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An investigation of whether agonist-selective receptor conformations occur with respect to M_2 and M_4 muscarinic acetylcholine receptor signalling *via* $G_{i/o}$ and G_s proteins

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1 A range of muscarinic acetylcholine (mACh) receptor agonists (methacholine (MCh), oxotremorine-M (OXO-M), oxotremorine (OXO), arecoline (AREC), bethanechol (BETH), pilocarpine (PILO)) have been investigated with respect to their binding to, and activation of, M_2 and M_4 mACh receptors, recombinantly expressed in Chinese hamster ovary cells, to explore the possibility that these agonists may differentially affect mACh receptor–$G_{i/o}$ and -G_s coupling.

2 M_2/M_4 mACh receptor coupling to the adenylyl cyclase/cyclic AMP signalling pathway has been explored in intact cells. $G_{i/o}$-mediated negative coupling to adenylyl cyclase was explored functionally by assessing the ability of the mACh receptor agonists to inhibit forskolin-stimulated enzymic activity. Following pertussis toxin treatment (100 ng ml^{-1}, 18–20 h) to inactivate $G_{i/o}$ proteins, each agonist caused a G_s-mediated enhancement of forskolin-stimulated adenylyl cyclase activity.

3 At both M_2 and M_4 mACh receptors, all agonists tested were more potent in mediating $G_{i/o}$- *versus* G_s-coupled responses. This difference (determined as the pIC_{50} ($G_{i/o}$ coupling) minus pEC_{50} (G_s coupling) value) was greatest for AREC (65–75-fold) and least for BETH and PILO ($\leqslant$10-fold).

4 Using apparent binding affinities (pK_B), and potency (EC_{50}/IC_{50}) and responsiveness (E_{max}/I_{max}) estimates, relative efficacy (e_{rel}) values for each agonist with respect to M_2 and M_4 mACh receptor coupling to $G_{i/o}$- and G_s-mediated signalling were also calculated. While the e_{rel} values obtained for MCh and OXO-M in CHO-m2 cells were similar, OXO-M behaved as a 'super-agonist' at the M_4 mACh receptor giving greater e_{rel} values for both $G_{i/o}$ and G_s coupling relative to MCh.

5 The experimental data indicate that while interesting differences between agonists with respect to M_2/M_4 mACh receptor activation and receptor–$G_{i/o}$ and -G_s coupling can be discerned, no clear examples of agonist trafficking of signal have emerged.
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Abbreviations: AREC, arecoline; BETH, bethanechol; CHO, Chinese hamster ovary; GPCR, G protein-coupled receptor; mACh, muscarinic acetylcholine; MCh, methacholine; NMS, *N*-methylscopolamine; OXO, oxotremorine; OXO-M, oxotremorine-M; PILO, pilocarpine; PTx, pertussis toxin; TCA, trichloroacetic acid

Introduction

The muscarinic acetylcholine (mACh) receptor family can be functionally subdivided into M_1, M_3 and M_5 mACh receptors, which couple preferentially to $G_{q/11}$ proteins, and M_2 and M_4 mACh receptors, which couple preferentially to $G_{i/o}$ proteins (Caulfield & Birdsall, 1998). M_2 mACh receptors are widely expressed and have been implicated in a variety of cellular functions, including the control of heart rate and presynaptic modulation of neurotransmitter release (Caulfield, 1993). M_4 mACh receptors, while having a more limited distribution, appear to be the predominant and functionally important mACh receptor subtype in some brain regions (Hersch *et al*., 1994). It is common for cells to express more than one mACh receptor subtype, and currently available mACh receptor agonists and antagonists are insufficiently selective to allow pharmacological dissection of the roles of the subtypes (Caulfield, 1993; Caulfield & Birdsall, 1998). Therefore, the generation of knockout mice has played a key role in clarifying and increasing our understanding of mACh receptor function (Wess, 2004), and in particular, M_2 and M_4 mACh receptor actions in the central nervous system and peripheral tissues (Gomeza *et al*., 1999a, b; Stengel *et al*., 2000; Zhang *et al*., 2002).

Preferential coupling of M_2 and M_4 mACh receptors to $G_{i/o}$ proteins allows these receptors to mediate an array of effects on ion channels to modify K^+ and Ca^{2+} fluxes, as well as an inhibitory action on adenylyl cyclase activity (Caulfield, 1993). In addition, some reports have provided evidence for both M_2 (Vogel *et al*., 1995; Michal *et al*., 2001) and M_4 (Jones *et al*., 1991; Dittman *et al*., 1994) mACh receptors linking to adenylyl

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cyclase stimulatory pathways. A similar dual coupling has been observed for other G protein-coupled receptors (GPCRs) that preferentially link to $G_{i/o}$, including α_2-adrenoceptors (Eason *et al*., 1992) and cannabinoid receptors (Bonhaus *et al*., 1998; Glass & Northup, 1999). In the majority of cases, the mechanism has been shown to involve GPCR-G_s coupling, as $G_{i/o}$ protein inactivation by pertussis toxin (PTx) reveals a robust coupling to adenylyl cyclase *via* G_s (Jones *et al*., 1991; Eason & Liggett, 1995; Bonhaus *et al*., 1998).

Exploration of the $G_{i/o}/G_s$ coupling of α_2-adrenoceptors (Eason & Liggett, 1995; Brink *et al*., 2000) and $G_i/G_o/G_s$ coupling of cannabinoid receptors (Bonhaus *et al*., 1998; Glass & Northup, 1999) has led to the further observation that different agonists can display differing intrinsic efficacies with respect to the different signalling outcomes, a phenomenon referred to as 'agonist trafficking of receptor signal' (Kenakin, 1995). Clearly, the identification of agonists that stabilize different active receptor conformations to traffic the signal towards a specific, or subset of the potential signalling outcomes may provide a mechanism whereby selectivity with respect to signalling pathway activation can be achieved through an agonist action at single receptor subtype.

