Altered Expression of Gq/11α Protein Shapes mGlu1 and mGlu5 Receptor-Mediated Single Cell Inositol 1,4,5-Trisphosphate and Ca2+ Signaling

Peter J. Atkinson, Kenneth W. Young, Steven J. Ennion, James N. C. Kew, Stefan R. Nahorski, and R. A. John Challiss

Department of Cell Physiology & Pharmacology, University of Leicester, Leicester, United Kingdom (P.J.A., K.W.Y., S.J.E., S.R.N., R.A.J.C.); and Psychiatry Centre of Excellence for Drug Discovery, GlaxoSmithKline, Harlow, United Kingdom (J.N.C.K.)

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ABSTRACT

The metabotropic glutamate (mGlu) receptors mGlu1 and mGlu5 mediate distinct inositol 1,4,5-trisphosphate (IP3) and Ca2+ signaling patterns, governed in part by differential mechanisms of feedback regulation after activation. Single cell imaging has shown that mGlu1 receptors initiate sustained elevations in IP3 and Ca2+, which are sensitive to agonist concentration. In contrast, mGlu5 receptors are subject to cyclical PKC-dependent uncoupling and consequently mediate coincident IP3 and Ca2+ oscillations that are largely independent of agonist concentration. In this study, we investigated the contribution of Gq11, α protein expression levels in shaping mGlu1/5 receptor-mediated IP3 and Ca2+ signals, using RNA interference (RNAi). RNAi-mediated knockdown of Gq11α almost abolished the single-cell increase in IP3 caused by mGlu1 and mGlu5 receptor activation. For the mGlu1 receptor, this unmasked baseline Ca2+ oscillations that persisted even at maximal agonist concentrations. mGlu5 receptor-activated Ca2+ oscillations were still observed but were only initiated at high agonist concentrations. Recombinant overexpression of Gqα enhanced IP3 signals after mGlu1 and mGlu5 receptor activation. It is noteworthy that although mGlu5 receptor-mediated IP3 and Ca2+ oscillations in control cells were largely insensitive to agonist concentration, increasing Gqα expression converted these oscillatory signatures to sustained plateau responses in a high proportion of cells. In addition to modulating temporal Ca2+ signals, up- or down-regulation of Gq11α expression alters the threshold for the concentration of glutamate at which a measurable Ca2+ signal could be detected. These experiments indicate that altering Gq11, α expression levels differentially affects spatiotemporal aspects of IP3 and Ca2+ signaling mediated by the mGlu1 and mGlu5 receptors.

Activation of the phospholipase C (PLC) pathway via coupling of G protein-coupled receptors (GPCRs) to G proteins of the Gq11 family results in inositol 1,4,5-trisphosphate (IP3) production and mobilization of intracellular calcium (Ca2+). Receptor activation can initiate spatially and temporally unique Ca2+ signals and thereby regulate an array of cellular processes (Berridge et al., 2000). In this study, we have investigated the contribution of Gq11, α protein expression in shaping receptor-initiated IP3 and Ca2+ signaling patterns.

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ABBREVIATIONS: PLC, phospholipase C; GPCR, G protein-coupled receptor; IP3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; eGFP, enhanced green fluorescent protein; eGFP-PHPLC, pleckstrin homology domain of PLC51 tagged with enhanced green fluorescent protein; CICR, Ca2+-induced Ca2+-release; mGlu, metabotropic glutamate; mACh, muscarinic acetylcholine; RNAi, RNA interference; CHO, Chinese hamster ovary; HEK, human embryonic kidney; siRNA, small interfering RNA; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; KHB, Krebs-Henseleit buffer; RFU, relative fluorescent units; AM, acetoxymethyl ester; RGS, regulator of G protein signaling.

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structural/permisive role for the phosphorylation of an adjacent residue (Ser\(^{839}\)) by PKC (Kim et al., 2005). Similar PKC-dependent Ca\(^{2+}\) oscillations have also been described after glutamate activation of astrocytes (Codazzi et al., 2001) and activation of another family C GPCR, the Ca\(^{2+}\)-sensing receptor (Young et al., 2002). Use of the pleckstrin homology domain of phospholipase C\(\delta\) tagged with enhanced green fluorescent protein (eGFP-PH\(_{PLC\delta}\)) has enabled IP\(_3\) oscillations underlying mGlu5a receptor-activated Ca\(^{2+}\) oscillations to be observed (Nash et al., 2001, 2002; Nahorski et al., 2003). These PKC-dependent Ca\(^{2+}\) oscillations (referred to as “dynamic uncoupling”) are distinct from regenerative Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR), which is generated through an intrinsic property of the IP\(_3\) receptor (Thomas et al., 1996; Taylor and Thorn, 2001). CICR oscillations can be maintained with a relatively low steady-state increase in IP\(_3\), as observed after activation of the M\(_3\) muscarinic acetylcholine (mACH) receptor with a low agonist concentration in the same cell background (Nash et al., 2001). Our previous studies exploring the determinants of mGlu5 receptor signaling led us to propose a model in which Ca\(^{2+}\) oscillation frequency is dependent on receptor expression levels but is largely independent of agonist concentration (Nash et al., 2002). However, the importance of receptor-G protein coupling efficiency in group I mGlu receptor-mediated Ca\(^{2+}\) signaling has not yet been investigated. It is clear that regulation and localization of G\(\alpha_{1}\) proteins could be a key, influencing factor in shaping the Ca\(^{2+}\) signals produced.

