Prediction of functionally selective allosteric interactions at an M3 muscarinic acetylcholine receptor mutant using *Saccharomyces cerevisiae*.

Gregory D. Stewart, Patrick M. Sexton and Arthur Christopoulos.

Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences & Department of Pharmacology, Monash University, Parkville, Victoria, Australia, 3052.
Abstract

*Saccharomyces cerevisiae* (*S. cerevisiae*) is a tractable yeast species for expression and coupling of heterologous G protein-coupled receptor (GPCRs) with the endogenous pheromone response pathway. Whilst this platform has been used for ligand screening, no studies have probed its ability to predict novel pharmacology and functional selectivity of allosteric ligands. As proof-of-concept, we expressed a rat M₃ muscarinic acetylcholine receptor (mAChR) bearing a mutation (K₇.₃₂E) recently identified to confer positive cooperativity between acetylcholine and the allosteric modulator, brucine, in various strains of *S. cerevisiae*, each expressing a different human Gα/yeast Gpa1 protein chimera, and probed for G protein-biased allosteric modulation. Subsequent assays performed in this system revealed that brucine was a partial allosteric agonist and positive modulator of CCh when coupled to Gpa1/G₉ proteins, a positive modulator (no agonism) when coupled to Gpa1/G₁₂ proteins, and a neutral modulator when coupled to Gpa1/Gᵢ proteins. Importantly, these results were validated at the human M₃K₇.₃₂E mAChR expressed in a mammalian (CHO) cell background by determination of calcium mobilization and membrane ruffling as surrogate measures of G₉ and G₁₂ protein activation, respectively. Furthermore, the combination of this functionally selective allosteric modulator with G protein-biased yeast screens allowed us to ascribe a potential G protein candidate (G₁₂) as a key mediator for allosteric modulation of M₃K₇.₃₂E mAChR-mediated ERK1/2 phosphorylation, which was confirmed by small interfering RNA (siRNA) knockdown experiments. These results highlight how the yeast platform can be used to identify functional selectivity of allosteric ligands and to facilitate dissection of convergent signaling pathways.
Introduction

The five subtypes (M1-M5) of muscarinic acetylcholine (ACh) receptors (mAChRs) are prototypical members of Family A G protein-coupled receptors (GPCRs) (Caulfield, 1993). The M1, M4 and M5 mAChRs are predominantly expressed in the CNS, whilst M2 and M3 mAChRs are expressed widely in the CNS and periphery (Wess et al., 2007). Of these receptors, the M3 mAChR currently represents the predominant subtype with respect to established clinical therapies, having proven a tractable drug target for conditions such as chronic obstructive pulmonary disease and overactive bladder disorder (Wess et al., 2007). Nonetheless, subtype-selective targeting of mAChRs remains difficult due to the high degree of sequence conservation in the orthosteric binding site (Caulfield, 1993; Gregory et al., 2007). One approach to circumvent this issue is to target alternative, allosteric, sites on these receptors (Birdsall and Lazareno, 2005).

Although it is known that mAChRs possess more than one allosteric site (Christopoulos et al., 1998; Gregory et al., 2007), most studies to date have focused on the so-called ‘prototypical’ allosteric site, which binds neuromuscular-blocking agents, such as gallamine and alcuronium, alkane-\textit{bis}-ammonium compounds, including heptane-1,7-bis-dimethly-3’-pthalimidopropyl ammonium bromide, and alkaloid derivatives, such as brucine. This prototypical mAChR allosteric site is thought to encompass regions of the receptor’s 2\textsuperscript{nd} and 3\textsuperscript{rd} extracellular loops, and the top of transmembrane (TM) domain VII (Avlani et al., 2007; Gnagey et al., 1999; Gregory et al., 2007; May et al., 2007). In particular, amino acid residue 523 (7.32; Ballesteros-Weinstein (1992) nomenclature), at the junction of TMVII and the 3\textsuperscript{rd} extracellular loop of the human mAChRs, plays an important role in the binding and cooperativity of prototypical allosteric ligands (Gnagey et al., 1999; Jakubik et al., 2005;
Krejci and Tucek, 2001). Interestingly, a recent study by Iarriccio (2008) found that substitution of K$^{7.32}$ on the $M_3$ mAChR with the $M_1$ mAChR equivalent, E$^{7.32}$, resulted in positive cooperativity between the allosteric modulator, brucine, and the endogenous agonist, ACh, when compared to the wild-type $M_3$ mAChR, where brucine displays almost neutral cooperativity with the agonist.

The ability of a single amino acid substitution to profoundly change the nature of an allosteric interaction between a small molecule modulator and the endogenous orthosteric agonist is consistent with the highly dynamic nature of GPCRs, which exhibit substantial pleiotropy with respect to both extracellular ligands and intracellular effector pathways. An important paradigm associated with this conformational plasticity of GPCRs is the phenomenon dubbed “functional selectivity”, whereby different ligands can promote unique conformations that bias the receptor stimulus towards certain pathways while excluding others (Kenakin, 1995; Urban et al., 2007). Since allosteric ligands, by their very nature, alter the conformation of the receptor in the absence and presence of an orthosteric ligand, it may be expected that such molecules will have the propensity to engender functional selectivity in the actions of orthosteric ligands (Leach et al., 2007). However, the detection of functionally selective ligands, be they orthosteric or allosteric, poses a substantial challenge to modern drug discovery due to the general necessity to probe different signaling pathways in different cell backgrounds.

Recently, we have explored the utility of the yeast system, *Saccharomyces cerevisiae*, as a sensor of GPCR-G protein coupling preferences that may be predictive of signaling patterns operative in mammalian cellular backgrounds (Stewart et al., 2009; 2010). The utility of
using this system to study mammalian GPCR/G protein interactions was first demonstrated in studies of $\beta_2$-adrenergic receptor coupling to mammalian G$\alpha_s$ proteins (King et al., 1990). Since then, further modifications to the yeast system have been made to accommodate mammalian GPCR signaling. One pivotal modification is the expression of a chimera consisting Gpa1 (yeast G$\alpha$ protein) with a five C-terminal amino acid substitution from the mammalian G$\alpha$ protein of choice (Brown et al., 2000; Dowell and Brown, 2002). Using this approach, we uncovered novel G protein-biased signaling of M$_3$ mAChR ligands previously classed as traditional orthosteric ‘antagonists’, such as atropine, that was subsequently validated in mammalian cells (Stewart et al., 2010). This exciting finding prompted the current study, where we investigated whether the *S. cerevisiae* platform could also be used to identify allosteric ligand-mediated functional selectivity, at the level of the G protein. As proof-of-concept, we focused on the brucine-CCh interaction at the K$^{7.32}$E M$_3$ mAChR mutant. We reveal that the yeast system is indeed capable of identifying G protein pathway-selective allosteric modulation that is predictive of behavior in mammalian cells, as well as being a potentially useful tool to help unravel networks of convergent signaling in mammalian cells.
MOL #64253

