Ligand Regulation of the Quaternary Organization of Cell Surface M₃ Muscarinic Acetylcholine Receptors Analyzed by Fluorescence Resonance Energy Transfer (FRET) Imaging and Homogeneous Time-resolved FRET*

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Flp-In™ T-REx™ 293 cells expressing a wild type human M₃ muscarinic acetylcholine receptor construct constitutively and able to express a receptor activated solely by synthetic ligand (RASSL) form of this receptor on demand maintained response to the muscarinic agonist carbachol but developed response to clozapine N-oxide only upon induction of the RASSL. The two constructs co-localized at the plasma membrane and generated strong ratiometric fluorescence resonance energy transfer (FRET) signals consistent with direct physical interactions. Increasing levels of induction of the FRET donor RASSL did not alter wild type receptor FRET-acceptor levels substantially. However, ratiometric FRET was modulated in a bell-shaped fashion with maximal levels of the donor resulting in decreased FRET. Carbachol, but not the antagonist atropine, significantly reduced the FRET signal. Cell surface homogeneous time-resolved FRET, based on SNAP-tag technology and employing wild type and RASSL forms of the human M₃ receptor expressed stably in Flp-In™ T-REx™ 293 cells, also identified cell surface dimeric/oligomeric complexes. Now, however, signals were enhanced by appropriate selective agonists. At the wild type receptor, large increases in FRET signal to carbachol and acetylcholine were concentration-dependent with EC₅₀ values consistent with the relative affinities of the two ligands. These studies confirm the capacity of the human M₃ muscarinic acetylcholine receptor to exist as dimeric/oligomeric complexes at the surface of cells and demonstrate that the organization of such complexes can be modified by ligand binding. However, conclusions as to the effect of ligands on such complexes may depend on the approach used.

Monomeric G protein-coupled receptors (GPCRs) have the capacity to bind and activate G proteins (1–3). Despite this, GPCRs also have the capacity to exist as dimers and/or oligomers in living cells (4–7). Such quaternary organization has been suggested, among other functions, to play a key role in effective folding and cell surface trafficking of GPCRs (8–10). However, a series of key questions related to GPCR quaternary structure remain to be explored fully. These include the proportion of a GPCR that is dimeric/oligomeric at steady state, how this may be affected by receptor expression level, if this is regulated at different stages in the life cycle of a GPCR, and the ability of receptor ligands to alter GPCR-GPCR interactions.

Five individual muscarinic GPCRs respond to the neurotransmitter acetylcholine (11, 12). Despite overlapping tissue distribution and very limited selective ligand pharmacology, studies on mouse lines that have selective knock-out of individual muscarinic receptor genes mean that a good deal is known about the roles of the individual subtypes (12). For example, the M₃ receptor mediates a wide variety of functions, including vasodilation, bronchoconstriction, stimulation of pancreatic insulin and glucagon release, modulation of salivary gland function, and smooth muscle contraction. An alternative means to study the function of individual muscarinic receptor subtypes is based on the generation of receptor mutants that are often designated Receptors activated solely by synthetic ligand (RASSLs) (13, 14). The introduced mutation(s) result in loss of affinity for the endogenous ligand but enhanced affinity for one or more small synthetic ligands that have little affinity/potency at the wild type receptor (15, 16). In the case of muscarinic receptors, introduction of mutations at conserved amino acids in transmembrane domains III and V greatly alters the agonist potency ratio between acetylcholine and the synthetic ligand clozapine N-oxide (17, 18). Expression of such a modified receptor, in either cell lines or in animals via transgenesis, can allow selective activation and analysis of the function and regulation of the RASSL variant.

The quaternary structure of muscarinic receptor family members has been explored extensively (19–24). However, whether ligand binding regulates such interactions is an area of considerable controversy. Using bioluminescence resonance energy transfer (BRET)-based approaches, Goin and Nathanson (21) did not observe short term agonist regulation of either homo- or hetero-interactions involving M₁, M₂, or M₃ receptors. By contrast, Ilien et al. (25) have reported that the selective M₁ receptor antagonist pirenzepine enhances M₁...
receptor dimerization, from a situation where M₁ receptor monomers predominate in the absence of ligand but dimerize upon pirenzepine binding. This is of particular interest because Hern et al. (26) have recently employed modified forms of the antagonist ligand telenzepine in single molecule tracking studies in Chinese hamster ovary cells expressing the M₁ receptor. These studies concluded that at any given time, ~30% of the receptor molecules exist as dimers and that this is a dynamic process with the M₁ receptor undergoing inter-conversion between monomers and dimers within seconds. However, if antagonist ligand binding inherently alters such receptor-receptor interactions, then data interpretation may be difficult in studies that rely on imaging of a bound ligand. GPCR quaternary structure has been explored by a number of approaches, but in recent times, these have been dominated by combinations of intact cell BRET and fluorescence-resonance energy transfer (FRET). These techniques are based upon energy transfer between either two autofluorescent proteins (in FRET studies) or an autofluorescent protein and an enzyme able to generate bioluminescence (in BRET studies). This takes advantage of the capacity of small proteins based on mammalian O₂-alkylguanine-DNA-alkyltransferase to be covalently modified with fluorescent and other small molecule labels (31). Such “SNAP” tagging can allow detection of cell surface, as well as intracellular protein-protein interactions. As a proof of concept, this has been used to explore aspects of GPCR quaternary structure, with particular focus on members of the class C, metabotropic glutamate receptor family (32).

