biosensor assays were performed similarly using 200 ng LgBiT-IP3R2-SmBiT plasmid. For IP3 measurements, basal luminescence was read on a PheraStar

FSX (BMGLabtech) for 8 min, then agonist added, and plates read for a further 30 min.

Gene expression analysis

Analysis of gene expression in parathyroid, kidney and pancreatic tissues was performed using the Gene Expression Omnibus (GEO) website (<https://www.ncbi.nlm.nih.gov/geo/>) using the search term 'parathyroid', 'kidney', 'pancreas' and 'human', accession date August 2020. Two datasets, #GSE83421 (Balenga *et al.* 2017) and #GSE2193 (Shyamsundar *et al.* 2005), were identified and used in analyses using GEO2R for parathyroid tissue. These comprised data from 9 normal parathyroids and 25 adenoma tissues from patients with primary hyperparathyroidism. For kidney and pancreas expression the following datasets were used: GSM12660, GSM12661, GSM12663, GSM12659, GDS181, GDS1085, GDS1663 (Su *et al.* 2002, Shyamsundar *et al.* 2005). Gene expression values for a panel of housekeeping genes: actin, beta (*ACTB*); phosphoglycerate kinase 1 (*PGK1*); peptidylprolyl isomerase A (*PPIA*); beta-2-microglobulin (*B2M*); transferrin receptor (*TFRC*); glucuronidase, beta (*GUSB*); hypoxanthine phosphoribosyltransferase 1 (*HPRT1*); TATA box binding protein (*TBP*); and tubulin, beta (*TUBB*) were acquired and the mean value used for normalisation using Microsoft Excel 2013. Expression of each G protein subunit was expressed relative to the geometric mean of the housekeeping genes in each sample and plotted using GraphPad Prism 7. A cut-off of representation in at least four datasets was used for analysis of expression between genes. To compare expression within each G protein subfamily, the relative expression of each gene was expressed as a fold-change to a representative gene (*GNA11*, *GNAI1*, *GNA12*, *GNAS*). Statistical analyses were performed using ANOVA between two or more datasets and correction for multiple testing applied. A value of $P < 0.05$ was considered significant. Comparisons between the gene expression data and proteome datasets could not be made as there is insufficient proteomic data available on parathyroid tissues.

Results

CaSR can couple to all members of the $G_{q/11}$ subfamily

Previous studies have indicated that CaSR is able to couple promiscuously to multiple G proteins (Kifor *et al.* 1997, Brown & MacLeod 2001, Hofer & Brown 2003, Huang *et al.* 2004). However, these studies are largely based on

measurements of second messengers or changes in gene transcription, which can only indicate the G protein subfamily and not necessarily the specific G protein activated. Furthermore, as many signalling pathways share downstream signal components, it may be difficult to differentiate the effects of different G proteins. We therefore used an unbiased approach to determine which specific G proteins are activated by CaSR using a recently described NanoBiT G protein dissociation assay system (Inoue *et al.* 2019). NanoBiT utilises a split nano-luciferase (NanoLuc) in which the large part of NanoLuc (LgBiT) is tagged to the $G\alpha$ subunits and the small part (SmBiT) is tagged to the $G\beta$ -subunit. Under basal conditions, the heterotrimeric $G\alpha\beta\gamma$ protein is stable and the LgBiT and SmBiT are associated with yielding high levels of luciferase. On activation of the GPCR, $G\alpha$ and $G\beta\gamma$ dissociate and luciferase levels reduce.

To investigate G protein activation by the CaSR, we first assessed $G\alpha$ - $G\beta3$ dissociation with six different concentrations of Ca^{2+}_e in adherent HEK293 (AdHEK) transiently expressing the CaSR. Robust concentration-dependent increases in G protein dissociation, including within the physiological range of Ca^{2+}_e (~1–3 mM), were observed for several members of the $G\alpha_{q/11}$ and $G\alpha_{i/o}$ families (Supplementary Fig. 1). However, G protein dissociation was only observed for some G proteins ($G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{12}$) at concentrations >3 mM Ca^{2+}_e . Therefore, for subsequent studies, we used 5 mM Ca^{2+}_e , as this concentration was the closest to the physiological range that would allow us to assess dissociation of the largest number of G proteins. G protein dissociation in response to 5 mM Ca^{2+}_e was not observed in AdHEK cells lacking the CaSR (Supplementary Fig. 2). Overexpression of other untagged $G\alpha$ proteins did not affect G protein coupling of NanoBiT constructs (Supplementary Fig. 3), nor did deletion of other $G\alpha$ proteins (Supplementary Fig. 4).

To assess G protein coupling and dissociation between 12 $G\alpha$ and 5 $G\beta$ subunits we generated a AdHEK cell-line stably expressing the CaSR with an N-terminal FLAG tag (Supplementary Fig. 5). These cells were demonstrated to overexpress CaSR and to activate IP3 accumulation and cAMP depletion on stimulation with increasing doses of Ca^{2+}_e (Supplementary Fig. 5). All subsequent assays were performed in one clone of this cell-line (clone A, named AdHEK-CaSR). Overexpression of NanoBiT constructs had no effect on CaSR total or cell surface expression, nor on the expression of other G proteins (Supplementary Fig. 6). Assays were performed with the 12 available $G\alpha$ -LgBiT-subunits in combination with 5 $G\beta$ -SmBiT-subunits. Cells were exposed to low concentrations of Ca^{2+}_e (0.1 mM) or an activating concentration of 5 mM Ca^{2+}_e .

The $G_{\alpha_{q/11}}$ family was first investigated as *GNA11* is known to be highly expressed in parathyroid tissue and mutations in this gene cause FHH2 and ADH2 (Mannstadt *et al.* 2013, Nesbit *et al.* 2013a). The four G_{α} proteins (q, 11, 14, 15) were assessed with all G_{β} -subunits. Cells in which the relative luminescence units (RLU) were below 1000 were excluded as this value was not significantly greater than background luminescence (Supplementary Table 1). Analyses of the $G_{q/11}$ family demonstrated that all four α -subunits could be activated following exposure of cells to 5 mM Ca^{2+}_e (Fig. 1A, B, C, D and Supplementary Fig. 7). CaSR produced greater activation responses with G_{α_q} and $G_{\alpha_{11}}$ than the other family members (Fig. 1A, B, C, D, E and Supplementary Table 2), and therefore it is more likely that these proteins are activated by the receptor when present in CaSR expressing tissues.