Studies examining the role of G\(\alpha_{1}\) proteins generally, and in conjunction with mGlu receptor signaling, have been facilitated greatly by the generation of G\(\alpha_{1}\) knockout mice (for review, see Offermanns, 2003). However, gene deletion studies are limited by the mortality of G\(\alpha_{i}/G\alpha_{11}\) double-knockout mice and also by the possibility that the phenotype of the cells studied may adapt to compensate for the loss of a particular G\(\alpha\), as observed for the deletion of other G protein subtypes, including G\(\alpha_{i}\) (Greif et al., 2000) and G\(\alpha_{11}\) (Davignon et al., 2000). Other investigators have successfully used antisense methods to reduce G\(\alpha_{11}\) expression; however, these studies often rely on microinjection, making an accurate determination of endogenous G\(\alpha_{i}\) and G\(\alpha_{11}\) protein expression difficult to ascertain (Macrez-Lepretre et al., 1997; Haley et al., 1998). Determining the relative G\(\alpha_{i}\) and G\(\alpha_{11}\) expression levels after knockdown is clearly desirable for defining the nature of the response observed.

### Materials and Methods

#### Cell Culture and Plasmid Transfection

CHO cells stably expressing the human mGlu1a or mGlu5a receptor under the control of the inducible LacSwitch-II system (Stratagene, La Jolla, CA) were maintained as described previously (Hermann et al., 1998; Nash et al., 2002) and are denoted as CHO-lac-mGlu1 or CHO-lac-mGlu5. HEK cells stably expressing the M\(_3\) mACH receptor (HEK-m3) were created and maintained as described previously (Tovey and Williams, 2004). Plasmid containing the fusion construct between eGFP and the pleckstrin homology domain of PLC\(\delta\) (eGFP-PH\(_{PLC\delta}\)) was kindly donated by T. Meyer (Stanford University, Stanford, CA).

For single cell imaging experiments, CHO-lac-mGlu1/5 or HEK-m3 cells were grown on 25-mm coverslips and cotransfected 72 h before experimentation with 1.8 \(\mu\)g of G\(\alpha_{11}\)-RNAi, control RNAi, or full-length human G\(\alpha_{i}\) and 0.2 \(\mu\)g of eGFP-PH\(_{PLC\delta}\) (for IP\(_3\) imaging) or eGFP (for Ca\(^{2+}\) imaging) using 6 \(\mu\)l of GeneJuice (Novagen/EMD Biosciences, Madison, WI) per coverslip. For induction of maximal mGlu receptor expression in CHO cells, the medium was replaced with fresh culture medium containing 100 \(\mu\)M IPTG 18 to 20 h before experimentation. For standard SDS-PAGE immunoblotting, HEK-m3 cells were transfected in six-well plates 72 h before experimentation with 2 \(\mu\)g of G\(\alpha_{11}\)-RNAi or control RNAi using Lipofectamine 2000. CHO cells were transfected in flasks (175 cm\(^2\)) with 10 \(\mu\)g of G\(\alpha_{11}\)-RNAi or control RNAi using 30 \(\mu\)l of GeneJuice and after 24 h, cells were seeded into six-well plates for a further 48 h. For receptor biotinylation, real-time PCR and 6 M ureaSDS-PAGE analysis, CHO cells were transfected using the Nucleofector system (Amaxa Biosystems, Gaithersburg, MD), according to the manufacturer’s optimized protocol. In brief, 5 \(\times\) 10\(^5\) cells were transfected with 2 \(\mu\)g of control- or G\(\alpha_{11}\)-RNAi and Program U-23 on the Nucleofector, before seeding cells into six-well plates 72 h before experimentation.

#### RNAi Design and Preparation

To design an RNAi plasmid expressing G\(\alpha_{11}\)-specific small interfering RNA (siRNA), the mRNA sequences for human G\(\alpha_{i}\) (GenBank accession number NM_002072) and G\(\alpha_{11}\) (GenBank accession number NM_002067) were aligned to identify potential target sequences. Candidate 19-base pair sequences, homologous for both human G\(\alpha_{i}\) and G\(\alpha_{11}\) genes (and containing a G/C content of 40–60%) were identified, and gene specificity was checked using the BLASTn algorithm to search the GenBank sequence database. RNAi-expressing constructs for five selected sequences were created according to the manufacturer’s instructions (using the pSilencer 1.0-U6 expression system; Ambion (Austin, TX)), and the knockdown of recombinantly expressed CFP-labeled G\(\alpha_{i}\) was used to assess the effectiveness of these constructs (data not shown). The target sequence selected for G\(\alpha_{11}\) silencing was 5’-GATGTTCCGGAACCTGAAAC-3’, corresponding to positions 932 to 950 relative to the start codon of human G\(\alpha_{11}\) and G\(\alpha_{3}\) (denoted “G\(\alpha_{11}\)-RNAi”). Furthermore, an additional control RNAi construct was generated (using the nucleotide sequence 5’-GCTGACCCCTGGAGAGTCTAC-3’), and is denoted “control RNAi”.

#### Immunoblot Analysis

Levels of endogenous G\(\alpha_{11}\)-protein expression in HEK-m3 and CHO-lac-mGlu1a cells were determined by a standard Western blot protocol (Willets and Kelly, 2001) using a G\(\alpha_{11}\)-specific antibody at 1:5000 dilution (CQ, kindly donated by G. Milligan, University of Glasgow, Glasgow, UK). Antibodies against G\(\alpha_{11}\) (1:2000; Sigma, St. Louis, MO) were used as controls for RNAi specificity and protein loading. Antibodies against the C-terminal region of the mGlu1 receptor (1:1000; Chemicon International, Temecula, CA) and the
C-terminal region of the mGlu5 receptor (1:1000; Upstate Biotechnology, Lake Placid, NY), respectively, were used to detect mGlu1α and mGlu5α receptor expression. All primary antibody incubations were made at room temperature for 2 h or overnight at 4°C. Resolution of both Gαq and G11α with an antibody (CQ) was achieved using SDS-PAGE gels containing 12.5% acrylamide and 6 M urea, as described previously (Milligan, 1993). The relative mobility of each Gα subunit was confirmed using cell lysates obtained from CHO-lac-mGlu cells recombinantly expressing human Gαq or G11α using equivalent gels.