EXPERIMENTAL PROCEDURES

Materials

Materials for cell culture were from Sigma or Invitrogen. Clozapine N-oxide was from Biomol International (Exeter, Devon, UK). Other drugs used in this study were from Sigma. Antibodies recognizing the different epitope tags were obtained as listed: anti-c-Myc antibody (Cell Signaling, Hitchin, Hertfordshire, UK), anti-SNAP antibody (ThermoFisher Scientific, Epsom, Surrey, UK), and monoclonal anti-FLAG M2-peroxidase and anti-FLAG M2 monoclonal antibodies were from Covalys Biosciences AG/New England Biolabs (Hitchin, UK) and monoclonal anti-FLAG M2-peroxidase and anti-FLAG M2 monoclonal antibodies were from Sigma. The antiserum directed against VSV-G epitope was obtained as listed: anti-c-Myc antibody (Cell Signaling, Hitchin, Hertfordshire, UK), anti-SNAP antibody (ThermoFisher Scientific, Epsom, Surrey, UK), and monoclonal anti-FLAG M2-peroxidase and anti-FLAG M2 monoclonal antibodies were from Sigma. The antiserum directed against VSV-G epitope was obtained as listed: anti-c-Myc antibody (Cell Signaling, Hitchin, Hertfordshire, UK), anti-SNAP antibody (ThermoFisher Scientific, Epsom, Surrey, UK), and monoclonal anti-FLAG M2-peroxidase and anti-FLAG M2 monoclonal antibodies were from Sigma.

Molecular Constructs

**Generation of the hM₃RASSL by Site-directed Mutagenesis—** cDNA corresponding to the human M₃ muscarinic acetylcholine receptor (hM₃) (GenBank™ accession number AF498917) was obtained from the Missouri S&T cDNA Resource Center. This was used as a template to generate the mutated RASSL receptor by substitution of the tyrosine at position 149 with a cysteine (Y149C), and the alanine in position 239 with a glycine (A239G) as described by Armbruster et al., (17). These mutations were introduced by two sequential rounds of mutagenesis according to the QuiKChange II (Stratagene, La Jolla, CA) method using the following primers: Y149C mutation, 5'-GCTTGCCATTGACTGCAGCCACGAAATTG-3' (forward) and 5'-CATTGGCTGCTACGCTAAATGCG-CAGC-3' (reverse); A239G mutation, 5'-GCACAGCCATTGCGTGTATTATATGCTG-3' (forward) and 5'-CAGGCATATAAAACCAGCATGGCTGTGC-3' (reverse). At least eight clones were screened by DNA sequencing and positive selections and used for further studies.

**FLAG-hM₃WT-Citrine and myc-hM₃RASSL-Cerulean cDNA Constructs—** FLAG-hM₃WT-Citrine and myc-hM₃RASSL-Cerulean constructs were engineered by the introduction by PCR of FLAG or c-Myc as N-terminal epitopes. This was followed by subsequent in-frame ligation of the resulting PCR fragments into the EcoRI site of pcDNA3 (Invitrogen) vectors harboring citrine- and cerulean-fluorescent proteins, respectively. Both FLAG-hM₃WT-Citrine and myc-hM₃RASSL-Cerulean constructs were also ligated into pcDNA5/FRT/TO (Invitrogen) to generate stable inducible Flp-In™ T-Rex™ 293 cell lines. In both cases, the stop codon of the receptor was removed to allow transcription of the fusion protein. The full length of the fusion constructs was confirmed to be correct by nucleotide sequencing.

**VSV-G-SNAP-hM₃WT and VSV-G-SNAP-hM₃RASSL cDNA Constructs—** The plasmid pSEM51-26m (SNAP tag) as supplied by Covalys Biosciences AG/New England Biolabs (Hitchin, UK) was modified by the addition of a small linker region encoding the metabolotropic glutamate 5 receptor (mGlur5) signal sequence and an epitope tag (VSV-G) between the ClaI and EcoRI sites of the multiple cloning site upstream of the SNAP tag (MCS1). The linker was made by annealing two complementary primers containing the sequences described above with the addition of a Kozak sequence, start codon, and appropriate nucleotides to generate Clal and EcoRI “sticky” ends. The primers were annealed by combining 1 ng of each with 1 μL of “multicore” buffer (Promega Corp.) in a final volume of 50 μL. This was then heated to 100 °C in a boiling water bath for 5 min, after which the bath was then turned off and allowed to cool overnight. The annealed fragment was then purified by gel extraction and ligated into the plasmid by standard techniques. The receptor sequences were PCR-amplified using primers designed to add BamHI (5'-CGCGGATCCGGCAATGACC-CTTGCAAAATACAGT-3') and NotI (5'-TTTTTTTCCGGGCGCCCTACAAGGGCTGCGGGTG-3') sites to the fragment termini. These were then ligated into the multiple cloning site downstream of SNAP tags (MCS2) of the modified plasmids described above. To create constructs that could be used to make Flp-In™ T-Rex™ 293-inducible stable cell lines of these constructs, the entire insert from the Clal site to the NotI site was cut out and ligated into a modified version of pcDNA5/FRT/TO (Invitrogen) with a Clal site added to the multiple cloning site using a linker formed from two annealed primers as described previously.
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**Generation of Stable Flp-In™ T-REx™ 293 Cells**