CaSR activates several members of the $G_{i/o}$ subfamily but not G_{α_z}

CaSR has been described to reduce cAMP in many studies, and signalling by these pathways has been demonstrated to play a role in PTH secretion in parathyroid cells (Brown *et al.* 1978). Five members of the $G_{i/o}$ subfamily were investigated by NanoBiT dissociation assays. CaSR robustly

activated $G_{\alpha_{i1}}$ when combined with any G_{β} -subunit, and G_{α_o} similarly activated all G_{β} -subunits, although activation, when combined with β_4 , was slow (Fig. 2 and Supplementary Fig. 8). $G_{\alpha_{i2}}$ and $G_{\alpha_{i3}}$ were activated only when combined with one G_{β} -subunit and G_{α_z} was not activated at all (Fig. 2). This was not due to inactivity of the LgBiT- G_{α_z} plasmid as other GPCRs could activate this G protein (Supplementary Fig. 9). Thus, activation of the $G_{i/o}$ family in CaSR expressing cells is unlikely to occur by G_{α_z} and activation of some family members ($G_{\alpha_{i2}}$ and $G_{\alpha_{i3}}$) will be dependent on β -subunit expression.

Other G protein families are activated slowly by the CaSR

CaSR has been reported to activate $G_{12/13}$ inducing cytoskeletal changes in some cells, while other studies were unable to show any signalling, indicating the pathway may be cell type specific (Pi *et al.* 2002, Huang *et al.* 2004, Davies *et al.* 2006). NanoBiT dissociation assays in AdHEK-CaSR cells showed $G_{\alpha_{12}}$ was activated by CaSR when combined with three β -subunits, β_1 , β_2 and β_3 (Fig. 3 and Supplementary Fig. 10). However, activation of $G_{\alpha_{12}}$ with G_{β_2} and G_{β_3} took more than 12 min, indicating it is unlikely rapid CaSR activation is induced by coupling

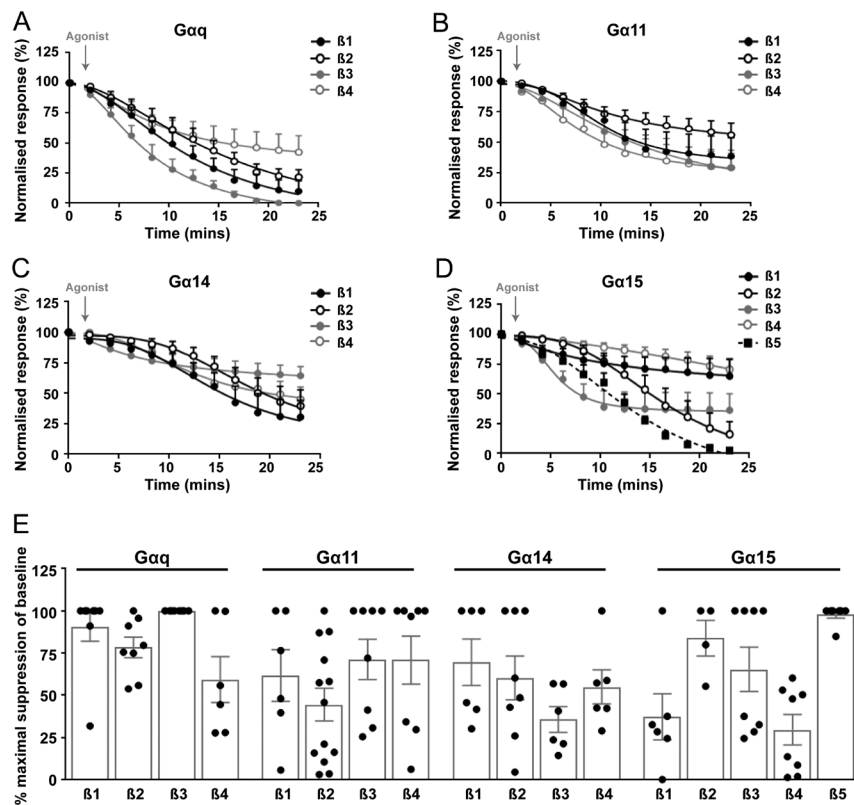
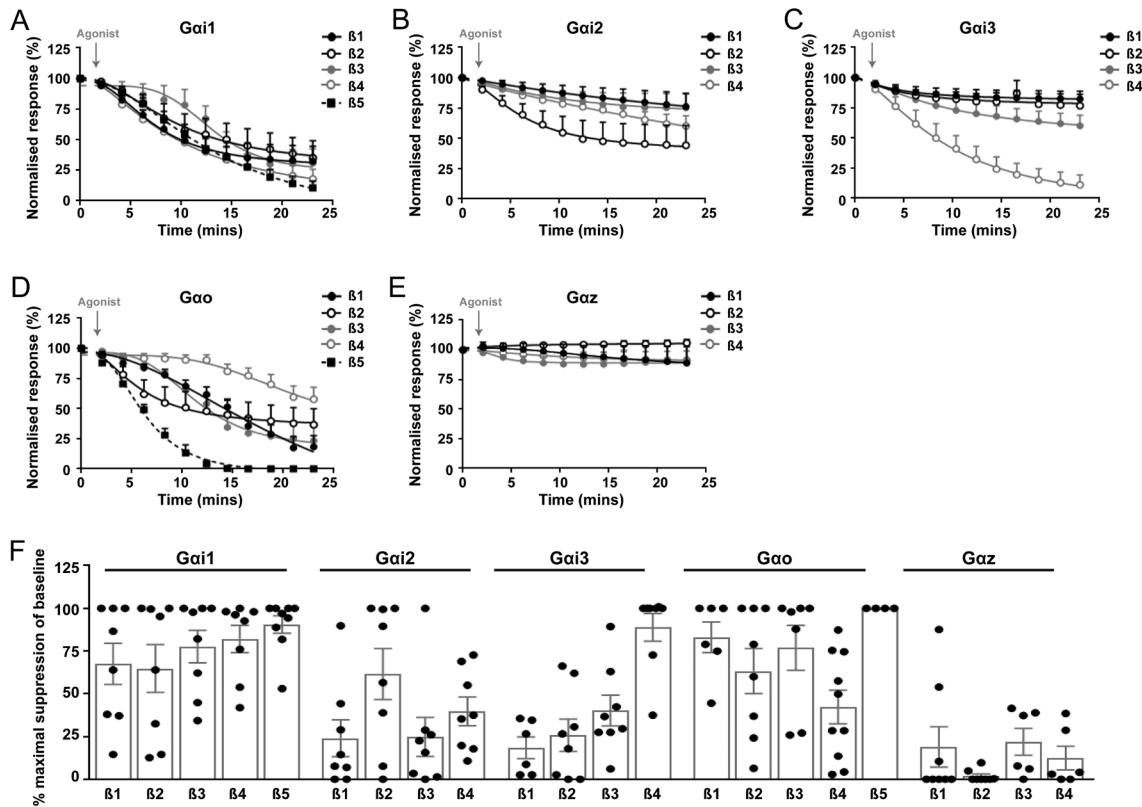