**Cell-Surface Biotinylation of mGlu1α and mGlu5α Receptors.** CHO-lac-mGlu1α and -mGlu5α cells were transiently transfected with Gαq/11α-RNAi, control RNAi, or full-length human Gαq using the Amaza Nucleofection system, seeded into six-well plates, and induced 48 h later. After 72 h, cells were washed twice with PBS at room temperature and labeled with membrane-impermeant EZ-Link Sulfo-NHS-biotin (1 mM in PBS; Pierce, Rockford, IL) for 30 min at room temperature. Cells were washed twice with ice-cold PBS, once with 500 mM Tris/HCl, pH 7.4, and twice more with ice-cold PBS. Cells were then lysed for 10 min with solubilization buffer (10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 500 mM EGTA, 1% Igepal, and 0.1% SDS, pH 7.4) and centrifuged at 14,000 g for 5 min. The cleared supernatant (900 μl) was then incubated with 200 μl of streptavidin/agarose beads (diluted 1:10 in solubilization buffer) for 2 h with constant rotation at 4°C. The remaining supernatant was retained to assess receptor expression in whole-cell lysates (see below). Beads were recovered by centrifugation during two washes with 1 ml of solubilization buffer and two washes with 1 ml of PBS. Immuno complexes were dissociated with 50 μl of 2× sample buffer (125 mM Tris/HCl, 50 mM dithiothreitol, 4% SDS, 20% glycerol, 0.01% bромphenol blue, pH 6.8), heated at 90°C for 5 min, and then resolved by SDS-PAGE (as described above). For total cell extracts, 20 μl of the retained supernatant was diluted (1:1) with 2× sample buffer before SDS-PAGE.

**PCR Amplification of the RNAi Target Region from CHO Cells.** To determine the sequence of the RNAi target region of Gαq and G11α derived from CHO cells, total RNA was isolated from CHO cells using the RNAeasy kit (QIAGEN, Valencia, CA). Samples (200 ng of RNA) were reverse-transcribed into cDNA using the Omniscript RT-PCR kit (QIAGEN). Primers specific for Gαq and G11α were reverse-transcribed into cDNA using the Omniscript RT-PCR kit (QIAGEN). Primers specific for Gαq and G11α were reverse-transcribed into cDNA using the Omniscript RT-PCR kit (QIAGEN). Primers specific for Gαq and G11α were reverse-transcribed into cDNA using the Omniscript RT-PCR kit (QIAGEN). Primers specific for Gαq and G11α were reverse-transcribed into cDNA using the Omniscript RT-PCR kit (QIAGEN). Primers specific for Gαq and G11α were reverse-transcribed into cDNA using the Omniscript RT-PCR kit (QIAGEN). Primers specific for Gαq and G11α were reverse-transcribed into cDNA using the Omniscript RT-PCR kit (QIAGEN).

**Quantitative Real-Time PCR.** After RNAi transfection, total RNA and cDNA were prepared from samples in triplicate, as described above. An RT-negative control was included for each triplicate to control for genomic DNA contamination. Real-time PCR using SYBR-green fluorescence (Applied Biosystems, Foster City, CA) was carried out using an ABI PRISM 7700 sequence detection system as described previously (Medhurst et al., 2000). PCR parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Real-time PCR data were captured using Sequence Detector software (Applied Biosystems) to obtain threshold cycle values for the gene of interest. Values were normalized against a housekeeping gene, cyclophilin, and expressed as percentage of control. All measurements were performed in triplicate for three separate transfections using the Amaza Nucleofection system.

**Single Cell Imaging of IP3.** Increases in cellular IP3 were detected by measuring the translocation of eGFP-PHPLCβ2 from the plasma membrane to the cytosol as described previously (Nash et al., 2002; Young et al., 2003). Cells were transfected as described above, and coverslips were mounted on the stage of an Olympus IX70 inverted epifluorescence microscope and perfused (5 ml/min) at 37°C with Krebs-Henseleit buffer (KHB): 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.3 mM CaCl2, 1.2 mM KH2PO4, 4.2 mM NaHCO3, 10 mM HEPES, and 11.7 mM glucose, pH 7.4) using a Gilson Minipulser 2 pump (Gilson, Inc., Middleton, WI). Confocal images were collected after excitation at 488 nm using an Olympus FV500 laser scanning confocal microscope at a scan rate of 1.5 to 2.5 Hz. eGFP-PHPLCβ translocation was measured by creating a region of interest in the cytosol and plotting the average pixel intensity in that region versus time. Data are expressed in relative fluorescent units (RFU) by subtraction of background fluorescence followed by dividing the fluorescent intensity at a given time by the initial fluorescence within each region of interest (F/F0). Drug applications were made using the perfusion line as indicated.