Previous studies have provided some evidence for the differential activation of $G_{i/o}$ proteins by M_2 and M_4 mACh receptors (Migeon & Nathanson, 1994; Migeon *et al*., 1995), and our own recent data have indicated differences in $G_{i/o}\alpha$ protein [^{35}S]GTPγS binding stimulated by methacholine (MCh) and pilocarpine (PILO) in membranes prepared from CHO-m2 and CHO-m4 cells (Akam *et al*., 2001). Therefore, in the present study, we have examined whether a range of commonly used and structurally diverse mACh receptor agonists exhibit differences in $G_{i/o}$ *versus* G_s coupling in intact Chinese hamster ovary (CHO) cells recombinantly expressing M_2 or M_4 mACh receptors.

Methods

Materials

Cell culture reagents were obtained from GIBCO-Invitrogen (Paisley, Scotland). Arecoline (AREC), bethanechol (BETH), MCh, oxotremorine (OXO), oxotremorine-M (OXO-M), PILO, atropine, forskolin, cyclic AMP and PTx were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset). *N*-methyl-[^{3}H]-scopolamine (NMS) (2.2–3.1 TBq mmol^{-1}) and [2,8–^{3}H]cyclic AMP (1.1–1.85 TBq mmol^{-1}) were from Amersham Biosciences UK Ltd (Chalfont St Giles, Bucks). All other reagents were of analytical grade.

Cell culture

CHO cells stably expressing recombinant M_2 or M_4 mACh receptors (CHO-m2 and CHO-m4 cell lines, respectively) were originally obtained from Professor Noel Buckley (then at NIMR, Mill Hill, London). CHO cell lines were grown in minimum essential medium-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 a humidified atmosphere of 5% CO_2 : air. For PTx treatment, cells were plated onto 24 multiwell plates and 100 ng ml^{-1} PTx was added 18–20 h prior to experimentation.

[^{3}H]NMS binding

All binding experiments were carried out on *intact* CHO-m2 and CHO-m4 cell monolayers plated out in 24 multiwells. To assess M_2/M_4 mACh receptor density (B_{max}) and affinity (K_D), various dilutions of [^{3}H]NMS (0.05–5 nM) in Krebs–Henseleit buffer (KHB (in mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; $CaCl_2$, 1.3; HEPES, 10; glucose, 11; pH 7.4 after equilibration with O_2/CO_2 95 : 5) were added to cells in the absence or presence of 1 μM atropine for 60 min at 37°C. Bound and free radioligand was determined by transfer of plates to an icebath and rapid washing of multiwells with 4 × 1 ml ice-cold KHB. Finally, cell monolayers were dissolved in 0.1 M NaOH and radioactivity was determined by scintillation counting.

To assess agonist binding affinities, CHO-m2 and CHO-m4 cell monolayers were incubated with appropriate concentration ranges of MCh, OXO, OXO-M, AREC, BETH or PILO in the presence of approximately 0.3 nM [^{3}H]NMS for 4 h at 4°C. The low temperature ensures that agonist-induced receptor internalization does not occur and the longer time period is to ensure that equilibrium binding is achieved. At the end of the incubation period, bound and free ligand were separated and quantified as described above.

Cell suspension experiments

Flasks of CHO-m2 or CHO-m4 cell monolayers approaching confluence were briefly rinsed with HBS-EDTA (10 mM HEPES, 0.9% NaCl, 0.2% EDTA, pH 7.4) and then approximately 20 ml HBS-EDTA was added to each flask to lift the cells. After 3–5 min at room temperature, suspended cells were centrifuged (200 × *g* for 4 min) and the pellet resuspended in KHB. Following a further centrifugation (200 × *g* for 4 min), cells were resuspended in KHB at a concentration of 1–2 mg protein ml^{-1}. The cell suspension (50 μl; approx. 5×10^5 cells) was added to 100 μl KHB containing appropriate agonist additions, incubated for 10 min at 37°C, and then forskolin was added to a final concentration of 10 μM for a further 5 min. Reactions were terminated by addition of 200 μl ice-cold 1 M trichloroacetic acid (TCA) and transfer to an icebath. Acid extraction and cyclic AMP determinations were carried out as described below.

Cell monolayer experiments

Confluent CHO-m2 and -m4 cell monolayers in 24 multiwell plates (approx. 5×10^5 cells) were briefly rinsed with KHB and preincubated at 37°C for 15–30 min in KHB. Unless otherwise stated, antagonist and agonist additions were made 30 and 10 min, respectively, before forskolin (10 μM) addition. In each experiment, a maximal response for the reference agonist (MCh, 100 μM in control cells and MCh, 300 μM in PTx-treated cells) was obtained. Incubations were continued for 10 min before rapid aspiration and addition of ice-cold 0.5 M TCA and transferal to an icebath. Extracts were neutralized using the freon/tri-*n*-octylamine method and cyclic AMP concentration was determined exactly as described previously (Brown *et al*., 1971).

Data analysis

All data are presented as means ± s.e.mean for the indicated number of separate experiments performed with the indicated individual experiment replication. Radioligand binding data and agonist concentration–effect curves were analysed using a commercially available program (GraphPad Prism version 3.0; GraphPad Software, San Diego, CA, U.S.A.). IC_{50} values obtained for agonist displacement of [^{3}H]NMS binding were converted to K_B values using the method of Cheng & Prusoff (1973). Where responses were biphasic, data were fitted to the sum of two logistic equations as described previously (Hornigold *et al*., 2003).

Relative efficacy (e_{rel}) was calculated according to the equation of Ehlert (1985):

$$e_{rel} = 0.5E_{max}/E_{max\text{-}A}(1 + K_B/EC_{50})$$

where E_{max} is the maximal response for a particular agonist, $E_{max\text{-}A}$ is the maximal response of the system (defined as the maximal response to MCh), K_B is the apparent affinity of the agonist obtained from radioligand binding/[^{3}H]NMS displacement experiments, and EC_{50} is the half maximal concentration of agonist needed to either inhibit or enhance forskolin-stimulated adenylyl cyclase activity in control and PTx pretreated CHO-m2 and -m4 cells, respectively. Thus, e_{rel} values were obtained for agonists with respect to $G_{i/o}$ or G_s coupling in the mACh receptor-expressing cell lines. The e_{rel} value describes the relative ability of an agonist to activate a particular signalling pathway in a specified test system and the equation used to calculate this value is based on two assumptions: (i) that the functional response (inhibition/stimulation of the forskolin-cyclic AMP response) is mediated through a single receptor type and that the binding of agonist to the receptor follows the law of mass action; and (ii) that the binding constant K_B accurately reflects the true affinity value of the agonist for the receptor under the conditions of the functional assay (Quock *et al*., 1999). Note that e_{rel} values can be less than one and also reach very high values. An e_{rel} of 1 is indicative of an agonist that is just capable of eliciting a full maximal response and exhibits an EC_{50} value approx. equal to the K_D value.

