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$G_{q/11}$ and $G_{i/o}$ activation profiles in CHO cells expressing human muscarinic acetylcholine receptors: dependence on agonist as well as receptor-subtype

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1 Profiles of G protein activation have been assessed using a [^{35}S]-GTPγS binding/immunoprecipitation strategy in Chinese hamster ovary cells expressing either M_1, M_2, M_3 or M_4 muscarinic acetylcholine (mACh) receptor subtypes, where expression levels of M_1 and M_3, or M_2 and M_4 receptors were approximately equal.

2 Maximal [^{35}S]-GTPγS binding to $G_{q/11}\alpha$ stimulated by M_1/M_3 receptors, or $G_{i1-3}\alpha$ stimulated by M_2/M_4 receptors occurred within approximately 2 min of agonist addition. The increases in $G_{q/11}\alpha$-[^{35}S]-GTPγS binding after M_1 and M_3 receptor stimulation differed substantially, with M_1 receptors causing a 2–3 fold greater increase in [^{35}S]-GTPγS binding and requiring 5 fold lower concentrations of methacholine to stimulate a half-maximal response.

3 Comparison of M_2 and M_4 receptor-mediated $G_{i1-3}\alpha$-[^{35}S]-GTPγS binding also revealed differences, with M_2 receptors causing a greater increase in $G_{i1-3}\alpha$ activation and requiring 10 fold lower concentrations of methacholine to stimulate a half-maximal response.

4 Comparison of methacholine- and pilocarpine-mediated effects revealed that the latter partial agonist is more effective in activating $G_{i3}\alpha$ compared to $G_{i1/2}\alpha$ for both M_2 and M_4 receptors. More marked agonist/partial agonist differences were observed with respect to M_1/M_3-mediated stimulations of $G_{q/11}\alpha$- and $G_{i1-3}\alpha$-[^{35}S]-GTPγS binding. Whereas coupling to these Gα subclasses decreased proportionately for M_1 receptor stimulation by these agonists, pilocarpine possesses a greater intrinsic activity at M_3 receptors for $G_i\alpha$ versus $G_{q/11}\alpha$ activation.

5 These data demonstrate that mACh receptor subtype and the nature of the agonist used govern the repertoire of G proteins activated. They also provide insights into how the diversity of coupling can be pharmacologically exploited, and provide a basis for a better understanding of how multiple receptor subtypes can be differentially regulated.

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Abbreviations: CHO, Chinese hamster ovary; DTT, dithiothreitol; GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-[γ-thio]triphosphate; mACh, muscarinic acetylcholine; MCh, methacholine; MEM, minimal essential medium; NMS, *N*-methyl-scopolamine; PLC, phospholipase C; PTx, pertussis toxin

Introduction

The muscarinic acetylcholine (mACh) receptor family consists of five members (M_1-M_5) and belongs to the G protein-coupled receptor (GPCR) superfamily. A characteristic of GPCRs is that ligand binding, the initial step in receptor signalling, elicits a conformational change in the receptor, leading to the activation of one or more heterotrimeric G proteins (Neer, 1995). An accumulation of evidence suggests that M_1, M_3 and M_5 mACh receptors couple preferentially to the activation of phospholipase C (PLC) *via* pertussis toxin (PTx)-insensitive G proteins of the $G_{q/11}$ family (Caulfield, 1993; Felder, 1995). For example, reconstitution experiments have shown that M_1 mACh receptors can activate PLC-β1 *via* $G_{q/11}$ (Berstein *et al*., 1992). In contrast, M_2 and M_4 mACh receptors couple preferentially to the inhibition of adenylyl cyclase *via* PTx-sensitive G proteins of the G_i family (Caulfield, 1993). However, recently it has become clear that several GPCRs, including mACh receptors can behave promiscuously and can interact with several different G proteins to influence multiple effector activities. Although the implications of such promiscuity to signal transduction *in vivo* are as yet unknown they may provide an unsuspected diversity of signalling (Kenakin, 1997).

The coupling between mACh receptors and G proteins has been assessed quite extensively using agonist-stimulated [^{35}S]-GTPγS binding (Hilf *et al*., 1989; Lazareno & Birdsall, 1993; Lazareno *et al*., 1993; Burford *et al*., 1995a). Although valuable for determining the potency and efficacy of various agonists acting at mACh receptors, the [^{35}S]-GTPγS binding methodology measures GDP/[^{35}S]-GTPγS exchange on all Gα subunits. Some indication of GPCR-G protein coupling partner preferences can be gained by the use of PTx.

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However, since multiple subtypes of $G_i\alpha$ and $G_o\alpha$ are PTx-sensitive, and it is increasingly evident that single GPCRs can productively couple to many different G protein subtypes (Offermanns & Schultz, 1994; Gudermann *et al*., 1996), it is becoming increasingly important to specify more precisely the initial receptor-G protein activation step.

Offermanns *et al*. (1994) used subtype-specific immunoprecipitation of G protein α-subunits photolabelled with [α-^{32}P]-GTP-azidoanilide to reveal selective coupling of activated mACh receptors to G protein subtypes. Here we have used an alternative approach in which we have immunoprecipitated specific G protein α-subunits labelled with [^{35}S]-GTPγS. To facilitate interpretation of such data we have used CHO cell clones recombinantly expressing either M_1, M_2, M_3 or M_4 mACh receptor subtypes, where expression levels of M_1 and M_3, or M_2 and M_4 receptors are approximately equal. We show that mACh receptor subtypes display differing G protein activation profiles, and furthermore that these profiles are dependent on both receptor subtype and the agonist used for activation, indicating that mACh receptors may interact with a limited or expanded G protein population.

Methods

Materials

GDP, GTP, Igepal CA-630, methacholine and pilocarpine were from Sigma Chemical Co. Ltd (Poole, U.K.). [^{35}S]-guanosine 5′-[γ-thio]triphosphate ([^{35}S]-GTPγS) and the G protein antisera raised against $G_{i1}\alpha/G_{i2}\alpha$ and $G_{i3}\alpha/G_o\alpha$ were from New England Nuclear (Brussels, Belgium). G protein antisera raised against $G_{i1}\alpha/G_{i2}\alpha$ $G_{i3}\alpha/G_o\alpha$ and $G_q\alpha/G_{11}\alpha$ were also purchased from Calbiochem (CN Biosciences U.K., Nottingham, U.K.) and used for Western blotting. Further G protein antisera, G_i common, $G_q\alpha/G_{11}\alpha$, $G_o\alpha$ and $G_s\alpha$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.)