**Ca2+ Imaging.** Cells were transfected as described, and then loaded with fura-2 AM (Invitrogen) in 1 ml of KHB (final concentration, 5 μM) for 60 to 90 min. The cells were mounted on an inverted epifluorescence microscope (Diaphot; Nikon, Tokyo, Japan) with an oil immersion objective (40×) and then excited at 340 and 380 nm (for fura-2 AM) and 488 nm (for eGFP) using a Spectramaster II monochromator (PerkinElmer Life and Analytical Sciences, Boston, MA) at a sample rate of 0.7 Hz. GFP-containing cells were identified (as a marker of Gαq/11α-RNAi/control RNAi/Gαqα-transfected cells), and sequential images were then captured from GFP-transfected cells at wavelengths above 510 nm after drug applications made using the perfusion line as indicated. Ca2+ signals are expressed as 340 nm/380 nm ratios.

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**TABLE 1**

**Oligonucleotides**

Oligonucleotide sequences are shown for sense (s) and antisense (as) primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession-Number</th>
<th>Primer</th>
<th>Sequence 5'-3'</th>
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</thead>
<tbody>
<tr>
<td>Rat cyclophilin</td>
<td>NM_017101</td>
<td>as</td>
<td>TGGGCCAGGTTGTTGACCT</td>
</tr>
<tr>
<td>For real-time PCR</td>
<td></td>
<td>as</td>
<td>CCACACGTCAATAGGCTGTT</td>
</tr>
<tr>
<td>Gαq</td>
<td>NM_031036</td>
<td>s</td>
<td>AGTCCAGTTCCACACACACAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as</td>
<td>CCTCTTCACTCACTACCTTTGAA</td>
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<tr>
<td></td>
<td></td>
<td>s</td>
<td>GGTGAGTCCGACACACAGA</td>
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<tr>
<td></td>
<td></td>
<td>as</td>
<td>GGAGTACGTGCCTGTTCC</td>
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<tr>
<td>For sequencing</td>
<td></td>
<td>s</td>
<td>GAGAAAGATTTATATTATCC</td>
</tr>
<tr>
<td>Gαq</td>
<td>NM_031036</td>
<td>as</td>
<td>TCTGTTTGTCGTCGACACAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as</td>
<td>AGACAGAGTCCTGCCACATCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as</td>
<td>TCGGGTGTCGCAGACAGA</td>
</tr>
</tbody>
</table>
Data Analysis. Curve fitting of data and calculation of EC50 values was carried out using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). Statistical differences between data sets were determined by one-way analysis of variance for multiple comparisons, followed by Bonferroni’s multiple-range test at p < 0.05 (using Prism 3.0 software), or Student’s t test (unpaired; p < 0.05 being considered significant).

Results

RNAi-Mediated Knockdown of Gq/11α Protein Expression and Function in HEK Cells Stably Expressing the M3 mACh Receptor. RNAi constructs designed using 19-base pair sequences targeting homologous regions of human Gqα and G11α protein expression were initially tested in HEK-m3 cells. Immunoblotting experiments revealed robust silencing of Gq/11α 72 h after transfection with Gq/11α-RNAi compared with control RNAi. In these experiments, the transfection efficiency typically achieved was ~50%, suggesting a high degree of Gq/11α silencing in individual HEK cells. Antibodies detecting γ-tubulin and G protein α-subunits, G11α, confirmed equal protein loading (Fig. 1A).

Single-cell IP3 imaging, using cells transfected with eGFP-PHPLCΔ5 alone, showed a concentration-dependent increase in cytosolic fluorescence in response to 30-s applications of methacholine, with a best-fit EC50 of 0.9 μM. This was unaffected by cotransfection with control RNAi but was inhibited by Gq/11α-RNAi, as demonstrated by a rightward shift in the concentration-response curve (EC50 6 μM) and a suppressed maximal response (2-fold, compared with 3.5-fold over basal in control cells, shown in Fig. 1B). Representative images (Fig. 1B) showed a clear suppression of eGFP-PHPLCΔ5 translocation from the plasma membrane to the cytosol in Gq/11α-RNAi-containing cells compared with control RNAi and also indicate that these cells display no obvious morphological changes.

RNAi-Mediated Knockdown of Gq/11α Protein Expression in CHO Cells. Having identified a functional Gq/11α-RNAi construct in HEK cells, we tested the same construct in CHO cell lines stably and inducibly expressing mGlul1 and mGlul5 receptors. Immunoblotting showed a significant inhibition of endogenous Gq/11α expression 72 h after transfection with Gq/11α-RNAi compared with control RNAi constructs (Fig. 2A). The transfection efficiency typically achieved in CHO cells was 40 to 50% (determined using green fluorescent protein; data not shown), again suggesting highly efficient Gq/11α protein silencing. It is noteworthy that G1α–3, G12α, and γ-tubulin protein expression levels were unaffected (Fig. 2A). Further experiments confirmed that induction of mGlul1 and mGlul5 receptor expression was unaffected by RNAi treatment (Fig. 2B).

After the identification of an RNAi construct with Gq/11α silencing activity in both human and CHO cell backgrounds, the 19-base pair RNAi target sequence within Gqα and G11α derived from the CHO cell line was investigated. No sequence information for Chinese hamster Gq/11α was available in the GenBank/EMBL databases. We therefore amplified the appropriate regions from CHO cells by PCR with Gqα- and G11α-selective primers, based on rat sequence information (Table 1). Sequencing of the ampiclons from CHO cells indicated two base pair differences (corresponding to C→T changes at positions 9 and 21 in Fig. 3A) in G11α compared with CHO Gqα and human Gq/11α.