Methods

Materials

The Surefire™ ERK1/2 phosphorylation kit was kindly donated by Dr Michael Crouch (TGR Biosciences, SA, Aust.). The p416GPD rM₃Δi3 mAChR was a generous gift from Dr Jürgen Wess (NIH, Bethesda, MD). The yeast strains were a kind gift from Dr Simon Dowell (GSK, Stevenage, UK). AlphaScreen™ beads and N-methyl scopolamine [³H], ([³H]-NMS), were purchased from Perkin Elmer, (Boston, MA). Flp-In™ Chinese hamster ovary (CHO) cells, Gateway™ plasmids, BP clonase kit, LR clonase kit, hygromycin B, zeocin, Fluo-4-AM, S. cerevisiae EasyComp™ transformation kit, Lipofectamine 2000 reagent and fluorescein di(β-D-galactopyranoside) (FDG) were obtained from Invitrogen (Carlsbad, CA). Fluo-4-AM, Hoechst 33342 and Alexa™ 568-conjugated phalloidin were purchased from Molecular Probes (Carlsbad, CA). cDNA constructs of the human M₃ mAChR were purchased from the Missouri University of Science and Technology (Missouri S&T), http://cdna.org (Rolla, MO). Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Gaithersburg, MD) and JRH Biosciences (Lenexa, KS), respectively. Gα₉ or Gα₄₁₂ siRNAs were acquired from Applied Biosciences/Ambion (Austin, TX). All other reagents were purchased from Sigma Aldrich (St Louis, MO).

Yeast transformations and signaling assay

Saccharomyces cerevisiae strains expressing chimeras of five C-terminal amino acids of human Gα protein with Gpa1 (1-467) have been previously described in Brown et al. (2000). The yeast strains were further transformed with a p416GPD vector containing the gene encoding the rat M₃ muscarinic acetylcholine receptor (rM₃Δi3 mAChR) with an intracellular
3rd loop deletion, described in Erlenbach et al. (2001), using the *S. cerevisiae* EasyComp™ transformation kit in accordance with manufacturer’s instructions. The K7.32E (K522E) mutation was introduced by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) by annealing the following oligo-deoxynucleotide pair into the open vector: 5’ GACAGCTGCATAC-CCGAAACCTATTGGAATC 3’ and 5’ GATTCCAATAG-GTTTCGGGTATGCAGCTGTC 3’, the vector containing the mutated gene was then transformed in the same manner as described above.

The conditions for the signaling component of the assay have also been previously described (Olesnicky et al., 1999). Briefly, single colonies were cultured overnight at 30°C in synthetic complete (SC) medium, lacking amino acids required for plasmid maintenance. Cells were pelleted and diluted to 0.02 OD600 mL⁻¹ in SC medium, lacking amino acids, but supplemented with 0-10mM 3-aminotriazole, 1μM fluorescein di(β-D-galactopyranoside) and 0.1M sodium phosphate, pH 7.3. Cell suspensions were diluted into 96-well plates with various ligands and incubated for 18-24 h at 30°C. Fluorescence was measured in a Flexstation™ (Molecular Devices) using 485nm excitation and 520nm emission wavelengths.

**Transfections and cell culture**

The cDNA sequence of the human M3 mAChR was amplified by PCR and cloned, using classical cloning methods, into the Gateway entry vector, pDONR201, using the BP clonase kit according to manufacturer’s instructions. The M3 mAChR construct was subsequently transferred into the Gateway destination vector, pEF5/FRT/V5-dest, using the LR clonase kit in accordance with manufacturer’s instructions. The construct was then transfected into Flp-In CHO cells using methods described previously (Nawaratne et al., 2008). The same processes were applied to generate a vector containing the gene encoding the human
M3K7.32E mACHr, however, prior to the BP clonase reaction, a mutation was introduced using the QuikChange mutagenesis kit (Stratagene) by applying the following oligonucleotides: 5’ GTGACAGCTGCATACCCGAGACCTTTTGGAATCTGG 3’ and 5’ CCAGAT-TCCAAAAGGTCTCGGGTATGCAGCTGTCAC 3’ to the open vector, then following the manufacturer’s instructions. Flp-In CHO cells stably expressing the M3 mACHR (CHO M3 mACHR cells) or the M3K7.32E mACHR (CHO M3K7.32E mACHR), were cultured at 37°C in 5% CO2 in DMEM supplemented with 5% (v/v) FBS, 16mM HEPES and were selected using 400μg mL−1 hygromycin B, but maintained using 200μg mL−1 hygromycin B. For small interfering RNA (siRNA) experiments, cells were transfected with lipid alone, Gαq or Gα12 siRNA at a concentration of 100nM per well (96-well format), using Lipofectamine 2000 according manufacturer’s instructions.

Membrane preparation

CHO M3 mACHR (wild type) or M3K7.32E mACHR cells were grown to 90% confluence, harvested and centrifuged at 300 × g for 3 min. The intact cell pellet was suspended in homogenization buffer (20mM HEPES; 10mM EDTA; 0.1mg mL−1 saponin, pH 7.7) and further centrifuged (300 × g, 3 min). Cells were then resuspended in homogenization buffer and homogenized using a Polytron PT1200 homogenizer for two 10 s intervals at maximum setting (6), with 30 s cooling periods on ice between each burst. The homogenate was then centrifuged (40,000 × g, 1 h, 4°C). The resulting pellet was resuspended in 5 ml of HEPES buffer (100mM NaCl; 20mM HEPES; 10mM MgCl2, pH 7.4), and the protein content determined using a BCA assay kit (Pierce Biotechnology) according to the manufacturer’s instructions, using bovine serum albumin as a standard. The homogenate was then divided into 1 ml aliquots and either used immediately or stored frozen at −80°C until required.
Radioligand binding assays

Saturation and interaction binding assays were performed using 15µg of membrane expressing the M₃ mACHR or M₃K⁷.₃₂E mAChR receptors. For saturation binding assays, membranes were incubated with the orthosteric antagonist [³H]NMS in HEPES buffer (20mM HEPES, 100mM NaCl, and 10mM MgCl₂, pH 7.4) at 37°C for 1 h before termination of the assay by rapid filtration onto GF/B grade filter paper (Whatman, Maidstone, UK) using a Brandel harvester, followed by three 2 mL washes with ice-cold NaCl (0.9%). Nonspecific binding was defined in the presence of 10µM atropine and radioactivity was determined by liquid scintillation counting. For interaction binding studies, membranes were incubated in HEPES buffer containing 100µM GppNHp with increasing concentrations of CCh in the absence or presence of brucine (3, 10 or 30µM) and [³H]NMS at a concentration equal to its equilibrium dissociation constant for each receptor (approximately 0.7nM for both receptors) as determined from saturation binding experiments. Determination of non-specific binding and termination of the experiment were as described above.