Cell culture

Chinese hamster ovary cells (CHO-K1) transfected with cDNAs encoding human m1, m3 or m4 mACh receptors (CHO-m1, CHO-m3 and CHO-m4, respectively) were obtained from Dr N. Buckley (NIMR, Mill Hill, U.K.). Cells transfected with a cDNA encoding the human m2 mACh receptor (CHO-m2) were obtained from Dr S. Lazareno (MRC Collaborative Centre, Mill Hill, London, U.K.). CHO cell clones were grown in minimum essential medium-α (MEM-α) supplemented with 10% newborn calf serum, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} amphotericin B. Cells were maintained at 37°C in an humidified atmosphere of 5% CO_2:air.

Membrane preparation

Confluent monolayers of CHO-transfects were briefly washed with HBS-EDTA (10 mM HEPES, 0.9% NaCl, 0.2% EDTA, pH 7.4), and cells lifted from the flask by addition of HBS-EDTA for approximately 15 min. A cell pellet was recovered by centrifugation at $200 \times g$ for 4 min. The cell pellet was homogenized on ice in Buffer 1 (10 mM HEPES, 10 mM EDTA, pH 7.4) using a Polytron homogenizer (4×5 s bursts at 60% of max. speed, separated by approximately 30 s). The homogenate was centrifuged ($40{,}000 \times g$, 15 min, 4°C) and re-homogenized and re-centrifuged as described above in Buffer 2 (10 mM HEPES, 0.1 mM EDTA, pH 7.4). The final membrane pellet was resuspended in Buffer 2 at a concentration of 1 mg protein ml^{-1}, rapidly frozen in liquid nitrogen, and stored at −80°C until required for either [^{3}H]-NMS binding or immunoblotting.

[^{3}H]-NMS binding

Saturation binding was performed as described by Lambert *et al*. (1989) using a range of concentrations of [^{3}H]-NMS (0.07 – 3 nM; specific activity 83 Ci mmol^{-1}) in the absence and presence of atropine (1 μM; to define the non-specific binding) in assay buffer (mM): HEPES 10, NaCl 100, $MgCl_2$ 10 (pH 7.4) for 60 min at 37°C. Bound and free [^{3}H]-NMS were separated by rapid vacuum filtration and radioactivity quantified by liquid scintillation spectrometry.

Immunoblotting of Gα proteins

Membrane samples were prepared as detailed above and then mixed with an equal volume of sample buffer (100 mM Tris, 200 mM dithiothreitol (DTT), 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min. Samples were electrophoresed on a 12%, 0.75 mm thick SDS – PAGE minigel, with a 5% stacking gel. Samples were run at around 100 V for approximately 1 h (running buffer; 25 mM Tris/HCl, 250 mM glycine, 0.1% SDS pH 8.0). Transfer to nitrocellulose was achieved using semi-dry apparatus with a transfer buffer consisting of 20 mM Tris, 150 mM glycine, 0.037% SDS and 10% methanol, at 0.65 mA cm^{-2}. Primary antibodies against specific Gα proteins (rabbit, polyclonal) were used in 1% milk at a dilution of 1 : 1000. The secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Sigma) was also used in 1% milk at a dilution of 1 : 1000. The ECL reagent kit from Amersham (Aylesbury, U.K.) was used to develop the blot.

[^{35}S]-guanosine 5′-[γ-thio]triphosphate ([^{35}S]-GTPγS) binding and immunoprecipitation of [^{35}S]-GTPγS bound Gα subunits

[^{35}S]-GTPγS binding to G-proteins and the subsequent immunoprecipitation was performed according to a modification of the methodologies described previously by Friedman *et al*. (1993), Wang *et al*. (1995) and Burford *et al*. (1998). CHO-cell transfects were grown to confluence and each 175 cm^2 confluent flask washed with HBS-EDTA, the cells were then lifted by the addition of HBS-EDTA for approximately 10 – 15 min. CHO-cell transfects expressing the same receptor were pooled and centrifuged at about $200 \times g$ for 5 min. Pelleted cells were homogenized in the presence of hypotonic lysis buffer (10 mM EDTA, 10 mM HEPES, pH 7.4). Disruption of the cells was achieved by using a Polytron homogenizer (4×5 s bursts, 70% max. setting). The homogenate was then centrifuged at $500 \times g$ for 5 min and the resulting supernatant further centrifuged at $36{,}000 \times g$ for 30 min. The final membrane pellet was

resuspended in freezing buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4) at a protein concentration of 5–9 mg ml^{-1} and rapidly frozen in liquid nitrogen. Membranes were then stored at −80°C until used.

Frozen membrane aliquots were diluted in assay buffer (mM): HEPES 10, NaCl 100, $MgCl_2$ 10 (pH 7.4) to give a final protein concentration of 75 μg per 50 μl. Membranes (75 μg) were added to 50 μl of assay buffer containing (final concentrations) 1 nM [^{35}S]-GTPγS (1250 Ci mmol^{-1}) and 1 or 10 μM GDP (as stated in Results) and incubated at 30°C for 2 min (unless otherwise stated). Incubations were terminated by the addition of 900 μl of ice-cold assay buffer and immediate transfer to an ice-bath. Cell membranes were recovered from the reaction mixture by centrifugation at 20,000 × *g* for 6 min with the resulting supernatant removed. Membrane pellets were solubilized by the addition of 50 μl of ice-cold solubilization buffer (mM): Tris/HCl 100, NaCl 200, EDTA 1, 1.25% Igepal CA 630 (pH 7.4) containing 0.2% SDS and vortex-mixing. Once the protein was completely solubilized, an equal volume of solubilization buffer without SDS was added to each tube.

The solubilized protein was pre-cleared with normal rabbit serum (1 : 100 dilution) and 30 μl of protein A beads (protein A-sepharose bead suspension 30% w v^{-1} in TE buffer (10 mM Tris/HCl, 10 mM EDTA, pH 8.0)) for 60 min at 4°C. The protein A beads and any insoluble material were collected by centrifugation at 20,000 × *g* for 6 min, then 100 μl of the supernatant was transferred to a fresh tube containing G protein antiserum (1 : 100 dilution). Samples were vortex-mixed and rotated for 90 min at 4°C. To each sample tube was added 70 μl of protein A-sepharose bead suspension and the samples again vortex-mixed and rotated for 90 min at 4°C. Protein A-sepharose beads were then pelleted at 20,000 × *g* and the supernatant removed by aspiration. The beads were washed three times with 500 μl solubilization buffer (−SDS) and after the final wash the recovered beads were mixed with scintillation cocktail and counted. Non-specific binding was determined in the presence of 10 μM GTPγS.