Cells were maintained in Dulbecco’s modified Eagle’s medium without sodium pyruvate, 4500 mg/liter glucose, and l-glutamine, supplemented with 10% (v/v) fetal calf serum, 1% penicillin/streptomycin mixture, and 10 μg/ml blasticidin in a humidified atmosphere. To generate Flp-In™ T-REx™ 293 cells able to inducibly express the different cDNA constructs, cells were transfected with a 1:9 mixture of cDNA in pcDNA5/FRT/TO vector and the pOG44 vector (Invitrogen) using Effectene (Qiagen, West Sussex, UK), according to the manufacturer’s instructions and as described previously (33–35). After 48 h, the medium was changed to medium supplemented with 200 μg/ml hygromycin B (Roche Diagnostics) to initiate selection of stably transfected cells. To constitutively co-express other variants of the hM3 receptor in the inducible cell lines, these were transfected with the appropriate cDNA construct as described above, and resistant clones were selected using 1 mg/ml G418. All stable cell lines were initially screened by fluorescence microscopy for receptor expression and subsequent specific binding of [3H]QNB in cell membranes.

**Cell Membrane Preparation**

Pellets of cells were frozen at −80 °C for a minimum of 1 h, thawed, and resuspended in ice-cold 10 mM Tris, 0.1 mM EDTA, pH 7.4 (TE buffer), supplemented with Complete protease inhibitors mixture (Roche Diagnostics). Cells were homogenized on ice by 40 strokes of a glass on a Teflon homogenizer followed by centrifugation at 1000 × g for 5 min at 4 °C to remove unbroken cells and nuclei. The supernatant fraction was removed and passed through a 25-gauge needle 10 times before being transferred to ultracentrifuge tubes and subjected to centrifugation at 50,000 × g for 45 min at 4 °C. The resulting pellets were resuspended in ice-cold TE buffer. Protein concentration was assessed, and membranes were stored at −80 °C until required.

**Radioligand Binding Assays**

Saturation binding curves were initiated by the addition of 1 (hM3 WT) or 5 μg (hM3 RASSL) of membrane protein to assay buffer (20 mM HEPES, 100 mM NaCl, and 10 mM MgCl₂, pH 7.4) containing varying concentrations of [3H]QNB (50.5 Ci/mmol). Nonspecific binding was determined in the presence of 10 μM atropine. Reactions were incubated for 90 min at 25 °C, and bound ligand was separated from free by vacuum filtration through GF/C filters (Brandel Inc., Gaithersburg, MD). The filters were washed twice with assay buffer, and bound ligand was estimated by liquid scintillation spectrometry.

**Cell Lysates and Western Blotting**

Cells were washed once in cold PBS and harvested with ice-cold RIPA buffer (50 mM HEPES, 100 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate, 10 mM NaF, 5 mM EDTA, 10 mM Na₂HPO₄, 5% ethylene glycol, pH 7.4) supplemented with Complete protease inhibitors mixture (Roche Diagnostics). Extracts were performed with 3% (w/v) nonfat dried skimmed milk powder plus 0.15% Triton X-100 in TBST (1% nonfat dried skimmed milk powder in TBST, and subsequently incubated with the required primary antibody overnight at 4 °C. Incubation with the appropriate horseradish peroxidase-linked IgG secondary antiserum was performed for 2 h at room temperature. Immunoblots were developed by application of enhanced chemiluminescence solution (Pierce).

**[Ca²⁺]i Mobilization Assays**

Cells expressing receptor constructs were grown in clear-bottom black 96-well plates (Greiner Bio-One Ltd., Stonehouse, UK) for the required length of time. Cells were incubated at 37 °C in the dark with the Ca²⁺-sensitive dye Fura-2 diluted to 3 μM in Dulbecco’s modified Eagle’s medium for 30 min. Cells were washed twice with HEPES physiological saline solution (130 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, and 10 mM D-glucose, pH 7.4) and transferred to a FLEXstation II (Molecular Devices, Sunnydale, CA) where they were stimulated with different drugs, and mobilization of intracellular calcium was recorded as changes of Fura-2 340:380 nm ratio.

**Immunocytochemistry**

Cells grown on poly-d-lysine-coated coverslips (number 0) were rinsed twice with PBS, and cell nuclei were stained by incubating cells for 15 min at 37 °C with fresh PBS containing 10 μg/ml of the nuclear DNA-binding dye Hoechst 33342 (Invitrogen). Samples were then washed 3–4 times with PBS, fixed in a 4% paraformaldehyde/PBS solution for 10 min, and washed three times with ice-cold PBS prior to the blocking step performed with 3% (w/v) nonfat dried skimmed milk powder in PBS (nonpermeabilized cells) or 3% (w/v) nonfat dried skimmed milk powder plus 0.15% Triton X-100 in PBS (permeabilized cells) for 10 min at room temperature. Cells were incubated with the appropriate primary antibody dilution for 1 h (22 °C) and subsequently washed twice with PBS. Cells were then incubated for a further 1 h with a dilution of secondary antibody at room temperature. After washing with PBS, coverslips were mounted on to glass slides and viewed using an epifluorescence microscope.

**Epifluorescence Imaging of SNAP-tag Proteins in Live Cells**

Cells stably expressing the receptor of interest were grown on coverslips pretreated with 0.1 mg/ml poly-d-lysine. SNAP-tag specific substrates were diluted in complete Dulbecco’s modified Eagle’s medium from a stock solution yielding a labeling solution of 5 μM dye substrate. The medium on the cells expressing a SNAP-tag fusion protein was replaced with the labeling solution and incubated at 37 °C, 5% CO₂ for 30 min. Cells were washed three times with complete medium and a further time with HEPES physiological saline solution (130 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, and
10 mM D-glucose, pH 7.4). Coverslips were then transferred to a microscope chamber where they were imaged using an inverted Nikon TE2000-E microscope (Nikon Instruments, Melville, NY) equipped with a ×40 (numerical aperture-1.3) oil-immersion Pan Fluor lens and a cooled digital photometrics CoolSnap-HQ charge-coupled device camera (Roper Scientific, Trenton, NJ). For internalization studies, ligands were added to the microscope chamber, and fluorescent images were acquired at different time intervals for 40 min.