Statistical differences between data sets were assessed using either Student's *t*-test, or by one-way analysis of variance followed by Bonferroni's multiple-comparison testing at $P<0.05$ using GraphPad Prism Software. For agonist ranking orders, the symbol '>' indicates a statistically significant difference between values, while '≈' is used where significance is not reached.

Results

Receptor expression in CHO-m2 and CHO-m4 cells

[^{3}H]NMS saturation binding analysis in adherent cell monolayers demonstrated that CHO-m2 cells possess an M_2 mACh receptor density of 1040 ± 104 fmol mg^{-1} protein (~125,000 receptors per cell). In contrast, CHO-m4 cells expressed approx. 50% more M_4 mACh receptors (1598 ± 145 fmol mg^{-1} protein; ~190,000 receptors per cell).

Comparison of agonist effects on adherent and nonadherent CHO-m2 and CHO-m4 cells

One striking feature of previous studies is that some of the most pronounced examples of dual M_2/M_4 mACh receptor coupling to $G_{i/o}$ and G_s have been observed in cells in suspension or broken cell preparations (Vogel *et al*., 1995; Michal *et al*., 2001). Therefore, we initially compared the effects of MCh and PILO on forskolin-stimulated cyclic AMP accumulation in suspensions or adherent monolayers of CHO-m2 and CHO-m4 cells. In both adherent and nonadherent cells, forskolin (10 μM) stimulated marked (>40-fold) increases in cyclic AMP accumulation in CHO-m2 and -m4 cells (Table 1). Slightly (approx. 20%) greater responses to forskolin were observed in CHO-m2 compared to CHO-m4 cells, and for both cell lines nonadherent cell responses to forskolin were ~75% of those of adherent cells (Table 1).

To assess whether adherence to a substratum influences mACh receptor–$G_{i/o}$/G_s coupling, concentration–effect curves were generated for the mACh receptor agonists MCh and PILO in CHO-m2 and -m4 cells. Evidence for a biphasic cyclic AMP response was only found for MCh in nonadherent CHO-m4 cells (Figure 1a). Analysis of these more complex curves yielded K_H and K_L values of 83 nM (62–105 nM; 95% confidence limits) and 7.4 μM (4.1–10.8 μM), respectively. In

Table 1 M_2 and M_4 mACh receptor densities, and the effects of methacholine (MCh) and pilocarpine (PILO) on forskolin-stimulated cyclic AMP responses in adherent and nonadherent CHO-m2 and CHO-m4 cells

	CHO-m2		*CHO-m4*	
	Adherent	*Nonadherent*	*Adherent*	*Nonadherent*
[^{3}H]NMS saturation binding				
B_{max} (fmol mg^{-1} protein)	1040 ± 104		1598 ± 145	
K_D (nM)	0.30 ± 0.01		0.31 ± 0.01	
Basal and forskolin-stimulated cyclic AMP accumulations (pmol mg^{-1} protein)				
Basal	5.7 ± 1.6	7.1 ± 1.6	5.4 ± 0.7	10.2 ± 1.8
+ Forskolin	651.9 ± 20.0	467.3 ± 28.1	532.4 ± 19.2	383.3 ± 16.7
mACh receptor agonist inhibitory potencies (pIC_{50} values)				
MCh	6.63 ± 0.10	6.87 ± 0.05	6.96 ± 0.10	Biphasic
PILO	4.78 ± 0.27	4.94 ± 0.03	5.00 ± 0.08	5.61 ± 0.02

All data are shown as means ± s.e.mean for three experiments (saturation binding) or four experiments (cyclic AMP experiments).

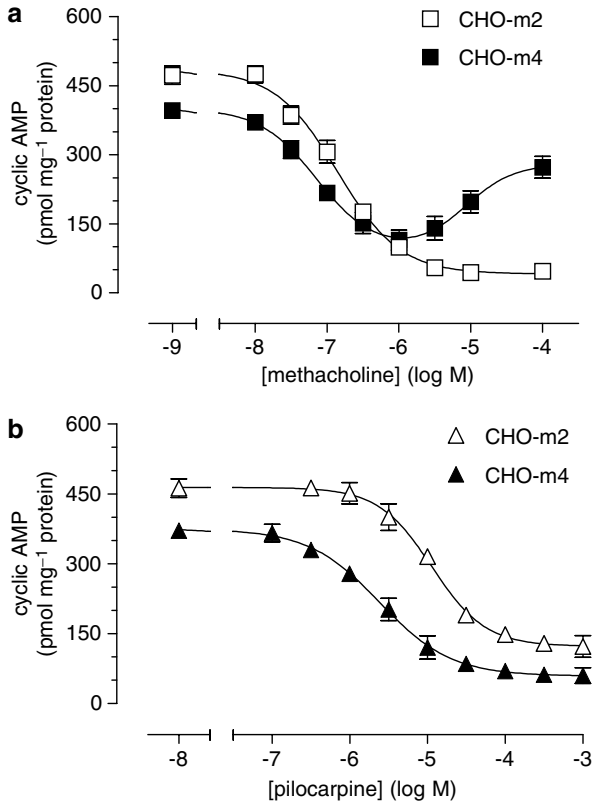

Figure 1 Concentration-dependent effects of MCh and PILO on forskolin-stimulated cyclic AMP accumulations in CHO-m2 and CHO-m4 cells in suspension. Cell suspensions of CHO-m2 (open symbols) and CHO-m4 (closed symbols) were preincubated with the indicated concentrations of MCh (a) or PILO (b) for 10 min before addition of forskolin (10 μM). After 10 min, incubations were terminated and cyclic AMP levels determined as described in Methods. Data points are shown for means±s.e.mean for four experiments each performed in duplicate.

contrast, MCh caused only a monophasic, inhibitory response in CHO-m2 cell suspensions, and the partial agonist PILO similarly caused only inhibitory effects in both CHO-m2 and -m4 cell suspensions (Figure 1b). In both suspended and adherent CHO-m2 cells, maximally effective concentrations of MCh caused >90% inhibitions of forskolin-stimulated cyclic AMP accumulation, whereas the maximal inhibitory effect of PILO was greater in suspended cells (75% of maximal MCh inhibition) compared to adherent cells (55% of MCh maximum).