Data analysis

Data are shown as means ± s.e.mean for the indicated number of experiments. Log concentration-response curves were analysed by non-linear regression using a commercially available programme (Prism 3.0, GraphPad Software, San Diego, U.S.A.) to generate pEC_{50} values. Statistical significance was assessed using Student's *t*-test (for paired observations) on untransformed datasets to assess basal versus agonist-stimulated differences.

Results

Characterization of mACh receptor-expressing CHO cell-lines

Expression levels of mACh receptor, assessed using [^{3}H]-NMS saturation binding to cell membranes, were 2.39 ± 0.19 and 2.52 ± 0.10 pmol mg^{-1} protein (n = 5) for CHO-ml and -m3, and 0.91 ± 0.02 and 1.51 ± 0.10 pmol mg^{-1} protein (n = 5) for CHO-m2 and -m4 cell-lines, respectively.

Identification of different Gα proteins present in CHO-m1/-m2/-m3/-m4 cells

Initial immunoblot experiments were performed on cell membranes prepared from CHO-m1, -m2, -m3, -m4 and untransfected CHO cells to examine the relative levels of different Gα proteins. Across all the transfected CHO cells no major differences in the levels of any specific Gα protein were observed and the cell-lines expressed each of the Gα proteins examined – $G_{q/11}α$, $G_{i1/2}α$, $G_{i3/o}α$, $G_oα$ and $G_sα$ – at similar levels (Figure 1).

Immunoprecipitation of [^{35}S]-GTPγS-bound Gα proteins

The experimental approach used here confirms several recent reports in other receptor models (Al-Aoukaty *et al*., 1997; Barr *et al*., 1997; Fukushima *et al*., 1998) that [^{35}S]-GTPγS-bound G protein α-subunits can be immunoprecipitated specifically, and furthermore that agonist-stimulated binding can be reproducibly observed. The ability of various dilutions of the $G_{q/11}α$ antiserum to immunoprecipitate [^{35}S]-GTPγS binding was totally insensitive to PTx pre-treatment of cells, whereas the $G_{i1/2}$ and $G_{i3/o}α$ antibodies immunoprecipitated binding that was abolished by PTx (data not shown). Furthermore, the ability of the $G_{q/11}α$ antiserum to reveal a dramatic increase in [^{35}S]-GTPγS binding following M_1- and M_3-mACh receptor activation, but not following M_2- and M_4-mACh receptor activation, provides strong evidence of the specificity of the antibody under these conditions.

This point is further supported by the reciprocal finding that agonist-stimulated binding mediated by M_2- and M_4-mACh receptors is only observed when measured with $G_{i/o}α$, and not $G_{q/11}α$, antisera. Furthermore, methacholine did not stimulate [^{35}S]-GTPγS binding to any Gα protein in untransfected CHO cell membranes. Therefore, we feel confident, that the comparative changes in Gα-[^{35}S]-GTPγS binding immunoprecipitated by the specific antisera faithfully delineates differences in activation mediated by different agonists at various mACh receptor subtypes. More caution is needed, however, in making any quantitative comparisons of the relative activation of different G protein α-subunits since the immunoprecipitating efficiency of each antiserum is unknown. With respect to this, it is encouraging that there was a good quantitative correlation of [^{35}S]-GTPγS immunoprecipitated by the $G_iα$ 'common' antiserum and that revealed by the sum of $G_{i1/2}α$ and $G_{i3/o}α$ antisera over a series of experiments (see Figure 4).

A time-dependent increase in methacholine-stimulated specific [^{35}S]-GTPγS binding to $G_{i1-3}α$ was observed in M_2- (Figure 2a) and M_4- (Figure 2b) mACh receptor-expressing CHO cell membranes. Agonist-stimulated [^{35}S]-GTPγS binding could be observed in the absence of GDP, but optimal signal-to-noise was achieved using 10 μM GDP. $G_{i1-3}α$ activation occurred rapidly in both CHO-m2 and -m4 cell membranes and was maximal 1–2 min after agonist addition. At this time-point [^{35}S]-GTPγS binding to $G_{i1-3}α$ was somewhat greater in M_2-compared to M_4-mACh receptor-expressing cells.

A time-dependent increase in methacholine-stimulated specific [^{35}S]-GTPγS binding to $G_{q/11}α$ was also seen in M_1- (Figure 3a) and M_3- (Figure 3b) mACh receptor-expressing cell membranes. Agonist-stimulated [^{35}S]-GTPγS binding