Figure 1

CaSR activation of the $G_{q/11}$ family by NanoBiT G protein dissociation assays. NanoBiT dissociation assays of AdHEK-CaSR cells transiently transfected with: (A) LgBiT- G_{α_q} , (B) LgBiT- $G_{\alpha_{11}}$, (C) LgBiT- $G_{\alpha_{14}}$, (D) LgBiT- $G_{\alpha_{15}}$ with SmBiT- G_{β} subunits (G_{β_1-5}) and unlabelled G_{γ_2} following treatment with 5 mM Ca^{2+}_e . Pre-calcium baselines are not shown. The first baseline value is shown. (E) Percentage suppression of baseline from A, B, C and D. Statistical analyses comparing values of all G_{α} - G_{β} dissociations are shown in Supplementary Table 1. For all panels only those G_{α} and G_{β} pairs that yielded relative luminescence units (RLU) over a threshold of 1×10^3 (i.e. above background luminescence) are shown. All responses were normalised to those under basal conditions (0.1 mM Ca^{2+}_e). Dissociation curves for 5 mM Ca^{2+}_e are shown (curves with 0.1 mM Ca^{2+}_e are shown in Supplementary Fig. 7). Curves show mean \pm s.e.m. for $n = 6-11$ independent assays.

**Figure 2**

CaSR activation of the $G_{1/o}$ family by NanoBIT G protein dissociation assays. NanoBIT dissociation assays of AdHEK-CaSR cells transiently transfected with: (A) LgBiT- $G\alpha_{11}$, (B) LgBiT- $G\alpha_{12}$, (C) LgBiT- $G\alpha_{13}$, (D) LgBiT- $G\alpha_o$ and (E) LgBiT- $G\alpha_z$ with SmBiT- $G\beta$ subunits ($G\beta 1-5$) and unlabelled $G\gamma 2$ following treatment with 5mM Ca^{2+}_e . Pre-calcium baselines are not shown. The first baseline value is shown. (F) Percentage suppression of baseline from A, B, C, D and E. Statistical analyses comparing values of all $G\alpha$ - $G\beta$ dissociations are shown in Supplementary Table 1. For all panels only those $G\alpha$ and $G\beta$ pairs that yielded relative luminescence units (RLU) over a threshold of 1×10^3 (i.e. above background luminescence) are shown. All responses were normalised to those under basal conditions (0.1 mM Ca^{2+}_e). Dissociation curves for 5 mM Ca^{2+}_e are shown (curves with 0.1 mM Ca^{2+}_e are shown in Supplementary Figure 8). Curves show mean \pm s.e.m. for $n = 6-10$ independent assays.

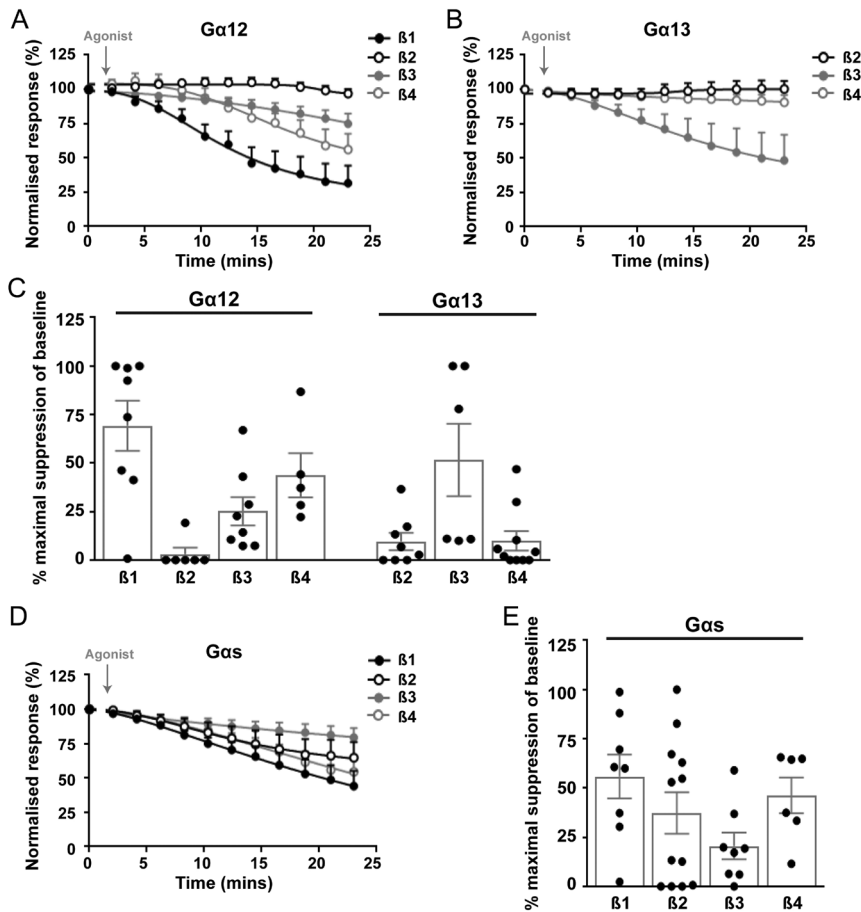
between these subunits. Similarly, $G\alpha_{13}$ could only be activated when combined with $G\beta 4$. Thus, activation of $G\alpha_{12/13}$ by CaSR may only occur when specific β -subunits are present in cells and may explain some of the discrepancies observed between published studies investigating CaSR signalling by this pathway.