All responses evoked by MCh or PILO in CHO-m2 and -m4 cells were antagonized by atropine. This mACh receptor antagonist was without effect on either basal or forskolin-stimulated cyclic AMP responses in either cell line (data not shown). To facilitate further analysis, we chose to study agonist effects in adherent CHO-m2 and CHO-m4 cells.

Estimation of mACh receptor apparent agonist affinities for M_2 and M_4 mACh receptors

To determine binding affinities for the six selected agonists, intact cell monolayers were incubated with an approx. K_D concentration of [^{3}H]NMS in the presence of different agonist concentrations at 4°C for 4 h. Under these conditions, equilibrium binding could be achieved without depletion of ligand through internalization (see Thompson & Fisher, 1990), and accurate estimates of equilibrium binding constants (K_B) for each agonist at the M_2 and M_4 mACh receptor were obtained (Table 2). In all cases, fitted agonist displacement isotherms had slope factors not significantly different to unity (data not shown). Preliminary experiments were performed to assess the effect of temperature on apparent agonist affinity estimates for two selected agonists (MCh and PILO) in CHO-m2 cells. Using a 60 min incubation at 37°C, only small (⩽2-fold) differences in K_B values were obtained, with the apparent affinity of MCh increasing and PILO decreasing. Thus, in intact cells, the effect of temperature on agonist K_B estimates appears to be less than has been reported previously in tissue membrane studies (Gies *et al*., 1986).

Table 2 Binding data, cyclic AMP responses and $G_{i/o}/G_s$ coupling efficiencies for six muscarinic acetylcholine (mach) receptor agonists assessed in intact CHO-m2 and CHO-m4 cells preincubated in the absence or presence of PTx

Agonist	*Binding* pK_B	*Inhibition of Fk-stimulated AC (−PTx)* pIC_{50}	I_{max}	*Enhancement of Fk- stimulated AC (+PTx)* pEC_{50}	E_{max}
CHO-m2 cells					
MCh	4.74±0.04	6.64±0.10	100	5.37±0.08	100
OXO-M	5.30±0.11	7.14±0.04	101.7±0.4	5.64±0.11	95.5±7.9
AREC	4.40±0.14	5.96±0.02	97.5±3.0	4.15±0.07	95.1±5.5
BETH	3.59±0.03	4.79±0.12	101.2±1.2	3.94±0.08	93.2±3.6
OXO	5.74±0.07	6.88±0.08	98.9±2.3	5.37±0.12	70.2±5.3*
PILO	3.70±0.27	4.58±0.05	54.7±5.7*	3.71±0.05	9.7±5.1*
CHO-m4 cells					
MCh	4.68±0.09	6.96±0.10	100	5.77±0.10	100
OXO-M	5.50±0.04	8.20±0.13	99.7±4.2	6.81±0.10	103.0±7.1
AREC	4.77±0.03	6.79±0.08	97.8±3.9	5.00±0.06	96.3±1.5
BETH	3.34±0.02	5.32±0.06	103.0±1.1	4.34±0.05	69.6±4.6*
OXO	6.01±0.01	7.70±0.09	103.1±1.0	6.30±0.05	97.5±3.8
PILO	4.07±0.02	5.00±0.08	90.5±7.1	3.96±0.10	35.3±3.1*

All data are presented as means±s.e.mean, the number of independent experiments performed in duplicate are given in the legends to Figures 2 and 3. Statistical analyses of potency ranking orders are presented in the main text. For I_{max}/E_{max} values, statistically significant differences from the MCh response are indicated as $*P<0.05$.

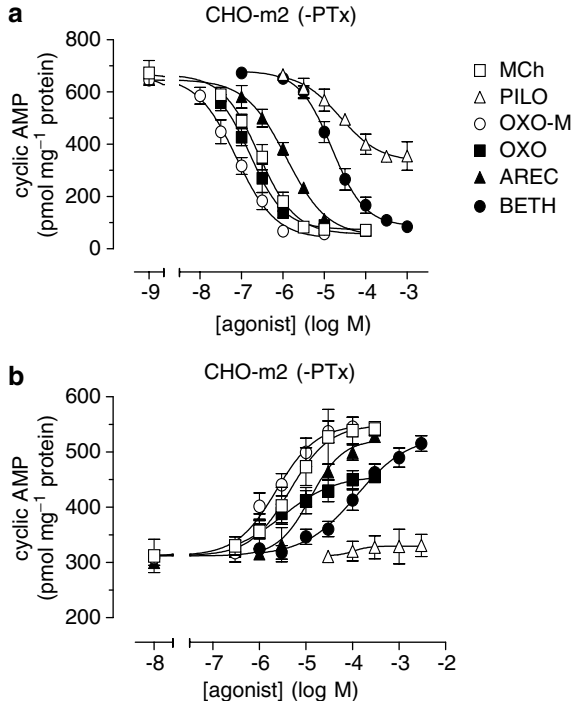

Figure 2 Concentration-dependent effects of different mACh receptor agonists on forskolin-stimulated cyclic AMP accumulations in CHO-m2 cells preincubated in the absence or presence of PTx. Subconfluent CHO-m2 cell monolayers were incubated in the absence (a) or presence (b) of PTx (100 ng ml^{-1}) for 20–24 h. The indicated concentrations of MCh, OXO-M, OXO, AREC, BETH or PILO were added 10 min prior to forskolin (10 μM) addition. After 10 min, incubations were terminated and cyclic AMP levels determined as described in Methods. Data points are shown for means ± s.e.mean for either three (−PTx, OXO-M, OXO, AREC, PILO) or four (−PTx, MCh, BETH; +PTx, all agonists) experiments each performed in duplicate.