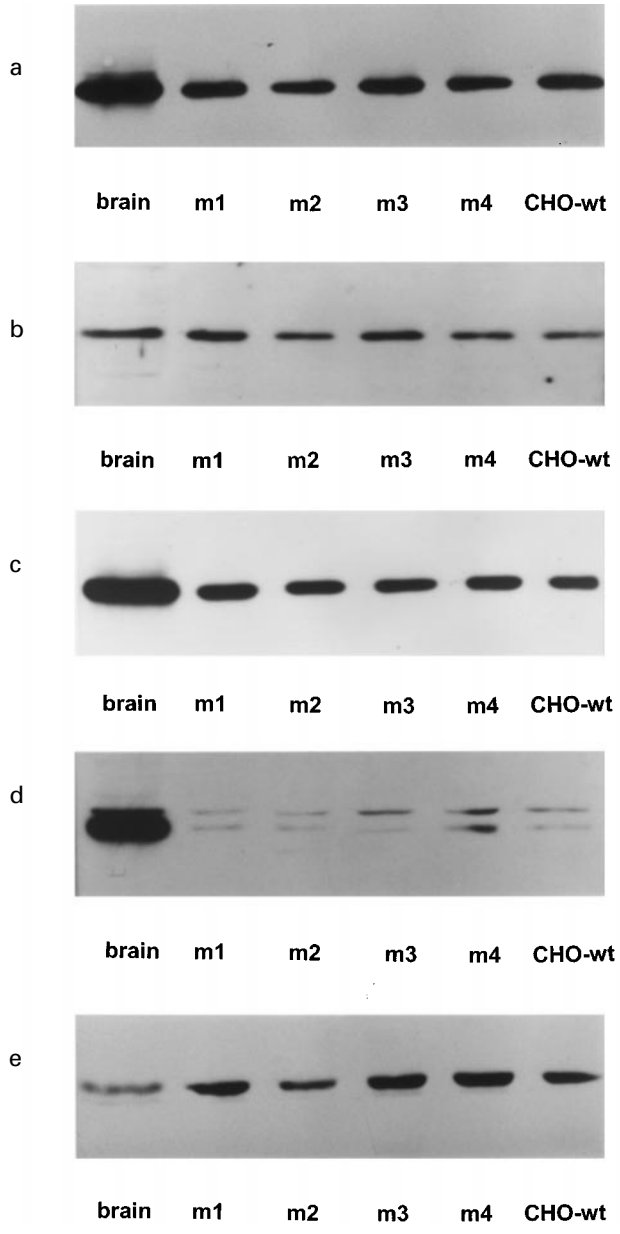

Figure 1 Expression of Gα proteins in CHO-m1, -m2, -m3, -m4, and untransfected CHO cell membranes. Cell membranes (20 μg protein) were solubilized, proteins separated and transferred to nitrocellulose for immunoblotting as described in Methods. Gα proteins were identified using 1 : 1000 dilutions of antisera to $G_{q/11}\alpha$ (a), $G_{i1/2}\alpha$ (b), $G_{i3/0}\alpha$ (c), $G_0\alpha$ (d), or $G_s\alpha$ (e). All Gα proteins migrated on electrophoresis consistently with the expected molecular weight (40–45 kDa) for each Gα protein compared to M_r standards. For each blot, lane 1 (brain) = crude rat brain homogenate, lanes 2–5 = CHO-m1 to CHO-m4 membranes respectively and lane 6 (CHO-wt) = untransfected CHO cell membranes.

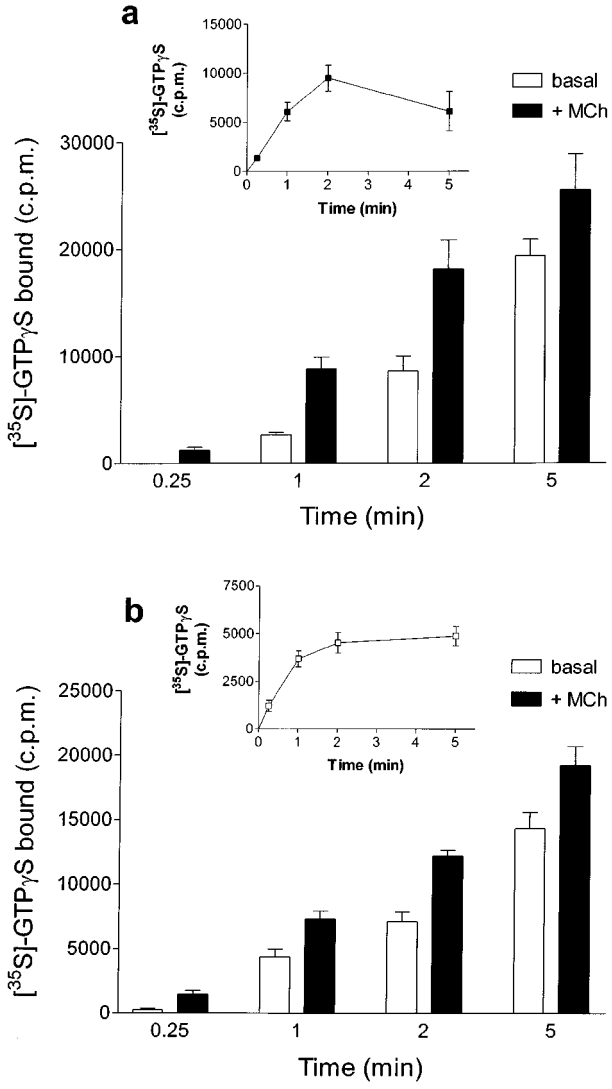

Figure 2 Time-course of methacholine-stimulated [^{35}S]-GTPγS binding to $G_{i1-3}\alpha$ in CHO-m2 and CHO-m4 cell membranes. Cell membranes prepared from CHO-m2 (a) or CHO-m4 (b) cells were incubated ([GDP] = 10 μM) in the absence (basal) or presence of methacholine (MCh, 1 mM) for the times indicated at 30°C. Insert panels illustrate the net change in [^{35}S]-GTPγS bound-over-basal. Data are shown as means ± s.e.mean for five separate experiments carried out in duplicate. Agonist-stimulated [^{35}S]-GTPγS binding was significantly greater than basal binding ($P < 0.05$) for both CHO-m2 and CHO-m4 membranes for all time-points beyond 0.25 min.

could be observed in the absence of GDP, but optimal signal-to-noise was achieved using 1 μM GDP. Comparisons between membranes prepared from CHO-m1 and CHO-m3 cells revealed that the extent of agonist-stimulated $G_{q/11}\alpha$-[^{35}S]-GTPγS binding was greater in the former (by approximately 3 fold) and although maximal binding could be observed at 2 min for both mACh receptor subtypes, activation in CHO-m1 membranes appeared to occur more rapidly. Overall, from these initial experiments, optimal assay conditions were defined as 2 min incubations with agonist at 30°C in the presence of 1 μM GDP for the M_1 and M_3, or 10 μM GDP for the M_2 and M_4 mACh receptor subtypes.

Pre-addition of atropine (10 μM, 15 min) prevented methacholine-stimulated [^{35}S]-GTPγS binding in all cases. It should also be noted that atropine had no effect on either basal $G_{q/11}\alpha$-[^{35}S]-GTPγS binding in CHO-m1/m3, or $G_{i1-3}\alpha$-[^{35}S]-GTPγS binding in CHO-m2/m4 cell membranes, demonstrating that atropine appears to be devoid of inverse agonist activity in this system (data not shown).