In view of the contrasting reports of RNAi specificity in the literature, it was imperative to assess the knockdown of Gqα relative to G11α. Using the NucleoFect system to achieve optimal plasmid transfection (>80% determined using GFP;
data not shown) and real-time PCR with Gqα- and G11α-specific primers (Table 1), a robust knockdown (~75%) of Gqα mRNA in both mGlu1 and mGlu5 receptor-expressing CHO-lac cells was observed (Fig. 3B). We were surprised to observe almost complete knockout of Gqα protein, as described under Materials and Methods. Under these conditions, Gqα migrates farther than Gα, an observation confirmed using lysates obtained from CHO-lac-mGlu1 cells recombinantly expressing human Gqα and G11α in equivalent gels (Fig. 3C). If the antibody immunoreactivity for both Gα subunits is comparable, these data suggest that expression levels of Gqα and G11α proteins under control conditions in CHO-lac-mGlu1a cells are similar. The relative reduction of Gqα and G11α protein expression was similar to the reduction in Gqα and G11α mRNA levels determined by real-time PCR. A reduction of Gqα mRNA by ~75% corresponds to an almost complete knockout of Gqα protein; similarly, a reduction in G11α mRNA by ~60% led to a slightly less efficient knockdown of G11α protein expression. The relationship between mRNA and protein knockdown is dependent on the turnover of Gqα and G11α proteins. A previous study in CHO cells reported that the half-time of Gqα is 18 h (Mitchell et al., 1993). Levels of G12α, G13α, and γ-tubulin protein expression in these experiments were unaffected (data not shown). These experiments demonstrate the effectiveness of RNAi in silencing both Gqα and G11α and so provide evidence that mismatches in the RNAi target sequence are tolerated, at least for these closely related gene products.

**Effects of Manipulating Gqα/11α Expression on Cell-Surface Receptor Expression.** To examine the possibility that up- or down-regulation of Gqα/11α protein expression can influence mGlu1/5 receptor trafficking to the plasma membrane, we used a cell-surface protein biotinylation strategy. In both CHO-lac-mGlu1a (Fig. 4A) and mGlu5a (Fig. 4B) cells, Gqα/11α protein expression was increased or decreased by transient transfection with human recombinant Gα or Gqα/11α-RNAi, respectively. For mGlu1a and mGlu5a receptors, doublet immunoreactive bands were evident at ~150

**Fig. 2.** RNAi-induced knockdown of Gqα/11α protein expression in CHO cells. To assess the activity of the Gqα/11α-RNAi construct for use in CHO cell lines (where Gqα and G11α sequence information was not available) stably expressing mGlu1 and mGlu5 receptors, cells were transfected (using GeneJuice) with control RNAi or Gqα/11α-RNAi 72 h before experimentation, as described under Materials and Methods. A, representative immunoblot showing reduction of Gqα/11α protein expression relative to control RNAi-transfected CHO cell lysates. Equal amounts of each sample were loaded in duplicate as indicated after probing for Gqα, G11α, and γ-tubulin. B, representative immunoblot showing the induction of mGlu1 and mGlu5 receptor expression by 100 μM IPTG in control RNAi and Gqα/11α-RNAi transfected cells. Data are representative of at least three separate transfections.

**Fig. 3.** Determining the specificity of Gqα/11α-RNAi-mediated knockdown of Gqα and G11α mRNA and protein expression in CHO cells. A, sequencing Gqα and G11α PCR products amplified from CHO cells. After the identification of an RNAi construct with Gqα/11α silencing activity in both human and CHO cell backgrounds, the 19-base pair RNAi target sequence within Gqα and G11α derived from the CHO cell line was confirmed. This region was amplified from CHO cells by PCR with Gqα and G11α-selective primers, based on rat sequence information (see Table 1). Shown is an alignment of the Gqα and G11α PCR products from CHO cells with the equivalent human sequences to which the Gqα/11α-RNAi construct was targeted. B, quantitative real-time PCR analysis of Gqα and G11α mRNA after Gqα/11α-RNAi transfection into CHO-lac-mGlu1 and -mGlu5 cells. Data are expressed as percentage of control after normalization to cyclophilin (mean ± S.E.M.; *, p < 0.05 compared with control). All measurements were performed in triplicate after three separate transfections using the Amaxa Nucleofection system. C, determining the relative knockdown of Gqα and G11α protein expression levels after Gqα/11α-RNAi transfection. Lysates from the same transfected cells described in B were resolved by SDS-PAGE using a gel system containing 6 M urea to allow separation of Gα from G11α (see Materials and Methods). Immuno- dot blotting was carried out using antiserum CQ recognizing the conserved C-terminal region of both protein subunits. Shown are representative immunoblots, repeated at least three times, for Gqα or G11α overexpression in CHO-lac-mGlu1 cells (slot on left), and Gqα or G11α knockdown in the same experiments. Gqα and G11α knockdown was normalized to cyclophilin mRNA levels, which were unaffected after Gqα/11α-RNAi transfection. To complement the mRNA analysis, SDS-PAGE gels containing 6 M urea were used to resolve Gqα and G11α proteins in these cells (see Materials and Methods).
kDa. mGlu1a and mGlu5a receptor cell-surface biotinylation indicated that the mature, higher molecular weight protein (top band) is enriched at the plasma membrane (Fig. 4, A and B). It is noteworthy that altering Gq/11 expression levels did not affect cell-surface mGlu1a and mGlu5a receptor, or indeed total receptor expression in whole-cell extracts, indicating that the trafficking/localization of these receptors to the plasma membrane is independent of Gq/11 protein expression.