**Homogeneous Time-resolved FRET Studies**

Cells expressing the receptors of interest were grown to 100,000 cells per well in solid black 96-well plates (Greiner Bio-One) pretreated with 0.1 mg/ml poly-D-lysine. The growth medium was replaced with 100 µl of a mixture containing the fixed optimal concentrations of donor and acceptor, Tag-Lite™ SNAP-Lumi4-Tb and Tag-Lite™ SNAP-Red (Cisbio Bioassays, Bagnoles-sur-Céze, France). Plates were incubated for 1 h at 37 °C, 5% CO2 in a humidified atmosphere and subsequently washed four times in labeling medium (Cisbio Bioassays). Plates were either read directly after this or further processed to test the effect of receptor ligands. For the later experiment, several drug concentrations were added to the plates after being washed in labeling medium; they were then incubated at set temperatures and time and read out on a PheraStar FS (BMG Labtechnologies, Offenburg, Germany) HTRF compatible reader. Both the emission signal from the Tag-Lite™ SNAP-Lumi4-Tb cryptate (620 nm) and the FRET HTRF compatible reader. Both the emission signal from the Tag-Lite™ SNAP-Lumi4-Tb and Tag-Lite™ SNAP-Red (Cisbio Bioassays, Bagnoles-sur-Céze, France). Plates were incubated for 1 h at 37 °C, 5% CO2 in a humidified atmosphere and subsequently washed four times in labeling medium (Cisbio Bioassays). Plates were either read directly after this or further processed to test the effect of receptor ligands. For the later experiment, several drug concentrations were added to the plates after being washed in labeling medium; they were then incubated at set temperatures and time and read out on a PheraStar FS (BMG Labtechnologies, Offenburg, Germany) HTRF compatible reader. Both the emission signal from the Tag-Lite™ SNAP-Lumi4-Tb cryptate (620 nm) and the FRET signal resulting from the acceptor Tag-Lite™ SNAP-Red (665 nm) were recorded. Finally, the specific fluorescent signal was calculated by subtracting from the total 665 nm signal that was obtained from cells labeled but not expressing the receptor (uninduced cells), and calculating the 665:620 ratio.

**Live Cell Epifluorescence Intermolecular Cerulean-Citrine FRET Microscopy**

Cells were grown on poly-D-lysine-treated glass coverslips (number 0) and induced with doxycycline to express the appropriate cerulean- and citrine-tagged receptor fusion proteins and tandem positive control constructs. Cells were placed into a microscope chamber containing physiological HEPES-buffered saline solution (130 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 20 mM HEPES, and 10 mM D-glucose, pH 7.4). Cells were then imaged using an inverted Nikon TE2000-E microscope (Nikon Instruments) equipped with a ×40 (numerical aperture = 1.3) oil immersion Fluor lens and a cooled digital photometrics CoolSnap-HQ (Roper Scientific, Trenton, NJ), attached to the left-hand microscope port or a CoolSnap-HQ2 connected to the bottom port. Excitation light was generated from a computer-controlled Optoscan monochromator (Cairn Research, Faversham, Kent, UK), which was coupled to an ultra-highpoint intensity 75-watt xenon arc Optosource lamp (Cairn Research). Monochromator was set to 430 and 500 nm for the sequential excitation of cerulean- and citrine-tagged receptors or fused and nonfused cerulean and citrine, respectively. Excitation light was reflected through the Fluor objective lens using a cyan/yellow fluorescent protein dual band dichroic filter (Semrock; Rochester, NY, catalog no. FF440/520-Di01).

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Using the multiple dimensional wavelength acquisition module of MetaMorph, acceptor, FRET, and donor channel, images were acquired using the same exposure time (175 or 250 ms) and were detected using either a high speed emission filter wheel (Prior Scientific Instruments, Cambridge, UK), coupled to a 12-bit mode CoolSnap-HQ digital camera, (left hand port), or a Quadvie 2 (QV2), image splitting device attached to a CoolSnap-HQ2 camera operated in 14-bit mode (bottom port). Using the emission filter wheel configuration, acceptor, FRET, and donor signals were sequentially detected using 542/27 and 472/30 nm emitters supplied by Semrock. Detection of equivalent signals using the QV2 image splitter was achieved using the following Chroma (Chroma, Brattleboro, VT), ET series dichroic and emitters: ET t505LPXR dichroic, ET535/30m, ET 470/30m. The image splitting device had the advantage that the FRET and donor signals could be acquired simultaneously without any potential threat of motion. Exposure time, binning (2 × 2), and camera gain were kept constant for all acquisitions taken during each experiment. Computer control of all electronic hardware and camera acquisition was achieved using Metamorph software (version 7.6.3 Molecular Devices, Sunnydale, CA).