Ca²⁺ mobilization assay

CHO M₃ or M₃K⁷.₃₂E mAChR cells were cultured overnight in 96-well plates at 37°C in 5% CO₂. Cells were washed twice in Ca²⁺ assay buffer (150mM NaCl, 2.6mM KCl, 1.2mM MgCl₂, 10mM dextrose, 10mM HEPES, 2.2mM CaCl₂, 0.5% (w/v) BSA and 4mM probenecid), then replaced with Ca²⁺ assay buffer containing 1µM Fluo-4-AM and incubated for 1 h at 37°C in 5% CO₂. Cells were washed twice more and replaced with 37°C Ca²⁺ assay buffer. Whilst fluorescence was measured, brucine was added for 1 min, CCh was subsequently added and the response was measured further for 3 min in a Flexstation™
MOL #64253

(Molecular Devices) using 485 excitation and 520 emission wavelengths. Peak fluorescence was measured as a marker for Ca$^{2+}$ mobilization and used in further analyses.

**Cytoskeletal rearrangement assay and image analysis**

CHO M3 and M3K$^{7,32}$E mAChR cells were cultured overnight in 96-well plates at 37°C in 5% CO$_2$. Samples were serum-starved 4 h prior to assaying then treated with ligand at indicated time points (CCh: 2 min, brucine: 15 min, determined by separate time-course assays). The assays were terminated, fixed, stained and analyzed as per Stewart et al. (2010). Briefly, samples were fixed in 4% paraformaldehyde and permeabilized in 0.3% (v/v) Tween20 in PBS. Samples were stained with 0.2 $\mu$g mL$^{-1}$ Hoechst 33342 and 2U mL$^{-1}$ Alexa 568-phalloidin, and imaged using an IN Cell analyzer 1000 (GE Healthcare). For the cytoskeletal analysis component, the images were randomized and blinded, and analyzed manually to detect the number of cells that exhibited membrane ruffling. That number was subsequently normalized to the nuclei content per image, which were counted using IN Cell Developer software. Each concentration-response curve data point represents one image performed in duplicate over the number of times indicated in the figure legends. On average, approximately 200 cells were present in each image.

**Extracellular signal-regulated kinase (ERK) 1/2 phosphorylation assays**

Initial ERK1/2 phosphorylation time-course experiments were performed to determine the time at which ERK1/2 phosphorylation was maximal after stimulation by each agonist at a single concentration, in addition to co-administration of brucine (10$\mu$M) with CCh (1$\mu$M). Cells were seeded into transparent 96-well plates at $5 \times 10^4$ cells per well and grown overnight. The cells were then washed twice with phosphate-buffered saline (PBS) and
incubated in serum-free DMEM at 37°C for at least 4 hrs. For interaction studies between CCh and brucine, cells were then stimulated with brucine for 1 min prior to CCh stimulation for 5 min and incubated at 37°C in 5% CO₂. For experiments where PTX pretreatment was required, cells were treated with 100ng mL⁻¹ PTX in serum-free DMEM overnight. For all experiments, 10% (v/v) FBS was used as a positive control, and vehicle controls were also performed. The reaction was terminated by removal of media and drugs, the cells were then lysed with 100μl of SureFire lysis buffer (as provided by the manufacturer). The lysates were agitated for 1 to 2 min and were diluted at a ratio of 4:1 (v/v) lysate/Surefire activation buffer in a total volume of 50μL. Under low light conditions a 1:240 (v/v) dilution of AlphaScreen beads: Surefire reaction buffer was prepared and this was mixed with the activated lysate mixture in a ratio of 6:5 (v/v), respectively, in a 384-well opaque Optiplate. Plates were incubated in the dark at 37°C for 1 h before the fluorescence signal was measured using a Fusion-α plate reader (Perkin Elmer) using standard AlphaScreen settings. Data were normalized to the maximal response elicited by 10% (v/v) FBS at the same time point.

Data analysis

All data were analyzed using Prism 5.02 (GraphPad Software, San Diego, CA). For radioligand saturation binding data, nonspecific and total binding data were fitted to the following equation.

\[ Y = \frac{B_{\text{max}} \cdot [A]}{[A] + K_A} + \text{NS} \cdot [A] \]  

(1)

where \( Y \) is radioligand binding, \( B_{\text{max}} \) is the total receptor density, \([A]\) is the radioligand concentration, \( K_A \) is the equilibrium dissociation constant of the radioligand, and \( \text{NS} \) is the
fraction of nonspecific radioligand binding. For interaction binding experiments, the shifts of the carbachol versus \[^{3}H\]NMS competition binding curves obtained in the absence or presence of brucine were fitted to the following allosteric binding model (Christopoulos, 2000):

\[
Y = \frac{B_{\text{max}} \cdot [A]}{[A] + K_{\text{App}}} \quad (2)
\]

where

\[
K_{\text{App}} = \frac{K_{A} \cdot K_{B}}{\alpha \cdot [B] + K_{B}} \left[ I + \frac{[I]}{K_{I}} + \frac{[B]}{K_{B}} + \frac{\alpha' \cdot [I] \cdot [B]}{K_{I} \cdot K_{B}} \right] \quad (3)
\]

and \([I]\) denotes the concentration of (orthosteric) competitor, \([B]\) denotes the concentration of allosteric modulator, \(K_{A}, K_{B}\) and \(K_{I}\) denote the equilibrium dissociation constants of the radioligand, modulator and competitor, respectively, \(\alpha\) denotes the modulator-radioligand binding cooperativity factor, which is a measure of the magnitude and direction of the allosteric effect the modulator exerts on the affinity of radioligand, and \(\alpha'\) defines the binding cooperativity factor between the allosteric ligand and orthosteric competitor. Values of \(\alpha\) or \(\alpha'\) > 1 denote positive cooperativity, values < 1 (but greater than 0) denote negative cooperativity, values = 1 denote neutral cooperativity, and values approaching zero denote inhibition that is indistinguishable from competitive (orthosteric) antagonism.

For some experiments (see Results) concentration-response data generated from functional assays were fitted to the following logistic equation:

\[
E = \text{Basal} + \frac{E_{\text{max}} - \text{basal}}{1 + 10^{\left(\text{pEC}_{50} - \log[A] - \log\delta\right)}} \quad (4)
\]
where \( E \) is effect, \( E_{\text{max}} \) and basal are the top and bottom asymptotes of the curve, respectively, Log[A] is the logarithm of the agonist concentration, pEC\(_{50} \) is the negative logarithm of the agonist concentration that gives a response halfway between \( E_{\text{max}} \) and basal (i.e., -ve log. EC\(_{50} \)) in the absence of allosteric modulator, and Log\( \delta \) is the logarithm of the difference between the EC\(_{50} \) of an agonist in the presence of a maximal concentration of allosteric modulator to that determined in the absence of modulator (see Results); the determination of Log\( \delta \) as a directly fitted parameter, rather than as a calculated ratio of two separately derived EC\(_{50} \) values, facilitated the statistical comparison of agonist curve shifts in the presence of modulator between various treatment groups (see Results).