Previous studies of CaSR in cancer cell-lines, including MCF-7 human breast cancer cells and mouse pituitary AtT20 cells, have shown that CaSR switches from preferentially coupling to $G_{q/11}$ and $G_{1/o}$ pathways to exclusively signalling by a G_s pathway (Mamillapalli *et al.* 2008, Mamillapalli & Wysolmerski 2010). Combinations of $G\alpha_s$ with $G\beta 1-4$ achieved luminescence values above the threshold, indicating efficient activation. However, agonist-induced responses were only significantly greater than basal responses from 12 min after stimulations in cells transfected with $G\beta 1$ and after 15 min of stimulation in cells expressing $G\beta 2$ and $G\beta 4$ (Fig. 3 and Supplementary Fig. 11). Therefore, CaSR may activate $G\alpha_s$ and $G\alpha_{12/13}$ more slowly than other

$G\alpha$ proteins, and this could explain why *in vitro* assays often fail to detect CaSR activation of these pathways.

$G\alpha_{11}$, $G\alpha_{11}$ and $G\alpha_z$ are most abundantly expressed in human parathyroid tissue

Our NanoBIT dissociation studies have demonstrated which G proteins are capable of coupling to CaSR. However, CaSR coupling in native human tissue will be dependent on the expression of the individual G proteins in that tissue. For example, previous studies using qRT-PCR have indicated that the gene encoding $G\alpha_{11}$ (*GNA11*) is expressed at higher levels than that of $G\alpha_q$ (*GNAQ*) (Nesbit *et al.* 2013a); while in bovine parathyroid, genes for $G\alpha_{11}$, $G\alpha_s$, $G\alpha_{12}$, $G\alpha_{12}$ and $G\alpha_z$ were highly expressed (Varrault *et al.* 1995). We sought to verify these findings in a larger dataset and investigate G protein expression in the three tissues in which CaSR is most abundantly expressed: the parathyroid glands, the kidneys and the pancreas.

**Figure 3**

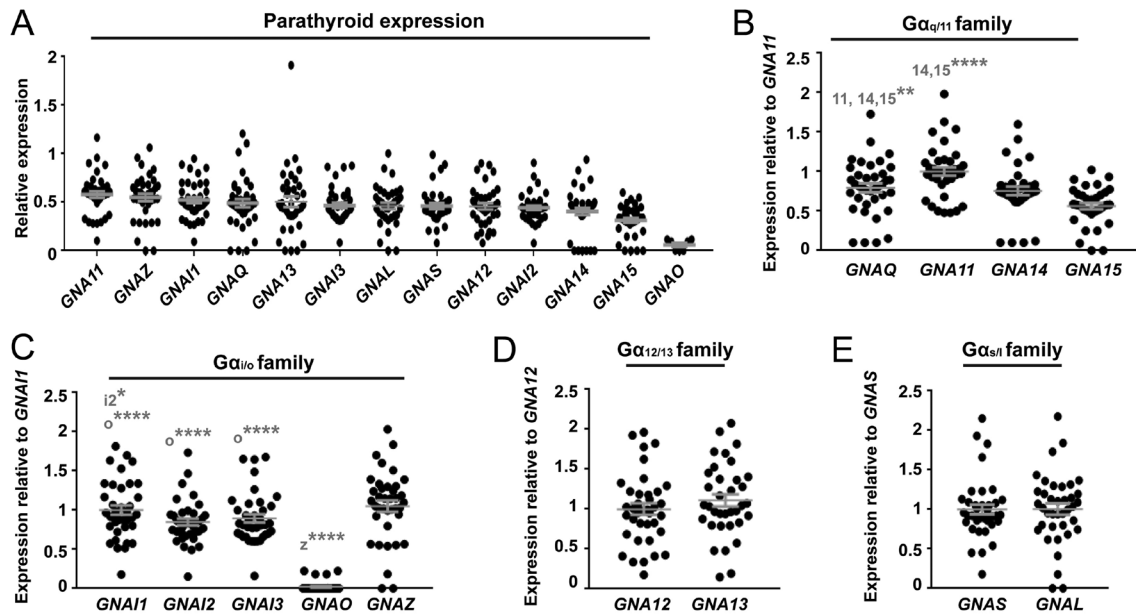
CaSR activation of the $G_{12/13}$ family by NanoBIT G protein dissociation assays. NanoBIT dissociation assays of AdHEK-CaSR cells transiently transfected with: (A) LgBiT- $G\alpha_{12}$ and (B) LgBiT- $G\alpha_{13}$ with SmBiT-G β subunits ($G\beta 1-5$) and unlabelled Gy2 following treatment with 5 mM Ca^{2+}_e . Pre-calcium baselines are not shown. The first baseline value is shown. (C) Percentage suppression of baseline from A and B. (D) NanoBIT dissociation assays of AdHEK-CaSR cells transiently transfected with: LgBiT- $G\alpha_s$, SmBiT-G β subunits ($G\beta 1-5$) and unlabelled Gy2 following treatment with 5 mM Ca^{2+}_e . Pre-calcium baselines are not shown. The first baseline value is shown. (E) Percentage suppression of baseline from D. Statistical analyses comparing values of all $G\alpha$ -G β dissociations are shown in Supplementary Table 1. For all panels only those $G\alpha$ and G β pairs that yielded relative luminescence units (RLU) over a threshold of 1×10^3 (i.e. above background luminescence) are shown. All responses were normalised to those under basal conditions (0.1 mM Ca^{2+}_e). Dissociation curves for 5 mM Ca^{2+}_e are shown (curves with 0.1 mM Ca^{2+}_e are shown in Supplementary Figs 10 and 11). Curves show mean \pm s.e.m. for $n = 6-12$ independent assays.