**FRET Signal Correction and Normalization of the FRET Signal to Donor and Acceptor FP Expression Levels**

Saved acceptor, FRET, and donor channel images were background-subtracted, and net-corrected FRET values from raw FRET images were corrected using Youvan’s fully specified bleed through algorithm (36) as follows: net corrected FRET = raw FRET - (acceptor - (DA × donor)) × (AF) - (donor - (AD × acceptor)) × (DF), where DA and AD represent the proportion of donor or acceptor bleed through into the acceptor and donor channel, respectively. AF and DF represent the amount of acceptor and donor contamination in the raw FRET signal channel. All bleed through coefficients were calculated from control cells expressing cerulean or citrine alone.

Net corrected FRET values were ratiometrically normalized to the amount of donor and acceptor FP expressed to generate a final ratiometric value (RFRET), which was dependent on protein expression levels. For this purpose, the equation RFRET = raw FRET/(acceptor - (DA × donor)) × (AF) + (donor - (AD × acceptor)) × (DF) was used. Knowing the expected bleed through of the donor and acceptor into the FRET channel, therefore in the absence of energy transfer, RFRET, will have a predicted value of 1. Values greater than 1 reflect the occurrence of FRET. Quantified RFRET values provided markedly better data quality compared with other ratiometric FRET metric algorithms (37).

**RESULTS**

The human M3 muscarinic acetylcholine receptor was modified at the N terminus to incorporate the FLAG epitope tag and at the C terminus via in-frame fusion of the modified yellow fluorescent protein citrine. This generated the FLAG-hM3WT-Citrine construct. Incorporation of Y149C (position 3.33) and A239G (position 5.46) mutations has been reported to generate a form of the M3 muscarinic receptor with substantially reduced potency for the endogenous agonist acetylcholine but
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that is able to bind and respond to the synthetic ligand clozapine N-oxide (25). This form of the M₃ muscarinic receptor was modified at the N terminus to incorporate the c-Myc epitope tag and at the C terminus via in-frame fusion of the modified cyan fluorescent protein cerulean. This generated the myc-hM₃RASSL-Cerulean construct. Each of these constructs was inserted into the Flp-In locus of Flp-In™ T-REx™ 293 cells and pools of positive cells were selected. No visible expression of either construct was observed in the absence of the antibiotic doxycycline (A and B, - dox and bright field images), but cell surface delivery of both was produced after addition of doxycycline (A and B, + dox). Elevation of [Ca²⁺], in response to varying concentrations of either carbachol (Cch) or clozapine N-oxide (CNO) was assessed in both uninduced and doxycycline-treated cells (C and D), means ± S.E., n = 4.

FIGURE 1. Generation and pharmacological characterization of Flp-In™ T-REx™ 293 cells able to express forms of the wild type or RASSL M₃ muscarinic acetylcholine receptor on demand. FLAG-hM₃WT-Citrine (A and C) or myc-hM₃RASSL-Cerulean (B and D) were inserted into the Flp-In locus of Flp-In™ T-REx™ 293 cells, and pools of positive cells were selected. No visible expression of either construct was observed in the absence of the antibiotic doxycycline (A and B, - dox and bright field images), but cell surface delivery of both was produced after addition of doxycycline (A and B, + dox). Elevation of [Ca²⁺], in response to varying concentrations of either carbachol (Cch) or clozapine N-oxide (CNO) was assessed in both uninduced and doxycycline-treated cells (C and D), means ± S.E., n = 4.

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Cells harboring myc-hM₃RASSL-Cerulean at the Flp-In locus were subsequently further transfected with FLAG-hM₃WT-Citrine. Clones expressing this construct constitutively and stably were then isolated. FLAG-hM₃WT-Citrine was predominantly present at the plasma membrane (Fig. 2A), whereas treatment with doxycycline was required for turn-on of expression of myc-hM₃RASSL-Cerulean in these cells (Fig.
agonist in both the absence (EC$_{50}$ = $2.4 \pm 1.3 \times 10^{-8}$ M, mean ± S.E., n = 3) and presence (EC$_{50}$ = $3.7 \pm 0.14 \times 10^{-8}$ M, mean ± S.E., n = 3) of doxycycline (Fig. 2C). By contrast, clozapine N-oxide was more than 650-fold more potent (EC$_{50}$ = $5.5 \pm 0.8 \times 10^{-8}$ M, mean ± S.E., n = 3) following treatment with doxycycline (Fig. 2C).

Merging of images of the location of FLAG-hM$_3$WT-Citrine and myc-hM$_3$RASSL-Cerulean in cells induced to express the RASSL construct indicated almost perfect overlap of localization of the two forms at the cell surface (Fig. 2D).

Such “co-localization” studies can only define proximity within a distance of some 300 nm because of the current limits of light microscopy. To explore potential direct interactions between these two forms of the hM$_3$ receptor, FRET imaging studies (Fig. 3) were performed and both corrected and ratiometric FRET values calculated (Fig. 3, A and B). Both of these calculations indicated that the visual overlap of distribution of the two hM$_3$ receptor variants was consistent with the formation of FLAG-hM$_3$WT-Citrine-myc-hM$_3$RASSL-Cerulean dimers/oligomers. The ability of differing concentrations of doxycycline to induce varying amounts of the FRET energy donor myc-hM$_3$RASSL-Cerulean was assessed by each of cell imaging (Fig. 3A), direct measures of fluorescence intensity corresponding to the cerulean fluorescent protein (Fig. 3C), and anti-c-Myc immunoblotting studies (Fig. 3D). Maintained levels of FLAG-hM$_3$WT-Citrine in these cells were similarly confirmed by each of cell imaging (Fig. 3A), assessing fluorescence intensity corresponding to the cerulean fluorescent protein (Fig. 3C) and anti-c-Myc immunoblotting (Fig. 3D). Both corrected FRET and ratiometric FRET were then calculated at varying acceptor to donor ratios in these cells. These initially increased, from a lack of measurable signal in the absence of donor to reach a peak after exposure to 100 ng·ml$^{-1}$ doxycycline, after which FRET signals were reduced with turn-on of higher levels of myc-hM$_3$RASSL-Cerulean (Fig. 3B). Interestingly, addition of carbachol ($1 \times 10^{-3}$ M), but neither clozapine

2A). Induction of expression of myc-hM$_3$RASSL-Cerulean was time-dependent, reaching maximal levels within 24 h (Fig. 2B).