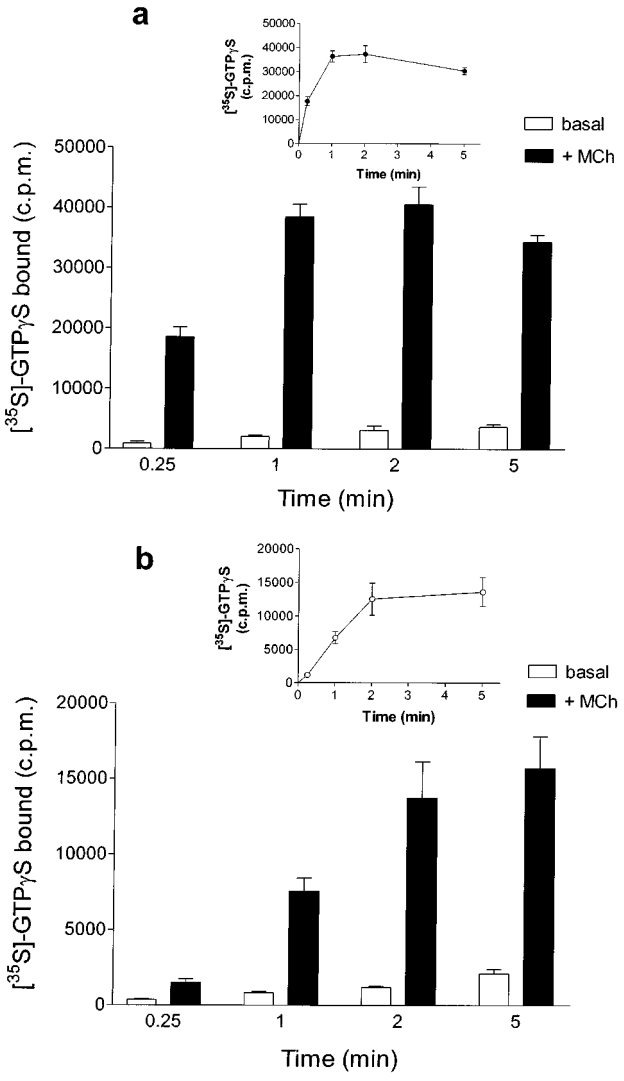

Figure 3 Time-course of methacholine-stimulated [^{35}S]-GTPγS binding to $G_{q/11}\alpha$ in CHO-m1 and CHO-m3 cell membranes. Cell membranes prepared from CHO-m1 (a) or CHO-m3 (b) cells were incubated ([GDP] = 1 μM) in the absence (basal) or presence of methacholine (MCh, 1 mM) for the times indicated at 30°C. Insert panels illustrate the net change in [^{35}S]-GTPγS bound-over-basal. Data are shown as means ± s.e.mean for five separate experiments carried out in duplicate. Agonist-stimulated [^{35}S]-GTPγS binding was significantly greater than basal binding ($P<0.05$) for both CHO-m1 and CHO-m3 membranes for all time-points.

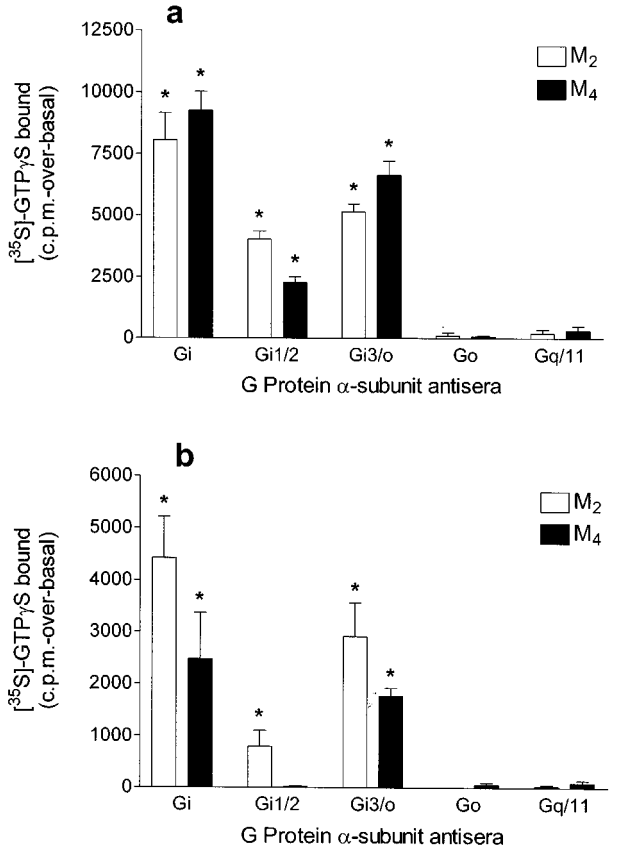

Figure 4 Quantitation of agonist-stimulated [^{35}S]-GTPγS binding to specific Gα protein subtypes in CHO-m2 and CHO-m4 cell membranes by immunoprecipitation with subtype-specific G protein antisera. Cell membranes prepared from CHO-m2 or CHO-m4 cells were incubated ([GDP] = 10 μM) in the absence or presence of methacholine (1 mM, panel a) or pilocarpine (1 mM, panel b) for 2 min at 30°C. Data are shown as means ± s.e.mean for five separate experiments carried out in duplicate. Statistically significant increases in [^{35}S]-GTPγS binding caused by agonist addition are indicated as $^*P<0.05$.

Further experiments were performed to assess the relative abilities of methacholine and the partial agonist pilocarpine to activate Gα proteins in CHO cell membranes expressing the different mACh receptor subtypes. Activation of M_2 and M_4 mACh receptors by either methacholine (Figure 4a) or pilocarpine (Figure 4b) resulted in significant stimulations of [^{35}S]-GTPγS binding to $G_i\alpha$, but not $G_o\alpha$ or $G_{q/11}\alpha$ proteins. The use of $G_{i1/i2}\alpha$- and $G_{i3/o}\alpha$-specific antisera revealed that, at M_2 receptors, both methacholine and pilocarpine caused significant activations of both $G_{i1/i2}\alpha$ and $G_{i3/o}\alpha$, whereas at M_4 receptors pilocarpine appeared only to activate $G_{i3/o}\alpha$. Comparisons of activation patterns across Gα protein subsets could, of course, be complicated by variable immunoprecipitating efficiencies of the various antisera used. However, there was a good quantitative correlation of [^{35}S]-GTPγS immunoprecipitated by the pan-$G_i\alpha$ antiserum compared to the sum of the radioactivities recovered associated with the $G_{i1/i2}\alpha$ and $G_{i3/o}\alpha$ antisera (Figure 4). Thus, we can propose with some confidence that there is a predominant activation of $G_{i3}\alpha$ by methacholine and pilocarpine in both CHO-m2 and -m4 cell membranes. Furthermore, the partial agonist pilocarpine appears to activate selectively $G_{i3}\alpha$, as $G_{i1/2}\alpha$ activation by this agonist is undetectable in CHO-m4 cell membranes (Figure 4b).