The Effects of Manipulating Gq/11 Protein Expression on Single Cell IP3 Levels. Measurements of IP3 production in cells transfected with Gq/11-RNAi, control RNAi, or full-length human Gqα were made possible by cotransfection of the IP3 biosensor eGFP-PHPLCδ. In all of the control RNAi CHO-lac-mGlu1a cells examined, a graded increase in eGFP-PHPLCδ translocation was observed in response to incremental glutamate concentrations, each applied for 30 s (Fig. 5), with a best-fit EC50 of 8.4 μM for the peak IP3 response. In CHO-lac-mGlu1a cells transfected with Gq/11-RNAi, responses to glutamate were almost abolished, with no translocation of eGFP-PHPLCδ observed in several individual cells. In contrast, when cells were transfected with full-length human Gqα, the EC50 for the peak IP3 response left-shifted from 8.4 to 2.0 μM glutamate (p < 0.05), whereas the maximal responsiveness was not significantly affected (p > 0.05; Fig. 5).

Single-cell mGlu5a receptor-induced IP3 responses underlying oscillatory Ca2+ signals have been studied extensively in CHO-lac-mGlu5a cells (Nash et al., 2002; Nahorski et al., 2003; Young et al., 2003). To examine the effect of manipulating Gq/11 protein levels, cells were transfected with Gq/11-RNAi, control RNAi, or full-length human Gqα. Responses to a single maximal concentration (30 μM) of glutamate were subsequently examined over a 300-s time period for each treatment. Within a population of control cells, mGlu5a receptor-induced IP3 increases in response to glutamate eGFP-PHPLCδ translocation were not detected in 38% of cells (denoted “non-responders” in Fig. 6, A and B). In control RNAi cells in which eGFP-PHPLCδ translocation was measurable, the predominant response was a peak elevation of IP3 followed by repetitive oscillations until agonist washout (denoted as oscillatory in Fig. 6, A and B). In several cells (24% of the total number of cells), no detectable change in IP3 was detected after the initial peak increase (denoted as a single spike in Fig. 6A). In CHO-lac-mGlu5a cells transfected with Gq/11-RNAi, repetitive oscillations in IP3 were still observed. However, the number of nonresponders and single spike responses increased to 86% (from 62%) of the total number of cells. In contrast, after full-length Gqα transfection, the number of nonresponding cells decreased to 25% of the total population, and only a relatively low number of cells (8%) exhibited IP3 oscillations. Instead, in 50% of Gqα-transfected cells, IP3 signals were manifested as an initial peak followed by a sustained plateau response until agonist wash-out (denoted as saturating in Fig. 6, A and B). In addition, the mean increase in peak IP3 production was at least 2-fold higher in Gqα-transfected cells (0.37 ± 0.07 RFU; p < 0.05), compared with control (0.17 ± 0.03 RFU) and RNAi-transfected cells (0.13 ± 0.05 RFU) (Fig. 6C).

Gq/11 Protein Expression Levels Influence Stimulus Strength and the Temporal Profile of mGlu Receptor-Mediated Ca2+ Mobilization. Previous studies from our laboratory have made real-time concurrent measurements of...
mGlu5a-receptor-mediated IP$_3$ and Ca$^{2+}$ in single cells (Nash et al., 2001). During prolonged activation, these receptors give an initial transient peak in IP$_3$ production followed by a sustained plateau phase that is closely mirrored by a peak and sustained plateau Ca$^{2+}$ response. In contrast, the mGlu5a receptor produces oscillatory IP$_3$ and Ca$^{2+}$ signals (Nash et al., 2001, 2002; Nahorski et al., 2003). In the current study, the effects of manipulating G$_{q/11}$,α protein levels on the glutamate concentration-dependence and temporal profile of mGlu1a and mGlu5a receptor-mediated Ca$^{2+}$ signaling were investigated. Receptors were therefore exposed to incremental increases in glutamate (1 to 100 µM, 200 s at each concentration) to determine the role of G$_{q/11}$,α expression levels on complex calcium signals.

In control RNAi and G$_{q/11}$,α-expressing CHO-lac-mGlu1a cells, ~60% of transfected cells responded to glutamate with an initial peak elevation in Ca$^{2+}$ followed by a sustained plateau phase. After G$_{q/11}$,α-RNAi transfection, however, 84% (66 of 79) of cells elicited transient peak increases in Ca$^{2+}$ mobilization without a sustained phase even at maximal concentrations of glutamate (Fig. 7C, denoted as "non-saturating"). These nonsaturating responses most often occurred as a single baseline spike coincident with agonist addition (as illustrated with the representative trace in Fig. 7A).

On occasion, however, additional intermittent spikes were evident, perhaps signifying oscillations occurring as a result of regenerative CICR. The few cells that actually initiated a sustained peak and plateau did so only at high concentrations of agonist (7 of 66 cells over four experiments). Changing levels of G$_{q/11}$,α-protein expression altered the glutamate concentration threshold for the onset of mGlu1a receptor-induced Ca$^{2+}$ responses (Fig. 8A). Thus, in control RNAi cells, the initial onset of Ca$^{2+}$ mobilization occurred at either 3 or 10 µM glutamate (35 and 50% of the total number of cells, respectively), which correlates with the IP$_3$ measurements made in these cells (Fig. 5). In G$_{q/11}$,α-RNAi-transfected cells, a greater agonist concentration was required to produce significant increases in IP$_3$ (around 10 µM glutamate), reflecting a shift to higher agonist concentrations for the onset of Ca$^{2+}$ mobilization (only 14% responded to 3 µM but 53% responded to 10 µM). Furthermore, 25% of G$_{q/11}$,α-RNAi-transfected cells compared with 8% of control cells responded only to concentrations greater than 10 µM glutamate. The shift in stimulus threshold was further exemplified in experiments using G$_{q/11}$,α overexpression, where 3 µM glutamate mediated a large increase in IP$_3$ (Fig. 5), so that 74% (25 of 34) of cells at this concentration of glutamate were able to initiate a Ca$^{2+}$ signal compared with 35% of control cells (Figs. 7 and 8A). In the mGlu1a model system, G$_{q/11}$,α expression levels clearly regulate the efficacy and the nature of agonist-mediated receptor activation. This is manifested as altered levels of IP$_3$ production and hence differential Ca$^{2+}$ signals in response to increasing agonist concentrations. In contrast, mGlu5a receptor signaling is relatively independent of stimulus-strength over a wide agonist concentration range (Nash et al., 2002), and it was not clear what effect alterations in G$_{q/11}$,α protein levels would have.