We first searched the Gene Expression Omnibus (GEO) repository to identify existing gene expression datasets from human parathyroid tissue. This identified: one dataset from expression profiling by array of normal parathyroid glands ($n=6$) and adenoma tissue from patients with primary hyperparathyroidism ($n=25$) (GEO accession #GSE83421) (Balenga *et al.* 2017); and one dataset from expression profiling of normal human tissues ($n=3$ parathyroid tissues) (GEO accession #GSE2193) (Shyamsundar *et al.* 2005). The expression of G protein α , β or γ -subunits was normalised to a panel of housekeeping genes. As no differences were detected between expression levels of any of these genes in the normal vs adenoma tissues, all data were combined. This demonstrated 13 $G\alpha$ -subunits, five $G\beta$ -subunits and 9 $G\gamma$ -subunits were expressed in the majority of parathyroid tissues (Figs 4 and 5). The expression of all $G\alpha$ subunits was compared in each sample by expression as a ratio of abundance. No $G\beta$ - or $G\gamma$ -subunits were consistently overexpressed (Fig. 5). However, these studies confirmed that *GNA11* has greater expression than *GNAQ* (Fig. 4A, B and Supplementary Table 3). Additionally, three other G protein subunits (*GNAZ*, *GNA11* and *GNA13*) had comparable expression to

GNA11 (Fig. 4 and Supplementary Table 3). As the NanoBIT dissociation assays demonstrated no or very little coupling to *GNAZ* and *GNA13* (Figs 2 and 3), it is unlikely that CaSR signals via these G proteins in human parathyroid glands; however, this remains to be verified in parathyroid cells. Thus, the higher expression of genes encoding $G\alpha_{11}$ and $G\alpha_{11}$ may, in part, explain the propensity for CaSR to activate Ca^{2+}_i and reduce cAMP signalling pathways, and the inability of other $G\alpha_{q/11}$ family members to fully compensate for mutant $G\alpha_{11}$ in FHH2 and ADH2 patients.

$G\alpha_s$ is the most abundantly expressed G protein in human kidney and pancreatic tissue

Similar analyses were conducted investigating G protein expression in human kidney tissue in the GEO repository. Only datasets investigating expression in non-diseased tissue were included in analyses. Whole kidney samples were used, as datasets for individual kidney cell types were not available. *GNAS* was the most abundantly expressed G protein in kidney tissue (Fig. 6 and Supplementary Table 4). When the data were reanalysed to focus on individual subfamily members, *GNAQ* and *GNA11* were similarly

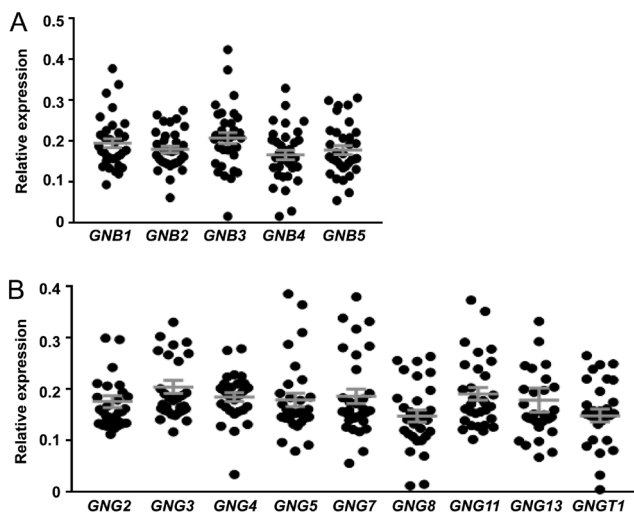
**Figure 4**

Expression of G protein α -subunits in human parathyroid tissue. (A) The mRNA expression of 13 α -subunits in human parathyroid tissue shown as a relative fold-change compared to the geometric mean of control genes. Statistical analyses comparing expression between subunits is shown in Supplementary Table 1. (B, C, D and E) To compare expression of genes within subfamilies, each gene was expressed as a fold-change compared to either: (B) *GNA11*; (C) *GNA11*; (D) *GNA12*; and (E) *GNAS*. Statistical analyses for panels B, C, D, E and F were performed by one-way ANOVA with multiple comparisons and subunits with which comparisons are made are indicated in grey. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