Data from functional experiments measuring the interaction between CCh and brucine at the M\(_3\) and M\(_3K^{7-32}\)E mAChR were also fitted to the following operational model for the interaction between a full orthosteric agonist and an allosteric modulator (Leach et al., 2010):

\[
E = \text{Basal} + \frac{(E_m - \text{Basal})\left(\frac{[A](K_B + \alpha \beta[B]) + \tau_B[B][EC_{50}]}{[EC_{50}](K_B + [B]) + ([A](K_B + \alpha \beta[B]) + \tau_B[B][EC_{50}])}\right)}{(5)}
\]

where \( E \) denotes the effect, \( A \) denotes the agonist, \( B \) denotes the allosteric modulator, \( \alpha \beta \) denotes a composite cooperativity factor that quantifies the change in affinity (\( \alpha \)) and signaling efficacy (\( \beta \)) imparted to the receptor by the agonist as a result of the presence of allosteric modulator, \( E_m \) denotes the maximal response of the system, EC\(_{50} \) is the agonist concentration that gives a response halfway between \( E_m \) and basal, and \([A]\) and \( K_B \) are as defined for equation 3. The key advantage of the operational model is in its ability to be directly fitted to experimental data to derive parameter estimates of allosteric modulator and agonist effects. However, it should also be noted that the model is mechanistically limited in
that it cannot derive parameters that describe the molecular properties underlying agonism and allosteric modulation of signaling efficacy; the resulting parameters are composite values that also reflect the influence of receptor density and stimulus-response coupling on the observed responses.

All parametric measures of potency, affinity, operational efficacy, and cooperativity were estimated as logarithms (Christopoulos, 1998). Statistical comparisons between parameters were performed using a Student’s $t$-test or one-way analysis of variance (ANOVA) with a Newman-Keul’s multiple comparison post-test, as appropriate, and $p < 0.05$ taken as indicating significance.
Results

Effect of brucine on carbachol (CCh) signaling at the rM3Δi3 and rM3Δi3K7.32E mAChR in yeast

Previous studies have demonstrated that the introduction of a 3rd intracellular loop deletion into the rat M3 mAChR (rM3Δi3 mAChR) yields a construct that displays robust cell-surface expression in yeast, whilst retaining pharmacological characteristics of the wild-type receptor (Erlenbach et al., 2001). Therefore, this mutant receptor was utilized for all our studies in the S. cerevisiae strains reported herein. To ascertain whether allosteric modulation could be detected in yeast signaling assays, interaction studies were performed between CCh and brucine at the rM3Δi3 and rM3Δi3K7.32E mAChR in yeast strains expressing Gpa1/Gαq, Gpa1/Gα12 or Gpa1/Gα1/2 (Figure 1). In all yeast strains expressing the rM3Δi3 mAChR, brucine had no appreciable effect on the carbachol concentration-response curve. However, in strains expressing the rM3Δi3K7.32E mAChR, brucine exhibited differential effects on carbachol signaling in a G protein-dependent manner. Specifically, brucine displayed both agonism and potentiation of the carbachol response when coupled to Gpa1/Gαq, whereas it displayed no agonism but a robust sinistral shift of the carbachol concentration-response curve when coupled to Gpa1/Gα12. When the rM3Δi3K7.32E mAChR was coupled to Gpa1/Gα1/2, brucine had no effect on carbachol responsiveness. Additionally, from the data in yeast expressing the rM3Δi3K7.32E mAChR and Gpa/Gαq or Gpa/Gα12, we derived brucine affinity (Log K_B), efficacy (Log τ_B) and cooperativity (Log αβ) estimates using equation 5 (Table 1).
Differential effects of brucine on carbachol and [3H]-NMS binding at human M<sub>3</sub> and M<sub>3K<sup>7.32E</sup></sub> mAChRs in mammalian cell membranes

Radioligand saturation binding studies were performed on CHO cell membranes expressing either the full-length M<sub>3</sub> wild type or M<sub>3K<sup>7.32E</sup></sub> mAChR, from which B<sub>max</sub> and [3H]-NMS affinity values were derived using equation 1 (Table 2). These studies revealed that there was no difference between the affinity of [3H]-NMS for each receptor, and that receptor expression did not change between cell lines. Interaction binding studies were also performed between [3H]-NMS, CCh and brucine to determine the effect of brucine on the affinity of the radioligand and the non-radiolabeled orthosteric competitor (Figure 2). From these data, brucine affinity and cooperativity values were derived using equation 2 (Table 3). Affinity values for [3H]-NMS, CCh and brucine derived from the binding assays were similar across the M<sub>3</sub> and M<sub>3K<sup>7.32E</sup></sub> mAChRs, suggesting that the mutation had little effect on the orthosteric or allosteric ligand binding pockets. Interestingly, however, the modulatory effect of brucine on orthosteric ligand binding was altered by the K<sup>7.32E</sup> mutation, since brucine displayed slight negative cooperativity for [3H]-NMS and CCh binding at the M<sub>3</sub> mAChR, but modest positive cooperativity at the M<sub>3K<sup>7.32E</sup></sub> mAChR.

Validation of brucine pharmacology in mammalian cells expressing the human M<sub>3</sub> and M<sub>3K<sup>7.32E</sup></sub> mAChR

Data generated in the yeast assays suggested that brucine had no effect on CCh signaling at the rM<sub>3</sub>Δi3 mAChR, but exhibited agonism in the Gpa/G<sub>αq</sub> yeast strain and robust potentiation of CCh function in the Gpa/G<sub>α12</sub> strain at the rM<sub>3</sub>Δi3K<sup>7.32E</sup> mAChR. To ascertain whether these findings were relevant to a mammalian cell background, experiments
were performed in CHO cells expressing either the human M₃ or M₃K₇.32E mAChR, using Ca²⁺ mobilization and membrane ruffling as surrogate assays for G₉ and G₁₂, respectively (Brown et al., 2006; Ridley, 2006; Yuan et al., 2006).

Interaction studies were initially performed in CHO cells expressing the M₃ or M₃K₇.32E mAChR, with Ca²⁺ mobilization as a functional endpoint (Figure 3). Similar to what was observed in Gpa1/Gαq yeast strains expressing the rM₃Δi3 mAChR, brucine had no effect on the CCh concentration-response curve in CHO cells expressing the M₃ mAChR. However, brucine exhibited both agonism and potentiation of the CCh concentration-response curve in CHO cells expressing the M₃K₇.32E mAChR. Application of equation 5 to the M₃K₇.32E mAChR data yielded the operational model parameter estimates shown in Table 4.