In control RNAi mGlu5a receptor-expressing cells, increasing concentrations of glutamate produced nonsaturating Ca$^{2+}$ oscillations (Fig. 7, B and D). Although in many cells the frequency of Ca$^{2+}$ oscillations was initially sensitive to the increase in agonist concentration, in all cases, the Ca$^{2+}$ frequency quickly became insensitive to agonist concentration, which is indicative of oscillations driven by PKC-dependent feedback on the receptor (see Nash et al., 2002; Fig. 7B, representative trace). Similar observations were made from...
mGluate receptor-expressing cells transfected with G_{q/11}-RNAi, but in the majority of cells, the onset of response occurred at higher concentrations of agonist (Fig. 8B; 10 μM versus 3 μM glutamate in control cells); consequently, non-saturating Ca²⁺ oscillations in most G_{q/11}-RNAi-transfected cells could not be identified until concentrations of ≥10 μM were used (Fig. 7, representative trace). In CHO-lac-mGluate cells transfected with G_{q/11} 75% (52 of 69 cells) responded at 1 μM glutamate. A key effect of G_{q/11} overexpression was the observation of mGluate cells exhibiting saturating Ca²⁺ responses to increasing concentrations of glutamate (Fig. 7, B and D). Thus, although oscillatory responses were observed at low glutamate concentrations, these rapidly converted to a sustained plateau response upon increases in agonist concentrations. In this way, the Ca²⁺ response mirrored the IP₃ signals produced by the mGluate receptor in recombinant G_{q/11}-expressing cells (Fig. 6B).

**Discussion**

Complex G_{q/11}-coupled receptor-mediated Ca²⁺ signals enable a wide range of cellular processes to be regulated (Berridge et al., 2000). Low levels of receptor occupancy are known to induce small steady-state increases in IP₃ that evoke oscillatory Ca²⁺ signals, the frequency of which is sensitive to agonist concentration. Higher levels of IP₃ generation caused by more intense receptor stimulation can lead to larger, peak-and-plateau–type Ca²⁺ responses. Studies using the IP₃ biosensor eGFP-PHPLCδ have since shown that Ca²⁺ oscillations can also occur in synchrony with IP₃ oscil-
The fine-tuning of Ca\textsuperscript{2+} signals by these factors may account for contrasting patterns of mGlu5 receptor-driven Ca\textsuperscript{2+} oscillations in different cell backgrounds (Codazzi et al., 2001; Dale et al., 2001; Nash et al., 2002). In the current study, we have extended these investigations by assessing the contribution of G\textsubscript{q11}\alpha protein expression levels in generating distinct IP\textsubscript{3} and Ca\textsuperscript{2+} signaling patterns mediated by group I mGlu receptors. Using RNAi or recombinant G\textsubscript{a} expression in combination with an IP\textsubscript{3} biosensor or Ca\textsuperscript{2+} sensitive dye, we have been able to study the concentration-dependence and the temporal profile of IP\textsubscript{3} and Ca\textsuperscript{2+} in single cells in real-time.

The use of RNAi as an approach to gene silencing is now extensive, although its mechanism is not yet fully understood (Dykxhoorn et al., 2003; Hannon and Rossi, 2004; Meister and Tuschi, 2004). The enormous interest in this technique has also highlighted potential limitations, in particular with respect to RNAi specificity (Hannon and Rossi, 2004; Snow and Holen, 2004). Here, we have shown a robust RNAi-induced silencing of G\textsubscript{q11}\alpha protein expression in HEK-m3 cells that resulted in a suppression of the methacholine-induced IP\textsubscript{3} signal. Very recently, effective silencing of G\textsubscript{q11}\alpha proteins in stress fiber formation (Barnes et al., 2005), but not chemotaxis (Hunton et al., 2005), after angiotensin II AT\textsubscript{1}A receptor activation.

Effective G\textsubscript{q11}\alpha silencing was also observed in CHO-lac cell lines stably expressing mGlu1 or mGlu5 receptors, with no effect on the expression levels of G\textsubscript{i1–3} and G\textsubscript{12,13} proteins or the receptors themselves. Despite the presence of a 2-base-pair mismatch in the RNAi target region of G\textsubscript{i1}α compared with G\textsubscript{i}α in the Chinese hamster cell model, real-time PCR and a pan-G\textsubscript{q11}α antibody revealed knockdown of both G\textsubscript{i1}α and G\textsubscript{i1}α. Several reports have described similar tolerance of mismatches between siRNA and target mRNA, especially when these occur at the periphery of the target sequence (Amarzguioui et al., 2003; Vickers et al., 2003; Snow and Holen, 2004). Other studies also indicate that siRNAs containing mismatches can act as endogenous micro-RNAs to inhibit translation, but not to cause significant mRNA degradation (Doench et al., 2003; Saxena et al., 2003). However, this was not the case here, in that we observed a clear reduction in mRNA and protein expression. The effect therefore seems to be specific to G\textsubscript{i}α and G\textsubscript{i1}α as a result of the close homology of the two sequences in the target region, instead of generic knockdown of unrelated proteins. In this study, we have therefore been able to use the RNAi construct to assess the effects of combined G\textsubscript{q11}\alpha knockdown, but in doing so also provide evidence for a “cross-reactivity” of siRNA targeting closely related genes.