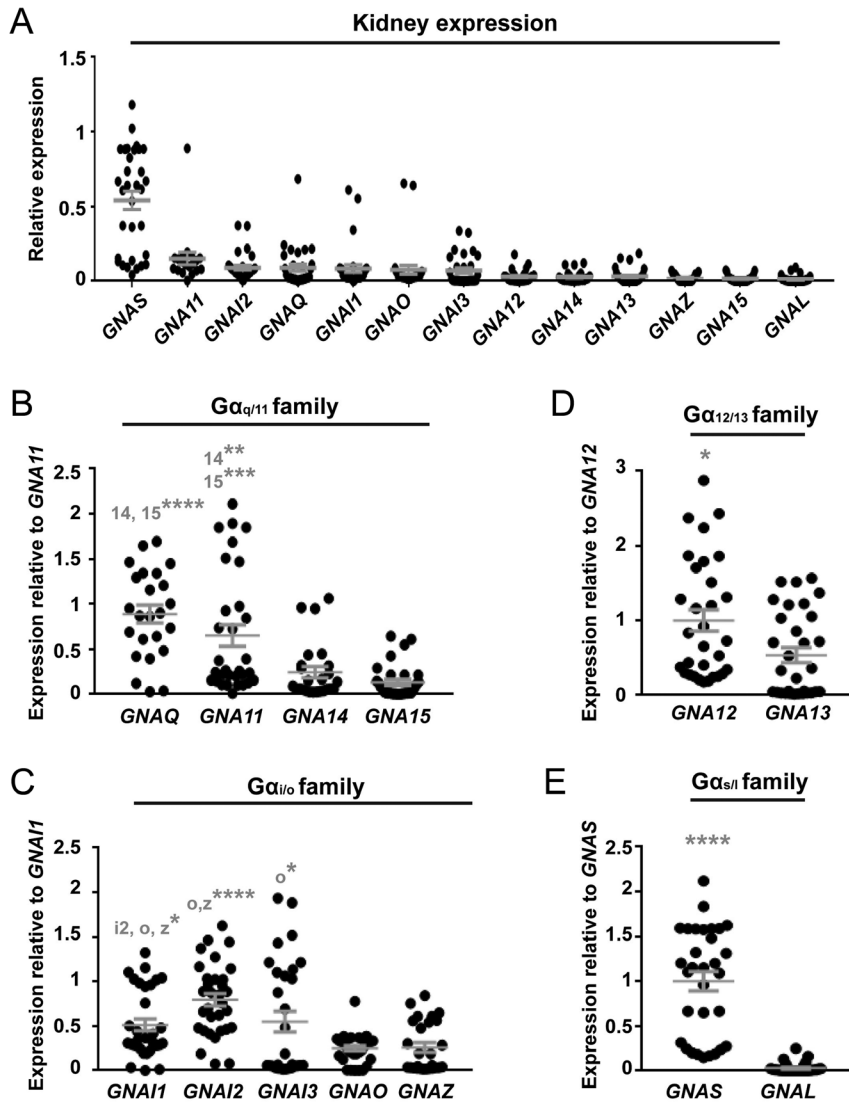
expressed, and both were more abundant than *GNA14* and *GNA15* (Fig. 6B). The *GNAL*, *GNAO* and *GNAZ* genes were poorly expressed, and *GNA12* was expressed at significantly greater levels than *GNA13* (Fig. 6C and D). Thus, within the

kidney, CaSR may be able to signal by G_{α_q} or $G_{\alpha_{11}}$, and may explain why patients with FHH2 and ADH2 mutations have milder symptoms than patients with CaSR mutations in FHH1 or ADH1 (Nesbit *et al.* 2013a, Gorvin *et al.* 2018a). Additionally, CaSR may signal by several other G proteins that are similarly expressed in kidney tissue.

The CaSR is highly expressed in pancreatic tissue, and roles have been described in α - and β -cells (Squires *et al.* 2000, Regard *et al.* 2008). A number of gene expression datasets are available in the GEO repository, including from the whole pancreas and individual cell types. We chose to analyse whole pancreas samples rather than individual cell types as: CaSR is expressed in multiple cell types; these samples were collected from non-disease tissue; and there were enough samples to perform statistical analyses on. These analyses demonstrated that *GNAS* is the most highly expressed G protein in pancreatic tissue (Fig. 7), while members of the $G_{i/o}$ and $G_{q/11}$ families were expressed at similar levels (Fig. 7 and Supplementary Table 5). Analyses within subfamilies demonstrated *GNAQ* and *GNA11* to be more abundantly expressed than *GNA14* and *GNA15*, with *GNAQ* most highly expressed (Fig. 7B). Few differences were observed between subfamily members in the $G_{i/o}$ family, while $G_{\alpha_{12}}$ was more highly expressed than *GNA13* (Fig. 7). Therefore, CaSR could couple to many G proteins within pancreatic tissue, and this may explain why no

**Figure 5**

Expression of G protein β -subunits in human parathyroid tissue. The mRNA expression of (A) all five G_{β} -subunits and (B) nine G_{γ} -subunits in human parathyroid tissue shown as a relative fold-change compared to the geometric mean of control genes. Statistical analyses were performed by one-way ANOVA and subunits with which comparisons are made are indicated in grey. ** $P < 0.01$, * $P < 0.05$.

**Figure 6**

Expression of G protein α -subunits in human kidney tissue. (A) The mRNA expression of 13 $G\alpha$ -subunits in human kidney tissue shown as a relative fold-change compared to the geometric mean of control genes. Statistical analyses comparing expression between subunits is shown in Supplementary Table 2. (B, C, D and E) To compare expression of genes within subfamilies, each gene was expressed as a fold-change compared to either: (B) *GNA11*; (C) *GNAI1*; (D) *GNA12*; and (E) *GNAS*. Statistical analyses were performed by one-way ANOVA and subunits with which comparisons are made are indicated in grey. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