Subsequent time-course studies in CHO M₃ and M₃K₇.32E mAChR cells revealed that the CCh-induced membrane ruffling response peaked at 2 min, and that brucine did not alter the time-course profile of CCh nor did brucine exhibit agonism for this pathway in either cell line (data not shown). CCh concentration-response curves were then constructed in the absence and presence of brucine in membrane ruffling assays in CHO cells expressing the M₃ or M₃K₇.32E mAChR (Figures 4 and 5). The results were consistent with those found in the yeast signaling assay for Gpa1/Gα₁₂ coupling, whereby brucine had no effect on the CCh concentration-response curves at the rM₃Δi3 mAChR, but was able to robustly potentiate CCh-induced signaling at the rM₃Δi3K₇.32E mAChR, without displaying any allosteric agonism. Table 4 shows the Log K_B, Log αβ and Log τ_B values that were derived by applying equation 5 to the M₃K₇.32E mAChR data from membrane ruffling assays.
Use of modulator profiling in yeast to delineate possible modes of convergent pathway signaling at the M₃K³²E mAChR

In conjunction with generating a G protein profile for receptor-ligand interaction, we reasoned that another possible utility of pairing the yeast signaling assay with a functionally selective modulator could be to aid in the dissection of intracellular G protein mediators of a convergent signaling pathway. For the purposes of the current study, we chose the phosphorylation of ERK1/2 as one such pathway (Werry et al., 2006). Specifically, the profile of the effect of brucine at different G protein subtypes may be used to predict which G protein (if any) underlies the predominant mode of coupling that leads to M₃K³²E mAChR-mediated phosphorylation of ERK1/2. Therefore, the effect of brucine on ERK1/2 phosphorylation was investigated. Time-course studies were performed in CHO M₃ and M₃K³²E mAChR cells, which demonstrated that maximal CCh-induced ERK1/2 phosphorylation occurred approximately 5 min after stimulation (data not shown). From this, it was also noted that brucine did not display agonism and did not alter the time point of the peak CCh response. Interaction studies between carbachol and brucine at M₃ and M₃K³²E mACh receptors were then performed to determine the effect of brucine on the potency of CCh at both receptors (Figure 6). The results revealed that brucine had little effect on CCh concentration-response curves from CHO M₃ mAChR cells, whereas brucine potentiated the CCh response at the M₃K³²E mAChR in a concentration-dependent manner. If ERK1/2 phosphorylation was downstream of G protein coupling, then the lack of agonism displayed by brucine suggested an absence of G_q contribution to M₃ mAChR-mediated ERK1/2 phosphorylation. Moreover, the yeast assays also predicted no coupling to G_ι/₀ proteins. To confirm the latter, the same ERK1/2 phosphorylation assays were performed in the presence
of PTX pretreatment (Figure 6). The lack of effect of PTX on M₃K⁷.₃²E mAChR-mediated ERK1/2 phosphorylation suggested that Gᵢₒ proteins had no contribution to this pathway, as predicted. Table 4 shows operational model parameter estimates derived by applying equation 5 to the ERK1/2 phosphorylation data from studies at the M₃K⁷.₃²E mAChR in the absence and presence of PTX.

Given the absence of contribution by Gᵣ and Gᵢₒ proteins to the allosteric modulation by brucine of M₃K⁷.₃²E mAChR-mediated ERK1/2 phosphorylation, we speculated that this allosteric effect is potentially mediated selectively via G₁₂ activation converging into the ERK1/2 stimulus-response chain. To more directly examine this hypothesis, we determined the effects of 30 μM brucine on CCh-mediated signaling in mammalian cells in the absence and presence of siRNA directed against mRNA of Gᵣ or G₁₂ (Figure 7). Although the degree of direct allosteric agonism mediated by brucine itself in Ca²⁺ mobilization assays was reduced by the presence of transfection lipid, robust potentiation of CCh-mediated signaling was still evident in the absence of siRNA (Figure 7A) as well as in the presence of G₁₂ siRNA (Figure 7C), but was significantly attenuated in upon transfection of Gᵣ siRNA (Figure 7B; Table 5), as expected. In contrast, selective knockdown of Gᵣ had minimal effect on the capacity of brucine to potentiate the CCh-stimulated ERK1/2 phosphorylation (Figures 7D, 7E), whereas knockdown of G₁₂ virtually abolished the allosteric potentiation (Figure 7F; Table 5), consistent with a selective role of G₁₂ activation in mediating the allosteric modulation of the ERK1/2 response.
Discussion

To our knowledge, this study is the first to use the yeast system to detect functional selectivity of an allosteric ligand, and to show that brucine is capable of exhibiting pathway selectivity. Furthermore, using the yeast system and the unique properties of brucine at the K7.32E mutant as pharmacological tools allowed us to determine a putative G protein candidate for brucine biased modulation of M3K7.32E mAChR-mediated ERK1/2 phosphorylation pathway in mammalian (CHO) cells.

There have been numerous studies investigating the properties of allosteric ligands that bind at mAChRs (Gregory et al., 2007), with the majority focusing on ligands that bind to the ‘prototypical’ binding site. Indeed, mutagenesis studies have mapped extracellular regions of mAChRs to determine amino acids residues that are pivotal for allosteric binding of the prototypical modulator, gallamine, and associated ligands (Buller et al., 2002; Gnagey et al., 1999). However, most of these studies focused on the effects of mutagenesis on radioligand binding, or only used a single signaling endpoint to define functional pharmacology (e.g. Jakubik et al. (1996); Iarriccio (2008)). In contrast, our current study investigated the ability of brucine to engender functional selectivity at the M3K7.32E mAChR by adopting the use of the yeast signaling assay as a predictive screen in conjunction with a multi-platform approach for mammalian system validation. The results produced in all of the yeast strains expressing the rM3Δi3 mAChR showed that brucine had no effect on CCh signaling, which was confirmed in CHO M3 mAChR cells. This result is consistent with what was found by Iarriccio (2008). The data generated from the yeast signaling assays at the K7.32E mutant, however, suggested that brucine was an agonist with modest enhancement of CCh signaling when coupled to Gq-mediated pathways. These data are concordant with those generated for CCh-induced Ca^{2+} signaling in CHO M3K7.32E mAChR cells, and also indicate that the use of...
a truncated rat receptor in yeast did not lead to spurious findings that could not be validated at the full length human receptor in mammalian cells. In both assay types, brucine induced a progressive sinistral shift of the CCh concentration-response curve of approximately 0.5 Log units, as well as displaying its own agonism. The degree of brucine agonism varied between the yeast and CHO cell assays, as quantified by the operational model parameters, $\tau_B = 1.4$ and 2.6, respectively, which may be due to a lower receptor expression in the yeast system compared to CHO $\text{M}_3\text{R}^{7,32}\text{E mAChR}$ cells.

Interaction binding assays between CCh and brucine in membranes expressing the $\text{M}_3$ and $\text{M}_3\text{K}^{7,32}\text{E mAChR}$ demonstrated that the $\text{K}^{7,32}\text{E}$ mutation did not greatly affect the binding of $[^3\text{H}]$-NMS, CCh or brucine, but rather weakly enhanced the cooperativity of brucine with the orthosteric ligands. These binding cooperativity profiles are consistent with the recent study by Iarriccio (2008), and the almost neutral cooperativity exhibited by brucine at wild-type $\text{M}_3$ mAChR is also consistent with evidence from previous studies at the same receptor (Lazareno et al., 1998). However, despite the cooperativities being similar, there was a discrepancy between the affinity estimates of brucine at the unoccupied $\text{M}_3$ mAChR from binding studies (Table 3), compared to the values derived by Iarriccio (2008; Log $K_B = -3.89$) and Lazareno et al. (1998; Log $K_B = -3.52$); this may be due to different assay conditions.