In contrast to the emerging picture for M_2 and M_4 subtypes, activation of M_1 and M_3 mACh receptors resulted in enhanced [^{35}S]-GTPγS binding not only to $G_{q/11}\alpha$, but also to $G_{i1/2}\alpha$ and $G_{i3/o}\alpha$ proteins (Figure 5). With respect to $G_{q/11}\alpha$-[^{35}S]-GTPγS binding, methacholine caused a much larger increase Gα activation in M_1, compared to M_3, mACh receptor-expressing cell membranes, despite the matched expression levels between the cell-lines. In contrast, methacholine stimulated comparable increases in $G_{i/o}\alpha$-[^{35}S]-GTPγS binding, although a significant activation of all $G_{i/o}$ proteins ($G_{q/11}\alpha$, $G_{i1/2}\alpha$, $G_{i3/o}\alpha$ and $G_o\alpha$) was only seen in CHO-m1 membranes (Figure 5).

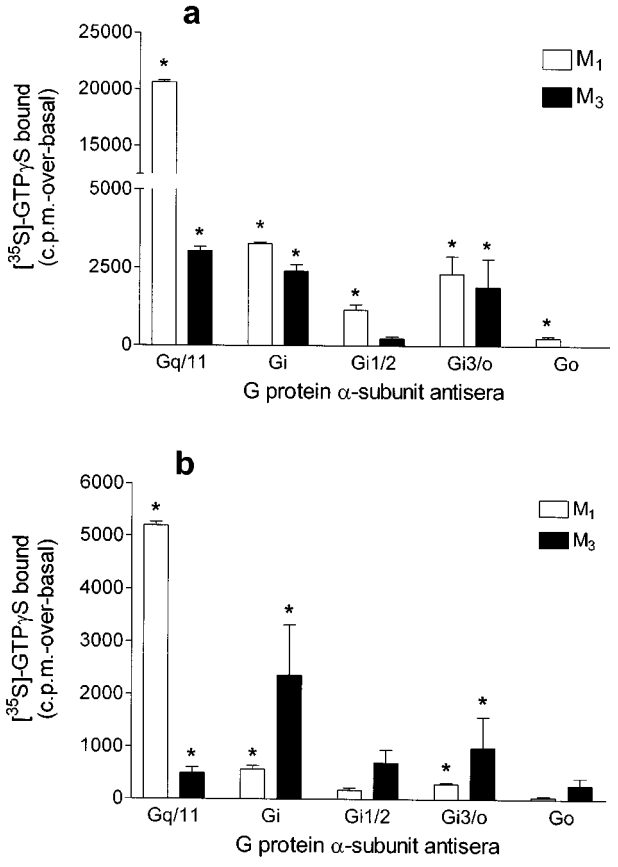

Figure 5 Quantitation of agonist-stimulated [^{35}S]-GTPγS binding to specific Gα protein subtypes in CHO-m1 and CHO-m3 cell membranes by immunoprecipitaion with subtype-specific G protein antisera. Cell membranes prepared from CHO-m1 or CHO-m3 cells were incubated ([GDP] = 1 μM) in the absence or presence of methacholine (1 mM, panel a) or pilocarpine (1 mM, panel b) for 2 min at 30°C. Data are shown as means ± s.e.mean for five separate experiments carried out in duplicate. Statistically significant increases in [^{35}S]-GTPγS binding caused by agonist addition are indicated as $^*P < 0.05$.

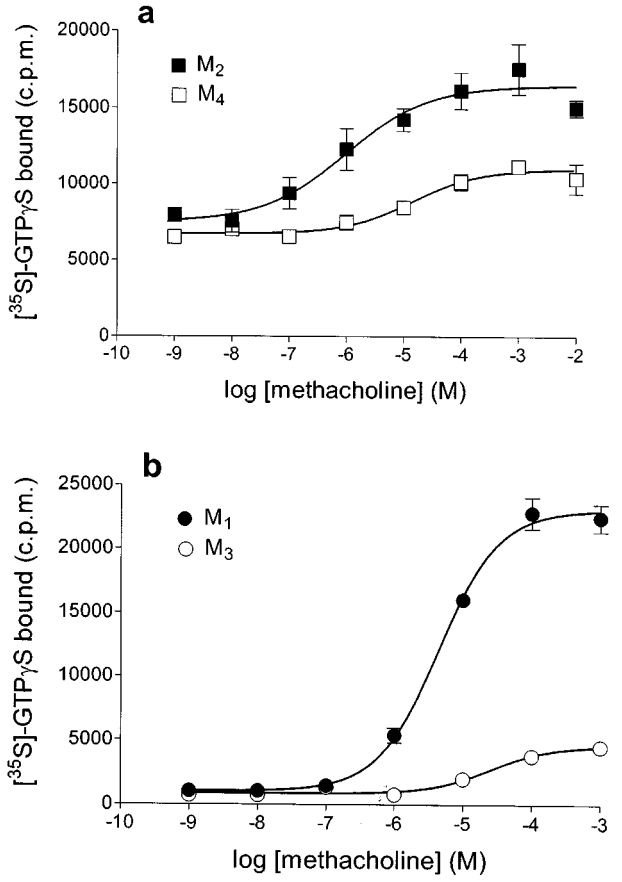

Figure 6 Concentration-dependencies of methacholine-stimulated [^{35}S]-GTPγS binding to specific Gα proteins in CHO-m1, -m2, -m3 and -m4 cell membranes. CHO cell membranes were incubated with methacholine (10^{-9}–10^{-3} M) under optimal assay conditions. (a) shows concentration-response curves for [^{35}S]-GTPγS-$G_{i3/o}$α binding in CHO-m2 and CHO-m4 cell membranes, while (b) shows similar data for [^{35}S]-GTPγS-$G_{q/11}$α binding in CHO-m1 and CHO-m3 cell membranes. Data are shown as means ± s.e.mean for 3–5 separate experiments carried out in duplicate.