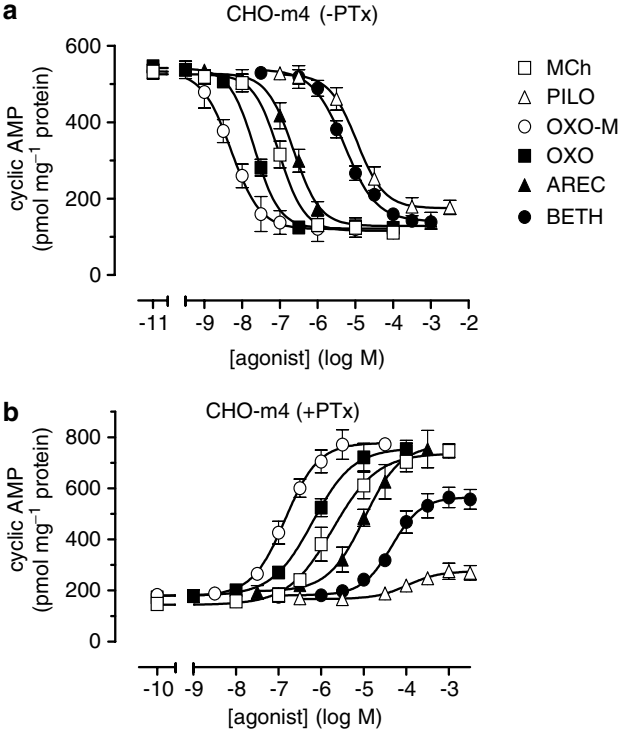

Figure 3 Concentration-dependent effects of different mACh receptor agonists on forskolin-stimulated cyclic AMP accumulations in CHO-m4 cells preincubated in the absence or presence of PTx. Subconfluent CHO-m4 cell monolayers were incubated in the absence (a) or presence (b) of PTx (100 ng ml^{-1}) for 20–24 h. The indicated concentrations of MCh, OXO-M, OXO, AREC, BETH or PILO were added 10 min prior to forskolin (10 μM) addition. After 10 min, incubations were terminated and cyclic AMP levels determined as described in Methods. Data points are shown for means ± s.e.mean for at least three experiments each performed in duplicate.

For both cell lines, OXO and OXO-M had the highest affinity and PILO and BETH the lowest. For OXO-M, OXO, AREC and PILO, K_B values at M_2 receptors were 2–3-fold higher compared to values obtained for M_4 mACh receptor binding. In contrast, the M_2 receptor K_B value was approx. 2-fold lower for BETH. The apparent binding affinity ranking order varied only slightly at the two receptors: at M_2, it was OXO > OXO-M > MCh ≈ AREC ≈ PILO ≈ BETH (note MCh > PILO/BETH; AREC > BETH), while for M_4 it was OXO > OXO-M > AREC ≈ MCh > PILO > BETH (see Table 2).

Analysis of receptor–$G_{i/o}$ coupling in CHO-m2 and CHO-m4 cells

In non-PTx-treated cells, each agonist caused a concentration-dependent inhibition of forskolin-stimulated cyclic AMP accumulations. OXO, OXO-M, MCh AREC and BETH all caused similar maximal inhibitory effects, which for MCh were 92 ± 2% in CHO-m2 cells and 80 ± 1% in CHO-m4 cells. In CHO-m2 cells, PILO was clearly a partial agonist with respect to this response (Figure 2a), whereas the maximal inhibition by PILO approached that caused by the other agonists in CHO-m4 cells (Figure 3a). The potency ranking order for agonist-mediated inhibition of cyclic AMP accumulation differed only slightly between the two cell lines: in CHO-m2, it was OXO-M ≈ OXO ≈ MCh > AREC > BETH ≈ PILO (note OXO-M > MCh), whereas in CHO-m4 cells, it was OXO-M > OXO > MCh ≈ AREC > BETH ≈ PILO.

Analysis of receptor–G_s coupling in CHO-m2 and CHO-m4 cells

To reveal M_2/M_4 mACh receptor–G_s coupling, cells were pretreated with PTx to uncouple receptor–$G_{i/o}$ signalling. PTx pretreatment *per se* decreased cyclic AMP accumulations stimulated by 10 μM forskolin by 54 ± 3 and 61 ± 5% compared to responses in nontoxin-treated CHO-m2 and -m4 cells, respectively (see Figures 2 and 3). MCh caused concentration-dependent enhancements of forskolin-stimulated cyclic AMP accumulation in both cell lines: in CHO-m2 cells, the maximal enhancement was 2.1 ± 0.3-fold, while in CHO-m4 cells a more marked 4.7 ± 0.8-fold enhancement was observed (see Figures 2b and 3b).

In CHO-m2 cells, OXO-M, MCh, AREC and BETH all caused similar maximal responses, whereas OXO (70%) and PILO (10%) were partial agonists with respect to the enhancement of forskolin-stimulated cyclic AMP accumula-

Table 3 $pIC_{50}-pEC_{50}$ differences and relative efficacy (e_{rel}) values for six mACh receptor agonists assessed in intact CHO-m2 and CHO-m4 cells preincubated in the absence or presence of PTx

Agonist	*$pIC_{50}-pEC_{50}$*	*e_{rel} ($G_{i/o}$)*	*e_{rel} (G_s)*
CHO-m2			
MCh	1.27±0.06	40.2	2.63
OXO-M	1.50±0.08	35.7	1.52
AREC	1.87±0.06*	18.2	0.74
BETH	0.86±0.10**	8.52	1.51
OXO	1.51±0.09	7.32	0.50
PILO	0.92±0.10**	2.35	0.10
CHO-m4			
MCh	1.19±0.06	95.8	6.65
OXO-M	1.39±0.08	250.3	11.0
AREC	1.81±0.10*	51.7	1.30
BETH	0.97±0.10***	49.7	3.83
OXO	1.40±0.05	25.8	1.43
PILO	1.04±0.03***	4.30	0.31