Importantly, interaction studies between CCh and brucine at the $\text{rM}_3\Delta i3\text{K}^{7,32}\text{E}$ in the Gpa1/G$\alpha_{12}$ yeast strain were able to predict the functional profile of brucine in membrane ruffling in CHO $\text{M}_3\text{K}^{7,32}\text{E mAChR}$ cells, where brucine treatment resulted in a concentration-dependent sinistral shift of the CCh concentration-response curve. Unlike G$q$ signaling, brucine did not display agonism, suggesting that the modulator is a selective (direct)
allosteric agonist for $G_q$-coupling at the mutant receptor but a selective positive allosteric modulator for $G_{12}$-mediated CCh signaling. There was a good accord in the rank order of $\alpha\beta$ and $\tau$ values derived from yeast G protein assays and their surrogate CHO cell assay, suggesting that the yeast assay is predictive of allosteric ligand pharmacology in mammalian cells.

As previously mentioned, there are now numerous studies that have investigated mAChR allosteric binding site mutations and allosteric ligand pharmacology, but with one very recent exception (Leach et al., 2010), studies have not generally probed for evidence of functional selectivity. However, given a surge in findings of functional selectivity induced by orthosteric ligands at various GPCRs (Baker et al., 2003; Galandrin et al., 2008), some evidence that allosteric ligands may also induce pathway selective signaling has recently emerged at other GPCRs. For example, prostaglandin D$_2$ receptor- (PGD$_2$R) mediated phosphoinositol signaling is not affected by the allosteric ligands, 1-(4-ethoxyphenyl)-5-methoxy-2-methylindole-3-carboxylic acid and $N_\alpha$-tosyltryptophan, but both abrogate PGD$_2$R-mediated arrestin recruitment via a non-G protein dependent mechanism (Mathiesen et al., 2005). Likewise, the allosteric ligand LPI805 was able to potentiate NKA-induced Ca$^{2+}$ mobilization, whilst allosterically inhibiting NKA-induced cAMP accumulation, at the tachykinin NK$_2$ receptor (Maillet et al., 2007). Thus it should not be surprising that this phenomenon may occur at other pleiotropically coupled receptors, such as the M$_3$ mAChR.

Given that the purpose of the current study was predominantly to provide proof of concept in the utility of pairing a yeast screening assay with a functionally selective allosteric ligand, we also attempted to ascertain whether this pairing can provide insights into a convergent
signaling pathway; in essence, to perform a ligand-dependent G protein “fingerprint” for likely candidates coupling the M_{3}K^{7.32}E mACHR to ERK1/2 phosphorylation. In yeast, brucine was a selective agonist for Gpa1/G_{α}q coupling, and the fact that brucine alone did not elicit an ERK1/2 phosphorylation response suggests a lack of involvement of G_{q} M_{3}K^{7.32}E mACHR-mediated ERK1/2 phosphorylation in CHO cells. The pretreatment of CHO M_{3}K^{7.32}E mACHR cells with PTX did not affect the potency of CCh or cooperativity of brucine, implying a lack of G_{i/o}-mediated signaling as predicted by the yeast assay. Interestingly, the knockdown of G_{α}q or G_{α}12 proteins did not diminish the potency of CCh, indicating that M_{3}K^{7.32}E mACHR-mediated ERK1/2 activation by this orthosteric agonist involves additional, e.g. G protein-independent components, and/or that the degree of knockdown was insufficient to overcome the high efficacy of the agonist for receptor activation. However, the abolishment of brucine’s allosteric effect on the CCh response in ERK1/2 phosphorylation assays by G_{α}12 siRNA clearly indicated that the allosteric modulator can promote a unique conformation that recruits G_{α}12 to converge on receptor coupling to the ERK1/2-response, further validating the use of the yeast assay as a predictor of this novel property.

Results from this study also suggest that residue 7.32 is not necessarily vital for either orthosteric ligand or brucine binding; instead it may be an important region for maintaining the flexibility, and hence possibly activation, of the receptor. Although the finer points of GPCR activation are still largely unknown, there is evidence that some residues in TMVII can form intramolecular interactions with residues in TMIII, to increase the stability of the receptor in an inactive state in the angiotensin II type 1 receptor and opsin, suggesting that TMVII may contribute, in part, to the activation of the receptor (Groblewski et al., 1997; Rosenbaum et al., 2009). Furthermore, it has been shown through disulphide cross-linking
studies that amino acid residues in TMI interact with residues at the bottom of TMVII, and that a large conformational change occurs at the bottom of TMVII upon application of agonist (Wess et al., 2008). There is also evidence that basic amino acid residues (such as lysine) in membrane proteins ‘snorkel’ in the lipid, and potentially interact with the charged head-groups in the phospholipid membrane (Mishra et al., 1994). Furthermore, a lysine residue at the juxtamembrane region of a TM helix has been shown to be important for coordinating the helix with the membrane and is also a determinant for the helical tilt (de Planque et al., 1999; Ozdirekcan et al., 2005). Therefore, perhaps, the K^{7.32}E mutation in the M₃ mAChR alters the interaction of TMVII with the plasma membrane and, in turn, increases the propensity of the receptor to be activated/modulated by brucine. Irrespective of the mode of receptor activation induced by ligands acting at the K^{7.32}E mutation, it is clear that the cooperativity between brucine and CCh is increased by the presence this mutation.

In conclusion, this study has provided evidence that the yeast signaling assay is a tractable and valuable platform for the determination of GPCR ligand-G protein functional selectivity profiles mediated by an allosteric ligand, as well as the provision of pharmacological parameters such as affinity, cooperativity and relative efficacy estimates. It is envisaged that this approach should be applicable to any GPCR than can be successfully expressed in yeast.

**Acknowledgments**

We are grateful to Dr. S. Dowell, GSK, UK, for provision of the yeast strains, Dr. J. Wess (NIH-NIDDK) for provision of the rat rM₃Δi3 mAChR construct, and Dr. Michael Crouch, TGR Biosciences, Adelaide, Australia, for provision of the ERK1/2 assay kits.
References


Iarriccio L (2008) Allosteric interactions at the M<sub>3</sub> muscarinic acetylcholine receptor, in *Chemical Engineering and Physical Biochemistry*, Polytechnic University and National Institute of Medical Research.


MOL #64253


Footnotes

This work was funded by the National Health and Medical Research Council (NHMRC) of Australia [Program Grant No. 519461]. Arthur Christopoulos is a Senior, and Patrick Sexton a Principal, Research Fellow of the NHMRC. Gregory Stewart is the recipient of an Australian Postgraduate Award (Industry) from the Australian Research Council.
Figure legends

**Figure 1** Effects of brucine on CCh concentration-response curves in yeast. CCh concentration-response curves were determined in yeast strains expressing the rM3Δi3 mAChR and A. Gpa1/Gαq, B. Gpa1/Gαi1/2 or C. Gpa1/Gα12, or the rM3Δi3K7.32E mAChR and D. Gpa1/Gαq, E. Gpa1/Gαi1/2 or F. Gpa1/Gα12, in the absence and presence of brucine. Data points are expressed as mean percentage of the basal activity in the absence of brucine + S.E.M. obtained from three to five experiments performed in duplicate. (RFU – relative fluorescence units).