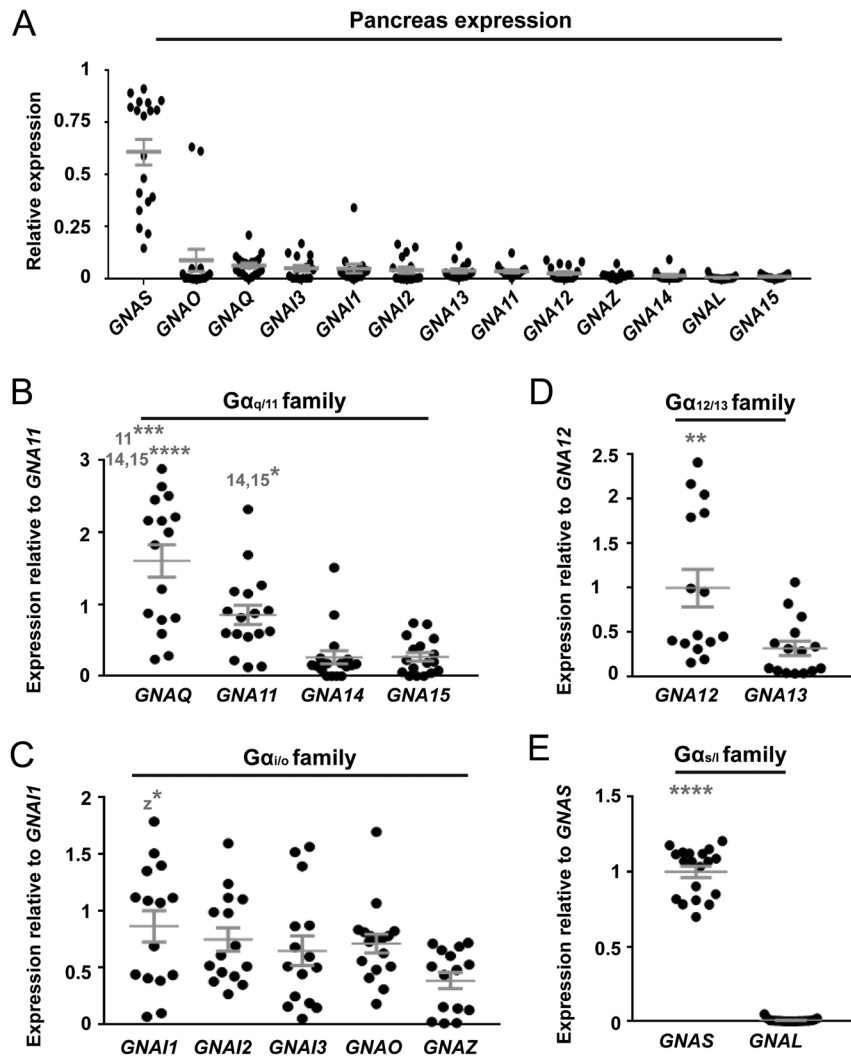
overt pancreatic phenotypes (e.g. diabetes, variations in blood glucose or insulin levels) have been reported in patients with *GNA11* mutations.

Discussion

Our studies confirm that the CaSR can activate multiple G proteins, and this may account for the increasingly varied functions that have been described for the receptor in a wide range of tissues (Hofer *et al.* 2000, Rossol *et al.* 2012, Yarova *et al.* 2015, Zietek & Daniel 2015). These studies indicate that GPCR coupling in different tissues is most likely governed by the ability of the receptor to activate an individual G protein, as well as the expression of G proteins in different tissues. Thus, in parathyroid tissue, in which CaSR has its predominant role, *GNA11* and *GNAI1* are two of the most abundantly expressed G

proteins, and have amongst the highest G protein activity, consistent with previous studies that indicate that parathyroid cells typically activate $G_{q/11}$ and $G_{i/o}$ pathways (Ca^{2+}_i mobilisation and reductions in cAMP, respectively) (Kifor *et al.* 1997, Brown & MacLeod 2001, Hofer & Brown 2003). Other G proteins that are highly expressed in parathyroid tissue, such as $G\alpha_z$, were not activated by CaSR in the NanoBiT dissociation assays, indicating it is unlikely to be important in calcium-mediated responses. These findings would need to be replicated in parathyroid cells to determine whether $G\alpha_z$ is activated by CaSR in this cell type.

These studies showed that CaSR activation of some members of the $G_{i/o}$ and $G_{q/11}$ subfamilies is comparable, indicating that these pathways may have similar importance in CaSR-mediated parathyroid activities, as indicated by early studies of PTH secretion (Conigrave

**Figure 7**

Expression of G protein α -subunits in human pancreatic tissue. (A) The mRNA expression of 13 G α -subunits in human pancreatic tissue shown as a relative fold-change compared to the geometric mean of control genes. Statistical analyses comparing expression between subunits is shown in Supplementary Table 3. (B, C, D and E) To compare expression of genes within subfamilies, each gene was expressed as a fold-change compared to either: (B) *GNA11*; (C) *GNA11*; (D) *GNA12*; and (E) *GNAS*. Statistical analyses were performed by one-way ANOVA and subunits with which comparisons are made are indicated in grey. **** $P < 0.0001$, *** $P < 0.001$, * $P < 0.05$.

2016). Despite this, many studies of CaSR variants assess only changes in Ca^{2+}_i , and thus disease-causing mutations in CaSR could be overlooked by these approaches. It is likely researchers have focussed on Ca^{2+}_i pathways rather than cAMP as fewer robust assays to assess cAMP were available until recent years. Newer assays, such as the cAMP Glosensor system, which allows cAMP to be assessed kinetically, have been increasingly used to assess cAMP signalling by GPCRs (Kumar *et al.* 2017, Lotta *et al.* 2019). However, these assays often still require forskolin treatment to elevate intracellular cAMP levels, and thus increase assay-to-assay variability. Therefore, the development of the NanoBiT dissociation assay will allow both $G_{q/11}$ and $G_{i/o}$ pathways to be compared in cells expressing CaSR variant proteins without the need to pre-treat cells with forskolin.

Our studies showed that CaSR could couple to $G_{\alpha_{12}}$ and $G_{\alpha_{13}}$, as indicated by previous studies (Huang *et al.*

2004), but that rates of activation were slower than with other G protein families. Slow rates of nucleotide exchange and GTP hydrolysis for $G_{12/13}$ have been described for other GPCRs, and it has been suggested that this may facilitate temporal control over cell processes that require prolonged signalling (Siehler 2009). These slower activation rates could explain why some laboratories have been unable to detect CaSR-mediated $G_{12/13}$ signalling. Alternatively, $G_{12/13}$ signalling may not have been detected in previous studies, as CaSR appears to activate only select combinations of $G_{12/13}$ and $G\beta$ -subunits (Fig. 3), despite coupling between $G_{\alpha_{12/13}}$ and $G\beta_{1-4}$ (Supplementary Table 1). Post-translational modifications, including palmitoylation and myristoylation, can affect G-protein recruitment and activation (Bhattacharyya & Wedegaertner 2000), and this may have affected the ability of $G_{\alpha_{12/13}}$ to activate some $G\beta$ subunits. Further studies investigating the effects of lipid modifications on CaSR signalling by $G_{12/13}$ would

be required to determine whether this could explain differences between activation within members of the same subfamilies.