The ($pIC_{50}-pEC_{50}$) differences were calculated for individual experiments and are shown here as means±s.e.mean for data obtained in three or four separate experiments. Statistical analysis of these data revealed that the ($pIC_{50}-pEC_{50}$) difference obtained for AREC in both CHO-m2 and CHO-m4 cells differed significantly from values obtained for the other agonists, $*P<0.05$. In addition, at the M_2 mACh receptor, differences for MCh, OXO-M and OXO were significantly greater than for BETH and PILO, $**P<0.05$); while at the M_4 mACh receptor, ($pIC_{50}-pEC_{50}$) differences for OXO-M and OXO were significantly greater than for BETH and PILO, $***P<0.05$), with the value for MCh being significantly different to AREC only. Relative efficacy (e_{rel}) values (Ehlert, 1985) were calculated as described in the Methods section using the pK_B, pIC_{50} and I_{max} data (e_{rel} ($G_{i/o}$)) or the pK_B, pEC_{50} and E_{max} data (e_{rel} (G_s)) shown in Table 2.

tion (Figure 2b; Table 2). In CHO-m4 cells, all agonists except BETH (70%) and PILO (35%) caused maximal responses (Figure 3b; Table 2). The fact that BETH elicits only a partial response (approx. 70% relative to the reference agonist MCh) in CHO-m4 cells is highly surprising given that the pEC_{50} value for this compound with respect to the G_s-mediated response is 10-fold lower that its apparent binding affinity (pK_B). Indeed, the pEC_{50}/pK_B difference is greater than observed for BETH in CHO-m2 cells where a response not significantly different to the E_{max} for MCh is achieved (Table 2). Further work established that prolonging the [^{3}H]NMS/BETH incubation time (18 h, 4°C), or determining pK_B values for BETH in PTx pretreated CHO-m4 cells yielded essentially similar pK_B values to that reported in Table 2. Therefore, at present, we can offer no explanation for the anomalous pharmacological behaviour of BETH with respect to M_4–G_s coupling.

The potency ranking order for stimulation of cyclic AMP accumulation was only slightly different between the two cell lines: in CHO-m2 cells, it was OXO-M≈OXO≈MCh> AREC≈BETH≈PILO (note AREC>PILO); whereas in CHO-m4 cells it was OXO-M>OXO>MCh>AREC>BETH ≈PILO.

Analysis of $G_{i/o}/G_s$ coupling differences at M_2 and M_4 mACh receptors

Comparison of IC_{50} and EC_{50} values provides a simple index of the $G_{i/o}$ *versus* G_s coupling potencies. As can be seen from Table 3, the difference values ($pIC_{50}-pEC_{50}$) were greatest for AREC in both CHO-m2 or -m4 cells. Thus, the difference in the concentrations of AREC required to cause 50% inhibitory (receptor–$G_{i/o}$ coupling) *versus* 50% stimulation (receptor–G_s coupling) of adenylyl cyclase activity (65–75-fold) is significantly greater than that for any of the other mACh receptor agonists studied. At the other end of the spectrum, BETH and

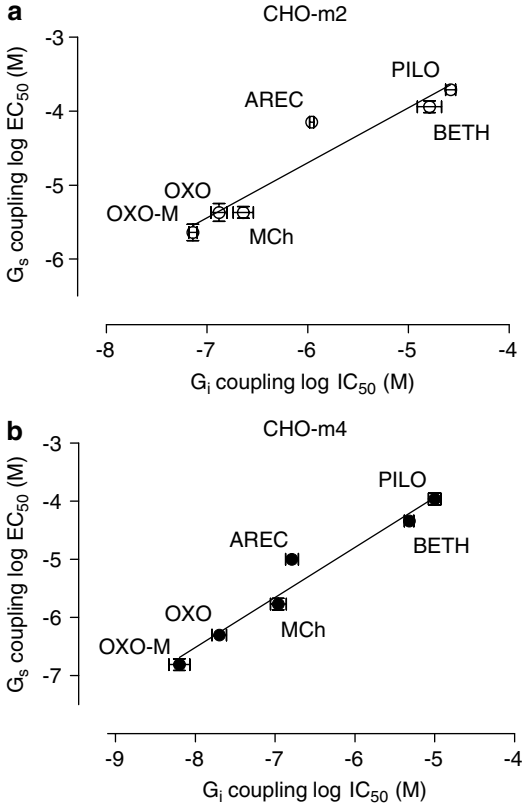

Figure 4 Correlation of IC_{50} and EC_{50} values obtained for inhibition or stimulation of forskolin-stimulated cyclic AMP accumulation by each agonist in CHO-m2 (a) and CHO-m4 (b) cells. Log IC_{50} (obtained in the absence of PTx) and log EC_{50} (obtained in PTx pretreated cells) values (see Table 2) for each of the six mACh receptor agonists studied are plotted. The line in each panel represents the best-fit linear regression for the data (CHO-m2, equation of line $y=0.744x–0.235$; goodness of fit $r^2=0.906$; CHO-m4, equation of line $y=0.860x–0.427$; goodness of fit $r^2=0.955$).