**Figure 2** The allosteric modulator brucine displays differential cooperativity at the M3 and M3K7.32E mACh receptors. Interaction between [3H]-NMS and carbachol at the A. M3 or B. M3K7.32E mAChR in the absence and presence of brucine. Dashed lines represent the curve fit for the CCh inhibition curve in the absence of brucine. Data points are represented as the mean percentage of specific [3H]-NMS binding in the absence of CCh or brucine + S.E.M. of three experiments performed in duplicate.

**Figure 3** Effect of brucine on CCh-induced Ca²⁺ mobilization in CHO cells. CCh concentration-response curves performed in CHO cells expressing A. the M3 or B. the M3K7.32E mAChR, in the absence and presence of brucine. Data points are expressed as mean percentage of the maximal CCh-induced Ca²⁺ mobilization response in the absence of brucine + S.E.M. obtained from four to six experiments performed in duplicate.
Figure 4  Representative images of membrane ruffling in CHO M3K7.32E mAChR cells.
CHO M3K7.32E mAChR cells fixed in paraformaldehyde and stained with Alexa-568-conjugated phalloidin (green) and Hoechst 33342 nuclear dye (blue), post treatment with
A. serum-free DMEM; B. 1μM CCh; C. 100μM brucine or D. 1μM CCh + 100μM brucine. Red arrows indicate regions of cell membranes that are ruffled.

Figure 5  Effect of brucine of carbachol-induced membrane ruffling in CHO cells.
Carbachol concentration-response curves performed in CHO cells expressing the A. M3 the B. M3K7.32E mAChR, in the absence and presence of brucine. Data points are presented as mean percentage of the maximal CCh-induced membrane ruffling response in the absence of brucine + S.E.M. obtained from three experiments performed in duplicate.

Figure 6  Effect of brucine on CCh-induced ERK1/2 phosphorylation in CHO cells.
CCh concentration-response curves performed in CHO cells expressing the A. M3 the B. M3K7.32E mAChR, in the absence and presence of brucine. These experiments were also performed after pre-incubation with PTX at the C. M3 the D. M3K7.32E mAChR. Data points are represented as mean percentage of the peak ERK1/2 phosphorylation response elicited by 10% FBS + S.E.M. obtained from three to six experiments performed in duplicate.

Figure 7  Impact of G protein-targeting siRNA on the ability of brucine to allosterically potentiate the CCh-stimulated response. CCh concentration-response curves performed in CHO M3K7.32E mAChR cells, in the absence (●) or presence (□) of 30μM brucine, in Ca2+ mobilization (A, B, C) and ERK1/2 phosphorylation assays (D, E, F). Experiments were
conducted 48 hrs post-transfection with transfection lipid alone (transfection control), G\(\alpha_q\) siRNA or G\(\alpha_{12}\) siRNA. Data are expressed as the mean RFU (Ca\(^{2+}\) mobilization) or ERK1/2 phosphorylation as a percentage of the peak CCh response from the control data (ERK1/2 phosphorylation) + S.E.M. collected from four experiments performed in duplicate.
Table 1  Operational model parameters for the interaction between CCh and brucine at the rM3A3i3K$^{7.32}$E mACHR in yeast. Data are expressed as the mean ± S.E.M. of three to five separate experiments performed in duplicate.

<table>
<thead>
<tr>
<th></th>
<th>Gpa1/Gαq</th>
<th>Gpa1/Gα12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log $K_B^a$</td>
<td>-4.48 ± 0.13</td>
<td>-4.89 ± 0.13</td>
</tr>
<tr>
<td>Log $\tau_B^b$</td>
<td>0.15 ± 0.07 (τB = 1.4)</td>
<td>-0.79 ± 0.17 $^{**}$ (τB = 0.16)</td>
</tr>
<tr>
<td>Log $\alpha\beta^c$</td>
<td>0.76 ± 0.17 ($\alpha\beta = 5.8$)</td>
<td>1.06 ± 0.10 ($\alpha\beta = 11.5$)</td>
</tr>
</tbody>
</table>

$^a$ logarithm of the dissociation constant of the allosteric modulator  
$^b$ logarithm of the operational efficacy of the allosteric modulator  
$^c$ logarithm of the cooperativity of the allosteric modulator on the potency of the orthosteric agonist  

$^{**}$ p<0.01 determined by Student’s $t$-test (compared to the same parameter in the Gpa1/Gαq strain)
Table 2  Saturation binding parameters for [³H]-NMS at the M₃ or M₃K⁷.3²E mAChR in CHO cell membranes. Data are presented as the mean ± S.E.M. of three separate experiments performed in duplicate.

<table>
<thead>
<tr>
<th></th>
<th>CHO M₃ mAChR</th>
<th>CHO M₃K⁷.3²E mAChR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log $K_A$</td>
<td>-9.14 ± 0.05</td>
<td>-9.20 ± 0.04</td>
</tr>
<tr>
<td>$B_{max}$ (fmol mg⁻¹ protein)</td>
<td>3425 ± 233</td>
<td>2940 ± 338</td>
</tr>
</tbody>
</table>

*Logarithm of the equilibrium dissociation constant of the radioligand

*Total number of binding sites, determined by specific binding of the radioligand
Table 3  Allosteric ternary complex model binding parameters for the interaction between [3H]-NMS, CCh and brucine at the M₃ and M₃K₇.₃₂E mAChRs in CHO cell membranes. Data are presented as the mean ± S.E.M. of three separate experiments performed in duplicate.

<table>
<thead>
<tr>
<th></th>
<th>CHO M₃ mAChR</th>
<th>CHO M₃K₇.₃₂E mAChR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log $K_B^a$</td>
<td>-5.95 ± 0.37</td>
<td>-5.06 ± 0.15</td>
</tr>
<tr>
<td>Log $K_I^b$</td>
<td>-5.48 ± 0.03</td>
<td>-5.32 ± 0.04</td>
</tr>
<tr>
<td>Log $\alpha^c$</td>
<td>-0.12 ± 0.03 ($\alpha = 0.75$)</td>
<td>0.10 ± 0.03** ($\alpha = 1.25$)</td>
</tr>
<tr>
<td>Log $\alpha'^d$</td>
<td>-0.35 ± 0.26 ($\alpha' = 0.45$)</td>
<td>0.58 ± 0.04* ($\alpha' = 3.80$)</td>
</tr>
</tbody>
</table>

*a* logarithm of the dissociation constant of the allosteric modulator  
*b* logarithm of the dissociation constant of the orthosteric inhibitor  
*c* logarithm of the cooperativity between the allosteric modulator on the radioligand  
*d* logarithm of the cooperativity between the allosteric modulator on the orthosteric inhibitor  

* $p<0.05$ determined by Student’s $t$-test (compared to the same parameter at the CHO M₃ mAChR)  
** $p<0.01$ determined by Student’s $t$-test (compared to the same parameter at the CHO M₃ mAChR)
Table 4 Operational model parameters for the interaction between CCh and brucine at the M₃K⁷-32E mACHR. Data are presented as the mean ± S.E.M. of three to six separate experiments performed in duplicate.