Pilocarpine caused a robust and relatively specific activation of $G_{q/11}$α-[^{35}S]-GTPγS binding in CHO-m1 cell membranes, although the maximal stimulation represented only 20–25% of that observed with the full agonist methacholine (Figure 5a,b). Interestingly, while pilocarpine stimulated only a small increase in $G_{q/11}$α-[^{35}S]-GTPγS binding in CHO-m3 cell membranes (Figure 5b), this agonist caused a relatively greater activation of [^{35}S]-GTPγS binding to G_iα proteins. These data indicate that methacholine and pilocarpine activate different populations of G proteins following M_3 mACh receptor stimulation.

Finally, the concentration-dependent effects of methacholine at M_2 and M_4 mACh receptors were assessed for $G_{i3/o}$α activation, and at M_1 and M_3 mACh receptors for $G_{q/11}$α activation (Figure 6). In agreement with previous data, methacholine stimulated a greater maximal response in CHO-m2 cells; furthermore this agonist was more potent at stimulating $G_{i3/o}$α-[^{35}S]-GTPγS binding in CHO-m2, compared to CHO-m4 cell membranes (pEC_{50} (M), M_2-, 5.76 ± 0.15; M_4-, 4.71 ± 0.11 ($n = 3$) – Figure 6a). Similarly, methacholine caused a 4–5 fold greater maximal stimulation of $G_{q/11}$α-[^{35}S]-GTPγS binding in CHO-m1 compared to CHO-m3 cell membranes, additionally this agent was more potent at the former receptor subtype (pEC_{50} (M), M_1-, 5.37 ± 0.13; M_3-, 4.63 ± 0.05 ($n = 3$) – Figure 6b).

Discussion

Several previous studies have reported the binding of the stable GTP analogue [^{35}S]-GTPγS to membranes as a functional assay for mACh receptor subtypes (Hilf *et al.*, 1989; Lazareno & Birdsall, 1993; Lazareno *et al.*, 1993; Offermanns *et al.*, 1994; Burford *et al.*, 1995a). This technique exploits a property essential to receptor-G protein communication, the accelerated exchange of GDP for GTP (or GTPγS), to provide an assessment of agonist-mediated receptor activation independent of effector activity. Using this approach, an interaction of M_1 and M_3 mACh receptors with PTx-sensitive and -insensitive G proteins has been demonstrated in CHO and HEK293 cells (Lazareno & Birdsall, 1993; Offermanns *et al.*, 1994; Burford *et al.*, 1995a). The present data explore the ability of various mACh receptor subtypes to couple to specific Gα subunits by selectively

immunoprecipitating [^{35}S]-GTPγS-Gα complexes using specific antibodies. This approach has been evaluated and characterized previously for other receptor subtypes in rat brain membranes (Friedman *et al*., 1993; Wang *et al*., 1995) and SF9 cells (Barr *et al*., 1997) and it provides a robust evaluation of receptor-G protein coupling without having to resort to purification and reconstitution of signalling components. Moreover, it has allowed evaluation of the coupling potential of mACh receptor subtypes expressed at comparable levels in a common cell background. Our results demonstrate that each mACh receptor subtype is capable of generating a distinct Gα protein activation profile, through stimulation by agonists and partial agonists.

[^{35}S]-GTPγS binding to both $G_i\alpha$ and $G_{q/11}\alpha$ proteins was rapid, agonist-sensitive and dependent on GDP concentration. GDP is included in the assay to discourage receptor-independent exchange with [^{35}S]-GTPγS, and conditions were established to obtain optimal agonist stimulation for particular Gα subunits. In common with previous studies, we found that different GDP concentrations were necessary for optimal receptor-$G_{q/11}$ and -G_i protein coupling (Breivogel *et al*., 1998; DeLapp *et al*., 1999). In CHO-m1 and -m3 cell membranes (in the presence of 1 μM GDP), dramatic agonist-stimulated increases in [^{35}S]-GTPγS binding to $G_{q/11}\alpha$ were observed as early as 15 s after agonist addition and reached maximal levels between 2 and 5 min. In the case of $G_{i1\text{-}3}\alpha$ in CHO-m2 and -m4 cell membranes, a similar kinetic profile was observed following agonist addition, but agonist-independent [^{35}S]-GTPγS binding was substantially greater. The latter was reduced by more than 70% by PTx pretreatment of CHO-m2 and -m4 cells indicating a possible constitutive coupling of the M_2 and M_4 mACh receptor subtypes to $G_i\alpha$.

It is generally accepted that the binding of [^{35}S]-GTPγS is essentially irreversible in the presence of Mg^{2+} as demonstrated for purified G proteins (Higashijima *et al*., 1987). However, Hilf *et al*. (1992) have shown that in cardiac membranes [^{35}S]-GTPγS binding is reversible upon addition of unlabelled guanine nucleotides, and moreover that agonist-activated M_2 mACh receptors can stimulate such release. Furthermore, activated Gα-subunits may dissociate from the plasma membrane, possibly through a change in lipidation status. Translocation/uncoupling of $G_s\alpha$ (Ransnas & Insel, 1988) and $G_{q/11}\alpha$ (Arthur *et al*., 1999) has been reported, although others have failed to observe this phenomenon (Huang *et al*., 1999). In our own studies, we have been unable reproducibly to detect immunoprecipitated [^{35}S]-GTPγS binding in supernatants of stimulated membranes (Bundey R. & Nahorski S.R., unpublished data). Thus, whether the rapid saturation of [^{35}S]-GTPγS binding seen at both $G_i\alpha$ and $G_{q/11}\alpha$ in the present experiments reflects a new steady-state resulting from associative/dissociative interactions, or that it results from a true, rapid uncoupling of mACh receptors from the Gα proteins remains to be established.

The isolation of [^{35}S]-GTPγS specifically bound to Gα protein species following M_1 and M_3 mACh receptor activation has allowed a number of previously unappreciated differences between these receptor subtypes to be highlighted. In particular, the magnitudes of $G_{q/11}\alpha$-[^{35}S]-GTPγS binding after M_1 and M_3 mACh receptor stimulation differ substantially, with M_1 mACh receptor activation causing a 4–5 fold greater increase in maximal [^{35}S]-GTPγS binding and requiring 5 fold lower concentrations of methacholine to stimulate a half-maximal response. These data contrast with our previous assessments of phosphoinositide turnover stimulated in receptor density-matched CHO-m1 and CHO-m3 cells, where essentially similar responses were observed with respect to both the magnitude and concentration-dependency of Ins(1,4,5)P_3 accumulation (Burford *et al*., 1995b). One possible explanation for this discrepancy between M_1 and M_3 mACh receptor-G protein coupling and effector activation might be the result of a greater reliance of the latter receptor subtype on a greater convergence of $G_{q/11}\alpha$ and $G_{i/o}$-derived $\beta\gamma$-subunits to cause effector activation (see Exton, 1997).