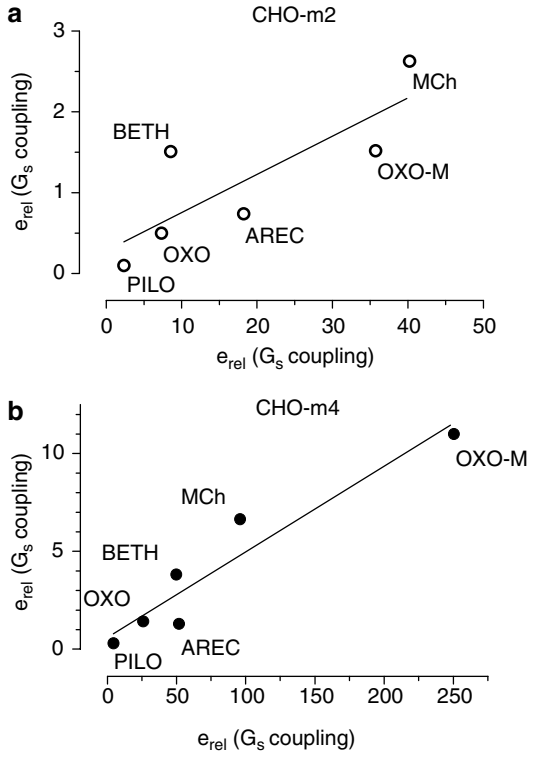

Figure 5 Correlation of relative efficacy (e_{rel}) values with respect to $G_{i/o}$ and G_s coupling in CHO-m2 (a) and CHO-m4 (b) cells. For each agonist, e_{rel} values were calculated using values obtained from [^{3}H]NMS binding and inhibitory (−PTx) or stimulatory (+PTx) effects on adenylyl cyclase activity (see Table 2). The line in each panel represents the best-fit linear regression for the data obtained for the six mACh receptor agonists (CHO-m2, equation of line $y = 0.047x + 0.282$; goodness of fit $r^2 = 0.675$; CHO-m4, equation of line $y = 0.044x + 0.604$; goodness of fit $r^2 = 0.909$).

PILO showed the smallest differences ($\leqslant$10-fold; Table 3). The relationship between the IC_{50} and EC_{50} values for the six agonists is also shown graphically in Figure 4, where it can be seen that AREC lies off the regression lines that best fit the complete datasets for all agonists in both CHO-m2 and CHO-m4 cells.

Relative efficacy as an index of differences in binding, potency and responsiveness between agonists

The relationship between agonist-receptor occupancy and the adenylyl cyclase functional responses has been assessed using the method of Ehlert (1985) by calculating relative efficacy (e_{rel}) values for each agonist under normal and PTx-treated experimental conditions (Table 3). Differences between mACh receptor agonists with respect to e_{rel} ($G_{i/o}$) and e_{rel} (G_s) values obtained in CHO-m2 and CHO-m4 cells are presented graphically in Figure 5.

With respect to agonist responses for receptor–$G_{i/o}$ coupling, recognized full agonists at the M_2 mACh receptor, such as OXO-M (40.2) and MCh (35.7), gave high e_{rel} values (Table 3), whereas this value was lower for AREC (18.2), BETH (8.52), OXO (7.32) and PILO (2.35). At the M_4 mACh receptor, e_{rel} values showed some interesting differences for particular agonists. Thus, OXO-M (250.3) appeared especially efficient in converting receptor occupation into a response relative to the reference agonist MCh (95.8), while the other four agonists showed a range of e_{rel} values (AREC (51.7), BETH (49.7), OXO (25.8), PILO (4.3)) (Table 3; Figure 5).

With respect to agonist responses for receptor–G_s coupling, MCh (2.63), OXO-M (1.52) and BETH (1.51) gave e_{rel} values greater than unity at the M_2 mACh receptor, whereas this ratio was lower for AREC (0.74) OXO (0.50) and PILO (0.10). For the M_4 mACh receptor, OXO-M (11.0) again gave a higher e_{rel} value than MCh (6.65), and the low relative efficacy for AREC (1.30) relative to BETH (3.83) highlighted both the anomalous behaviour of BETH (the contradictory finding of a receptor reserve for a partial agonist with respect to M_4–G_s coupling – see above) and the relatively poor ability of AREC to induce receptor–G_s coupling compared to receptor–$G_{i/o}$ coupling (Table 3; Figure 5).

Discussion

The present study was designed to assess whether mACh receptor agonists can selectively induce M_2/M_4 mACh receptor conformations that favour signalling *via* a specific subset of downstream pathways (i.e. 'agonist trafficking of receptor signal' (Kenakin, 1995)). Further, the experimental design has allowed differences in M_2 *versus* M_4 mACh receptor agonist pharmacology to be studied in a common cell background.

Since the concept of agonist-induced conformational pleiotropy was first formalized (Kenakin, 1995), a body of experimental evidence has accumulated that supports the concept (see Kenakin, 2003). Detailed pharmacological studies have demonstrated marked agonist-specific potency differences in directing receptor-coupling to distinct G proteins (Berg *et al*., 1998; Bonhaus *et al*., 1998; Glass & Northup, 1999; Brink *et al*., 2000), and biochemical studies have begun to provide a mechanistic basis for this pharmacological phenomenon. Thus, mutational studies have indicated that different receptor structural determinants may be involved in coupling to different G protein/effector pathways (Spengler *et al*., 1993; Eason & Liggett, 1995; Perez *et al*., 1996), additionally, it has been shown that mechanisms such as receptor phosphorylation may 'switch' preferential receptor–G protein-coupling (Daaka *et al*., 1997; Francesconi & Duvoisin, 2000), and methods such as fluorescence lifetime spectroscopy are beginning to allow ligand-induced conformational changes in GPCRs to be visualized (Ghanouni *et al*., 2001; Kobilka, 2002).

With respect to mACh receptors, a number of studies have demonstrated that both $M_1/M_3/M_5$ and M_2/M_4 mACh receptor subfamilies can couple to multiple G protein/effector pathways, and we have previously presented evidence for agonist-specific patterns of $G_{q/11}$ and $G_{i/o}$ protein activation in membranes prepared from CHO-m1 and -m3 cells using a [^{35}S]GTPγS/Gα-specific immunoprecipitation strategy (Akam *et al*., 2001). In the same paper, we provided some preliminary evidence for differences in how mACh receptor agonists may differentially promote $G_{i1}\alpha/G_{i2}\alpha/G_{i3}\alpha$-[^{35}S]GTP$\gamma$S binding. Interestingly, Kukkonen *et al*. (2001) have provided evidence for agonist trafficking of signal in HEL 92.1.7 cells that express the α_{2A}-adrenoceptor. This adrenoceptor subtype links to both adenylyl cyclase inhibition and Ca^{2+} elevation *via* PTx-sensitive G proteins in this cell type and investigation of a range of agonists has provided strong evidence for agonist-

dependent receptor-active states that differ markedly in their abilities to couple to the two responses. Contrary to these findings, a recent study using the baculovirus/Sf9 insect cell system to coexpress M_2 mACh receptors with either G_i ($G_{i1}\alpha\beta_1\gamma_2$) or G_o ($G_{o1}\alpha\beta_1\gamma_2$) heterotrimers failed to find any significant affinity or efficacy differences for M_2–G_i *versus* M_2–G_o coupling (Uustare *et al*., 2004). In the present study, we have utilized intact mammalian cells recombinantly expressing either M_2 or M_4 mACh receptors and a simple experimental approach to investigate whether different agonists can activate M_2/M_4 mACh receptors to promote G_i-adenylyl cyclase *versus* G_s-adenylyl cyclase interactions.