<table>
<thead>
<tr>
<th></th>
<th>Ca²⁺ mobilization</th>
<th>Membrane ruffling</th>
<th>ERK1/2 phosphorylation</th>
<th>ERK1/2 phosphorylation (+PTX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>log K_B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-5.17 ± 0.15</td>
<td>-4.61 ± 0.13</td>
<td>-5.21 ± 0.28</td>
<td>-5.30 ± 0.19</td>
</tr>
<tr>
<td>log τ_B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.41 ± 0.03&lt;sup&gt;++&lt;/sup&gt; (τ_B = 2.6)</td>
<td>-1.40 ± 0.48 (τ_B = 0.04)</td>
<td>-0.66 ± 0.16 (τ_B = 0.2)</td>
<td>-0.43 ± 0.16 (τ_B = 0.4)</td>
</tr>
<tr>
<td>log αβ&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.70 ± 0.17 (αβ = 5.0)</td>
<td>0.88 ± 0.10 (αβ = 7.6)</td>
<td>0.84 ± 0.06 (αβ = 6.9)</td>
<td>0.84 ± 0.07 (αβ = 6.9)</td>
</tr>
</tbody>
</table>

<sup>a</sup> logarithm of the dissociation constant of the allosteric modulator

<sup>b</sup> logarithm of the operational efficacy of the allosteric modulator

<sup>c</sup> logarithm of the cooperativity between the allosteric modulator and the orthosteric agonist

<sup>++</sup> p<0.01 determined by one-way ANOVA with Newman-Keuls’s multiple comparisons post-test across log τ_B values (statistically different to the log τ_B value derived for membrane ruffling)
Table 5  CCh potency (pEC$_{50}$) and curve-translocation (Log $\delta$) values for the interaction between CCh and brucine at the M$_3$K$^{7,32}$E mAChR after transfection with siRNA. Data are presented as the mean ± S.E.M. of four to five separate experiments performed in duplicate.

<table>
<thead>
<tr>
<th>Transfection control</th>
<th>G$\alpha_q$ siRNA</th>
<th>G$\alpha_{12}$ siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ca$^{2+}$ mobilization</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEC$_{50}$$^a$</td>
<td>7.16 ± 0.20</td>
<td>7.44 ± 0.12</td>
</tr>
<tr>
<td>Log $\delta$$^b$</td>
<td>1.14 ± 0.23</td>
<td>0.41 ± 0.12$^*$</td>
</tr>
<tr>
<td><strong>ERK1/2 phosphorylation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEC$_{50}$$^a$</td>
<td>6.33 ± 0.11</td>
<td>6.28 ± 0.12</td>
</tr>
<tr>
<td>Log $\delta$$^b$</td>
<td>0.82 ± 0.13</td>
<td>0.62 ± 0.14</td>
</tr>
</tbody>
</table>

$^a$ negative logarithm of concentration agonist that generates 50% of the maximal agonist response

$^b$ logarithm of the ratio of the EC$_{50}$ values of the allosteric modulator-treated and control CCh curves.

$^*$ $p<0.05$ determined by one-way ANOVA with Newman-Keuls’s multiple comparisons post-test between each transfection group in a single assay type.
Figure 1

\[ rM_3\Delta i3 \text{ mAChR} \]

\[ rM_3\Delta i3K^{7.32E} \text{ mAChR} \]

[A. Gpa1/G_q](#)  
[B. Gpa1/G_{i1/2}]  
[C. Gpa1/G_{12}]  
[D. Gpa1/G_q]  
[E. Gpa1/G_{i1/2}]  
[F. Gpa1/G_{12}]  

Log[Ch] (M)  
RFU (% of basal)  

[Brucine] (μM)  
- 0  
- 1  
- 3  
- 10  
- 30
Figure 2

A. M₃ mAChR

B. M₃K⁷.₃²E mAChR

% Specific[^3H]-NMS binding

Log[CCh] (M)

[Brucine] (μM) 0 3 10 30
Figure 3

A. $M_3$ mAChR

B. $M_3K^{7.32}E$ mAChR

[Brucine] (µM) 0, 1, 3, 10, 30
Figure 4

A.

B.

C.

D.

Figure 4
Figure 5

A. M₃ mAChR

B. M₃K⁷.₃₂E mAChR

[Brucine] (µM) 0 1 3 10 30 100
Figure 6

A. M₃ mAChR - PTX

B. M₃K⁷.₃²E mAChR

C. M₃ mAChR + PTX

D. M₃K⁷.₃²E mAChR

ERK 1/2 Phosphorylation (% of FBS Stimulation)

Log[CCh] (M)

[Brucine] (µM)  ● 0  ■ 1  ▲ 3  ○ 10  □ 30
Figure 7

**Ca^{2+}** mobilization

A. Transfection control

B. \( \alpha_q \) siRNA

C. \( \alpha_{12} \) siRNA

**ERK1/2 phosphorylation**

D. Transfection control

E. \( \alpha_q \) siRNA

F. \( \alpha_{12} \) siRNA

<table>
<thead>
<tr>
<th>RFU</th>
<th>Log [CCh] (M)</th>
<th>RFU</th>
<th>Log [CCh] (M)</th>
<th>RFU</th>
<th>Log [CCh] (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-12</td>
<td>0</td>
<td>-12</td>
<td>0</td>
<td>-12</td>
</tr>
<tr>
<td>5000</td>
<td>-11</td>
<td>1500</td>
<td>-11</td>
<td>5000</td>
<td>-11</td>
</tr>
<tr>
<td>1000</td>
<td>-10</td>
<td>2000</td>
<td>-10</td>
<td>1000</td>
<td>-10</td>
</tr>
<tr>
<td>5000</td>
<td>-12</td>
<td>2500</td>
<td>-12</td>
<td>5000</td>
<td>-12</td>
</tr>
</tbody>
</table>

ERK1/2 phosphorylation (% of CCh Emax)

D. Transfection control

E. \( \alpha_q \) siRNA

F. \( \alpha_{12} \) siRNA

<table>
<thead>
<tr>
<th>ERK1/2 phosphorylation (%)</th>
<th>Log [CCh] (M)</th>
<th>ERK1/2 phosphorylation (%)</th>
<th>Log [CCh] (M)</th>
<th>ERK1/2 phosphorylation (%)</th>
<th>Log [CCh] (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-12</td>
<td>0</td>
<td>-12</td>
<td>0</td>
<td>-12</td>
</tr>
<tr>
<td>50</td>
<td>-11</td>
<td>100</td>
<td>-11</td>
<td>50</td>
<td>-11</td>
</tr>
<tr>
<td>75</td>
<td>-10</td>
<td>125</td>
<td>-10</td>
<td>75</td>
<td>-10</td>
</tr>
<tr>
<td>150</td>
<td>-12</td>
<td>150</td>
<td>-12</td>
<td>150</td>
<td>-12</td>
</tr>
</tbody>
</table>