This, however, had little effect on the expression level of FLAG-hM$_3$WT-Citrine (Fig. 2B). As anticipated, based on the pharmacological characteristics of cells able to express each variant individually, in these cells carbachol was an effective and potent

FIGURE 2. Generation and characterization of Flp-In™ T-REx™ 293 cells able to express inducibly the RASSL M$_3$ muscarinic acetylcholine receptor in the presence of constitutive expression of wild type M$_3$ muscarinic acetylcholine receptor. Cells harboring myc-hM$_3$RASSL-Cerulean at the Flp-In locus as in Fig. 1 (blue) were subsequently further transfected with FLAG-hM$_3$WT-Citrine (yellow), and clones expressing this construct constitutively and stably were isolated. A shows the inducible nature of the RASSL variant (+ dox versus − dox); B, membranes from these cells were isolated after treating with doxycycline for varying periods of time, resolved by SDS-PAGE, and immunoblotted with anti-c-Myc (upper panel), anti-FLAG (middle panel), or anti-green fluorescent protein (GFP) (that identifies both cerulean and citrine fluorescent proteins) (lower panel). (Arrowheads show the position of the 105-kDa molecular mass marker.) C, elevation of [Ca$^{2+}$], in response to varying concentrations of either carbachol (Cch) or clozapine N-oxide (CNO) was assessed in both RASSL-uninduced (− Dox) and doxycycline-treated cells (+ Dox), means ± S.E., n = 4. D, images of citrine fluorescent proteins (green), cerulean fluorescent proteins (red), and merging of these images (merge) from doxycycline-induced cells were used to construct correlation analyses of color overlap (right-hand picture).
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FIGURE 3. FRET analysis of interactions between variants of the human M₃ muscarinic acetylcholine receptor. A and B, co-expression of myc-hM₃RASSL-Cerulean (donor) with FLAG-hM₃WT-Citrine (acceptor) resulted in raw and corrected (c) FRET signals. Such images were used to calculate ratiometric FRET signals (Fig. 3E). For both the VSV-G-SNAP-hM₃RASSL cell lines (Fig. 5A and B), for both the VSV-G-SNAP-hM₃WT and VSV-G-SNAP-hM₃RASSL cell lines (Fig. 5B), this was ~8 × 10⁻⁹ M

FIGURE 4. Generation and characterization of Flp-In™ T-REx™ 293 cells able to express SNAP-tagged variants of human M₃ muscarinic acetylcholine receptor. A, Flp-In™ T-REx™ 293 cells able to express in an inducible fashion VSV-G-SNAP-hM₃WT (left-hand panels) or VSV-G-SNAP-hM₃RASSL (right-hand panels) are shown. Following receptor induction, these were treated with the cell-permeant SNAP substrate SNAP-505 (upper panel and green), an anti-SNAP antibody labeled with Alexa-594 (middle panel and red), or such images were merged (lower panels). Cell nuclei are shown in blue. B, cells induced as in A (top, VSV-G-SNAP-hM₃WT; bottom, VSV-G-SNAP-hM₃RASSL) were labeled with the non-cell-permeable reagent Cell-Surface-SNAP-488 and treated with carbachol (1 × 10⁻⁷ M) or clozapine N-oxide (1 × 10⁻⁴ M) for 0 or 40 min and then imaged. Arrowheads focus attention on receptor populations that became internalized in the presence of ligand. Cch, carbachol; CNO, clozapine N-oxide.

N-oxide (1 × 10⁻⁴ M) nor atropine (1 × 10⁻⁵ M), reduced substantially the calculated ratiometric FRET signals (Fig. 3E). This is consistent with carbachol altering the organizational structure of the oligomeric complex.

FRET between pairs of fluorescent proteins linked to polypeptides of interest is a well-characterized means to observe protein-protein interactions in living cells (28, 29). However, a novel form of htrFRET, based on SNAP tagging, and marketed as Tag-Lite™ (32), has recently been introduced. This is suitable to monitor cell surface protein-protein interactions without the need for antibodies that, because of their potential to induce clustering, have been suggested to limit interpretation of more traditional time-resolved FRET techniques (28). We generated a generic plasmid to allow expression of constructs containing an N-terminal leader sequence, derived from the metabotropic glutamate 5 receptor, linked in-frame to the VSV-G epitope tag sequence, the 20-kDa SNAP tag (31), and then the receptor of interest. Both the wild type and the RASSL forms of the hM₃ receptor were cloned into this plasmid, and these were used to generate lines able to express, in an inducible fashion, VSV-G-SNAP-hM₃WT or VSV-G-SNAP-hM₃RASSL (Fig. 4A). Addition of an anti-SNAP-Alexa-594-labeled antibody to nonpermeabilized cells identified, in both cases, cell surface receptors following induction (Fig. 4A). Furthermore, addition of the cell-permeant SNAP-505 substrate, which links covalently to the SNAP tag, allowed detection of a small pool of intracellular receptors as well as confirming the extensive population of cell surface receptors (Fig. 4A). Merging of images derived from cells labeled with either the anti-SNAP antibody or SNAP-505 confirmed both cell surface and intracellular pools of the hM₃ receptor variants (Fig. 4A). Importantly, the presence of the N-terminal leader and SNAP tag did not alter basic pharmacological characteristics of the receptors. Saturation [³H]QNB binding studies indicated a Kₘ of 54 ± 5 pm (mean ± S.E., n = 6) for VSV-G-SNAP-hM₃WT and 2.44 ± 0.16 nm (mean ± S.E., n = 4) for VSV-G-SNAP-hM₃RASSL. Addition of carbachol (1 × 10⁻³ M) resulted in internalization of 17.9 ± 1.2% of cell surface VSV-G-SNAP-hM₃WT over a 40-min period, whereas clozapine N-oxide (1 × 10⁻⁴ M) produced loss of 17.6 ± 3.5% of VSV-G-SNAP-hM₃RASSL over the same time period (Fig. 4B). By contrast, treatment of either cell line with the agonist selective for the other receptor variant did not produce significant internalization (Fig. 4B).