Initial studies addressed the issue of whether dual G_i/G_s coupling could be observed in CHO-m2 or -m4 cells, as has been previously reported in a number of studies (Jones *et al*., 1991; Dittman *et al*., 1994; Vogel *et al*., 1995; Michal *et al*., 2001). In our hands we only observed concentration–effect curves where inhibition of forskolin-stimulated cyclic AMP accumulation at low agonist concentrations was superseded by activation at high agonist concentration in suspended CHO-m4 cells. In contrast, substratum-adherent CHO-m4 cells did not show this biphasic response suggesting that adenylyl cyclase activation may be influenced by factors beyond the type and expression level of the GPCR. Based on these data, it was decided to perform all further experiments on adherent cells and to use PTx to ensure that G_i proteins do not contribute (either positively or negatively) to the agonist-stimulated cyclic AMP responses.

With respect to G_i-mediated inhibitory responses, MCh, OXO-M, OXO, AREC and BETH caused similar, large maximal inhibitory effects, whereas PILO caused inhibitory effects that were 55 and 90% of maximal in CHO-m2 and -m4 cells, respectively. These data are in broad agreement with previous studies (Lazareno *et al*., 1993; Richards & van Giersbergen, 1995; van Giersbergen & Leppik, 1995). For G_s-mediated stimulatory responses, all agonist concentration–effect curves were right-shifted relative to the adenylyl cyclase inhibitory data. While OXO-M, MCh and AREC were again full agonists for this response, OXO and PILO were observed to be partial agonists with respect to adenylyl cyclase stimulation in CHO-m2 cells, whereas BETH and PILO were partial agonists in CHO-m4 cells.

Irrespective of whether inhibitory ($G_{i/o}$) or stimulatory (G_s) adenylyl cyclase responses were studied in CHO-m2 or -m4 cells, the agonist-potency ranking-order was essentially similar (OXO-M $\geqslant$ OXO $\geqslant$ MCh $\geqslant$ AREC $\geqslant$ BETH $\geqslant$ PILO). This strongly suggests that the agonists explored here do not generate distinct conformations with absolute preferences for receptor–$G_{i/o}$ or receptor–G_s coupling. Nevertheless, subtle agonist-dependent receptor-coupling effects have been observed.

A simple index of the relative coupling to G_i *versus* G_s signalling for each agonist is the difference between $pIC_{50}{}^{(Gi)}$ and $pEC_{50}{}^{(Gs)}$ values, and this and other measures have been used to indicate agonist trafficking of receptor signal (Berg *et al*., 1998; Bonhaus *et al*., 1998; Glass & Northup, 1999; Brink *et al*., 2000). While the mean ($pIC_{50}-pEC_{50}$) differences for the agonists are not sufficiently diverse to provide categoric evidence for agonist trafficking of receptor signal, significant divergence was seen. Thus, AREC showed the greatest preference for $G_{i/o}$ over G_s coupling (65–75-fold), while BETH and PILO showed the least ($\leqslant$10-fold) with $G_{i/o}$ *versus* G_s coupling rank orders being AREC > OXO $\approx$ OXO-M $\approx$ MCh > PILO $\approx$ BETH at M_2 mACh receptors, and AREC > OXO $\approx$ OXO-M $\approx$ MCh $\approx$ PILO $\approx$ BETH (note: OXO-M > PILO) at M_4 mACh receptors.

An alternative means of pharmacological analysis is to compare agonist efficacy values for G_i *versus* G_s-coupling, and between receptor subtypes. Relative efficacy values, termed e_{rel}, have been obtained using the method originally described by Ehlert (1985). These values are shown in Figure 5, where the e_{rel} with respect to G_i *versus* G_s coupling has been plotted for each agonist. The regression line on each panel reflects the relationship between M_2 and M_4–G_i/G_s coupling. Thus, the average efficacy difference between mACh receptor–G_i *versus* -G_s coupling was found to be 21.1-fold for M_2, and 22.9-fold for M_4 mACh receptors.

Figure 5 also highlights another significant difference in agonist actions at M_2 and M_4 mACh receptors: while OXO-M exhibits e_{rel} values that approach those of MCh at the M_2 mACh receptor, OXO-M can be classed as a 'super-agonist' (relative to MCh) at the M_4 mACh receptor. In addition, BETH exhibits the smallest (and AREC the greatest) differences between $e_{rel}{}^{(G_i)}$ and $e_{rel}{}^{(G_s)}$ values at both M_2 and M_4 mACh receptors. In CHO-m2 cells, BETH gives a $e_{rel}{}^{(G_s)}/e_{rel}{}^{(G_i)}$ ratio of 5.6 compared to a range of values of 14.6–24.6 for the other agonists. Whether the recent report (Liu & Rittenhouse, 2003) that shows BETH to exhibit a marked M_2 mACh receptor subtype selectivity with respect to mACh receptor modulation of voltage-gated Ca^{2+} channel modulatory activity in superior cervical ganglion cells relates at all to the present observations remains to be investigated.

In conclusion, we have failed to demonstrate definitive evidence of agonist trafficking of receptor signal for the selected agonists at either M_2 or M_4 mACh receptors. However, AREC behaves anomalously with respect to G_i-*versus* G_s-coupling at M_2 and M_4 mACh receptors, and we present evidence for OXO-M exhibiting 'super-agonist' behaviour with respect to both G_i- and G_s-coupling at M_4 mACh receptors with reference to the other mACh receptor agonists tested.

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