Activation of M_2 and M_4 mACh receptor subtypes with methacholine caused marked increases in [^{35}S]-GTPγS binding to $G_{i1\text{-}3}\alpha$, $G_{i1/2}\alpha$ and $G_{i3/o}\alpha$, but not to $G_{q/11}\alpha$ or $G_o\alpha$. In the majority of experiments, M_2 mACh receptor activation elicited greater increases in [^{35}S]-GTPγS-G_i binding, and approximately 10 fold lower concentrations of this agonist were required to stimulate a half-maximal increase in $G_i\alpha$ activation compared to responses in CHO-m4 cell membranes. The activation of $G_{i1}\alpha$, $G_{i2}\alpha$ and $G_{i3}\alpha$ by the M_2 mACh receptor has been reported previously (Offermanns *et al*., 1994; Migeon *et al*., 1995). However, in the latter study, preferential coupling of the M_4 mACh receptor to $G_{i2}\alpha$ and $G_o\alpha$, with a limited interaction with $G_{i1}\alpha$ and $G_{i3}\alpha$, was observed in JEG-3 cells (Migeon *et al*., 1995).

Perhaps the most intriguing data to arise from the present study concern differential responses elicited by methacholine and pilocarpine, agonists that display very different intrinsic activities at mACh receptors (e.g. see Richards & Van Giersbergen, 1995). Using the $G_{i1\text{-}3}\alpha$ antibody, a maximal concentration of the partial agonist pilocarpine stimulated 49 and 30% of the GDP/[^{35}S]-GTPγS exchange stimulated by methacholine in CHO-m2 and -m4 membranes, respectively. Moreover, this partial agonist appears to be markedly more selective for activation of $G_{i3/o}\alpha$ compared to $G_{i1/2}\alpha$, with pilocarpine stimulating no discernible increase in [^{35}S]-GTPγS-$G_{i1/2}\alpha$ binding in CHO-m4 membranes. Although activation of both $G_{i1/2}\alpha$ and $G_{i3/o}\alpha$ was still observed in CHO-m2 membranes, pilocarpine was again less efficacious at $G_{i1/2}\alpha$ compared to $G_{i3/o}\alpha$ (19% versus 58% of MCh-stimulated responses, respectively). Taken together, these findings suggest that selective agonist-activated receptor-G protein coupling can occur at both M_2 and M_4 mACh receptors.

An agonist-specific pattern of Gα protein activation was also observed for the PLC-coupled M_1 and M_3 mACh receptors. Maximal pilocarpine promoted substantially less [^{35}S]-GTPγS-$G_{q/11}\alpha$ binding compared to the full agonist in CHO-m1 and -m3 membranes (25 and 16% of MCh-stimulated values, respectively). In contrast, while GDP/[^{35}S]-GTPγS exchange was also similarly reduced at $G_{i1\text{-}3}\alpha$ proteins in CHO-m1 cells (to 17% of the MCh-stimulated value), pilocarpine behaved as a full agonist at the M_3 mACh receptor with respect to $G_{i1\text{-}3}\alpha$ protein activation. This G protein activation profile suggests that pilocarpine may stimulate a cellular response that is dominated by $G_{i/o}$, relative to $G_{q/11}$ protein-mediated effector regulation.

It is noteworthy that some studies have reported that different mACh receptor agonists can stimulate different

(functional) responses in cells. Although a number of explanations may underlie such differences between full and partial agonists (e.g. functional antagonism by partial agonists of endogenous agonist-mediated effects), differences in G protein activation profiles between agonists may account for, or at least contribute to, such phenomena (Yule *et al*., 1993; Gurwitz *et al*., 1994). The selective activation of Gα proteins by pilocarpine through the different mACh receptor subtypes reported here lends support to the idea that some agonists may be capable of inducing relatively selective coupling of the receptor to specific sub-populations of G proteins. This 'agonist trafficking' of receptor signals allows divergent signalling through separate active receptor states, selectively promoting G protein coupling in response to activation by different agonists (Kenakin, 1995; 1997) and experimental evidence has accrued for a number of GPCRs including, PACAP receptors (Spengler *et al*., 1993), cannabinoid receptors (Glass & Northup, 1999), 5-$HT_{2A/2C}$ receptors (Berg *et al*., 1998) and α_1- and β-adrenoceptors (Perez *et al*., 1996; Zuscik *et al*., 1998). The present data suggest that mACh receptors may also exhibit this pharmacologically exploitable property.

Alternatively, if only one agonist-liganded receptor state were to exist, the differential coupling of the mACh receptor subtypes seen upon pilocarpine stimulation suggests that reductions in the activation of specific Gα species are due to reductions in stimulus strength. Thus, if a single receptor differentially couples to multiple G proteins, high efficacy agonists will activate multiple G proteins, whereas low efficacy agonists may activate only the most efficiently coupled G protein species available within a signalling complex (Neubig, 1998). If the loss of $G_{i1/2}\alpha$ stimulation after M_4 mACh receptor activation, and the reduction of $G_{q/11}\alpha$ signal after activation of the M_3 mACh receptor subtype are interpreted as changes in the stimulus strength, and not as evidence of agonist trafficking, it leads to a different conclusion. Thus, as M_2 mACh receptor coupling to both $G_{i1/2}\alpha$ and $G_{i3/o}\alpha$ is stimulated by either methacholine or pilocarpine, this receptor subtype appears to possess a greater intrinsic activity than the M_4 receptor. In the case of the M_1 and M_3 mACh receptors, it would appear that the M_3 subtype is more strongly coupled to G_i-like G proteins, whereas the M_1 receptor preferentially couples to the G_q-family.

Overall, we believe this direct approach to evaluating G protein activation by different agonists holds advantages over the analysis of different effector responses that could be influenced by 'crosstalk' between effectors. Whatever model underlies the agonist and mACh receptor subtype-dependent coupling to G protein subtypes observed here, they may provide insights into how the diversity of coupling can be pharmacologically exploited, and provide a basis for a better understanding of how multiple receptor subtypes can be differentially regulated by a single physiological agonist.

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