To establish optimal conditions for the Tag-Lite™ htrFRET studies, cells induced to express either VSV-G-SNAP-hM₃WT or VSV-G-SNAP-hM₃RASSL were incubated with the terbium-cryptate energy donor SNAP-Lumi4-Tb. The extent of labeling with this reagent was dependent on the level of receptor expression because treatment of cells with concentrations of doxycycline between 0 and 10 ng·ml⁻¹ increased the binding of a range of concentrations of this label (5–30 × 10⁻⁵ M) in an essentially linear fashion (Fig. 5A and data not shown). Optimization of htrFRET signals corresponding to cell surface hM₃ dimers/oligomers was achieved by varying the added concentration of the corresponding energy acceptor, SNAP-Red, in the presence of a fixed concentration of SNAP Lumi4-Tb (Fig. 5, A and B). For both the VSV-G-SNAP-hM₃WT and VSV-G-SNAP-hM₃RASSL cell lines (Fig. 5B), this was ~8 × 10⁻⁹ M
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extent of the effect of carbachol was dependent on the level of receptor expression, with lower levels of doxycycline associated with a greater effect of carbachol (Fig. 6B). This reflected that at higher receptor expression levels the basal htrFRET signal was higher than at lower expression levels (Fig. 6B), and in this situation carbachol produced a more limited effect on the htrFRET signal (Fig. 6B). The effect of carbachol on the htrFRET signal corresponding to cell surface VSV-G-SNAP-hM₃WT dimers/oligomers was selective. Clozapine N-oxide was without effect on the htrFRET signal (Fig. 6A), and although atropine (1 × 10⁻⁵ M) produced a small reduction in the basal htrFRET signal in some experiments (Fig. 6A), this was not observed consistently. The effect of carbachol was concentration-dependent with EC₅₀ = 3.3 ± 1.1 × 10⁻⁴ M (mean ± S.E., n = 3) (Fig. 6C). Studies in which carbachol was allowed to compete with [³H]QNB to bind to VSV-G-SNAP-hM₃WT in membranes of cells induced to express this construct showed that this potency corresponded to the lower of two affinity states for carbachol (Fig. 6D).

Acetylcholine, the endogenous agonist of the M₃ muscarinic receptor, was also able to increase the htrFRET signal as extensively as carbachol in cells expressing VSV-G-SNAP-hM₃WT and was some 60-fold more potent than carbachol in so doing (EC₅₀ = 5.8 × 10⁻⁶ M) (Fig. 6E). Interestingly, in cells induced to express VSV-G-SNAP-hM₃RASSL, clozapine N-oxide increased the basal htrFRET signal in a concentration-dependent fashion (Fig. 7, left), whereas neither carbachol nor atropine had any effect (Fig. 7, right).

**DISCUSSION**

It is now well established that many, and perhaps all, rhodopsin-like family A GPCRs can form dimers and/or higher oligomers (4–7). However, the significance of this remains uncertain because it is not inherently required to allow interaction with a G protein (1–3). Indeed, there is evidence that monomeric family A GPCRs may actually mediate G protein-dependent signals more effectively than dimers (2). Signals from GPCRs may also be generated in G
A further topic that has attracted considerable attention is the question of whether ligands modulate the quaternary organization of GPCRs. Published data on this topic are highly variable (5–8). Given that interactions between individual protomers of class A receptor dimers/oligomers are not generally based on covalent interactions (4, 5), then there is clear potential for monomer-multimer equilibria to be altered by the binding of receptor ligands. This reflects that agonist binding must alter receptor conformation (40) to induce states of the receptor able to interact more effectively with G proteins and other GPCR-interacting proteins. Although many studies have suggested that ligand binding to receptors does not produce substantial effects on GPCR quaternary structure (4–7), a considerable number of observations of ligand modulation of GPCR dimers/oligomers exist in the literature. These include effects of an antagonist/inverse agonist drug to promote the production of a high affinity dimeric state of the M1 muscarinic receptor from receptor monomers (25) and the concept that the β2-adrenoreceptor exists predominantly in a basal tetrameric state that is unaffected by either agonist or antagonist ligands, whereas inverse agonists enhance tighter packing of the protomers and/or the formation of more complex oligomers by reducing conformational fluctuations in individual protomers (41). Other studies are consistent with agonist ligands promoting conformational alterations in pre-existing GPCR dimers/oligomers (40) and both enhancing and reducing dimerization (4–7, 25, 42–44).