Knockdown of G\textsubscript{q11}\alpha protein almost completely eliminated mGlu1a receptor-mediated IP\textsubscript{3} responses in single cells at maximal concentrations of agonist, whereas increased G\textsubscript{a} expression produced a significant enhancement in agonist sensitivity. Despite the ability of mGlu1a receptors to couple to other G proteins, such as G\textsubscript{a}, which can contribute to phosphoinositide turnover (Hermans and Challiss, 2001), our current data suggest that G\textsubscript{q11}\alpha coupling alone accounts for the majority of PLC activation in this expression system. Reducing the expression level of G\textsubscript{q11}\alpha clearly affected the

Fig. 8. Manipulating G\textsubscript{q11}\alpha expression alters the agonist-concentration threshold for mGlu1a and mGlu5a receptor-mediated signaling. mGlu receptor-mediated Ca\textsuperscript{2+} responses from the same experiments described in Fig. 7 were further analyzed to determine onset of Ca\textsuperscript{2+} signaling events. Histograms indicate the number of mGlu1 (A) or mGlu5 (B) receptor-expressing cells (as a percentage of the total) that are responding for the first time.
signaling properties of mGlu1a receptor, so that the subsequent Ca\textsuperscript{2+} response no longer resembled the characteristic sustained peak-and-plateau signal typical of this receptor subtype (Kawabata et al., 1996; Nash et al., 2001). Instead, the observedenerative CICR associated with small increments in IP\textsubscript{3} that PKC down-regulation can prevent IP\textsubscript{3} oscillations, such agonist for initiation. Although PKC dependence was not pathway (e.g., PLC \textgreek{m}Glu1a receptor (Nash et al., 2002). It is noteworthy that at lower concentrations of agonist, PKC-independent Ca\textsuperscript{2+} entry. Indeed, previous studies have demonstrated mGlu5 receptor-activated peak-and-plateau responses in the CHO-lac-mGlu5a cell-model after thapsigargin-induced depletion of intracellular Ca\textsuperscript{2+} stores (Nash et al., 2002). Using RNAi, we have shown that a reduction in mGlu5 receptor stimulus-strength restricts IP\textsubscript{3} production, even at maximal concentrations of agonist, and constrains Ca\textsuperscript{2+} responses to baseline oscillations that require higher concentrations of agonist for initiation. Although PKC dependence was not tested here, previous studies in the same cells have shown that PKC down-regulation can prevent IP\textsubscript{3} oscillations, such that maximal concentrations of agonist initiate sustained peak-and-plateau Ca\textsuperscript{2+} responses in a similar manner to the mGlu1a receptor (Nash et al., 2002). It is noteworthy that at lower concentrations of agonist, PKC-independent Ca\textsuperscript{2+} oscillations were seen that were sensitive to agonist concentration and may reflect the Ca\textsuperscript{2+} oscillations observed in some cells after RNAi treatment in the present experiments.

In conclusion, we have shown that altering G\textsubscript{q11a} expression markedly alters the temporal profile and agonist concentration-dependencies of IP\textsubscript{3} and Ca\textsuperscript{2+} signals generated after either mGlu1a or mGlu5a receptor activation. This work also demonstrates that regulating G\textsubscript{q11a} expression levels can fundamentally alter the signaling properties of the mGlu5 receptor subtype. For the mGlu5 receptor, it seems that the predominant dynamic uncoupling mechanism linking receptor activation to Ca\textsuperscript{2+} signaling instills particular emergent properties. Thus, whereas the receptor expression level primarily determines the Ca\textsuperscript{2+} oscillatory frequency, the transitions between CICR, dynamic uncoupling, and peak-and-plateau behaviors are modulated by the level of G\textsubscript{q11a} expression. It will be interesting to establish the effects of changing the levels of other intermediates in the signaling pathway (e.g., PLC\textbeta expression) or the presence of Homer proteins (Fagni et al., 2000; Kiselgov et al., 2003) on mGlu1/5 receptor signaling. It is also interesting to speculate on whether translocation of mGlu5 receptors to the plasma membrane from an intracellular locus (Hubert et al., 2001) and/or direct or indirect (e.g., through changes in RGS protein expression) changes in G protein expression/function can fundamentally alter neuronal Ca\textsuperscript{2+} signaling by this receptor subtype under altered physiological or pathophysiological conditions.

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References


Fagni L, Chavis P, Ango F, and Bockaert J (2000) Complex interactions between mGlu1/5 metabotropic glutamate receptors. It is also interesting to speculate on whether translocation of mGlu5 receptors to the plasma membrane from an intracellular locus (Hubert et al., 2001) and/or direct or indirect (e.g., through changes in RGS protein expression) changes in G protein expression/function can fundamentally alter neuronal Ca\textsuperscript{2+} signaling by this receptor subtype under altered physiological or pathophysiological conditions.


Address correspondence to: Prof. R. A. J. Challiss, Department of Cell Physiology and Pharmacology, University of Leicester, Maurice Shock Medical Sciences Building, University Road, Leicester, LE1 9HN, UK. E-mail: jc36@le.ac.uk