Previous studies have suggested that patients with FHH2 due to $G\alpha_{11}$ mutations may have a milder phenotype than patients with CaSR mutations, as other G proteins could compensate for $G\alpha_{11}$ functions (Nesbit *et al.* 2013a, Gorvin *et al.* 2018a). Our studies demonstrate that CaSR can activate all members of the $G_{q/11}$ subfamily, and therefore, in principle, these proteins could compensate for impaired $G\alpha_{11}$ functionality. However, consistent with previous studies, *GNA11* was expressed at significantly greater levels than *GNAQ* in human parathyroid tissue, which may explain why $G\alpha_q$ cannot entirely compensate for $G\alpha_{11}$, and patients with inactivating $G\alpha_{11}$ mutations develop mild hypercalcaemia (Nesbit *et al.* 2013a). In contrast to parathyroid tissue, expression of *GNA11* and *GNAQ* are not significantly different in kidney tissue, thus compensation by other subunits may be present in kidney cells, providing a reason why phenotypes may be milder than that observed in FHH1 and ADH1. Further investigation of CaSR coupling to mutant $G\alpha_{11}$ in cells depleted of other $G_{q/11}$ subfamily members may be required to investigate G α -protein compensation in greater detail.

In pancreatic tissue, expression of G α -subunits of the $G_{i/o}$ family were not significantly different to some members of the $G_{q/11}$ family. The NanoBiT dissociation assays demonstrated that CaSR robustly activates $G\alpha_{11}$ and $G\alpha_o$, both of which are expressed at comparable levels in the pancreas. Thus, CaSR-mediated functions in pancreatic cells may arise by coupling to either G protein family. Activation of the $G_{i/o}$ family that negatively modulates cAMP signalling and is important in reducing hormone secretion by other GPCRs (Braun 2014), could explain how CaSR activation impairs hormone secretion in pancreatic cells. However, previous studies in pancreatic β -cells failed to show a role for CaSR-mediated cAMP depletion in insulin secretion (Squires *et al.* 2000), and therefore further studies of CaSR coupling in pancreatic β -cells may be required.

Studies of GPCR signalling using a NanoBiT G protein dissociation assay system has several advantages over other methodologies to study GPCR activation. The ability to assess multiple G protein pathways in parallel reduces the need to perform different assays to assess individual signalling pathways, and avoids the need to chemically or genetically inhibit gene expression, allowing the researcher to directly compare responses by each G protein. This will revolutionise the ability to assess biased signalling by disease-causing CaSR mutations which has been described in several studies (Leach *et al.* 2012, Gorvin

et al. 2018b,c) and will simplify comparisons between CaSR allosteric modulators. Furthermore, the simplicity of the methodology and availability of these plasmids will allow researchers in different laboratories to directly compare results, increasing the robustness and reproducibility of data yielded from these assays. The ability to collect kinetic data easily, which many assays that have been used previously to measure GPCR signalling cannot do (e.g. IP-one, LANCE cAMP, pERK AlphaScreen, luciferase reporter genes), will increase our understanding of GPCR temporal signalling. This was demonstrated for the CaSR as our NanoBiT assays showed activation of $G\alpha_{12/13}$ and $G\alpha_s$ occurs more slowly than activation of other G α -proteins. If this assay system is to be adopted by multiple laboratories, it will be important that a uniform methodology is used. For example, the time between agonist addition and measurement of luminescence should always be the same in all laboratories, to allow rates of dissociation to be compared.

A disadvantage of this study is that it was conducted in a single cell-line, adherent HEK293 overexpressing CaSR, which could be criticised as an artificial system. We chose this cell-line as HEK293: are easy to grow and transfect; are available to most research labs so findings can be replicated; are routinely used in CaSR research; and cultures of parathyroid and kidney cell-lines are not widely available. Moreover, the purpose of this study was to determine whether CaSR can couple to all G protein subunits and establish a simple assay system to assess CaSR variants and potentially screen pharmacological compounds to determine their ability to engender signalling bias. Future studies investigating endogenous G protein activation by CaSR in specific cell types may become possible with recently published BRET constructs (Maziarz *et al.* 2020). However, these assays still require transfection and, therefore, may be difficult in primary cells. Our study could also be criticised for only assessing one CaSR ligand. We assessed Ca^{2+}_e as it is the best characterised ligand in *in vitro* assays of CaSR signalling and is the major physiological agonist of the receptor. Other studies have described CaSR activation by other ligands, including aromatic amino acids and polyamines (Conigrave *et al.* 2000, 2004, Thomsen *et al.* 2012). However, many of these act as modulators of Ca^{2+}_e -mediated responses, or physiological functions are unknown. Future studies could utilise the NanoBiT dissociation assay system to compare the effects of these ligands by a single methodology. Finally, the NanoBiT assay we describe will be unable to detect G protein activation in which G α -G $\beta\gamma$ dissociation does not occur. Studies have indicated that such activation in the absence of G α -G $\beta\gamma$ dissociation may occur in some cell types

(e.g. cone photoreceptors (Rosenzweig *et al.* 2007)). However, the number of GPCRs able to utilise such signalling is unknown, and it is possible that some studies that reported the absence of dissociation may not have measured signal kinetics, and thus may have missed slower dissociation rates that have been reported by some laboratories (Digby *et al.* 2008). The NanoBiT dissociation assay, will be able to detect these differences in dissociation rates as shown for $G\alpha_{12/13}$ and $G\alpha_s$.

In conclusion, we have demonstrated that CaSR differentially activates multiple $G\alpha$ proteins. Physiological effects of CaSR are likely mediated by a combination of G protein activation and expression of individual $G\alpha$ -proteins in different tissues. These studies highlight a simple, single assay system that can be used to robustly assess biased signalling and could be utilised in the development of new pharmacological compounds targeting the CaSR.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-21-0058>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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