It is clearly possible that such variation reflects intrinsic differences in the organization and affinity between protomers of different GPCRs. For example, fluorescence recovery after photobleaching microscopy has been applied to conclude that the β2-adrenoreceptor forms stable complexes, although the β1-adrenoreceptor displays only transient interactions (45). However, given the range of approaches used to

protein-independent fashion (39), but it remains to be established if such signals can also be transduced by monomeric GPCRs.
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FIGURE 7. Clozapine N-oxide promotes structural re-organization and/or enhanced dimerization of the RASSL M₃ muscarinic acetylcholine receptor. Clozapine N-oxide produced a concentration-dependent increase of htrFRET signal in cells induced to express VSV-G-SNAP-hM₃,RASSL after 40 min of treatment, although carbachol (Cch) and atropine (Atro) were without effect.

detect interactions between GPCRs and potential regulation by ligands, it is also possible that different techniques might be more or less well suited. Resonance energy transfer based approaches have dominated the field in recent years (27–29, 46), and studies have generally added energy acceptor and donor moieties to the intracellular C-terminal tail of the GPCR(s) being studied. Strengths and weaknesses of this have been reviewed (28) and debated (47, 48). One of the recent additions to the armory of approaches is an htrFRET method that takes advantage of the capacity of small proteins based on mammalian O⁶-alkylguanine-DNA-alkyltransferase to be covalently modified with FRET-competent energy donor and acceptors (31). Via addition of such a SNAP tag to the extracellular N terminus of class C GPCRs, Maurel et al. (32) were able to confirm the presence of cell surface dimers of metabotropic glutamate receptor subtypes. They also used this approach to provide some preliminary data on dimerization of class A receptors (32). Herein, we have extended this to explore the presence and regulation of dimeric/oligomeric complexes at the surface of cells stably expressing forms of the M₃ muscarinic acetylcholine receptor and compared the results with those obtained via more conventional FRET imaging-based studies. A key feature of the FRET imaging studies was to employ the inducible expression of DNA located at the Flp-In™ locus of Flp-In™ T-REx™ 293 cells (33–35). This allowed us to regulate expression of one form of the M₃ muscarinic receptor in the presence of an unaltered amount of a second form in the same cells, rather than attempting to control relative expression levels in a substantial series of transient co-transfection studies. Transient transfection into heterologous cell lines of GPCRs in general and, in particular, of the type of highly modified forms used for resonance energy transfer studies often results in incomplete folding and their retention in the endoplasmic reticulum and Golgi. This is a major issue in efforts to explore dimerization in so-called “saturation” resonance energy transfer studies (28). These require a series of measurements in cells expressing differing ratios of energy-donor and energy-acceptor species. Therefore, the linkage of the energy donor and acceptor species to the C-terminal tail of the GPCRs in question results in signal being recorded from intracellular locations as well as the cell surface. A second key feature of this study was the combined use of wild type and RASSL forms of the M₃ muscarinic receptor to take advantage of selective agonist pharmacology. One minor limitation of the M₃ muscarinic receptor RASSL is that it binds conventional antagonist ligands less well than the wild type receptor. This increased the concentration of [³H]QNB needed in the ligand binding studies that were used to define expression levels and receptor pharmacology. However, with good practice the pharmacological characteristics of this variant can be fully defined. This is important because although the affinity of [³H]QNB was 40-fold lower for the RASSL forms of the receptor compared with the wild type, variation in affinity for the widely used muscarinic receptor antagonist atropine was only 3-fold. Variation in ligand affinity at the modified receptor is therefore defined by the identity of the specific ligand and must be determined directly for each compound being studied.

Both wild type and RASSL forms of the M₃ muscarinic receptor suitable for FRET imaging were expressed predominantly at the cell surface of Flp-In™ T-REx™ 293 cells when they were induced. Furthermore, strong FRET signals between these forms at the cell surface membrane were obtained in cells engineered to allow their co-expression. However, it should be noted that increasing FRET donor amounts in the presence of a fixed level of FRET acceptor resulted in a bell-shaped curve of ratiometric FRET signal. This is not surprising. Higher levels of donor would be expected to result in a greater proportion of non-FRET-productive donor-donor interactions that are anticipated to eventually limit or out-compete productive donor-acceptor interactions. In these studies, carbachol caused a substantial reduction in ratiometric FRET signal. This is consistent with either alteration in the organizational structure of pre-formed WT-RASSL M₃ receptor complexes or the dissociation of such complexes. By contrast, the antagonist atropine did not modulate such signals substantially. Intramolecular GPCR FRET sensors, constructed in a single GPCR protomer, are often used to detect agonist-induced alterations in FRET signal corresponding to relative movements of the intracellular ends of transmembrane helices that are believed to promote interaction with a G protein (49–51). Furthermore, relative re-orientation of the transmembrane helices that are believed to be contact interfaces within GPCR dimers/oligomers have been detected upon addition of agonist by a number of means (41, 52, 53). However, in many studies employing FRET or BRET, limited overall effects of GPCR ligands have been noted and interpreted to indicate that GPCR dimers/oligomers are present constitutively and not regulated acutely (5, 21, 54). Surprisingly, unlike carbachol, clozapine N-oxide did not alter FRET signal in cells induced to co-express the hM₃ receptor variants. Given that FRET signals in these cells must reflect complexes containing at least one wild type and one RASSL mutant M₃ receptor, it might have been anticipated that agonist occu-
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